**Anti-tumor effect of ultrasound-induced Nordy-loaded microbubbles destruction**

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**ABSTRACT**

**Background:** Synthesized dl-Nordihydroguaiaretic acid (dl-NGDA or “Nordy”) can inhibit the growth of malignant human tumors, especially the tumor angiogenesis. However, its liposoluble nature limits its in vivo efficacy in the hydrosoluble circulation of human.

**Purpose:** We tried to use the ultrasonic microbubble as the carrier and the ultrasound-induced destruction for the targeted release of Nordy and evaluate its in vitro and in vivo anti-tumor effect.

**Methods:** Nordy-loaded lipid microbubbles were prepared by mechanical vibration. Effects of ultrasound-induced Nordy-loaded microbubbles destruction on proliferation of human umbilical vein endothelial cells (HUVECs), tumor derived endothelial cells (Td-ECs), and rabbit transplanted VX2 tumor models were evaluated.

**Results:** The ultrasound-induced Nordy-loaded microbubbles destruction inhibited the proliferations of HUVECs and Td-ECs in vitro, and inhibited the tumor growth and the microvasculature in vivo. Its efficacy was higher than those of Nordy used only and Nordy with ultrasound exposure.

**Conclusion:** Ultrasonic microbubbles can be used as the carrier of Nordy and achieve its targeted release with improved anti-tumor efficacy in the condition of ultrasound-induced microbubbles destruction.

**Background**

Synthesized dl-Nordihydroguaiaretic acid (dl-NGDA or “Nordy”), a natural product purified from Larrea divaricata and Guaiacum officinale, has been proved to exert multiple biological activities by inhibiting lipoxygenase during Arachidonic acid metabolism [1–3]. NDGA has been reported to be capable of down-regulating the expression of some vital proteins such as VEGF, VEGF receptor KDR, cyclinD1, and CDC2 protein [3,4], in addition to its multiple effects including inhibiting the growth of tumor cells and their grafts grown in mice, inducing the differentiation of rat and human glioma cells, and blocking the differentiation of myoblast cells. Nordy has also been shown to promote the differentiation of malignant glioma cells both in vitro and in vivo [1,5,6]. Furthermore, it has been suggested that Nordy inhibited angiogenesis in inflammation diseases and cancer [7]. Nordy may exhibit its anti-tumor activity through blocking the receptor mediated cell signaling at several levels. It can not only inhibit malignant gliomas and transplanted tumors in animals and decrease the expression and activation of gloma chemokine receptor (CXCRC4), VEGF, and formyl peptide receptor (FPR) but also inhibit the tubular structure formation of the primarily cultured microvascular endothelial cells of human malignant glioma in vitro [8–10]. In addition, it was proved that Nordy could inhibit the proliferation, migration, and tubular structure formation of endothelial progenitor cells [11]. Despite the remarkable anti-tumor angiogenesis effect of Nordy, its clinical application may be limited by its nature of lipid compound, so direct administering may not achieve satisfactory efficacy for its low solubility in the hydrosoluble blood circulation of human and the subsequent low drug concentration in the tumor region. Therefore, it is necessary to seek an efficient method for targeted delivery of Nordy, and the solution to this problem will lay a foundation for its clinical usage, while there is no reported research on drug targeting of Nordy yet so far.

Ultrasound micorbubbles, widely used in the contrast-enhanced ultrasonography (CEUS) in clinical as the contrast agent to improve the signal to noise ratio of ultrasonic imaging, have been proved to be a promising carrier for drugs and genes [12,13]. Microbubbles are constituted by the gas core and the outer shell encapsulating the gas, which is composed of lipids, proteins, surfactant or polymers, etc, and their high biocompatibility and stability endow the microbubbles with the ability to carry drugs, especially the hydrophobic drugs. Ultrasound-induced microbubbles destruction, by breaking drug-loaded microbubbles at the target site in the condition of ultrasound exposure with proper frequency and intensity, permits the targeted release of drugs. During this process, cavitation, including stable and inertial cavitations caused by the expansion, contraction and final rupture of microbubbles in the ultrasonic field, produces a lot of energy around through shock wave, microstream, microjet, etc, [14,15], and sonoporation causes the temporary improvement of cell membrane permeability, both of which help to increase the uptake of the released drugs by cells and improve the therapeutic efficacy [16,17].

In this present study, we tried to take the microbubbles as the carrier of Nordy, induce its targeted release by ultrasound exposure, and evaluate its effect on tumor growth in vitro and in vivo.
the microbubbles were (2.53 ± 0.61) μm, and + (17.52 ± 2.04) mV, respectively, measured by a Malvern spray analyzer (Zetasizer 3000, Malvern, England). The content of Nordy loaded in lipid microbubbles was detected by reversed-phase high performance liquid chromatography (Waters, Milford, MA). The encapsulation efficiency and the drug-loading rate were calculated.

Methods

Preparation of Nordy-loaded microbubbles

An appropriate amount of lipid mixture (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, diphenylphosphoryl azide, and dipalmitoyl phosphatidylethanolamine-polyethylene glycol-2000) (Lipoid, Germany) in a molar ratio of 47:10:5 and Nordy powder (Pathology Laboratory of Southwest Hospital, Chongqing, China) with a mass ratio of 18.8% to total phospholipid were dissolved in a solvent of chloroform and methanol (1:1, V/V). The solution was transferred to a round bottom flask and the solvent was removed by rotary vacuum evaporation at 60 °C. The resulting lipid film was hydrated in phosphate-buffered saline (PBS, pH 7.4), and afterward proportional glycerol (9:1, V/V) was added to produce a final lipid concentration of 16 mg/mL. The liposomal formulation underwent aqueous bathing at 60 °C for 0.5 h and was aliquoted at 450 μL in 1.5 mL round bottom tubes with rubber caps. Subsequently, the air in the tubes was replaced by perfluoropropane gas with a syringe through the rubber caps. After that, the dispersion was mechanically vibrated at 3200 rpm for 45 s and then Nordy-loaded microbubbles were obtained. The microbubbles were washed with PBS three times and free drug (not incorporated into the microbubbles) was separated by centrifugal flotation. The unloaded microbubbles were similarly prepared but without the addition of Nordy.

Microbubbles were rinsed with phosphate buffer 3–5 times and observed under microscope (Olympus CX41, Tokyo, Japan) (Figure 1). The concentration, particle size, and Zeta potential of the microbubbles were (2.53 ± 0.52) × 10^9/mL, (2.61 ± 0.32) μm, and + (17.52 ± 2.04) mV, respectively, measured by a Malvern spray analyzer. The introduction of Nordy-loaded microbubbles enhanced the echo of healthy rabbit liver noticeably, confirmed by contrast-enhanced ultrasound.

Effect of ultrasound-induced Nordy-loaded microbubbles destruction on proliferation of human umbilical vein endothelial cells (HUVECs) and tumor-derived endothelial cells (Td-ECs)

HUVECs from fresh fetal umbilical cord tissue was cultivated (Data S1), which was obtained from healthy hospitalized puerpera of Southwest Hospital affiliated to Third Military Medical University, in the context of the informed consent of patient. The successfully in vitro cultured HUVECs were divided into five groups: Group Control, no treatment; Group Nordy-MB, treated with Nordy-loaded microbubbles only; Group Nordy, treated with Nordy solution (200 μmol/L) only; Group Nordy + US, treated with Nordy solution (200 μmol/L) with simultaneous ultrasound exposure with a frequency of 1.5 MHz, mechanical index of 1.9 and exposure time of 120 s; and Group Nordy-MB + US, treated with Nordy-loaded microbubbles (5 μL) with simultaneous ultrasound exposure same as the previous group.

The inhibition to HUVECs proliferation was detected by methyl thiazolyl tetrazolium (MTT) method, according to Mao’s research [18]. The optical density (OD) was detected with an automatic enzyme linked immunological detector at a wavelength of 550 nm. The inhibition rate of cell proliferation was calculated as follows: inhibition rate = (OD value of control − OD value of experimental group)/OD value of control × 100%. The inhibition to three-dimensionally cultured vascular endothelial cells was also detected by an inverted phase contrast microscopy. In addition, the HUVECs that were passaged to the third-generation were cultivated with stoody rat tail collagen medium in 24-well cell culture plate and then treated with 4 wells in each group. The tubular structure of endothelial cells was observed under phase contrast microscope and its number was counted once every 2 d from the third day post cultivation. One tube was counted as 1, one branch was counted as 2, two branches were counted as 3, and so on. The counting was repeated three times in each well and the mean value was calculated.

HUVECs (EA.Hy 926 cell line) were induced to Td-ECs with human lung adenocarcinoma cells (A459 cell line) supernate, referring to Möbius’s work [19] (the cell lines were presented by Liujunze’s lab of Third Military Medical University). Tumor endothelial marker (TEM8) was detected with RT-PCR. The in vitro cultured Td-ECs were divided into five groups and treated as previously described in HUVECs. Cells were collected and RNA was extracted at 6 h, 12 h, and 18 h post-treatment, and the changes of TEM8 were compared among groups.

Effect of ultrasound-induced Nordy-loaded microbubbles destruction on rabbit transplanted VX2 tumor models

The transplanted VX2 tumor models were successfully established, by transplanting the tumor tissue into the leg muscle of 30 New Zealand rabbits purchased from Experimental Center of Third Military Medical University (Data S2). The rabbits weighed 2.3–2.8 kg (mean 2.5 ± 0.32 kg). The experiments were approved by the Animal Care and Use Committee of the Third Military Medical University. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States [20].

The VX2 tumor-bearing rabbits were randomly divided into six groups (five in each group) when the diameter of tumor was > 10 mm detected by ultrasonography 2 weeks post-tumor transplantation. Group control: no treatment. Group Nordy: Nordy solution was injected only. Group Nordy-MB: Nordy-loaded microbubbles were injected only. Group US: the tumor was treated with ultrasound exposure only. Group US + Nordy: the Nordy solution was injected with simultaneous ultrasound exposure. Group US + Nordy-MB: the Nordy-loaded microbubbles were injected with simultaneous ultrasound exposure. The dose of Nordy solution was 0.2 mg/kg, and the dose of microbubbles was 0.1 mL/kg. Ultrasound exposure was performed with an Acuson...
Sequoia 512 ultrasound diagnostic device (Siemens, Munich, Germany) with 4V1C-S transducer in mode of microbubble destruction (MBD) (frequency = 1.5 MHz, MI = 1.9, depth = 6 mm), in which the ultrasound was irradiated in an intermittent pattern with the interval time of 6 s and the total exposure time of 2 min.

The experimental procedures were performed as follows. The tumor-bearing rabbits were anesthetized and fixed on the operation table in prone position. The hair of right hind leg was shaved for ultrasound examination. An Acuson Sequoia 512 ultrasonic instrument (Siemens, Munich, Germany) with high-frequency linear-array transducer (14 MHz, 40 mm) was utilized for ultrasonography. As the gray scale image of tumor was clearly shown, the tumor volume was measured and calculated according to the formula: \( V = \frac{\pi}{6} \times a \times b \times c \) (\( V \), tumor volume; \( a \), length of major axis; \( b \), length of minor axis; \( c \), thickness). The tumor location and transducer orientation were marked with color at the surface of leg. The sonographic changes of transplanted tumor were observed at 3 d, 6 d, and 9 d post-transplantation. The growth rate (GR) of tumor was calculated according to the formula: \( GR = \frac{\text{volume 9 d post-treatment} - \text{volume before treatment}}{9 \text{ d post-treatment} \times 100\%} \).

When the experiments were finished, the tumor tissues were cut off, fixed with formalin, and embedded with paraffin. Part of each tissue was HE stained for pathological examination, and the other part was evaluated by CD34 monoclonal antibody staining for neovascular density (MVD), according to the method developed by Weidner with a commercial immunochemistry kit (BOSTER, China) [21]. For the microvessel counts, any brown-stained cell or cluster of endothelial cells clearly separate from tissue cells and other connective tissue elements was considered as a single vessel. First, the entire CD34-stained section was observed under \( \times 100 \) microscopy (OLYMPUS BX51, Tokyo, Japan) for regions of high capillary density (hotspots), and then \( \times 200 \) lens was used to quantify the number of vascular endothelial cell cluster stained with brown dye. The mean of five capillary quantifications observed with the \( \times 200 \) lens was used as the MVD value. All the pathological examinations were performed by two experienced pathologists who were blinded to the groups and the treatment results.

**Statistical analysis**

All the data were presented with mean ± standard deviation (\( \bar{x} \) ± s). The data were compared among groups with one-way analysis of variance (ANOVA), which was performed by the software of SPSS 16.0 (SPSS Inc., Chicago, IL) in a computer. A \( p \) value < 0.05 was considered statistically significant.

**Results**

**Effect of ultrasound-induced Nordy-loaded microbubbles destruction on proliferation of HUVECs**

After treatment, the OD values detected by MTT of the groups of Nordy (0.43 ± 0.08), US + Nordy (0.372 ± 0.07), and US + Nordy-MB (0.345 ± 0.06) were significantly lower than that of control (0.527 ± 0.11) (Figure 2), with \( p < 0.05 \) in the former two and \( p < 0.01 \) in the latter one, that is to say, the growth of HUVECs was inhibited in the groups of Nordy, US + Nordy and US + Nordy-MB, in which it was the most significant in the group of US + Nordy-MB.

Counting of cell tubular structure of endothelial cell in different groups is presented in **Table 1**. The formation of tubular structure was influenced in varying degrees in different groups from the third day, and it is very significant in the groups of US + Nordy and US + Nordy-MB (Supplemental Figure 1).

The EA.Hy926 cells cultured with A549 cells supernate presented an irregular shape with prominences protruding outwards under microscope at 48 h post cultivation (Supplemental Figures 2 and 3). The electrophoresis of RT-PCR amplification products showed that the cells expressed TEM8, the spatial antigen expressed by tumor endothelial cells, proving that the EA.Hy926 cells induced by A549 cells supernatant possessed the characteristics of tumor endothelial cells (Supplemental Figure 4). The TEM8 expressions at 6 h, 12 h, and 18 h post-treatment detected by RT-PCR (Supplemental Figure 5) are showed in **Table 2**. The cells grew well and TEM8 expressions had no significant change between different time points in the groups Nordy-MB and Control, and the TEM8 expression decreased at 12 h post-treatment in the groups Nordy, US + Nordy and US + Nordy-MB, among which it is the most remarkable in group US + Nordy-MB.

**Effect of ultrasound-induced Nordy-loaded microbubbles destruction on tumor growth and microvasculature of rabbit transplanted VX2 tumor models**

Ultrasoundography of the rabbit VX2 tumor models showed that the tumors presented as hypoechoic nodules in the leg muscle (Supplemental Figure 6). The volume changes, growth rates, and growth inhibition rates of tumors after treatment are showed in **Table 3**. Compared with the other groups, the size of tumor post-treatment was the smallest in group US + Nordy-MB (\( p < 0.05 \)). There was no significant difference among the other groups. And the inhibition rate of tumor growth was the highest in group US + Nordy-MB.

To quantify the change of angiogenesis in tumors, MVD was determined by staining tissue sections immunohistochemically for
Table 1. Inhibition of different treatments to tubular structure formation of endothelium.

| Group (d) | Control (n = 12) | Nordy-MB (n = 12) | Nordy (n = 12) | US + Nordy (n = 12) | US + Nordy-MB (n = 12) |
|-----------|------------------|-------------------|----------------|---------------------|------------------------|
| 3         | 5.69 ± 0.85      | 5.42 ± 0.86       | 4.34 ± 0.81    | 3.61 ± 0.71***     | 3.21 ± 0.94***         |
| 5         | 7.67 ± 0.37      | 7.44 ± 0.67       | 6.25 ± 1.25    | 5.60 ± 1.30***     | 4.93 ± 1.52***         |
| 7         | 10.57 ± 1.18     | 9.93 ± 0.79       | 8.36 ± 1.63*** | 7.15 ± 1.62***     | 6.10 ± 1.49***         |
| 9         | 13.03 ± 0.96     | 12.26 ± 0.55      | 10.81 ± 2.79***| 9.30 ± 2.49***     | 7.57 ± 2.00***         |

Compared with control at the same time point.
*<i>p</i> < 0.05,
**<i>p</i> < 0.01,
***<i>p</i> < 0.001.

Table 2. Effects of Td-ECs on expression of TEM8 with different treatments.

| Group (h) | Control (n = 4) | Nordy-MB (n = 4) | Nordy (n = 4) | US + Nordy (n = 4) | US + Nordy-MB (n = 4) |
|-----------|-----------------|------------------|--------------|-------------------|-----------------------|
| 6         | 0.9859 ± 0.0085 | 0.9796 ± 0.0062  | 0.8821 ± 0.0034 | 0.7897 ± 0.0041  | 0.7529 ± 0.0046       |
| 12        | 0.9886 ± 0.0036 | 0.9799 ± 0.0059  | 0.8462 ± 0.0072* | 0.7569 ± 0.0064* | 0.6876 ± 0.0098****   |
| 18        | 0.9827 ± 0.0022 | 0.9797 ± 0.0042  | 0.7730 ± 0.0025** | 0.7023 ± 0.0091** | 0.6204 ± 0.0103*****  |

Compared with the control at the same time point.
*<i>p</i> < 0.05,
**<i>p</i> < 0.01; Compared among different time points in the same group.
*<i>p</i> < 0.05.

Table 3. Volume change, growth rate, and growth inhibition rate of rabbit transplanted VX2 tumor in different groups.

| Group     | Volume before treatment (cm³) | Volume 3 d post treatment (cm³) | Volume 6 d post treatment (cm³) | Volume 9 d post treatment (cm³) | Growth rate (%) | Growth inhibition rate (%) |
|-----------|-------------------------------|---------------------------------|---------------------------------|-------------------------------|-----------------|---------------------------|
| Control   | 4.09 ± 0.43                   | 12.68 ± 3.67                   | 26.1 ± 13.19                    | 53.08 ± 4.34                  | 92.22 ± 1.17    | -                         |
| Nordy     | 2.95 ± 0.81                   | 6.41 ± 1.37                    | 15.49 ± 2.21                    | 26.17 ± 4.98                  | 88.76 ± 1.81    | 50.70                     |
| Nordy-MB  | 2.93 ± 1.01                   | 9.91 ± 2.42                    | 18.72 ± 3.08                    | 34.78 ± 2.25                  | 91.60 ± 2.65    | 34.68                     |
| US        | 3.53 ± 1.18                   | 11.38 ± 1.69                   | 25.25 ± 3.18                    | 46.04 ± 5.46                  | 92.12 ± 3.38    | 13.26                     |
| US + Nordy| 3.19 ± 1.09                   | 8.12 ± 1.94                    | 20.14 ± 4.81                    | 34.12 ± 13.79                 | 89.60 ± 4.72    | 35.72                     |
| US + Nordy-MB | 3.50 ± 1.08                | 5.88 ± 0.93                    | 13.74 ± 1.81                    | 21.91 ± 4.19*                 | 84.28 ± 2.62*   | 58.72                     |

*Compared with the other groups.
*p < 0.05.

Discussion

It has been well accepted that ultrasonic microbubbles, working as contrast agents in CEUS at present, are potential to be one of the promising carriers for drugs and genes, which has been proved effective in curing tumors, gene transfection, thrombolysis, etc. [22–25]. As the ultrasonic microbubbles are the gas-filled (usually) microspheres with a diameter of 1–10 μm, which serve as true intravascular non-diffusible indicators, thus providing imaging of the intrinsic spatial and temporal heterogeneity of tissue perfusion [26,27]. CEUS represents a significant advancement in the evaluation of blood perfusion and angiogenesis in tissue and organ, because these minute bubbles are capable of surviving transpulmonary passage to recirculate and produce useful systemic ultrasound enhancement, so act as “surrogate red blood cells” unlike larger molecules which are retained in vascular beds. The shell and the inner space of microbubble, which are suitable for taking drugs or genes through multiple ways, endows the microbubbles the ability of carrying drugs or genes but not affecting their activity [28]. At present, it is a mainstream to prepare the microbubbles with lipids as the shell materials. The lipid shell of microbubble has a good histocompatibility with lipophilic drugs that may be poorly soluble in water and blood, and it has been proved that microbubbles are capable of carrying lipophilic drugs such as paclitaxel [29,30]. In this present study, we succeeded in preparing the Nordy-loaded microbubbles and reserving its activity during drug releasing with ultrasound-induced microbubbles destruction, providing a unique solution for the limitation of Nordy’s low solubility in blood. This method is also of potential to be helpful for the clinical promotion of this new encouraging anti-tumor drug. The results of this in vitro study proved the definitive anti-tumor efficacy of ultrasound-induced Nordy-carrying microbubbles destruction. This approach inhibited the in...
vitro tumor cells growth and down-regulated the expression of TEM 8, better than Nordy alone. TEM is a newly found marker specifically expressed on tumor endothelial cells and TEM 8 is the only subtype expressed in tumor endothelial cells as a transmembrane receptor [31,32]. Furthermore, ultrasound-induced Nordy-carrying microbubbles destruction had a higher efficacy in inhibiting tumor growth and reducing tumor microvascular density in the VX2 tumor models of rabbits, compared with Nordy alone and Nordy with ultrasound exposure, while there are little influences in the other groups of Nordy-carrying microbubbles only, US only, and control. The results showed that microbubbles may deliver Nordy and release the drug targeted in tumor blood vessels under the ultrasound-induced microbubble destruction, inhibiting cell growth and gross growth of tumor as well as angiogenesis. We speculate that it may be the results of the combined effects of ultrasound-microbubbles cavitation, sonoporation to tumor tissue, and anti-tumor effect of drug. First, Nordy is entrapped in the microbubbles, which prevent them from the degradation of blood and keep the drug’s activity, and ultrasound-induced microbubbles destruction realizes the targeted release of Nordy at the tumor site. Second, sonoporation, caused by the interaction between microbubbles and cell membrane, is able to push and pull the membrane, inducing moderate and reversible changes at the cellular level, including opening cell walls, and loosening tight junctions. This increased permeability of membrane allows drug and drug mimic to enter the membrane [33–36]. Third, cavitation effects produced by ultrasound-microbubbles microbubbles destruction, including microjet, microstream, shock wave, etc., assist to drive the drug into tumor stroma and cells [37], and the upregulation of endocytosis stimulated by cavitation-related phenomena and subsequent increased uptake of the drug or drug-containing micelles into cells may also play an important role [38].

This research is still in infancy and there are some limitations in the experiment design and the results. First, the preparation technology should be further optimized, for the encapsulation efficiency and drug-loaded amount are relatively low, and it is the key to improve its anti-tumor efficacy and to be applied clinically eventually. Second, the treatment of microbubbles only was not included as a control in this study for we thought it had little effect referring to the relevant literature [39,40], and the efficacy of this technology has not been compared with those of the other drug delivery systems such as liposome and the other classical anti-tumor drugs, so its superiority has not been fully explored. Third, the mechanism of this drug delivery system in anti-tumor angiogenesis was mainly based on the speculation, short of support of experimental data, which will be the emphasis of our future research.

Conclusions

As a conclusion, ultrasound-induced Nordy-loaded microbubbles destruction can not only overcome the limitation of Nordy’s poor water-solubility through changing drug formulation but also realize its targeted release at tumor, improving local anti-tumor efficacy. This method provides a new way for the controlled release of Nordy, contributing to the potential clinical usage of this new anti-tumor drug.

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Disclosure statement

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