Conjugation of Dexamethasone to C60 for the Design of an Anti-Inflammatory Nanomedicine with Reduced Cellular Apoptosis

Yi Zhang,‡§ Lu Wang,‡§ Yanhong Sun,*† Ying Zhu,‡ Zengtao Zhong,*‡ Jiye Shi,‡∥ Chunhai Fan,‡ and Qing Huang‡

‡Laboratory of Physical Biology, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China
§College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China
∥University of Chinese Academy of Sciences, Beijing 100049, China
¶UCB Pharma, 208 Bath Road, Slough SL1 3WE, U.K.

ABSTRACT: Dexamethasone (DEX) is a well-known anti-inflammatory drug, whose widespread clinical use is nevertheless restricted by its serious side effects. By conjugation of DEX with C60, we found that this nanomedicine retained the anti-inflammatory activity of DEX while reducing side effects in the animal model. In mouse thymocytes, the CCK-8 assay showed that the cytotoxicity of DEX–C60 was significantly lower than that of free DEX. Flow cytometric studies revealed that incubation with DEX–C60 induced much less apoptotic thymocytes. Interestingly, such reduced cytotoxicity and apoptosis were not observed when equal moles of free C60 and free DEX were coincubated with thymocytes, suggesting that the conjugation alters the signal pathway of DEX. Indeed, we found that the binding of DEX–C60 and a glucocorticoid receptor (GR) was partially blocked in the thymocytes, which resulted in down-regulation of several apoptosis-related genes. These findings help understand the mechanism of beneficial effects of this new nanomedicine, DEX–C60, and promote its clinical applications.

KEYWORDS: dexamethasone, C60 glucocorticoid receptor, apoptosis, thymocytes

1. INTRODUCTION

Dexamethasone (DEX) is a synthetic glucocorticoid (GC) that is widely used to treat inflammatory and autoimmune diseases, including the inflammatory responses to cardiopulmonary bypass and acute infection.1–5 Unfortunately, DEX is associated with potentially serious side effects, including gastrointestinal dysfunction, adrenal suppression, and hypersensitivity in the central nervous system.6–9

Until recently, the mechanisms underlying the anti-inflammatory activity and the side effect of DEX have remained unclear.10–15 Nevertheless, it was demonstrated that DEX could induce apoptosis of many cell types, including mouse thymocytes.14,15 DEX increases the intracellular production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (H2O2), which increase oxidative stress.16,17 ROS play important roles in apoptosis induction in physiologic and pathologic conditions.18,19 Therefore, it is reasonable to think that the side effects of DEX are closely related to the generation of ROS.20–22

Many studies have showed that the anti-inflammatory properties and the side effects of DEX are associated with the glucocorticoid receptor (GR).23–25 The GR is an intracellular receptor that is mainly located in the cytoplasm in association with heat shock proteins (HSPs) in its inactive form.26–28 After binding to DEX, the DEX–GR complex translocates to the nucleus and binds to a glucocorticoid response element (GRE) and modulates the expression of target genes, including interleukin-6 and nuclear factor-κB.29,30 It is generally believed that the binding of DEX to GR is involved in DEX-induced apoptosis.31,32

C60 is a carbon nanomaterial with many potential biological applications, including drug delivery, neuroprotection, and enzyme inhibition. One of the most important features of C60 is its ability to scavenge ROS, which makes it an excellent antioxidant in vitro. C60 was also reported to reduce H2O2-induced cytotoxicity, free radical formation, and mitochondrial damage.33–35 Gharbi et al. reported that aqueous C60 suspensions could protect the rodent liver against free-radical-induced damage.36 Similarly, Cai et al. reported C60 is a mitochondrial protective antioxidant with direct radical scavenging activity.37 As ROS-induced apoptosis could be partly responsible for the side effects of DEX, it is reasonable to suggest that C60 could reduce the incidence of side effects. In our former study, we reported on the successful synthesis and characterization of DEX–C60, a new C60 derivative. Our experiments confirmed that the anti-inflammatory properties of...
DEX were retained when conjugated with C60. Furthermore, behavioral tests showed DEX–C60 did not affect central nervous system activity in mice, implying a low risk of side effects. These earlier studies also highlighted the broad clinical applications of DEX–C60.

In this paper, we determined the cytotoxicity of DEX–C60 in mouse thymocytes to identify the mechanism underlying its lower risk of side effects. We found that the reduction in thymocyte apoptosis associated with DEX–C60 was not related to the scavenging of ROS by C60. Instead, in vivo and in vitro experiments showed reduced binding of DEX–C60 to GR, which reduced the activity of GRE and conferred lower cytotoxicity.

2. EXPERIMENTAL SECTION

2.1. Reagents and Instruments. C60 (99.9% purity), DEX, GR, and RU-486 were obtained from Sigma-Aldrich Co. Ltd. The Cell Counting Kit-8 (CCK-8) assay was purchased from Dojindo Co. Ltd. Anti-DEX and anti-GR antibodies were obtained from Abcam Co. Ltd. Fetal bovine serum, penicillin, and streptomycin are purchased from Invitrogen Corporation (Carlsbad, CA). APC-annexin V and 7-amino-actinomycin (7-AAD) were purchased from BD Biosciences Co. Ltd. (Shanghai, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Preparation and Characterization of the DEX–C60 Suspension. DEX–C60 was prepared as described by Liu et al. C60 and DEX were dispersed in chloroform (18 mL), followed by the addition of dicyclohexyl carbodiimide (22 mg, 107 μM) and 4-dimethylaminopyridine (negligible amount) with gasification under N2 for 5 min. The resulting mixture was stirred for 7 days in the dark at room temperature. Then DEX–C60 was purified by column chromatography with 8:2 CH3COOC2H5/CS2; the purified DEX–C60 was a brown solid. DEX–C60 was dissolved in water and suspended in polyvinylpyrrolidone (PVP) by mixing 115 μM of DEX–C60 with 95 μM of PVP in a quartz mortar. The mixture was milled until the color turned dark brown. Suspensions of C60 and DEX were also prepared. The distribution of particle diameter of C60 and DEX–C60 was analyzed by dynamic light scattering (DLS), and the ζ potential was used to analyze dispersion stability.

2.3. Preparation of RU-486 and DEX. RU-486 (8.27 mg) was added to 551 μL of dimethyl sulfoxide (DMSO), and 28.64 μL of the RU-486–DMSO suspension was mixed with 971 μL of RPMI-1640 medium. The final concentration of RU-486 was 500 μM. DEX (56.29 mg) was dissolved in 56.29 mL of alcohol (100%), and the DEX–alcohol suspension was diluted with RPMI-1640 medium. The final concentration of DEX was 1 μM.

2.4. Thymocyte Preparation. All animal experiments were performed in accordance with guidelines from the local ethics committee. Female C57BL/6 mice (18–22 g) were obtained from Shanghai Experimental Animal Center. The animal room was maintained at 22 °C with lights on from 06:00 to 18:00. The mice were fed with a standard diet and were provided water. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee.

The thymus from C57BL/6 mice was milled until the color turned dark brown. Suspensions of C60 and DEX were also prepared. The distribution of particle diameter of C60 and DEX–C60 was analyzed by dynamic light scattering (DLS), and the ζ potential was used to analyze dispersion stability.

2.5. RT-PCR. The cells were washed three times with PBS and were collected. Each sample was lysed in 1 mL of Trizol Reagent and 0.2 mL of chloroform. The cell suspensions were shaken vigorously for 15 s and incubated for 3 min at 25 °C. The suspensions were centrifuged at 12000 rpm for 15 min. The aqueous supernatant was transferred to a new tube, and 0.5 mL of isopropyl alcohol was added. The tube was left at room temperature for 10 min to precipitate RNA. The resulting RNA was washed with 1 mL of 75% ethanol and dissolved in Na2SO4-free water. The RNA concentration was measured at 260 nm.

2.6. Enzyme-Linked Immunosorbent Assays (ELISAs). DEX–GR binding was measured with an ELISA using a monoclonal rabbit antimouse DEX antibody. First, 96-well plates were coated by incubation with 2 μg/mL of GR solution for 48 h. Then, the antibody was blocked with 1% bovine serum albumin/phosphate solution. The test samples were added to duplicate wells and incubated overnight after washing. DEX, DEX–C60, and C60 were added to independent wells and incubated with GR overnight. After washing, the antibody (diluted 1:500) was added to each plate for 6 h at room temperature. The complex was detected using horseradish peroxidase-conjugated goat antirabbit IgG with absorbance measured at 405 nm.

2.7. RT-PCR. The cells were washed three times with PBS and were collected. Each sample was lysed in 1 mL of Trizol Reagent and 0.2 mL of chloroform. The cell suspensions were shaken vigorously for 15 s and incubated for 3 min at 25 °C. The suspensions were centrifuged at 12000 rpm for 15 min. The aqueous supernatant was transferred to a new tube, and 0.5 mL of isopropyl alcohol was added. The tube was left at room temperature for 10 min to precipitate RNA. The resulting RNA was washed with 1 mL of 75% ethanol and dissolved in Na2SO4-free water. The RNA concentration was measured at 260 nm. Next, the RNA samples were heated to 70 °C for 5 min followed by 37 °C for 1 h in 25 μL of reaction mixture containing 2 μg of RNA, 0.5 μg of oligo-dT, 0.5 μL of each dNTP, 200 U of Moloney murine leukemia virus reverse transcriptase, and 5 μL of 5X reaction buffer. All samples were stored at −70 °C. The following primers were used: GADPH, 5'-GGGAGCCAAAAGGTACATCCTC-3' and 5'-CCATGCCAGTAGCTTCCGTTT-3'; TDAG8, 5'-AATGGATGTGATCCGGGAG-3' and 5'-GAGATTTAGACTAAGGAGGAGG-3'; GR, 5'-AATGGCCCAAAGCCGATAC-3' and 5'-TTGGCTCTTACAGACCTTC-3'; Tnpx, 5'-TGGAACGATGTCGACGACT-3' and 5'-GGGAAGGACAAGGCGAAG-3'; 2.8. Confocal Microscopy. For confocal microscopy, thymocytes were grown on glass coverslips in 24-well plates in 1 mL of RPMI-1640 medium containing 10% FBS. After incubating for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO2, the medium was replaced with 1 mL of fresh medium containing DEX and DEX–C60, respectively, and cultured for 2 h. The medium was then removed and washed twice with warm PBS and fixed in 4% paraformaldehyde with 4% sucrose for 30 min. Then, 3% bovine serum albumin and 0.2% Triton X-100 in PBS were added for 20 min. After washing twice with PBS, the cells were incubated with rabbit anti-GR antibody (1:1000) for 6 h, washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG for 1 h to stain nuclei. Cells were excited with a laser at 405 and 488 nm excitation.

2.9. Statistical Analysis. All of the variables were compared using one-way analysis of variance using GraphPad Prism version 5.0 for Windows. Differences are considered statistically significant for p < 0.05(*).
In culture experiments, we determined the distribution of particle diameters and ζ-potential of C60 and DEX–C60 in RPMI-1640. DLS was used to measure the mean distribution of particle diameters of DEX–C60, and >80% of DEX–C60 nanoparticles were 155–300 nm in diameter. The major distribution of particle diameters of C60 was 167.9 nm, and that of DEX–C60 was 213.4 nm (Figure 1a,b). The ζ-potential of C60 was −2.5 mV, and the ζ-potential of DEX–C60 was −7.9 mV (Figure 1c,d). These results indicated that the DEX–C60 complex was synthesized successfully. After the DEX–C60 nanoparticles were incubated with RPMI-1640 for 1 week, no megascopic aggregation of DEX–C60 was observed in a previous study. Although DEX–C60 was stable in RPMI-1640 medium, DEX–C60 appeared to aggregate after storage for 30 days at 4 °C. The color of the DEX–C60 suspension turned brown, and peaks corresponding to DEX–C60 (596.9 nm in diameter) were detected by DLS. In our experiments, DEX–C60 was used to incubate with cells as soon as it was prepared, and the dispersion of DEX–C60 was perfect.

3.2. Cytotoxic and Apoptotic Effects by DEX–C60

Although previous studies showed that DEX–C60 was associated with fewer side effects than DEX in an animal model, the underlying mechanism remained unknown. Here, we used mouse thymocytes as a cell model to compare the cytotoxic and apoptotic effects of DEX–C60 and DEX. DEX–C60 and DEX were separately dissolved in RPMI-1640 medium, and the concentrations of them were at gradient. Thymocytes were exposed to DEX–C60 or DEX at concentrations of 2, 1.0, 0.5, 0.1, and 0.01 μM (Figure 2a). Results of the CCK-8 assay indicated that DEX–C60 and DEX had dose-dependent cytotoxic effects on thymocytes. However, the cytotoxicity of DEX was greater than that of DEX–C60 at the same concentration. For example, following exposure to 0.01 or 0.1 μM DEX, 40% and 63% of cells died. By contrast, only about 50% of cells died when thymocytes were incubated with 1 μM DEX–C60. Even when the DEX–C60 concentration was increased to 2 μM, more than 40% cells were still alive. These results clearly indicated that the cytotoxicity of DEX–C60 was much weaker than that of DEX.

In previous studies, including the study by Liu et al.,38 C60 was used as an effective ROS scavenger. If C60 in DEX–C60 also protects against free radicals induced by DEX, coincubation of thymocytes with C60 may attenuate the reduction in viability caused by DEX. To test this hypothesis, thymocytes were pretreated with 50, 10, or 2 μM C60 followed by incubation with 100 nM DEX for 6 h. Cell viability was then determined using the CCK-8 kit. Interestingly, there were no significant differences of thymocyte viability between C60-pretreated cells at any concentration as compared with cells without C60 pretreatment as >60% of the thymocytes in each group had died after 6 h of culture (Figure 2b). These results indicated that C60 itself will not reduce DEX-induced cytotoxicity and that a reduction of ROS did not explain the reduced cytotoxicity of DEX–C60.

Wyllie et al. reported that DEX induces cellular apoptosis, which might be involved in its side effects.39 Therefore, we determined the effects of C60, DEX, and DEX–C60 on thymocyte apoptosis by flow cytometry (Figure 3). In a control group, the apoptotic rate of normal thymocytes was 5 ± 1.2%.

![Figure 1. Characteristics of the C60 and DEX–C60 suspensions in different phases. (a) Distribution of particle diameters of C60. (b) Distribution of particle diameters of DEX–C60. (c) ζ-potential of C60. (d) ζ-potential of DEX–C60.](image1)

![Figure 2. Viability of thymocytes treated with DEX or DEX–C60. (a) Viability of thymocytes treated with DEX–C60 or DEX at the indicated concentrations. (b) Effects of C60 pretreatment on thymocyte viability.](image2)
The apoptotic rate of thymocytes incubated with 100 nM DEX for 6 h was about 80%. DEX−C60 was associated with a low apoptotic rate of about 16%, consistent with the lower cytotoxicity of DEX−C60 compared with DEX. Interestingly, in cells pretreated with 50 μM C60 over 50% of cells were apoptotic, indicating that C60 itself does not reduce the cytotoxicity of DEX. The results of flow cytometry were very consistent with those of viability tests. Accordingly, it seems likely that the low cytotoxicity of the DEX−C60 is not due to the free radical scavenging activity of C60.

### 3.3. Effects of DEX−C60 on DEX−GR Binding

It was previously reported that DEX-induced apoptosis was related to the activity of the GR, a cytoplasmic receptor. After binding with DEX, the DEX−GR complex translocates from the cytoplasm to the nucleus, where it activate apoptosis signaling pathways. As DEX−C60 was associated with reduced thymocyte apoptosis compared with DEX alone, we hypothesized that the complex may interfere with binding and translocation of the DEX−GR complex.

RU-486 is a potent GC antagonist that is used to block the GR in vitro and in vivo. In our experiments, pretreatment of thymocytes with 50 μM RU-486 for 1 h prevented the loss of thymocyte viability caused by DEX, which was consistent with the results of earlier studies. However, pretreatment with RU-486 did not significantly affect the viability of cells treated with DEX−C60 (Figure 4a). These results suggest that DEX−C60 may improve viability through a mechanism similar to RU-486, by interfering with DEX−C60 binding to the GR.
To examine the binding between the GR and DEX–C$_{60}$, we performed ELISAs. Briefly, GR was coated onto the surface of 96-well plates, which was followed by the addition of DEX and DEX–C$_{60}$. After incubation overnight and washing, we added a DEX antibody to label DEX or DEX–C$_{60}$ that was still bound to the GR. The results of this assay showed that there was no DEX–C$_{60}$ bound to GR on the plate, whereas DEX was easily detected (Figure 4b). These data confirmed that DEX–C$_{60}$ lost the ability to bind to GR. By contrast, DEX was detected in wells that were coincubated with DEX and C$_{60}$.

After DEX is taken up by cells, it binds to cytoplasmic GR, which is translocated to the nucleus, where it activates the transcription of downstream target genes.$^{42,43}$ Therefore, we performed immunostaining to determine the expression and localization of GR in cells treated with DEX–C$_{60}$ or DEX. To visualize the GR, we stained cells with rabbit anti-GR antibody and FITC-labeled goat antirabbit IgG. As illustrated in Figure 5, the green fluorescent signal corresponding to FITC-labeled GR was detected in the cytoplasm and nucleus of cells treated with DEX–C$_{60}$ or DEX. Treatment with DEX–C$_{60}$ was associated with a much weaker signal for GR in the cytoplasm, indicating reduced GR expression. Furthermore, the signal for GR was negligible in the nucleus, which indicates the absence of GR translocation.

These results provide in vitro evidence that DEX–C$_{60}$ hardly bound to the GR. Meanwhile, the expression and translocation of GR in thymocytes coincubated with C$_{60}$ and DEX was not significantly different from that in cells incubated with DEX alone. These results provide further confirmation that C$_{60}$ itself does not reduce the cytotoxicity of DEX because it did not inhibit DEX–GR binding or receptor translocation.

3.4. Effects of the DEX–C$_{60}$ Complex on DEX-Induced Expression of Apoptosis-Related Genes. The proapoptotic effects of DEX involve GR-mediated activation of downstream genes, including genes involved in the mitochondrial apoptotic pathway. The DEX–GR complex binds to the GREs on its target genes and modulates their transcription.$^{44-46}$ Studies using mice expressing a dimerization-deficient GR mutant indicate that GC-induced thymocyte apoptosis requires the gene transactivation function of this receptor.$^{47}$ T cell death-associated gene 8 (TDAG8) is a G-protein-coupled receptor that is transcriptionally upregulated by DEX and overexpressed in DEX-induced apoptosis. TDAG8 was first identified by differential mRNA display during thymocyte apoptosis induced by T-cell receptor (TCR) engagement.$^{48}$ Recent studies have shown that TDAG8 expression was positively correlated with thymocyte apoptosis. Thioredoxin-interacting protein (TXNIP) is a regulator of metabolism and an inhibitor of the antioxidant thioredoxin and could mediate DEX-induced apoptosis.$^{49}$ The expression levels of these genes were also associated with caspase-3 activity and DEX-induced cell apoptosis.

In this study, we determined the gene expression levels of GR, TDAG8, and TXNIP by RT-PCR. As shown in Figure 6, the mRNA expression levels of TDAG8 and TXNIP in DEX-treated cells were 6-fold and 2-fold higher, respectively, than those in untreated cells. GR gene expression was also increased slightly in DEX-treated cells. Conversely, in cells treated with DEX–C$_{60}$, the mRNA expression levels of these genes were significantly lower than those in DEX-treated cells. These results indicate that DEX–C$_{60}$ inhibits DEX-induced expression of apoptosis-related genes, probably because the binding of this component to GR is reduced, thus preventing GR translocation and GR-mediated expression of TDAG8 and TXNIP. Consequently, this treatment attenuated the side effects of DEX, providing a novel method to reduce the side effects of DEX therapy.

The data presented in this paper provide compelling evidence that the C$_{60}$ component of the DEX–C$_{60}$ complex has a steric effect by blocking binding of DEX to GR. We found that DEX–C$_{60}$ is readily taken by thymocytes but fails to bind...
to GR, which prevents GR translocation to the nucleus. Consequently, GR is unable to activate the transcription of apoptosis-related genes. This pathway is illustrated in Figure 7.

4. CONCLUSIONS

In conclusion, we found that GR in thymocytes did not successfully bind to the DEX component of the DEX–C60 complex, preventing its translocation into the nucleus. Thus, treatment with the DEX component blocked interactions between the DEX component and the GR. Consequently, GR is unable to activate the transcription of apoptosis-related genes. This pathway is illustrated in Figure 7.

REFERENCES


5296  dx.doi.org/10.1021/am401153k | ACS Appl. Mater. Interfaces 2013, 5, 5291–5297