Eliminating Active Species by Endohedral Metallofullerenol *in vitro* and *in vivo*

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ABSTRACT

We previously demonstrated that gadolinium endohedral metallofullerenol $Gd@C_{82}(OH)_{22}$ nanoparticles had high inhibitory activity on growth of malignant tumor *in vivo* by uncertain mechanism(s). The activities of enzymes associated with the metabolism of reactive oxygen species (ROS) were decreased in the tumor-bearing mice by intraperitoneally injection of $Gd@C_{82}(OH)_{22}$ nanoparticles . In current study, systemic investigation of the potential function of $Gd@C_{82}(OH)_{22}$ nanoparticles found that it expressed direct scavenging activity toward active species. Electron spin resonance (ESR) spectroscopy, the state-of-art technique to measure chemical active species that have one or more unpaired electrons in parallel, was employed to measure free radicals scavenging activities of $Gd@C_{82}(OH)_{22}$ nanoparticles *in vitro*. Pre-treatment with $Gd@C_{82}(OH)_{22}$ nanoparticles significantly reduced ESR signal of various of reactive oxygen species. In addition, $Gd@C_{82}(OH)_{22}$ nanoparticles demonstrated extensively scavenging activities of active species measured *in vivo*, which was consistent with reduced progression of cancer cells treated with $Gd@C_{82}(OH)_{22}$ nanoparticles. In summary, results obtained in this study revealed strong active species-scavenging activities of $Gd@C_{82}(OH)_{22}$ nanoparticles *in vitro and in vivo.* Scavenging activities of hydroxylated $Gd@C_{82}(OH)_{22}$ nanoparticles was systemically measured in different milieu, and demonstrated the inhibition of tumor progression by treatment with $Gd@C_{82}(OH)_{22}$ nanoparticles likely due to eliminating active species.

Keywords: $Gd@C_{82}(OH)_{22}$, free radicals, scavenging activity

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1 INTRODUCTION

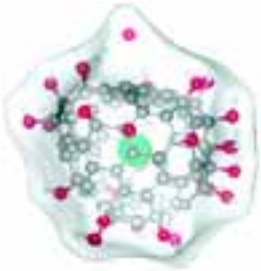

Figure 1. Diagram of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles based on theoretical calculation

$Gd@C_{82}(OH)_{22}$ is a functional fullerenol with metal gadolinium. The gadolinium atom is trapped inside fullerene cage and it was originally designed as magnetic resonance imaging (MRI) contrast agent for biomedical imaging (See Figure 1). In our previous publications, we have reported the chemical and physical properties of unique $Gd@C_{82}(OH)_{22}$ [1-3]. Recently, we also found that $Gd@C_{82}(OH)_{22}$ could inhibit tumor growth in tumor-bearing mice [4, 5]. Further studies indicated that its therapeutic effects are not due to toxicity to tumor cells, but a regulation process on oxidative-related enzymes [4].

Since free radicals are usually known as crucial factors of tumor development, more and more studies focus on the possible effects of antioxidants in cancer therapy. Various reagents, including C_{60} fullerene derivatives, have been found with antioxidative effects. Previous studies have demonstrated that hydroxylated fullerene derivatives and C_{60}-malonic acid are both effective in protecting nerve[6] and eliminating free radicals[7]. Dugan et al. showed that three-malonic acid modified C_{60} (*C3*) was a potent ROS-

scavenger, which had the activity to prevent the apoptosis of cultured cortical neurons [8].

To be hydroxylated fullerene derivatives, $Gd@C_{82}(OH)_{22}$ is similar to other hydroxylated C_{60}, hydroxyl groups asymmetrically distributed on the surface of $Gd@C_{82}$ could serve as electron reactive sites. The specific surface area of $Gd@C_{82}(OH_)_{22}$ presents it as optimized scavenger for free radicals. Although we have reported that $Gd@C_{82}(OH)_{22}$ are capable of modulating oxidative system in animal model [4], there is no thorough research on the molecular mechanism and it is far less known for the regulation details. Consequently, in this study we use ESR spin-trap technique to directly detect the scavenging activities of $Gd@C_{82}(OH)_{22}$ on different types of free radicals. We also determined the protection effects of $Gd@C_{82}(OH)_{22}$ on oxidative stress-induced cell damage by using human lung adenocarcinoma cells A549 and rat brain capillary endothelial cells (rBCECs). We found that $Gd@C_{82}(OH)_{22}$ could effectively reduce H_2O_2-induced free radical formation and mitochondrial damage, resulting in increasing the cell viability in cellular injury system.

2 MATERIALS AND METHODS

2.1 Materials

$Gd@C_{82}(OH)_{22}$ with high purity were synthesized as previously described protocol [9, 10]. The nanoparticles were characterized and the average size was about 22.4 nm in diameter, which was measured by high resolution atomic force microscopy and synchrotron radiation small angle scattering.

2.2 ESR Measurement

The ESR spectrum was measured using a Varian E-109 X-Band ESR Spectrometer (Varian Inc., Palo Alto, CA). To analyze the changes of free radicals captured by $Gd@C_{82}(OH)_{22}$, we used specific probes for measurement of various of free radicals in a cell-free system. The concentrations of $Gd@C_{82}(OH)_{22}$ used in these free radicals measure system are different to demonstrate the maximal radical scavenging efficacy. The intensity of the ESR spectra was recorded and all experiments are done at room temperature (27℃). Each experiment was repeated at least three times.

2.3 Cell Culture

Human lung adenocarcinoma A549 cells got from ATCC (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) at 37 ℃ in 5% CO_2. Primary brain capillary endothelial cells (rBCEC) was accomplished following a modified protocol of Deli et al.[11]. Four 85-90g Wistar rats were sacrificed by cervical dislocation, followed by forebrains collection. Then the meninges were removed and the tissue was minced and digested with a type Ⅱ collagenase. After centrifugation (1500 rpm, 5 min), the pellet was re-suspended in DMEM with FBS (20% w/v). Processes of suspension and centrifugation were repeated for three times, then the capillary pellet was collected and cell suspension were cultured in DMEM medium with 20% fetal bovine serum (FBS), supplemented with 10U/ml Heparin (Sigma-Aldrich Co, USA), 100U/ml penicillin-streptomycin solution and 150 μg /ml endothelial cell growth factor (ECGF). All experiments were done by using the third passage, and were repeated at least three times.

2.4 Protection on H_2O_2-induced Cell Damage

We used a cytotoxicity assay to determine the protection effects of $Gd@C_{82}(OH)_{22}$ on cultured Human lung adenocarcinoma A549 cells and Primary brain capillary endothelial cells (rBCEC). The assay was performed using water-soluble tetrazolium salt, a chemical measure kit named Cell Counting-8 Kit (CCK-8 assay kit, Dojindo Laboratories, Japan) according to the instructions of the manufacturer. $1x10^5$ cells were passaged in the 96-well plate before incubation. Then different concentrations of $Gd@C_{82}(OH)_{22}$ nanoparticles diluted in phosphate buffer solution (PBS) were added in cell medium. After 24 h incubation, the medium was replaced with fresh medium contained 50 μM H_2O_2. After treatment for 2h, the cells were washed with PBS for viability detection. One volume CCK-8 solution was added into 10 volume DMEM medium and each group was measured in triplicate. The samples were incubated at 37 ℃ for 1.5 h before collection the absorbance at 450nm. The H_2O_2 concentration that used on A549 and rBCECs cells was 10μM. 1.5μg/ml tea polyphenol was used as positive control.

2.5 Statistical Analysis

All data are expressed as mean ± S.D. Values for cell viability was shown as the percentage of the mitochondria dehydrogenase activity corresponding to that of control cells. The unpaired student *t*- test was applied to identify significant differences between the treated and non-treated control. For the ESR experiments, one-way analysis of variance (ANOVA) followed by a post hoc Fisher least significant difference test was applied. *P* less than 0.05 was considered to indicate a significant difference.

3 RESULTS AND DISCUSSION

We found the effective regulation of $Gd@C_{82}(OH)_{22}$ on superoxide dismutase (SOD) by using a liver tumor-bearing mice model[4]. The results demonstrated that there was obviously reduced SOD activities for mice treated with $Gd@C_{82}(OH)_{22}$ nanoparticles compared to that of saline

group (untreated group) (see Figure 2), which indicated that $Gd@C_{82}(OH)_{22}$ nanoparticles could effectively modulate the activities of enzymes-associated with oxidative stress.

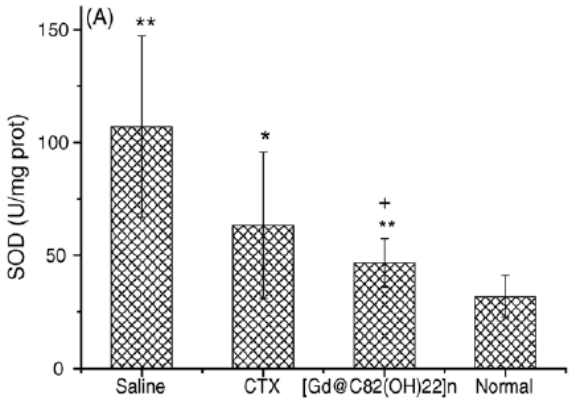

Figure 2. Activities of superoxide dismutase (SOD) in the liver of mice treated with $Gd@C_{82}(OH)_{22}$ nanoparticles [4].

The above *in vivo* results in Figure 1 demonstrated that there is a close relationship between $Gd@C_{82}(OH)_{22}$ nanoparticles and free radicals. ESR technique was employed in order to clarify the potential interaction of $Gd@C_{82}(OH)_{22}$ nanoparticles with several typical free radicals. Different free radical (DPPH, $O_2^{\bullet-}$, $HO^{\bullet}$, $^1O_2$) were generated and measured in a pure chemical reaction system with or without $Gd@C_{82}(OH)_{22}$ nanoparticles. The results demonstrated that free radicals were reduced in presence of $Gd@C_{82}(OH)_{22}$ (Table 1).

Free radicals	DPPH	$O_2^{\bullet-}$	$HO^{\bullet}$	$^1O_2$
Reduced rate (%)	**59.4**	**44.4**	**58.4**	**19.8**

Table 1: Significantly free radicals scavenging ability of $Gd@C_{82}(OH)_{22}$.

Active species are known to be associated with a wide range of acute and chronic human diseases, even including tumor initiation, therefore, it might be another effective way to inhibit tumor growth by reducing oxidative stress during tumor development. In this study, the employed ESR technique systematically provides direct evidence that $Gd@C_{82}(OH)_{22}$ nanoparticles can markedly scavenge different types of free radicals *in vitro and in vivo*. These results are consistent with the previously reported sparing effects of $Gd@C_{82}(OH)_{22}$ nanoparticles on oxidative damage in the livers of tumor-bearing mice [4]. The overall results in this study suggest that scavenging of reactive chemical species by $Gd@C_{82}(OH)_{22}$ nanoparticles could play a vital role in the inhibition of tumor growth.

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