Amino acid modified [70] fullerene derivatives with high radical scavenging activity as promising bodyguards for chemotherapy protection

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Despite the great efforts for tumor therapy in the last decades, currently chemotherapy induced toxicity remains a formidable problem for cancer patients, and it usually prohibits the cancer therapy from successful completion due to severe side effects. In general, the main side effects of chemotherapeutic agents are from the as-produced reactive oxygen species (ROS) that not only harm the tumor cells but also damage the patients’ organs. Here we report the application of amino acid derivatives of fullerene (AADF) in the chemotherapy which strongly scavenge the excess ROS to protect the tested mice against the chemotherapy-induced hepatotoxicity and cardiotoxicity. Two amino acids, i.e., L-lysine and β-alanine were separately employed to chemically modify C_{70} fullerene, and L-lysine derivative of fullerene (C_{70}-Lys) exhibits superior radical scavenging activity to β-alanine derivative of C_{70} (C_{70}-Ala). As expected, C_{70}-Lys show much better protective effect than C_{70}-Ala against the chemotherapy injuries in vivo, which is verified by various histopathological, haematological examinations and antioxidative enzyme studies. Moreover, the L-glutathione level is increased and the cytochrome P-450 2E1 expression is inhibited. They are potentially developed as promising bodyguards for chemotherapy protection.

Chemotherapy is currently the mainstream for clinical cancer treatments, but the severe side effects of chemotherapeutic drugs are extremely hard to reduce1–3. Doxorubicin abbreviated as DOX, an anthracycline antibiotic, is one of most effective chemotherapeutic agents for a wide variety of cancer treatments4–6. However, the clinical application of DOX is limited due to its acute and chronic adverse effects on liver, heart, kidneys and so on. It has been reported that the generation of excessive free radicals during DOX chemotherapy contributes to these side effects5–6, especially the serious hepatotoxicity9 and cardiotoxicity11. Certain free radicals know as reactive oxygen species (ROS) are considered to be an important factor for oxidative stress and damage, which leads to the imbalance of oxidant-antioxidant systems12,13. And a clinically strategy is to employ antioxidant agents simultaneously to alleviate the injury induced by oxidative stress14,15. Thus, it is of great significance to seek a chemical species with a high efficiency to scavenge free radicals, both for preserving our health and for utilization of cancer chemotherapy.

Fullerene, since its discovery in 1985, has been extensively studied in the biomedical field including its great potential in free radical scavenging16,17. The plenty of conjugated double bonds and the low lying lowest unoccupied molecular orbital (LUMO) of fullerenes give them the ability to efficiently capture electrons. Due to the insolubility of unmodified fullerenes in water, diverse chemical modifications have been applied to improve their...
water solubility and biocompatibility, such as the carboxyl-modified, hydroxylated (known as fullerenols), ethylenediamine-modified and amino acid modified fullerenes and metallofullerenes, all of which exhibit different degrees of antioxidant activities in vitro or in vivo. For example, we have recently reported another water-soluble gadofullerene nanoparticles, Gd@C82(EDA)2, based on ethylenediamine (EDA)-modified Gd@C82(EDA)2, which revealed a better cytoprotective effect on human epidermal keratinocytes-adult cells than Gd@C82(OH)24 nanoparticles. Chen et al. synthesized three kinds of water-soluble fullerenes (C60(COOH)2), C60(OH)2 and Gd@C82(OH)24 and demonstrated that both the surface chemistry and physical properties would influence their biological and biomedical activities. In addition, some other hydrophilic fullerene derivatives have also been studied for their protection against chemotherapy medicines induced free radical damage.

In this work, two water-soluble amino acid derivatives of fullerene have been prepared by a simple one-step reaction through the nucleophilicity of nitrogen atoms towards the carbon cage. In consideration of the possible mechanism of the reaction between amino acids and fullerenes, we speculate that the amino group plays a key role in the reaction. L-lysine and β-alanine were chosen for their simple and typical structures, which would not cause the steric hindrance with fullerene cage during the reaction. L-lysine is susceptible to react with fullerene compared to β-alanine owing to one extra amino group. We investigated the chemical and physical properties of the derivatives and discovered that the L-lysine derivative of fullerene (C70-Lys) exhibited better capability in scavenging ROS than the β-alanine derivative fullerene (C70-Ala). Furthermore, for the hepatoprotective and cardioprotective effects against DOX, as well as the cytoprotective effect on human umbilical vein endothelial cells (HUVECs), the C70-Lys were also more excellent than that of the C70-Ala, which attributed to the different modifications on the fullerene surface.

### Material and Methods

#### Chemicals

Solid C70 (99% purity) was obtained from Xiamen Funano Co. Ltd., China. L-lysine, β-alanine, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), doxorubicin and vitamin C (VC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H2O2) was obtained from Chemical Reagents Co., Beijing. The assay kits of ALT, AST, LDH, CK and α-HBDH were purchased from Toshiba Medical Systems Corporation. The assay kits of MDA, GSH, GSSG, GSH-Px, GR, CAT and SOD to detect the oxidative status in organs were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All reagents and solvents were obtained commercially and used without further purification.

#### Preparation of water-soluble amino acid derivatives of fullerene (AADF)

Highly purified L-lysine and β-alanine substitute derivatives of C70 were prepared by a method similar to Shu’s. Briefly, a solution was prepared by dissolving NaOH (0.412 g, 10 mmol) and β-alanine (0.868 g, 10 mmol) or L-lysine (1.462 g, 10 mmol) in deionized water (3 mL), and then ethanol (10 mL) was added. The resulting solution was added dropwise to 30 mL C70 (ca. 50 mg) toluene solution under stirring conditions, and the solution was stirred at 80 °C for ca. 24 h (for L-lysine) or 36 h (for β-alanine) to form a precipitate. The precipitate was collected by centrifugation and dialyzed against deionized Milli-Q water. Highly purified L-lysine and β-alanine derivatives of C70 were prepared by a method similar to Shu’s. Briefly, a solution was prepared by dissolving NaOH (0.412 g, 10 mmol) and β-alanine (0.868 g, 10 mmol) or L-lysine (1.462 g, 10 mmol) in deionized water (3 mL), and then ethanol (10 mL) was added. The resulting solution was added dropwise to 30 mL C70 (ca. 50 mg) toluene solution under stirring conditions, and the solution was stirred at 80 °C for 24 h (for L-lysine) or 36 h (for β-alanine) until the organic layer became completely colorless. After reaction, the aqueous layer was separated from the organic layer, concentrated, and dialyzed against deionized Milli-Q water (Millipore, Billerica, MA, USA) for 3 days before freeze drying.

#### Characterization of water-soluble AADF

Solid AADF were obtained after freeze drying, then characterized by elemental analysis (EA), thermogravimetric analysis (TGA) (Shimadzu DTG-60H, Japan), dynamic light scattering (DLS) and zeta-potential measurements. TGA was conducted under a nitrogen flow (50 mL/min) and the samples were heated from room temperature to 500 °C at a ramp rate of 5 °C/min. DLS and zeta-potential measurements were performed on a NanoZS ZEN3600, Malvern instrument at 25 °C. All of the samples were measured three times in Milli-Q water at pH 7.0 after filtering through a 0.22 μm pore size membrane.

#### Cryo-TEM characterization

Samples for cryo-TEM were prepared with a Leica EM GP immersion freezer (Leica Microsystems) as follows: 3 μL of each sample was loaded onto a glow discharged carbon-coated holey TEM grid (GIG) and blotted with a filter paper to obtain a thin liquid film on the grid. The grid was quickly plunged into liquid ethane (cooled by liquid nitrogen). The vitrified samples were then transferred to a cryogenic sample holder (626, Gatan) and examined with a TEM (JEM-2010, JEOL) at 94 K. Micrographs were captured with a CCD camera (Gantega, Olympus Soft Imaging Solutions).

#### Cell viability assessment

Cell viability was evaluated by the activity of mitochondrial dehydrogenase according to a protocol previously described. In brief, to detect the pre-protection of AADF against DOX-induced oxidative stress, the HUVECs were cultured with DOX for 1 h. Between DOX and AADF, the cells were washed with phosphate buffer saline (PBS) to remove the residue. After that, the medium was replaced with fresh medium and incubated in the dark at 37 °C for 24 h. A WST-8 assay with a Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan) was used to evaluate the cell viability. The absorption value at 450 nm was read using a 96-well plate reader (iMark microplate reader, Bio-RAD, USA). Each treatment was repeated three times. For the post-repair of AADF evaluation, the HUVECs were cultured with DOX for 1 h and followed by the incubation with AADF for 3 h. Other processing kept consistent with the pre-protection action. Experiments were performed at least three times.

#### Scavenging of hydroxyl radicals

Interception of hydroxyl radicals by AADF was determined by electron paramagnetic resonance (EPR) (JEOL, JEF FA200, Japan). Hydroxyl radicals were generated by H2O2 with the irradiation of ultraviolet light (UV), and DMPO, as a radical capturer, could immediately transfer them to DMPO-OH which would be detected by EPR. A solution containing 100 μL of DMPO (100 mM) and 50 μL of H2O2 (1 M) was mixed with 50 μL of ultrapure water, C70-Lys (100 μg/mL), and C70-Ala (100 μg/mL), separately.
The solutions were firstly irradiated with a 500 W UV lamp for 4 min, and then recorded the X band EPR spectrum in the dark. The scavenging effect was evaluated by comparison with the control group lacking AADF.

**Animals and treatments.** Six-week-old female BALB/c mice with body weights in range of 18.0 g to 20.0 g were purchased from Center for Experimental Animals, Institute of Process Engineering, Chinese Academy of Sciences (Beijing, China). All animal experiments complied with the protocols approved by the National Regulation of China for Care and Use of Laboratory Animals. Mice were randomly distributed into five groups \((n = 6)\) as follows: 1) control healthy group treated with saline only every day; 2) DOX + saline group; 3) DOX + VC group; 4) DOX + C70-Lys group; 5) DOX + C70-Ala group. The latter four groups were treated with intraperitoneal \((i.p.)\) injection of saline, VC \((10 \text{ mg/kg/day})\) and AADF \((10 \text{ mg/kg/day})\) once a day for eleven days, respectively. The choice for effective dosage was investigated by detecting a short-term change of haematological parameters and organ coefficients. And at the 4th day all the mice \(\text{(except control healthy group)}\) received a single intravenous injection \((i.v.)\) of DOX 30 minutes after the injection of saline, VC or AADF. DOX was injected at a dose of 20 mg/kg, which would induce cardiotoxicity and hepatotoxicity in mice.

**Coefficients of liver and heart.** The coefficients of liver and heart to body weight were measured by weighing the organs and calculating the ratio of organ tissues \((\text{wet weight, mg})\) to body weight \((\text{g})\).

**Hematology examination.** By quickly removing the eyeball on the eleventh day, the blood samples were divided into two parts, one for blood routine index examination and the other centrifuged for biochemical analysis. Some blood routine indexes were measured by a hematology analyzer \((\text{BC-5000VET, Shenzhen, China})\), including WBC, RBC, HGB, HCT, PLT, neutrophil and lymphocyte. The second portion was used to evaluate the toxicity towards liver and heart by measuring serum levels of ALT, AST, LDH, CK and \(\alpha\)-HBDH, using an automated biochemical analyzer \((\text{TBA-2000FR, Tochigi, Japan})\).

**Biomarker assay of liver and heart tissues.** Each liver and heart was quickly removed from the sacrificed mice and placed into liquid nitrogen for the analysis of enzymatic activity. They were minced and homogenized for the measurements of MDA and the assays of total protein concentration, LDH, GSH, GSSG, GSH-Px, GR, CAT and SOD. The levels of LDH, GSH and GSSG were measured by an automatic microplate reader \((\text{MULTISKAN MK3, Thermo, USA})\). And the spectrophotometry was used to determine the activities of GSH-Px, GR, CAT and SOD \((\text{UV-2000, Jinhua, China})\).

**Histopathological examination.** Immediately after surgical removal, the liver and heart tissues were harvested and fixed in 4% paraformaldehyde solution. Hematoxylin and eosin staining was conducted based on the standard manual. In brief, the tissues were immersed in paraffin blocks, sliced into 5\(\mu\)m sections, unfolded on the histological slides for staining. Histological observations and photomicrography of normal tissue slides were performed using an optical microscope and the sections of liver/heart were scanned by a scanner \((\text{Magscanner, KF-PRO-005, Japan})\).

**Western blotting.** Western blotting was performed to determine the protein expression of CYP2E1. A 200\(\mu\)g portion of each precleared, detergent-solubilized liver and heart tissues was used for total protein preparation. Bicinchoninic Acid \((\text{BCA})\) protein assay \((\text{Thermo Fisher Scientific, USA})\) was used to determine protein concentration. All operations were carried out in accordance with the manual specification.

**Statistical Analysis.** All data presented are means or means \(\pm\) standard deviations. According to a Student’s \(t\)-test the variance in different groups was statistically analyzed to evaluate its significance and \(P < 0.05\) were recognized as statistically significant \((^*\text{)}\) and \(P < 0.01\) were greatly statistically significant \((^{**\text{)}\})

**Results and Discussion**

**Preparation and Characterization of AADF.** Both of C70-Lys and C70-Ala were simply prepared by a facile one-step synthetic method with the addition of alkali and ethanol under heating condition \((\text{Scheme in Fig. 1a})\). With the higher number of amino group in L-lysine than that of \(\beta\)-alanine, L-lysine is susceptible to react with the carbon cage and needs less reaction time for the synthesis of C70-Lys. The obtained aqueous layers after reaction were concentrated and further purified by dialysis. It was well known that the conventional characterization methods for small molecules were not suitable for our fullerene derivatives. Thus a previously established method\(^{25,26}\), the elemental analysis along with a study for hydration water by TGA, was conducted to estimate their average structures \((\text{Table S1 and Fig. S1})\). The ratio of nitrogen to carbon \((\text{N/C})\) from the elemental analysis was used to determine their average structures \((\text{Table S1 and Fig. S1})\). The ratio of nitrogen to carbon \((\text{N/C})\) from the elemental analysis was used to determine their average structures \((\text{Table S1 and Fig. S1})\).

**Cryo-TEM characterization of AADF has indicated their sizes in aqueous solution. Both of them tend to aggregate into nanoparticles with a wide distribution \((20–70 \text{ nm})\), and the diameter of C70-Lys is larger than C70-Ala \((\text{Fig. 1b})\). The DLS investigation shows that the hydrodynamic diameters of AADF in aqueous solution are 155.2 nm \((\text{C70-Lys})\) and 139.8 nm \((\text{C70-Ala})\) \((\text{Fig. 1c})\), which accords with the result of Cryo-TEM. Both of them revealed a single peak, which was a stable form driven by the aggregation behavior and would be possible
for cellular uptake. They revealed high stability in physiological mediums without forming any aggregation even after centrifugation at 8000 rpm for 10 min (Fig. S2). It is worth mentioning that the zeta potential of C70-Lys (−37.0 mV) was more positive than that of C70-Ala (−45.4 mV), implying successful introduction of L-lysine onto C70 and exposing more amino groups outside (Fig. 1d). The distinctions in structure and zeta potential between these two samples would greatly influence their physicochemical properties, especially the radical scavenging ability and the behaviors at cellular level as discussed below.

**Hydroxyl radical scavenging capability.** Hydroxyl radicals (•OH), the most potent free radical produced from oxygen, are extremely powerful and active oxidants in the presence of biologic substrates, damaging their nuclear and mitochondria DNA, membrane lipids and carbohydrates. We chose hydroxyl radical as a representative to evaluate the radical scavenging capability of AADF by the EPR method. The effects of the two kinds of AADF on the EPR spectra of •OH are shown in (Fig. 1e,f, Fig. S3). The intensities of the DMPO-OH adduct were reduced by addition of C70-Lys or C70-Ala, indicating both of them possessing the radical scavenging capability. The radical scavenging properties of lysine and alanine were detected by the EPR method, which didn't show any capability in scavenging free radicals (Fig. S4). At the same concentration (100 μg/mL), C70-Lys scavenged about 87% of the generated hydroxyl radicals compared with reduction in signal intensity of 49% for C70-Ala. We attributed the different efficiency of scavenging abilities to the distinction of the fullerene cage integrity in accord with the structural properties of AADF. A better conjugation provided more reactive sites to attack the ROS, thus enhanced the efficiency of scavenging reactive species.

**Protective effects of AADF against DOX-induced oxidative stress using HUVECs.** Before the evaluation of the protective effects, we have investigated the cytotoxicity of the samples toward HUVECs by
incubation with AADF for 24 h (Fig. S5). Their negligible toxicity is an absolute and obvious prerequisite for their potential use in biomedicine. The protective effects of the two AADF against DOX-induced oxidization were investigated using HUVECs. For the study of pre-protection, the cells were treated with AADF for 3 h before the incubation with DOX for 1 h. While for the post-repair test, we exchanged the order of AADF and DOX treatments. As shown in Fig. 2, the concentration dependent protection and repair were observed and C70-Lys exhibited an apparent protective effect compared with C70-Ala. As the concentration increasing from 100 to 1000 μg/mL, the cell viability treated with C70-Lys displayed a steady rise, with respect to the mild increase of that treated with C70-Ala (pre-protection ratio: C70-Lys 21.5% versus C70-Ala 2.5%; post-repair ratio: C70-Lys 27.9% versus C70-Ala 10.4%). AADF also showed their superiority to C70 suspensions, which may be ascribed to the poor dispersity of C70 suspensions (Fig. S6). This result demonstrated a correlation between the cellular protective effects and their radical scavenging capability.

Protective effects of AADF against DOX-induced hepatotoxicity and cardiotoxicity in vivo.

The DOX-induced toxicities towards liver and heart have been widely reported, thus we choose DOX as a chemotherapeutic agent to investigate in vivo the potential hepatoprotective and cardioprotective effects of AADF during the chemotherapy. Firstly, we set two groups of mice treated with C70-Lys or C70-Ala for 7 days. We tested the haematological parameters (neutrophil and lymphocyte, the indicator of inflammation) at 3th and 7th day (Table S2), and the histopathological examinations of the main organs (heart, liver, spleen, lung and kidneys) were conducted by H&E staining (Fig. S7). Neither C70-Lys nor C70-Ala showed any toxicity towards the mice. For system study, thirty BALB/c mice were randomly distributed into five groups (n = 6): control healthy group, DOX + saline group, DOX + VC group, DOX + C70-Lys group and DOX + C70-Ala group. The control healthy group was treated with saline only and all the other four groups were received intravenously (i.v.) application of DOX (20 mg/kg of body weight) at the fourth day after the pre-treatments of saline, VC, C70-Lys or C70-Ala for 3 days respectively. Then they continued to be intraperitoneally (i.p.) injected with the drugs in the following six days (scheme Fig. 3a). The optimal dosage of AADF was investigated ahead by detecting a short-term change of haematological parameters (Table S3) and organ coefficients (Table. S4). We chose the concentration of 1.0 mg/mL for system studies in vivo. Allowing for high toxicity usually results in weight loss, the body weight of each mouse was checked every other day during the treatments. For the first three days before the injection of DOX, the body weights of all the mice exhibited a rapid growth, and among them the group treated with C70-Lys had the fastest growth rate. Besides, the DOX + C70-Lys and DOX + C70-Ala groups showed a greater resistance to the...
Toxicity caused by the DOX based on the decay rate of weight (Fig. 3b). By comparison, the group treated with VC, slightly resisted the toxicity induced the drop of body weight. The final body weights of the DOX-treated mice were more than 10% less with respect to the control healthy group. And significant different values of the coefficients for liver and heart were found between the AADF-treated groups and saline-treated group (Fig. 3c). For the AADF-treated groups, the coefficients were obviously lower compared with the DOX + saline group, implying the AADF had a certain improving effect on the hepatomegaly and enlarged hearts caused by DOX. This macroscopic variation has a good correlation with the symptom in DOX-induced liver and heart injury previously reported.

The histopathological examinations of the liver and heart were studied using hematoxylin&eosin (H&E) stained tissue slices. Compared with the control group, the DOX induced extensive and severe lesions, classified as hepatocellular ballooning degeneration, vacuolization of cytoplasm and single cell necrosis in the liver tissues (Fig. 4a). It also caused the interstitial myocardial edema, disordered arrangement of myocardial fibers with vesicular degeneration and loose cytoplasm of myocardial cell in the heart tissues (Fig. 4b). These histopathological changes qualitatively agreed with the macroscopic variation of the liver and heart coefficients, which implied the swelling of the organs treated with DOX. Although slight vacuolization of cytoplasm was observed in DOX + C70-Lys group and DOX + C70-Ala group, the degeneration of hepatocytes and myocytes induced by DOX were significantly suppressed. The protection of AADF from histologic observation reflected its advantage over the traditional antioxidant, VC. It’s worth mentioning that there were almost no hepatocellular ballooning degeneration and disordered arrangement of myocardial fibers in the mice treated with AADF.

We collected the blood samples to measure haematological parameters and some markers of cellular injury in serum. From the results of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), blood platelet (PLT), neutrophil and lymphocyte, a positive influence of AADF was found on the abnormality of haematological parameters after treatment with DOX (Table 1). And significant variation in almost all parameters was noted for the C70-Lys and saline treated DOX groups, especially the neutrophil and lymphocyte which represented the inflammation. For the healthy mice, about 80% of WBC were lymphocytes and 17% were neutrophils. It was obvious that the DOX treatment caused a decrease of lymphocyte and an increase of neutrophils. The neutrophil, a first responder to the xenobiotic, usually raised number as the acute inflammatory processes. The level of producing neutrophils of AADF-treated groups was lower than that of the DOX + saline group, which indicated the AADF had an improvement for the injury induced by DOX. The variation of RBC and WBC in groups treated with AADF also showed the positive effects on chemotherapy-induced myelosuppression. Compared comprehensively, DOX + C70-Lys group had a better chemoprotective effect than the DOX + C70-Ala group.

It is well known that creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) are usually used as serum enzymatic markers to detect hepatic and cardiac injury. ALT and AST are liver-specific enzymes and the ratio of AST to ALT is always used to evaluate the liver status, a high value of the ratio typically indicating the hepatocellular damage. According to our results, DOX treatment induced a highly significant increase in serum ALT

Figure 3. (a) Protective treatment schedule of AADF in DOX-induced hepatotoxicity and cardiotoxicity in mice. (b) Body weight curves after various treatments indicated. (c) The coefficients of liver and heart after sacrificing. *P < 0.05 and **P < 0.01 vs. DOX + saline group.
and AST levels in comparison with the control (Fig. 5a,b). Combined with the ratio of AST/ALT, it could be concluded that the DOX injection had induced marked hepatotoxicity. AST is also a heart-sensitive enzyme and its activity is approximately proportional to the extent of heart injury. In addition, the elevated level of CK, LDH or \( \alpha \)-HBDH is commonly the index of myocardial infarction, cardiovascular damage, anaemia or hepatitis\(^{35,36} \). With the high level of AST, the increase of CK and LDH activity in the serum of mice treated with DOX is expected (Fig. 5c,d), in accordance with the DOX-induced cardiac damage as previously reported\(^{37} \). Administration of AADF markedly lowered the serum ALT and AST levels, as well as the CK and LDH levels, which revealed that the AADF not only had the hepatoprotective but also cardioprotective effects. The \( \alpha \)-HBDH levels of the five groups were not statistically different when the mice were sacrificed (Fig. 5e). This phenomenon was also reported by Bjelogrlic\(^{38} \) and Injac\(^{36} \), which verified the occurrence of a high \( \alpha \)-HBDH level within 48h after an administration of chemotherapeutic agents. With recovery processing in our experimental procedure, the \( \alpha \)-HBDH activity had probably dropped to the relatively lower level after one week of DOX treatment. Comparing the AST/ALT and LDH/\( \alpha \)-HBDH ratios in Fig. 5f, we demonstrated the advantage of C\( \text{C}_{60}\)-Lys over C\( \text{C}_{60}\)-Ala in the protection of liver and heart, even better than the commercial liposomal formulations and earlier published fullerene derivative\(^{22} \) (Figs S8 and 9).

To further investigate oxidative status in the liver and heart, we tested various parameters of oxidative stress in the liver and heart tissues. Application of DOX significantly raised the activity of all the examined antioxidative enzymes in the liver with regard to the control group (Table 2). Pre- and post- AADF succeeded in preventing

![Figure 4.](image)

**Figure 4.** The histologic sections of the liver (a) and heart (b) of the mice in the control group, DOX + saline group, DOX + C\( \text{C}_{60}\)-Lys group, DOX + C\( \text{C}_{60}\)-Ala group, DOX + VC group. Scale bars are 100\( \mu \)m (in black) and 50\( \mu \)m (in red).

| Parameter | Groups          | Control | DOX + Saline | DOX + C\( \text{C}_{60}\)-Lys | DOX + C\( \text{C}_{60}\)-Ala | DOX + VC |
|-----------|----------------|---------|--------------|-----------------------------|-----------------------------|---------|
| WBC (\( \times 10^9/\)L) | 5.31 ± 1.27 | 4.33 ± 0.72 | 4.45 ± 0.66* | 4.36 ± 0.57 | 4.40 ± 0.99 |
| RBC (\( \times 10^{12}/\)L) | 7.31 ± 0.95 | 5.73 ± 0.39 | 7.15 ± 1.68* | 6.95 ± 0.92* | 5.82 ± 0.63 |
| HGB (g/L) | 121.25 ± 14.36 | 95.75 ± 5.74 | 119.50 ± 9.19* | 109.67 ± 18.77* | 97.67 ± 12.02 |
| HCT (%) | 39.63 ± 4.30 | 28.43 ± 2.34 | 37.55 ± 5.43* | 33.43 ± 7.09* | 28.05 ± 2.90 |
| PLT (\( \times 10^9/\)L) | 567.75 ± 87.17 | 741.67 ± 45.54 | 582.67 ± 57.74** | 595.75 ± 52.82** | 735.25 ± 78.79 |
| Neutrophil (%) | 17.60 ± 3.35 | 22.60 ± 3.52 | 15.70 ± 3.30** | 18.75 ± 1.44* | 24.27 ± 3.93 |
| Lymphocyte (%) | 81.82 ± 6.90 | 73.92 ± 3.67 | 81.63 ± 4.25** | 75.86 ± 4.01 | 72.75 ± 3.17 |

**Table 1.** Effects of AADF on haematological parameters with DOX-induced hepatotoxicity. *P < 0.05 and **P < 0.01 vs. DOX + saline group.
By meaningfully lowering the glutathione oxidized (GSSG), glutathione peroxidase (GSH-Px), glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD) levels in different degrees. The improvement of AADF in DOX-caused peroxidative alterations was also evident by the depletion of GSH and elevation of malondialdehyde (MDA) production. GSH exists in all kinds of organs, which can adjust the synthesis of protein and ribonucleotide and is closely related to the antioxidant capacity of the body. MDA is one of the well-known secondary products of lipid peroxidation, and it is widely used as an indicator of cell membrane injury. Their changes had the same trend with the antioxidative enzymes, which once again confirmed that AADF were potential hepatoprotectors against DOX-induced hepatotoxicity. Moreover, fullerene nanoparticles have been reported to own antineoplastic activities by some possible mechanisms, such as angiogenesis inhibition, cancer stem cell-specific inhibition or the regulation of the tumor microenvironment. AADF and DOX may have a synergistic effect to enhance the anti-tumor efficacy, without the impact on the anticancer properties of DOX.

To better understand the mechanism relevant with the protection of AADF, the CYP2E1 protein expression was measured by western blotting (WB) analysis. CYP2E1, a membrane protein expressed in high levels in the liver, plays an important role in several metabolic reactions, including the deactivation of endogenous or exogenous harmful substances accompanied by the depletion of GSH and elevation of malondialdehyde (MDA) production. GSH exists in all kinds of organs, which can adjust the synthesis of protein and ribonucleotide and is closely related to the antioxidant capacity of the body. MDA is one of the well-known secondary products of lipid peroxidation, and it is widely used as an indicator of cell membrane injury. Their changes had the same trend with the antioxidative enzymes, which once again confirmed that AADF were potential hepatoprotectors against DOX-induced hepatotoxicity. Moreover, fullerene nanoparticles have been reported to own antineoplastic activities by some possible mechanisms, such as angiogenesis inhibition, cancer stem cell-specific inhibition or the regulation of the tumor microenvironment. AADF and DOX may have a synergistic effect to enhance the anti-tumor efficacy, without the impact on the anticancer properties of DOX.

Table 2. Influence of AADF on the indexes of oxidative stress in the liver tissue of the mice.

| Groups | Control | DOX + Saline | DOX + C70-Lys | DOX + C70-Ala | DOX + VC |
|--------|---------|--------------|---------------|---------------|----------|
| GSH (μmol/g) | 22.03 ± 1.44 | 14.62 ± 3.94 | 19.61 ± 3.33" | 17.77 ± 2.93" | 16.60 ± 3.23 |
| GSSG (μmol/L) | 79.95 ± 11.96 | 111.92 ± 24.18 | 86.84 ± 12.62" | 82.61 ± 16.04" | 97.87 ± 9.35" |
| MDA (nmol/mg protein) | 0.94 ± 0.13 | 2.30 ± 0.29 | 1.37 ± 0.31" | 1.37 ± 0.23" | 1.45 ± 0.24" |
| GSH-Px (U/mg protein) | 353.66 ± 53.36 | 594.30 ± 56.84 | 334.56 ± 50.26" | 384.13 ± 32.27" | 428.31 ± 39.04" |
| GR (U/g protein) | 13.40 ± 3.70 | 22.81 ± 6.61 | 10.65 ± 2.28" | 13.99 ± 2.45" | 16.11 ± 3.62" |
| CAT (U/mg protein) | 8.34 ± 1.42 | 11.53 ± 1.71 | 6.71 ± 0.41" | 8.67 ± 1.69" | 9.15 ± 0.85 |
| SOD (U/mg protein) | 95.27 ± 13.98 | 153.89 ± 13.90 | 85.34 ± 5.44" | 97.25 ± 13.79" | 113.37 ± 11.18" |

Figure 5. (a–e) Serum ALT (a), AST (b), CK (c), LDH (d), α-HBDH (e) levels in the control and DOX treated mice. (f) The AST/ALT and LDH/α-HBDH ratios of the groups. *P < 0.05 and **P < 0.01 vs. DOX + saline group.
Conclusions

We have investigated the impact of different surface modifications on the physicochemical property of fullerene derivatives, as well as the radical scavenging capability. In vitro study of cytoprotection against the DOX-induced oxidative stress by using HUVECs demonstrated the superiority of C70-Lys compared with C70-Ala. We further explored the potential protective effects of AADF in mice with DOX-induced hepatotoxicity and cardiotoxicity, and the distinction between C70-Lys and C70-Ala in radical scavenging was more distinguishable in the in vivo models. These results clearly supported that the chemical functionalization of fullerene plays a significant role in their properties and specific surface modification is great benefit for the biomedical application of these materials.

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Figure 6. (a,b) GSH levels in liver and heart of the five groups. (c,d) CYP2E1 protein expression in the liver and heart. The blots were stripped and probed with the indicated antibody. GAPDH served as loading controls. *P < 0.05 and **P < 0.01 vs. DOX + saline group.
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**Acknowledgements**

We thank Dr Bo Guan and Yang Liu at the Center for Physiochemical Analysis and Measurement for the Cryo-TEM characterization. We acknowledge the financial support from the National Natural Science Foundation of China (Nos. 51472248, 51502301), National Major Scientific Instruments and Equipments Development Project (ZDYZ2015-2) and the Key Research Program of the Chinese Academy of Sciences (QYZDJ-SSW-SLH025).

**Author Contributions**

Y.Z., M.Z., C.S. and C.W. conceived and designed the research. Y.Z., M.G., T.Y., L.M., W.L. and J.Z. performed experiments. Y.Z., M.G., T.Y., M.Z. and C.W. analyzed the data and wrote the manuscript. All authors reviewed and commented on the manuscript.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-34967-7.

**Competing Interests:** The authors declare no competing interests.

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