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RGD-peptides modifying dexamethasone: To enhance the efficacy of anti-inflammation and limit the risk of osteoporosis

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Dexamethasone (Dex) is one of the most effective anti-inflammatory glucocorticoids, while the side effect, osteoporosis seriously limits its clinical use. Cell adhesion is involved in the onset of inflammation and osteoporosis, and RGD-peptides are well known as anti-adhesion peptides. To enhance the anti-inflammatory efficacy and limit the osteoporotic risk of Dex three novel conjugates of RGDV, RGDS and RGDF covalently modified Dex were presented here. On xylene-induced ear edema model the ear edema of the mice treated with the conjugates was significantly lower than that of the mice treated with Dex. Receiving 15-day therapy the total volumetric bone mineral density, the peripheral quantitative CT and the femur weight of the mice treated with the conjugates were significantly higher than those of the mice treated with Dex. Therefore covalently modifying Dex with RGDV, RGDS and RGDF not only increased the anti-inflammation activity but also decreased the osteoporotic risk of Dex. Besides, the enhanced anti-inflammation activity was collated with the down-regulated DNA expression of the conjugates.

Introduction

Dexamethasone (Dex) represents the most effective anti-inflammatory glucocorticoids in treating several chronic and acute inflammatory conditions,[1-3] peritoneal adhesion, cardio-pulmonary bypass and acute infection,[4-6] and rheumatoid arthritis.[7-11] However, the clinical efficacy of Dex is limited by a series of side effects.[12] Of the side effects osteoporosis is capable of weakening the trabecular bone and increasing the fracture risk of the spine, hip and rib.[13-15] To eliminate the osteoporotic risk Dex was converted to various preparations, such as the encapsulations and liposomes,[16] the hydrophilic gold nanoparticles,[17] the copolymer and N-(2-hydroxypropyl)-methacrylamide,[18] and was conjugated with β-cyclodextrins,[19] and polyethylene glycol.[20] Nevertheless the risk of osteoporosis remains to be the calamity of Dex therapy.

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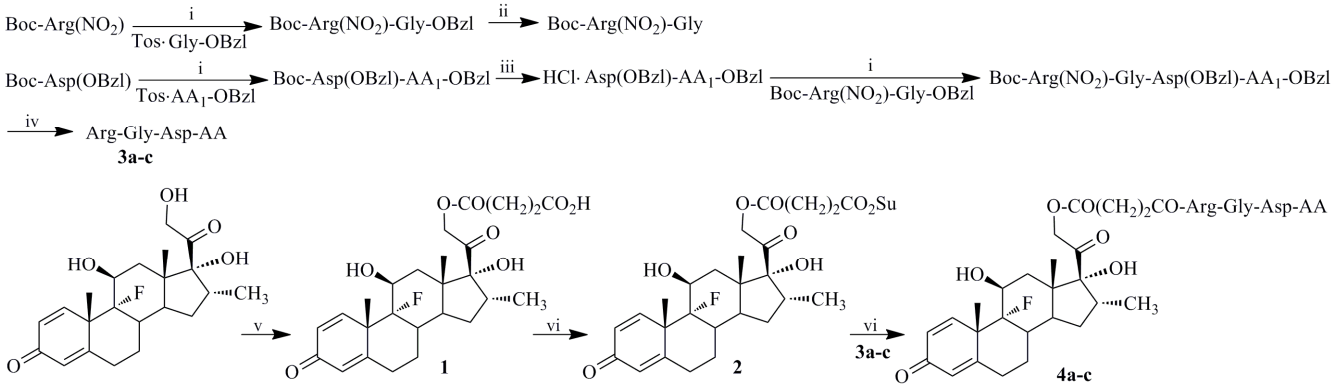
RGD-tetrapeptides, the well known anti-adhesion molecules, are the motif of integrins recognizing collagen, fibronectin, vitronectin, laminin, immunoglobulin superfamily and the plasma proteins. The motif like function of RGD-tetrapeptides to integrins has been widely used in drug design. The use of the anti-adhesion property of RGD-tetrapeptides to integrins resulted in the design of biomaterials such as the amphiphilic block copolymer,[21] hydroxyapatite biomaterials,[22] collagen tubes,[23] conjugates that promote cell adhesion and cell spreading,[24] mussel adhesive proteins,[25] as well as resulted in the anti-thrombotic agent design.[26-29]

Cell adhesion is involved in the onset of both inflammation and osteoporosis. In order to enhance the anti-inflammatory activity and limit the osteoporotic risk, the present paper covalently modified Dex with the anti-adhesion peptides RGDV, RGDS and RGDF to form three novel conjugates, to characterize their nano-structures, to evaluate their anti-inflammation activities, to estimate their osteoporotic risk and to explore the possible mechanism.

Results and discussion

Synthesis of the conjugates. By using 9-step procedure depicted in Scheme 1 RGD-peptide modified Dex, **4a** (RGDV-Dex), **4b** (RGDS-Dex) and **4c** (RGDF-Dex) were prepared in acceptable yields. The modified hydroxyl of Dex was identified

by ROESY 2D NMR spectra of **4a-c**, the cross-peaks were marked with blue rings and are shown in Figures S13-S15, which consistently gives a cross peak from -CO-CH2-O- (4.20 ppm) and -O-CO-CH_2-CH2-CO- (2.16 ppm). Therefore among the 11-hydroxy, 17-hydroxy and 17-(2-hydroxyacetyl) of Dex only the latter was modified by RGD-peptides. The details and the characteristic data are given as the Supporting Information.



Scheme 1 Synthetic route of **4a** (RGDV-Dex), **4b** (RGDS-Dex) and **4c** (RGDF-Dex). i) DCC (dicyclohexylcarbodiimide), HOBt, N-methylmorpholine; ii) CH_3OH, aqueous NaOH (2 N); iii) Hydrogen chloride in ethyl acetate (4 N); iv) CF_3CO_2H, trifluomethane sulfonic acid; v) Butanedionic anhydride, DMAP (dimethylaminopyridine); vi) N-hydroxylsuccimmide, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC); vii) DMF, H_2O. Wherein AA_1=Val, Ser(Bzl), Phe and AA=Val, Ser, Phe.

FT-Ms spectra evidence in water the conjugates forming trimer. The mass spectra of RGDV-Dex, RGDF-Dex and RGDS-Dex were tested on SolariX FT- ICR mass spectrometer (Bruker Daltonics) with ESI ion source and a superconductive magnet of 9.4 T. **Figure 1** shows that the FT-MS spectrum of the solution of RGDV-Dex or RGDS-Dex or RGDF-Dex in ultrapure water gives the ion peak of the trimer. **Figure 1** also shows that the spectrum gives the ion peaks of the dimer and the monomer, the qCID spectra demonstrate that both the dimer and the monomer are the fragmentation products of the trimer. Therefore the trimer is the existing form of RGDV-Dex, RGDF-Dex and RGDS-Dex in ultrapure water.

ROESY 2D NMR clarifies the trimerization manner. To reveal the manner of the trimer formation the ROESY 2D NMR spectra of RGDV-Dex, RGDF-Dex and RGDS-Dex were measured on 800 MHz in deuterated DMSO. **Figure2a** lists the Noesy 2D NMR spectra of 3 conjugates, and each givs one interesting cross-peak only, which are labeled with blue circles, and mirrors the interaction between the H of CO-CH_2-CH_2-CO of one molecule with the C-terminal carboxyl H of RGD-tetrapeptide moiety of another molecule. This means that the interaction of CO-CH_2-CH_2-CO and the C-terminal amino acid of the peptide moiety 3 molecules of RGDV-Dex or RGDF-Dex or RGDS-Dex approach each other and form the trimers. The related NMR spectra are also given as the Supporting Information. The 3D-features of the trimers were built with the energy optimized 3 monomers to approach each other in the manner defined by Noesy 2D NMR spectra. **Figure 2b** sows that the trimers look like the umbrella.

TEM image reveal the conjugates forming nanoparticles. The TEM images of 1 nM aqueous conjugates were measured. **Figure 3** shows that in ultrapure water RGDV-Dex, RGDF-Dex and RGDS-Dex form the nanoparticles of 18.2-98.2 nm, 54.4-145.7 nm and 20.2-71.4 nm in diameter, respectively. The diameter less than 150 nm and in the most case less than 100 nm means that the nano-particles can not be phagocytized by macrophage and should be safely delivered in blood circulation.

Relationship between trimers and nanoparticles. Mesocite module of the Materials Studio software was used to show the course of the trimers forming nanoparticles and to predict the number of the trimers involved in a definite nanoparticle, for which RGDV-Dex, RGDF-Dex and RGDS-Dex were built and optimized simply in the Visualizer window. The "beads" were constructed from atomistic simulations and placed at the center-of-mass of groups of atoms corresponding to particular parts of the molecules of RGDV-Dex, RGDF-Dex and RGDS-Dex. **Figures 3a'-3c'** shows that a nanoparticle of 5.67 nm in diameter of RGDV-Dex contains 109 trimers, a nanoparticle of 5.72 nm in diameter of RGDF-Dex contains 111 trimers, and a nanoparticle of 5.78 nm in diameter of RGDS-Dex contains 104 trimers. Unexpectedly, 3 simulated nanoparticles occur as the smallest nanoparticles in **Figures 3a-3c**. Therefore mesocite simulation can help us to understand the relationship of the trimer and the nanoparticle of RGDV-Dex, RGDF-Dex and RGDS-Dex.

RGD-tetrapeptide covalent modification enhances the anti-inflammatory activity. To examine the effect of RGD-tetrapeptide covalent modification on the clinical use of Dex in treating inflammatory diseases the anti-inflammatory activities of Dex and the conjugates were evaluated with xylene-induced ear edema assay. In brief, the mice were orally administered with 0.5% CMC-Na (blank control), or the suspension of 25.5 μmol/kg of Dex in 0.5 % CMC-Na, or the suspension of 25.5 μmol/kg of RGDV-Dex in 0.5 % CMC-Na, or the suspension of 25.5 μmol/kg of RGDF-Dex in 0.5 % CMC-Na, or the suspensions of 0.25, 2.5 and 25.5 μmol/kg of RGDS-Dex in 0.5 % CMC-Na, the ear edema of the mice were measured and are shown in **Figure 4B.** The data indicate that 25.5 μmol/kg Dex effectively exhibits xylene-induced ear edema, but the efficacy is significantly lower than that of 25.5 μmol/kg RGDV-Dex, RGDS-Dex and RGDF-Dex, and equals that of 2.55 μmol/kg RGDS-Dex. Therefore the anti-inflammatory activity of Dex is increased by 10 folds due to the covalent modification. **Figure 4B** also shows the anti-inflammation activities of 25.5, 2.55 and 0.255μmol/kg RGDS-Dex. As the representative of RGD-tetrapeptide covalent modification RGDS-Dex inhibits xylene-induced ear edema in a dose-dependent manner and the orally effective dose is 2.55μmol/kg.

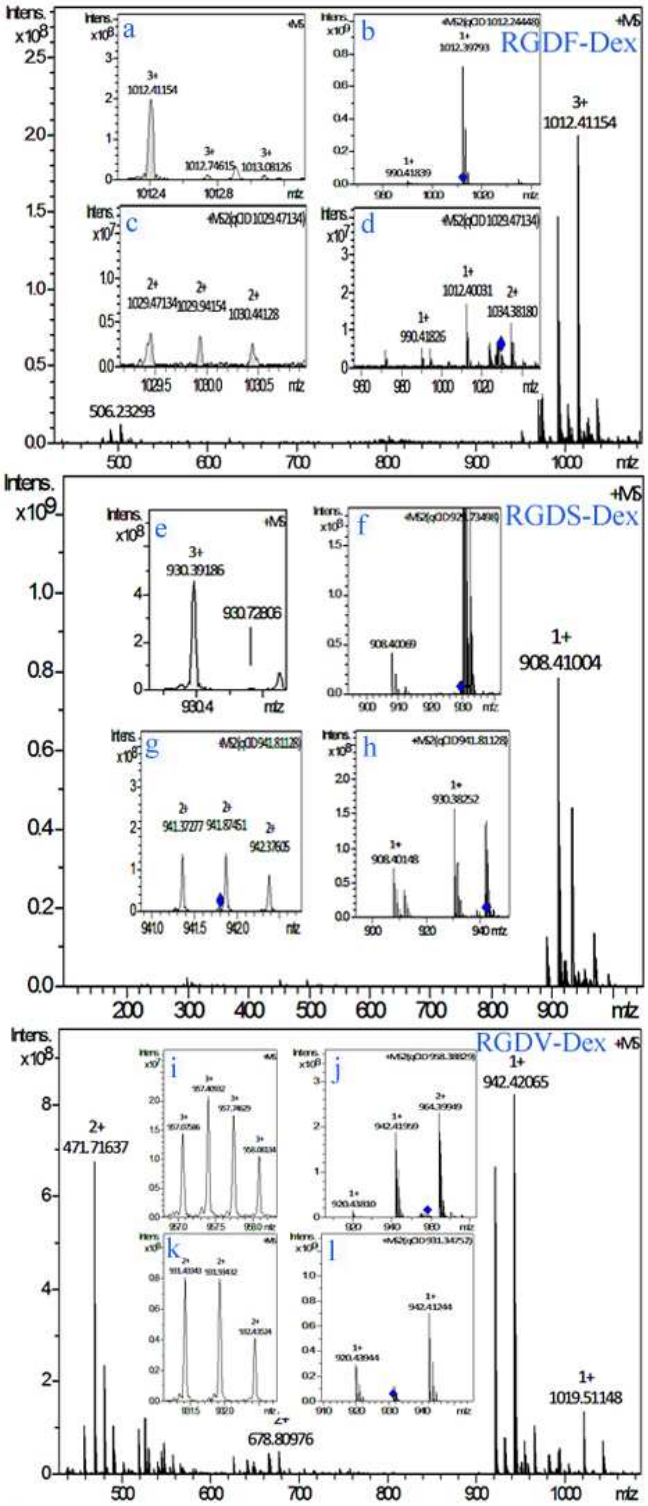


Figure 1 FT-MS spectra of the solution of RGDV-Dex, RGDF-Dex and RGDS-Dex in ultrapure water: Spectrum of RGDF-Dex gives an ion peak of the trimer plus 2Na and H at $[1012.41154]^{+3}$, an ion peak of the dimer plus H at $[1029.47134]^{+2}$ and an ion peak of the monomer plus H at $[990.41826]^{+1}$; Spectrum of RGDS-Dex gives an ion peak of the trimer plus 3Na at $[930.39186]^{+3}$, an ion peak of the dimer plus Na and K at $[941.37277]^{+2}$ and an ion peak of the monomer plus H at $[908.41004]^{+1}$; Spectrum of RGDV-Dex gives an ion peak of the trimer plus 3K at $[957.07586]^{+3}$, an ion peak of the dimer plus Na and H at $[931.43343]^{+2}$ and an ion peak of the monomer plus H at $[919.51148]^{+1}$. qCID spectra of the solution of RGDV-Dex, RGDF-Dex and RGDS-Dex in ultrapure water, which are inserted to identify the monomer and dimer are the fragments of trimer fragmentation in FT-MS condition.

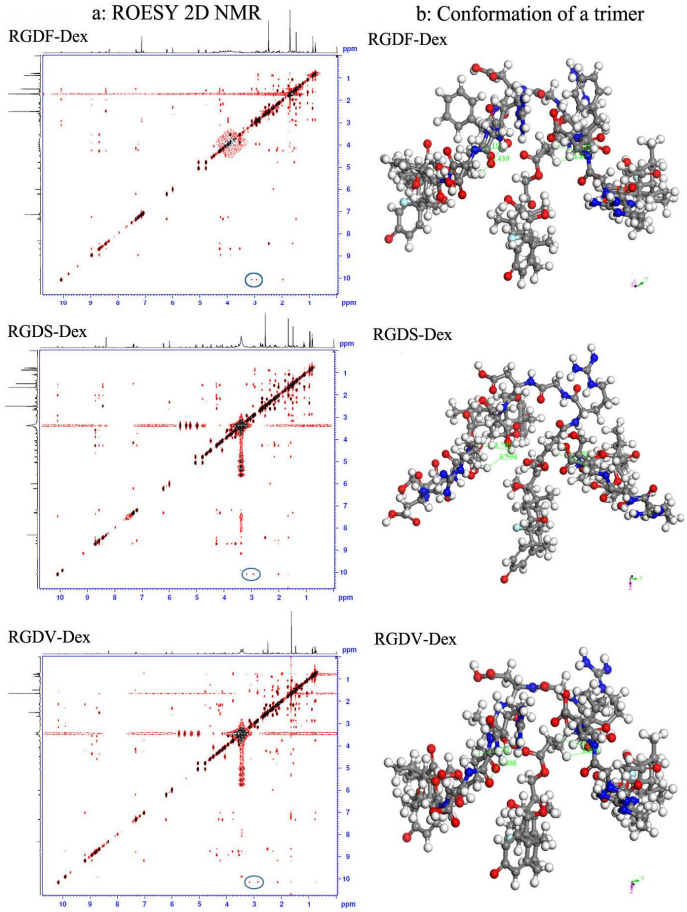


Figure 2 (a) ROESY 2D NMR spectra: The cross-peaks labeled with blue circles define the intermolecular interaction of RGDV-Dex, RGDF-Dex and RGDS-Dex ; (b) The 3D-features of the trimers of RGDV-Dex, RGDF-Dex and RGDS-Dex in minimal energy look like the umbrella.

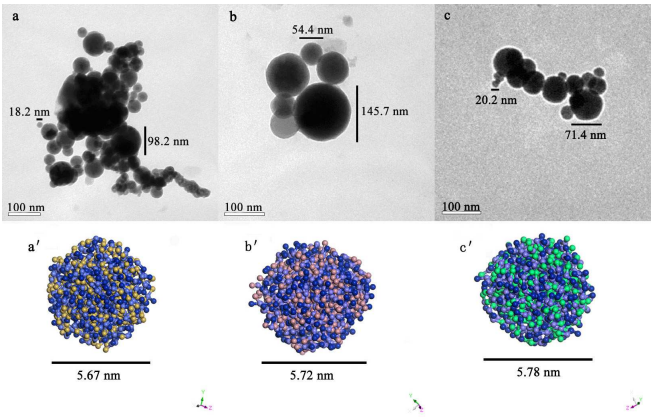


Figure 3 TEM images of 1 nM solution of the conjugates in ultrapure water: (a) TEM images of RGDV-Dex; (b) TEM images of RGDS-Dex; (c) TEM images of RGDF-Dex. The calculated nanoparticles of the conjugates: (a') The calculated nanoparticle of 5.67 nm in diameter of RGDV-Dex; (b') The calculate nanoparticle of 5.72 nm in diameter of RGDS-Dex); (c') The calculated nanoparticle of 5.78 nm in diameter of RGDF-Dex.

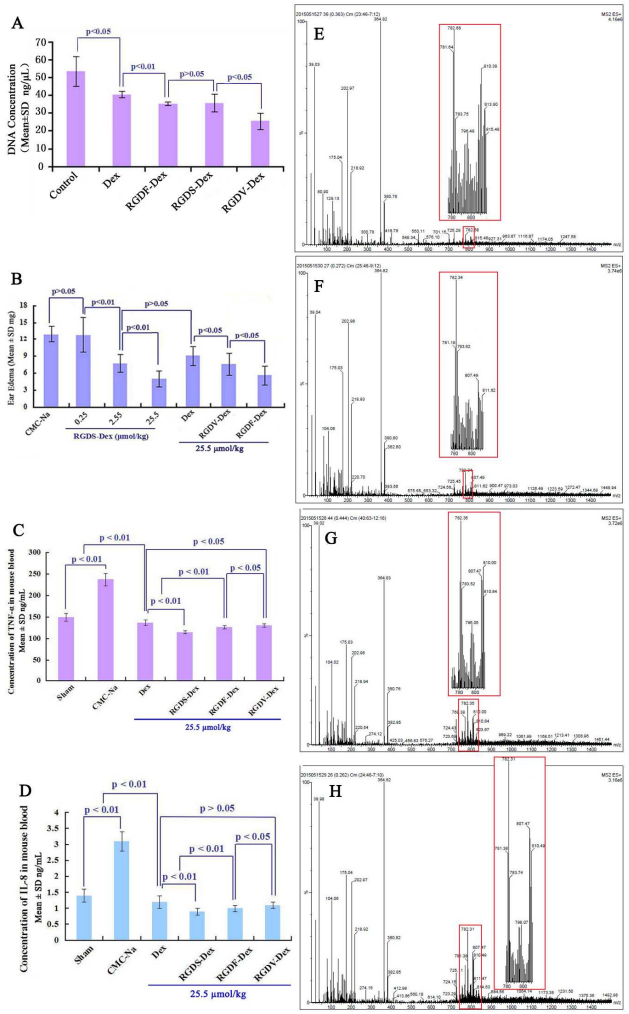


Figure 4 (A) DNA concentrations of the A549 cells treated with 1 μM Dex, RGDV-Dex, RGDS-Dex and RGDF-Dex, n = 4; (B) Anti-inflammatory activities of Dex, RGDV-Dex, RGDS-Dex and RGDF-Dex, n = 12; (C) Plasma TNF-α of the inflammation mice orally treated with CMC-Na, Dex, RGDV-Dex, RGDS-Dex and RGDF-Dex, n=12; (D) Plasma IL-8 of the inflammation mice orally treated with CMC-Na, Dex, RGDV-Dex, RGDS-Dex and RGDF-Dex, n=12; (E) ESI-MS spectrum of cytoplasm of A549 cells treated with NS, n=4; (F) ESI-MS spectrum of cytoplasm of A549 cells treated with 1μM RGDF-Dex, n=4; (G) ESI-MS spectrum of cytoplasm of A549 cells treated with 1μM RGDS-Dex, n=4; (H) ESI-MS spectrum of cytoplasm of A549 cells treated with 1μM RGDV-Dex, n=4.

RGD-tetrapeptide covalent modification enhances the anti-inflammatory activity. To understand the action mechanism of RGD-tetrapeptide covalent modification enhancing the anti-inflammatory activity the pro-inflammatory cytokines TNF-α and IL-8 of the plasma of xylene-induced ear edema of mice orally treated with 0.5% CMC-Na, or the suspensions of the conjugates in 0.5% CMC-Na were measured according to Supporting Information. **Figure 4C** indicates that the plasma TNF-α of 25.5 μmol/kg Dex treated inflammatory mice is significantly lower than those of NS treated inflammatory mice and Sham mice, but is significantly higher than those of 25.5 μmol/kg RGDS-Dex, RGDV-Dex and RGDF-Dex treated inflammatory mice. **Figure 4D** indicates that the plasma IL-8 of 25.5 μmol/kg Dex treated inflammatory mice is significantly lower than those of NS treated inflammatory mice and Sham mice, but is significantly higher than those of 25.5 μmol/kg RGDS-Dex, RGDV-Dex and RGDF-Dex treated inflammatory mice. Thus it is hypothesized that via dcreasing plasma TNF-α and IL-8 RGD-tetrapeptide covalent modification enhances the anti-inflammatory activity.

Down-regulating DNA replication and enhancing *in vivo* anti-inflammatory activity. A549 cells were widely used to respond the cross talk of inflammation and DNA replication,[30-32] and some anti-inflammatory agents were reported to be able to counteract both pro-inflammatory effect and DNA replication.[33] To correlate the enhanced *in vivo* anti-inflammatory activity with DNA replication the quantities of the DNA of treated A549 cells were measured. **Figure 4A** indicates that the DNA quantity of A549 cells treated with 1 μM Dex is significantly lower than that of A549 cells treated with NS ($p<0.05$), but is significantly higher than that of A549 cells treated with 1 μM RGDV-Dex, RGDS-Dex and RGDF-Dex ($p<0.01$). Thus down-regulating DNA replication may be one of the mechanisms of RGD-tetrapeptide modification to enhance the *in vivo* anti-inflammatory activity. This was further ensured by ESI-MS spectra of the cytosome of treated A549 cells. **Figures 4E-4H** indicate that the ESI-MS spectra of the cytosome of A549 cells treated by RGDV-Dex, RGDS-Dex and RGDF-Dex, but not by NS, give the ion peak of Dex, i.e. the ESI-MS spectrum of the cytosome of NS treated A549 cells gives no any Dex related ion peak (**Figure 4E**), while the ESI-MS spectra of the cytosome of A549 cells treated by 1 μM RGDV-Dex, RGDS-Dex and RGDF-Dex consistently give the ion peak at ~807.42, the mass of a dimer of Dex plus Na (**Figures 4F-4H**). This suggests that the trimers of the RGDV-Dex, RGDS-Dex and RGDF-Dex enter the cells, releases the dimers of Dex and down-regulate the replication of A549 cell DNA.

RGD-tetrapeptide covalent modification decreases osteoporotic risk. The effect of RGD-tetrapeptide covalent modification on the osteoporotic risk of Dex therapy was examined with mouse model. In brief, BALB/C mice (male, 14 weeks in age) were orally administered with 0.5 % CMC-Na or the suspension of 1.43 μmol/kg/day of Dex in 0.5 % CMC-Na or the suspension of 1.43 μmol/kg/day of the conjugates in 0.5 % CMC-Na for 15 consecutive days to record the total volumetric bone mineral density (vBMD) and the peripheral quantitative CT (pQCT) images of the femurs. **Figure 5** indicates that the femurs of the mice receiving 0.5 % CMC-Na, and 1.43 μmol/kg/day of RGDV-Dex or RGDS-Dex or RGDF-Dex have close total vBMD values and similar image, suggesting during 15-day treatments they induce no femur loss, while the total vBMD value and image of the femurs of the mice receiving 1.43 μmol/kg/day of Dex is significantly different from that of the mice receiving 0.5 % CMC-Na, suggesting during 15-day treatments Dex induces femur loss. Therefore RGD-tetrapeptide covalent modification effectively decreases osteoporotic risk of Dex therapy.

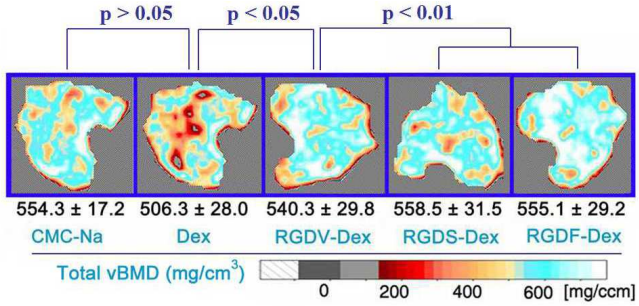


Figure 5 Total vBMD and pQCT images of the femurs of the mice treated with CMC-Na, Dex, RGDV-Dex, RGDS-Dex and RGDF-Dex; n=12.

The effect of RGD-tetrapeptide covalent modification on osteoporotic risk was further estimated with the femur weight of BALB/C mice (male, 14 weeks in age) orally receiving 0.5 % CMC-Na or 1.43 μmol/kg/day of Dex or the conjugates for 15 consecutive days. **Figure 6** indicates that the dry femur weight and the ash weight of the mice receiving 0.5 % CMC-Na, and 1.43 μmol/kg/day of RGDV-Dex or RGDS-Dex or RGDF-Dex have the close values, suggesting during 15-day treatments they induce no femur loss, while the dry femur weight and the ash weight of the mice receiving 1.43 μmol/kg/day of Dex are lower than that of the mice receiving 0.5 % CMC-Na, suggesting during 15-day treatments Dex induces femur loss (**Figure 6a,b**). The phenomena is also found in femur calcium, but not femur phosphor (**Figure 6c,d**). All data emphasize that RGD- tetrapeptide covalent modification effectively decreases the osteoporotic risk of Dex therapy.

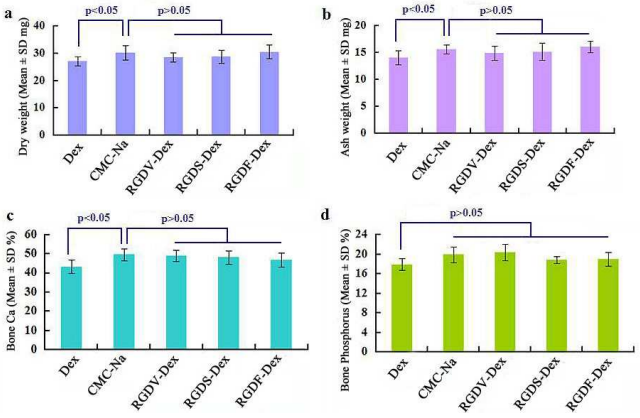


Figure 6 (a) Femur weight, (b) femur ash weight, (c) femur calcium and (d) femur phosphor of the mice orally treated with 0.5 % CMC-Na, 1.43 μmol/kg/day of Dex, 1.43 μmol/kg/day of RGDV-Dex, 1.43 μmol/kg/day of RGDS-Dex and 1.43 μmol/kg/day of RGDF-Dex for 15 days, n = 12.

Experimental

The detailed methodologies and data of all experiments are given as Supporting Information.

Conclusions

The covalent modification of Dex with RGDV, RGDS and RGDF can effectively increase the anti-inflammatory activity and lower the osteoporotic risk of Dex therepy, and therefore is a general strategy to improve the clinical anti-inflammatory therapy of glucocorticoids. The enhancement of the anti-inflammatory activity may attribute to the abilities of RGDV-Dex, RGDS-Dex and RGDF-Dex to cross membrane and down-regulate DNA replication. To clarify the responsible integrin for RGDV-Dex, RGDS-Dex and RGDF-Dex to have higher anti-inflammatory activity and lower side reaction is of pharmacological importance, is one of our interests and should be deeply investigated.

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Graphical Abstract