

## Original Research Article

# Investigation of nedaplatin and CpG oligodeoxynucleotide combination therapy in a mouse model of lung cancer

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### Abstract

**Purpose:** To investigate the anti-tumor effects of nedaplatin (NDP) and CpG oligodeoxynucleotide (CpG-ODN) combination therapy in a mouse-modeled lung cancer.

**Methods:** To evaluate the anti-tumor effects of NDP and CpG-ODN combination therapy, a lung cancer xenograft mouse model was established by subcutaneous injection of LA-795 cells. BALB/c mice were divided into four groups as follows: NDP, CpG-, NDP + CpG-ODN and untreated control group. The sections of lung cancer tissue were stained with hematoxylin and eosin (H&E) and morphologically examined. Spleen, body weight, and spleen index were measured. Flow cytometry was used to determine the proportions of CD3<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup> and CD4<sup>+</sup>/CD8<sup>+</sup> in mice blood cells. Serum levels of interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-12 (IL-12) were measured by enzyme-linked immunosorbent assay (ELISA).

**Results:** NDP + CpG-ODN therapy significantly reduced tumor volume and prolonged the survival time of tumor-bearing mice. NDP + CpG-ODN induced a change in cancer cell morphology, including large areas of necrosis which correlated with a reduction in tumor size. NDP + CpG-ODN significantly increased spleen weight/index and dramatically enhanced immune cell activation. This was evident in the increase serum levels of IFN- $\gamma$  and IL-12.

**Conclusion:** NDP and CpG-ODN combination therapy inhibits the growth of lung cancer and prolongs the survival time of tumor-bearing mice. This may result from the activation of immune cells and increased expression of IFN- $\gamma$  and IL-12.

**Keywords:** CpG ODN, NDP, Lung cancer, Combination therapy

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## INTRODUCTION

Lung cancer is a common malignant cancer with high morbidity and mortality rates. Lung cancer frequently results in organ metastasis, leading to death [1]. It is therefore necessary to develop new lung cancer therapy strategies [2].

Nedaplatin (NDP) is a platinum-based anti-tumor drug, which is widely used in chemotherapy to treat a variety of cancers. Its prominent anti-tumor effects are attributed to its interference with DNA replication, resulting in DNA damage [3]. However, NDP therapy shows some side effects, including bone marrow suppression, renal dysfunction, and immune dysfunction [4].

CpG oligodeoxynucleotides (CpG-ODNs), acting as immuno-stimulants, and are composed of single-stranded, non-methylated DNA molecules. Some researchers have demonstrated that CpG-ODN therapy significantly enhances humoral immunity, activating the patient's immune system, and promoting tumor clearance [5]. However, the effects of NDP + CpG-ODN combination therapy on lung cancer remains unknown. In order to better understand this relationship we established a lung cancer xenograft mouse model to evaluate the effects of NDP + CpG-ODN combination therapy, as well as, the mechanisms of NDP + CpG-ODN combination therapy.

## EXPERIMENTAL

### Animals

BALB/c mice (5 weeks old) were purchased from Luzhou Medical College (Animal Quality Certificate No. SCXK Chuan 2013-17). All animal experiments were performed in accordance with the guidelines of China Council on Animal Care and Use. All animals were maintained and experiments were performed in accordance with the European Community guidelines for the use of experimental animals (86/609/EEC) [6]. The animals were raised at the Department's Animal Experiment Center, The Affiliated Hospital of Shanxi University of Chinese Medicine. All animal procedures were approved by the Emory Institutional Animal Care and Use Committee (approval ref no. SZFYIEC-PJ-201421).

### Animal model

Each mouse was subcutaneously injected with LA-795 cells ( $10^6$ ) in 0.1 mL medium (mouse lung adenocarcinoma cell line, obtained from ATCC, Manassas, VA). When the tumor volume reached 80 mm<sup>3</sup>, sixty mice were randomly divided into 4 groups as follows: control group (no treatment), CpG-ODN group, NDP group, and NDP + CpG-ODN group. All mice received an intravenous injection every two days. In detail, in the control group, the mice received 0.1 mL PBS. In the CpG-ODN group, the mice received 0.1 mL CpG-ODN at a dose of 0.5 mg/mL. In the NDP group, the mice received 0.1 mL NDP (Aladdin Co., Shanghai, China) at a dose of 1 µg/mL. In the NDP + CpG-ODN combination therapy group, the mice received 0.1 mL CpG-ODN at a dose of 0.5 mg/mL and 0.1 mL NDP at a dose of 1 µg/mL. The sequence of non-methylated CpG-ODN was ATA ATC GAC GTT CAA GCA AG (Shenggong Co., Shanghai, China). After 22 days of treatment, all mice were held without food overnight. The next day the

mice were deeply anesthetized with CO<sub>2</sub> and their tissues were isolated.

### Therapeutic assessment

The long diameter/length (L) and short diameter/width (W) of the tumor was measured using vernier calipers. According to a previous report [7], tumor volume (V) was calculated as follows:  $V = (L \times W^2)/2$ . For the survival experiment, the treatment lasted 22 days. In addition, death of mice was defined by the following conditions: tumor volume > 2000 mm<sup>3</sup>, mouse weight loss > 20 %, or tumor diameter > 20 mm.

### Histological examination

Tumor tissues were fixed in 10% formalin before being embedded in paraffin and sectioned (10µm thickness) for histology. The sections were stained with hematoxylin and eosin (H&E) and images were captured by microscopy.

### Evaluation of spleen weight and index

The spleens were weighed and measured using the following formula: spleen index = spleen weight/body weight.

### Immunocyte assay and enzyme-linked immunosorbent assay (ELISA)

After 22 days of treatment, blood was also collected using a glass capillary tube from the retro-orbital venous plexus under light ether anesthesia. CD3+, CD8+, CD4+ and CD4+/CD8+ were analyzed by flow cytometry. The levels of IFN-γ and IL-12 in the serum were measured using ELISA.

### Statistical analysis

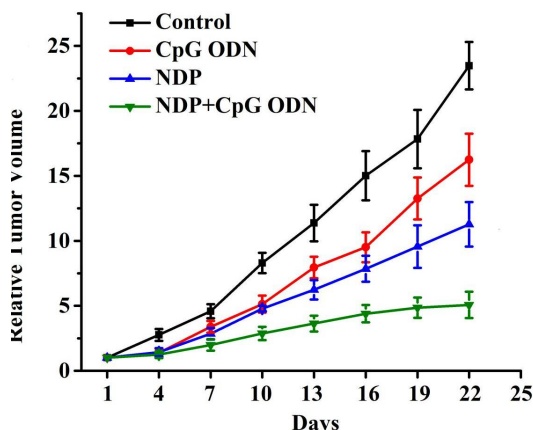
Statistical analyses were conducted using SPSS 11.5 (Chicago, IL, USA). All the data are presented as mean ± standard deviation (SD). Student's t-test and Welch's t-test were used for comparing two groups, while a one-way ANOVA, followed by SNK-q test was used for comparing multiple groups.  $P < 0.05$  was considered statistically significant.

## RESULTS

### NDP and CpG-ODN therapies attenuate tumor growth in tumor-bearing mice

All treated groups showed slower tumor growth compared to control group. However, from day 10 to day 22, there was a significant increase of

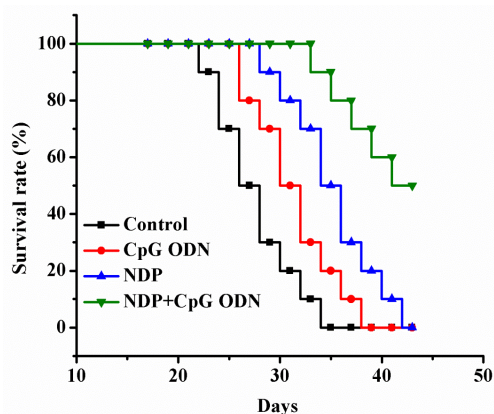
tumor volume in the NDP + CpG-ODN combination group, compared to the CpG-ODN group and NDP group.



**Figure 1:** NDP and + CpG-ODN inhibited tumor growth of lung cancer xenograft mice

**NDP and CpG-ODN therapies prolong the survival time of tumor-bearing mice**

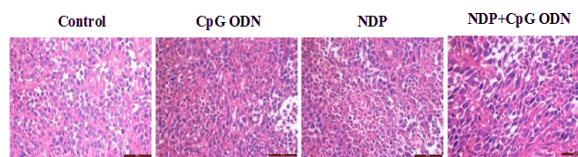
As shown in Figure 2, the CpG-ODN (31 days), NDP (35 days) or NDP + CpG-ODN combination (43 days) therapy could significantly prolong the survival time compared to the control groups, which averaged 27 days ( $p < 0.05$ ).



**Figure 2:** NDP and CpG-ODN therapies prolong the survival time of tumor-bearing mice

**Morphological changes in by NDP and CpG-ODN therapies**

As shown in Figure 3, the histopathological evaluation of tumor tissues showed that the tumor cells in the control group were closely arranged, in round shaped without any apoptotic morphological changes. However, in the treatment groups, the histopathological evaluation of tumor tissues showed obvious vacuoles and nucleus pyknosis.



**Figure 3:** H&E staining of tumor tissues in different groups (Scale bar = 10  $\mu$ m)

**CpG-ODN or NDP + CpG-ODN combination therapy improve spleen index**

As shown in Table 1, the average spleen weight in the CpG-ODN group ( $0.34 \pm 0.07$  g) was, significantly higher than controls ( $0.19 \pm 0.03$  g). However, there was no significant difference between the NDP group ( $0.17 \pm 0.03$  g) and the control group. In addition, the spleen weight in the NDP + CpG-ODN combination therapy group significantly increased compared to the control group and the NDP group. However, there was no significant difference between the NDP + CpG-ODN and CpG group. In addition, the spleen index in both CpG-ODN ( $20.55 \pm 2.62$  %) and NDP + CpG-ODN group ( $20.54 \pm 2.65$  %) significantly increased compared to controls ( $11.92 \pm 1.87$  %). There was no significant difference between NDP and the controls.

**Table 1:** Spleen index and spleen weight of tumor-bearing mice (mean  $\pm$  SD)

Group	Spleen weight (g)	Spleen index (mg/g)
Control	$0.19 \pm 0.03$	$11.92 \pm 1.87$
CpG-ODN	$0.34 \pm 0.07^a$	$20.55 \pm 2.62^a$
NDP	$0.17 \pm 0.03^b$	$11.89 \pm 1.88^b$
NDP+CpG-ODN	$0.33 \pm 0.06^{ac}$	$20.54 \pm 2.65^{ac}$

**Note:** Compared to the control group, <sup>a</sup> $p < 0.05$ ; compared to the CpG-ODN group, <sup>b</sup> $p < 0.05$ ; compared to the NDP group, <sup>c</sup> $p < 0.05$

**Effect of NDP and CpG-ODN therapies on activation of immune cells**

As shown in Table 2, the amount of CD4<sup>+</sup>, CD3<sup>+</sup> and NK cells and the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> in the CpG-ODN group were significantly increased compared to those in the control group ( $p < 0.05$ ), whereas the proportion of CD8<sup>+</sup> cells was significantly decreased ( $p < 0.05$ ). However, in the NDP-treated group, the proportion of CD8<sup>+</sup> cells was significantly increased, while the CD3<sup>+</sup>, CD4<sup>+</sup>, NK and ratio of CD4<sup>+</sup>/CD8<sup>+</sup> significantly decreased compared to those in the control group. In addition, all cells amount in the NDP + CpG-ODN combination therapy group were significantly higher compared to those in the control group ( $p < 0.05$ ).

**Table 2:** Comparison of immune cell populations in tumor-bearing mice in different groups (mean ± SD)

Group	CD3+ (%)	CD4+ (%)	CD8+ (%)	CD4+/CD8+ (Ratio)	NK (%)
Control	36.23±2.44	21.51±1.76	16.48±1.34	1.50±0.31	15.47±1.64
CpG-ODN	45.17±3.68 <sup>a</sup>	27.36±1.93 <sup>a</sup>	13.55±1.06 <sup>a</sup>	1.98±0.17 <sup>a</sup>	21.81±2.37 <sup>a</sup>
NDP	28.51±2.07 <sup>ab</sup>	16.24±1.54 <sup>ab</sup>	20.39±1.71 <sup>ab</sup>	1.19±0.19 <sup>ab</sup>	12.17±1.02 <sup>ab</sup>
NDP+CpG-ODN	41.04±2.987 <sup>abc</sup>	24.62±1.89 <sup>abc</sup>	17.04±1.45 <sup>abc</sup>	1.71±0.24 <sup>abc</sup>	18.83±1.94 <sup>abc</sup>

**Note :** Compared to the control group, <sup>a</sup>*p* < 0.05; compared to the CpG-ODN group, <sup>b</sup>*p* < 0.05; Compared to the NDP group, <sup>c</sup>*p* < 0.05

**Effect of NDP and CpG-ODN therapies on cytokine secretion**

The serum levels of IFN-γ in the CpG-ODN group were 132.47 ± 8.57 pg /mL. The serum levels of IFN-γ in the NDP + CpG-ODN combination therapy group were 115.02 ± 7.30 pg /mL. The levels of IFN-γ in serum in these two groups were significantly increased when compared with those in the control group, which were 64.23 ± 4.68 pg/mL (*p* < 0.05). However, the serum level of IFN-γ in the NDP group was 57.12 ± 3.98 pg/ mL, which was significantly decreased in comparison to that in the control group (*p* < 0.05) (Table 3).

The serum level of IL-12 in the CpG-ODN group was 451.94 ± 23.74 pg/mL, which was significantly increased compared to the controls, which were 244.37 ± 13.26 pg/mL (*p* < 0.05). The serum levels of IL-2 in the NDP group were also significantly increased compared to those in the control group, at 183.07 ± 10.55 pg/mL (*p* < 0.05). The serum level of IL-12 in the NDP + CpG-ODN combination therapy group was 378.41 ± 18.52 pg/mL, which was significantly increased compared with that of the control group (*p* < 0.05). Additionally, the serum levels of IL-12 in the NDP + CpG-ODN combination therapy group were significantly increased compared to those in the CpG-ODN group (*p* < 0.05).

**Table 3:** Serum levels of IFN-γ and IL-2 in tumor-bearing mice in different groups (mean ± SD)

Group	IFN-γ (pg/mL)	IL-12 (pg/mL)
Control	64.23±4.68	244.37±13.26
CpG-ODN	132.47±8.57 <sup>a</sup>	451.94±23.74 <sup>a</sup>
NDP	57.12±3.98 <sup>ab</sup>	183.07±10.55 <sup>ab</sup>
NDP+CpG-ODN	115.02±7.30 <sup>abc</sup>	378.41±18.52 <sup>abc</sup>

<sup>a</sup>*P* < 0.05, CpG-ODN & controls, <sup>b</sup>*p* < 0.05, Compared to NDP group, <sup>c</sup>*p* < 0.05

**DISCUSSION**

Lung cancer, as a malignant tumor, is characterized by uncontrolled cell division. It is

one of the more common forms of cancer and displays extremely high rates of morbidity and mortality. It is therefore necessary to develop new strategies for lung cancer therapy [8]. We found that NDP + CpG-ODN combination therapy had a significant anti-tumor effect. NDP + CpG-ODN significantly increased the survival time of tumor-bearing mice compared with the control. Moreover, the spleen index has been improved by treatment of NDP + CpG-ODN compared to both control and NDP-treated mice, indicating positive effects of the combination therapy.

NDP is a platinum-based drug, widely used in the treatment of bladder, lung and esophageal cancer. The anti-tumor effects result from an interaction between NDP and nucleosides, forming a nucleoside-platinum complex, which interferes with DNA replication, leading to DNA damage. The glycolate is cleaved as an “active species 1” by hydrolysis after NDP enters into the cells. There are balances among a series of active species, which means active species 1 might also be interconverted. The active species can inhibit both DNA replication and RNA transcription by binding to DNA. This process results in apoptosis and necrosis of cells [9-11]. However, because of a lack of specificity, NDP is not precisely related to cancer cells, which induces normal cell death severe side effects result, including immune suppression, bone marrow hematopoietic dysfunction, and hematologic toxicity.

CpG-ODN, as an immuno-modulator, contains the CpG sequence and effectively activates dendritic cells which secrete a variety of immunologic factors [12]. In our study, we found an increase in spleen weight in the CpG-ODN treated mice. In addition, the numbers of CD3<sup>+</sup>, CD4<sup>+</sup> and NK cells, as well as levels of IFN-γ and IL-12 increased in the CpG-ODN group. These findings demonstrated that CpG-ODN enhanced immune function in the tumor-bearing mice. CpG-ODN can activate the NF-κB signaling pathway and induce immuno-stimulation *in vivo* by binding with toll-like receptor 9 to enter into immune cells[13]. Previous studies also showed

that CpG-ODN might induce cytokines, such as IFN- $\gamma$  and IL-12, by activating JNK1/2 and p38 pathways [14,15]. IFN- $\gamma$  and IL-12 perform functions on activation of T cells by increasing B7-2 and CD69 expression and further promoting the proliferation and differentiation of T cells [16,17]. Additionally, CpG-ODN also activates NK cells [14,15].

This study investigated the combination therapeutic effects according to the advantage of both NDP and CpG-ODN. The combination of NDP and CpG-ODN showed the significant improvement of the efficiency of lung cancer therapy in animal models. Meanwhile, this combination may also decrease the side effects. We did not present a side effects investigation in this study, but we look forward to studying those mechanisms in the future.

## CONCLUSION

The results of this study show that NDP + CpG-ODN inhibits the tumor growth and prolonged the survival time of tumor-bearing mice via activation of immune cells. The therapeutic changes observed indicate that the combination therapy is a potential treatment strategy for the management of lung cancer and reduction in NDP resistance.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

## REFERENCES

1. Stolz AJ, Harustiak T, Simonek J, Schutzner J, Lischke R. Pneumonectomy for non-small cell lung cancer: predictors of early mortality and morbidity. *Acta Chir Belg*. 2014; 114(1): 25-30.
2. MacIntyre EA, Gehring U, Molter A, Fuertes E, Klumper C, Kramer U, Quass U, Hoffmann B, Gascon M, Brunekreef B, et al. Air Pollution and Respiratory Infections during Early Childhood: An Analysis of 10 European Birth Cohorts within the ESCAPE Project. *Environ Health Persp*. 2014; 122(1): 107-113.
3. Pang H, Feng T, Lu HL, Meng QW, Chen XS, Shen Q, Dong XQ, Cai L. Efficacy and Safety of Nedaplatin in *Advanced Breast Cancer Therapy*. *Cancer Invest*. 2016; 34(4): 167-172.
4. Vergara C, Martinez-Ferrer A, Fernandez M, Oller JE, De la Morena I, Ybanez A, Valls E, Robustillo M, Vicens E, Alegre JJ. Bone Manifestations in Patients with Gaucher Disease. *Annals of the Rheumatic Diseases*. 2014; 73: 1069-1070.
5. Gershan JA, Barr KM, Weber JJ, Jing WQ, Johnson BD. Immune modulating effects of cyclophosphamide and treatment with tumor lysate/CpG synergize to eliminate murine neuroblastoma. *Journal for Immunotherapy of Cancer*. 2016; 3: 24
6. Zhang J, Song S, Pang Q, Zhang R, Zhou L, Liu S, Meng F, Wu Q, and Liu C. Serotonin deficiency exacerbates acetaminophen-induced liver toxicity in mice. *Scientific reports*. 2015; 5, 12184.
7. Yang H, Wang J, Fan JH, Zhang YQ, Zhao JX, Dai XJ, Liu Q, Shen YJ, Liu C, Sun WD et al. Ilexgenin A exerts anti-inflammation and anti-angiogenesis effects through inhibition of STAT3 and PI3K pathways and exhibits synergistic effects with Sorafenib on hepatoma growth. *Toxicol Appl Pharmacol*. 2017; 315: 90-101.
8. Bai Y, Wang YL, Yao WJ, Guo L, Xi HF, Li SY, Zhao BS. Expression of miR-32 in human non-small cell lung cancer and its correlation with tumor progression and patient survival. *Int J Clin Exp Patho*. 2015; 8(1): 824-829.
9. Akiyama H, Yamamoto M, Sakashita C, Umezawa Y, Kurosu T, Murakami N, Miura O. Therapy-related Leukemia with Inv(16)(p13.1q22) and Type D CBF/MBP11 Developing after Exposure to Irinotecan-containing Chemoradiotherapy. *Internal Med*. 2015; 54(6): 651-655.
10. Qiu ZQ, Zhao K. Expression of ERCC1, RRM1 and LRP in Non-small Cell Lung Cancers and their Influence on Chemotherapeutic Efficacy of Gemcitabine Concomitant with Nedaplatin. *Asian Pac J Cancer P*. 2014; 15(17): 7303-7307.
11. Kawanishi K, Miyagi Y, Yamamoto J, Miyagi Y, Nakamura K, Kodama J, Hongo A, Yoshinouchi M, Kudo T. Cytocidal effect and DNA damage of nedaplatin in vitro by simulating pharmacokinetic parameters. *Cancer Chemotherapy and Pharmacology*. 2001; 47(4): 303-308.
12. Li JY, Li JY, Aipire A, Luo JJ, Yuan PF, Zhang FC. The combination of *Pleurotus ferulae* water extract and CpG-ODN enhances the immune responses and antitumor efficacy of HPV peptides pulsed dendritic cell-based vaccine. *Vaccine*. 2016; 34(31): 3568-3575.
13. Zhang YY, Lin A, Sui QJ, Hang C, Tian ZG, Zhang J. Phosphorothioate modification of the TLR9 ligand CpG ODN inhibits poly(I:C)-induced apoptosis of hepatocellular carcinoma by entry blockade. *Cancer Letters*. 2014; 355(1): 76-84.
14. Lee S, Hong J, Choi SY, Oh SB, Park K, Kim JS, Karin M, Lee SJ. CpG oligodeoxynucleotides induce expression of proinflammatory cytokines and chemokines in astrocytes: the role of c-Jun N-terminal

- kinase in CpG ODN-mediated NF-kappaB activation. *J Neuroimmunol.* 2004; 153(1-2): 50-63.
15. Ma C, Muranyi M, Chu CH, Zhang JH, Chu WM. Involvement of DNA-PKcs in the IL-6 and IL-12 Response to CpG-ODN Is Mediated by Its Interaction with TRAF6 in Dendritic Cells. *Plos One.* 2013; 8(3).
  16. Ruan M, Thorn K, Liu SW, Li SY, Yang WJ, Zhang CY, Zhang CP. The secretion of IL-6 by CpG-ODN-treated cancer cells promotes T-cell immune responses partly through the TLR-9/AP-1 pathway in oral squamous cell carcinoma. *International Journal of Oncology.* 2014; 44(6): 2103-2110.
  17. Pradhan P, Qin H, Leleux JA, Gwak D, Sakamaki I, Kwak LW, Roy K. The effect of combined IL10 siRNA and CpG ODN as pathogen-mimicking microparticles on Th1/Th2 cytokine balance in dendritic cells and protective immunity against B cell lymphoma. *Biomaterials.* 2014; 35(21): 5491-5504.