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3D QSAR of novel estrogen–RGD peptide conjugates: Getting insight into structural dependence of anti-osteoporosis activity and side effect of estrogen in ERT

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ABSTRACT

To explore the structural dependence of the oral potency and side effect of estrogen–RGD peptide conjugates, here six novel conjugates were prepared via introducing RGD-tetrapeptides into both 3- and 17-positions of estradiol, and introducing RGD-octapeptides into 3-position of estrone. In an ovariectomized mouse model they exhibited higher anti-osteoporosis activity and lower side effect than estrogen. For 3D QSAR analysis the anti-osteoporosis activities of nine known conjugates estrogen–RGD tetrapeptide conjugates were also provided. Using Cerius^2 module their 3D QSAR analysis was performed, four equations with high r^2 values were established, and the structural dependence of the oral potency and side effect of them was elucidated.

1. Introduction

Osteoporosis, the most frequent bone remodeling disease and defined as skeletal disorder, is characterized by a low bone mass and a high risk of fractures and has been a major health problem for elderly women. Ovarium atrophy leads postmenopausal women confronting with different level of osteoporosis.[1] Since more than 50% of the osteoporosis women may suffer from fracture and some will die, osteoporosis has being crushing pressure for our society.[2,3] It is well known that estrogen lack results from overium atrophy is responsible for the osteoporosis development of postmenopausal women, and estrogen replacement therapy (ERT) or estrogen/progestogen replacement therapy (HRT) is widely used to inhibit bone loss and consequently to decrease the risk of coronary heart disease for postmenopausal women.[4–7] The fracture risk of the postmenopausal women who receives HRT for at least 5 years could be decreased by 50%.[8] On the other hand however, when the dose-related side effect such as endometrial hyperplasia and the thromboembolic events are considered the efficacy of a long term ERT or HRT is usually discredited.[9–13] To improve the efficacy a lot of efforts have being given to the administration and/or the modification of estrogen.[14–22]

It is well established that osteoporosis relates not only to the decrease of the formation of osteoblast-modulated bone but also to the increase of the resorption osteoclast-modulated bone. The role of estrogen is directly up-modulating osteoblast activity and proliferation, and/or regulating the gene expression in osteoblasts and osteoclasts.[23–26] Bone resorption is regulated by osteoclasts binding to bone surface and depends on the adhesiveness of osteoclast, which is mediated by the transmembrane vitronectin receptor $\alpha_v\beta_3$ integrin and its recognition to RGD containing protein of osteoclast.[27] Since upregulation of $\alpha_v\beta_3$ integrin has been associated with osteoporosis, RGD peptides have been recognized as emerging potential therapeutic agents. This knowledge implies that the activity and proliferation of osteoblasts and the adhesiveness of osteoclasts can be simultaneously upregulated with estrogen and down-regulated with RGD peptide, respectively.

In this context, here six novel estrogen–RGD peptide conjugates were prepared by introducing RGD-tetrapeptides in to both 3- and 17-positions of estradiol, and by introducing RGD-octapeptides into 3-position of estrone. Based on the need of 3D QSAR, 9 known conjugates, estradiol-3-RGD tetrapeptides, estradiol-17-RGD tetrapeptides and estrone-3-RGD tetrapeptides, were also prepared as the reference conjugates. In an ovariectomized mouse model, these novel conjugates, reference conjugates, estradiol and estron (estrogen controls) were orally administered, and the important parameters reflected the bone health of the treated mice, such as the weight of femur and femur ash, calcium and phosphorous in femur and blood, the level of serum ALP, bleeding time and uterine weight, were measured. Using Cerius^2 module, the 3D QSAR analyses were performed, and four equations

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capable of predicting a comparatively exact anti-osteoporosis activity and side effect for estrogen–RGD peptide conjugates were established.[28]

2. Results and discussion

2.1. Preparation of RGD peptide moieties

The tetrapeptides and octapeptides of RGD used for preparing the conjugates were synthesized via solution method according to the route depicted in Scheme 1. Upon the coupling of Boc-Asp(OBzl) with L-Ser(Bzl)-OBzl, L-Val-OBzl and L-Phe-OBzl, as well as then removing Boc group HCl·Asp(OBzl)-Ser(Bzl)-OBzl, HCl·Asp(OBzl)-Val-OBzl and HCl·Asp(OBzl)-Phe-OBzl were obtained in 94%, 92% and 87% yield, respectively. Upon coupling Boc-Arg(NO_2) and Gly Boc-Arg(NO_2)-Gly was obtained in 84% yield. After the corresponding coupling and deprotection reactions HCl·Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl (**1**), HCl·Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl (**2**), HCl·Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl (**3**), HCl·Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl (**4**), HCl·Arg(NO_2)-Gly-Asp(OBzl)-Val-Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl (**5**) and HCl·Arg(NO_2)-Gly-Asp(OBzl)-Phe-Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl (**6**) were obtained in 89%, 76%, 81%, 82%, 66% and 69% yield, respectively. The procedure, synthetic data, and chemical physical data of all peptides are given in a SI file.

2.2. Preparing novel conjugates estradiol-3,17-di(Arg-Gly-Asp-AA)

The preparation of novel estradiol-3,17-di(RGD tetrapeptide) conjugates is shown with Scheme 2. Estradiol was alkylated with ethyl bromoacetate to provide 3-ethoxycabonylmethoxyestradiol (**7**) in 83% yield. By saponification **7** was converted into 3-carboxylmethoxyestradiol (**8**, 96% yield). Treating **8** with succinic anhydride 3-carboxymethoxyl-17β-carboxyethylcarbonyloxyestradiol (**9**) was provided in 81% yield, to which **1**–**3** were introduced and corresponding estradiol-3,17-di[Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl] **14**, **15** and **16** [AA = Ser(Bzl), Val and Phe] were obtained in 71%, 82% and 80% yield, respectively. Removing all protective groups by catalytic hydrogenation three novel conjugates estradiol-3,17-di(Arg-Gly-Asp-AA) (estradiol-3S,17S: AA = Ser; estradiol-3V,17V: AA = Val and estradiol-3F,17F: AA = Phe) were obtained in 38–88% yield.

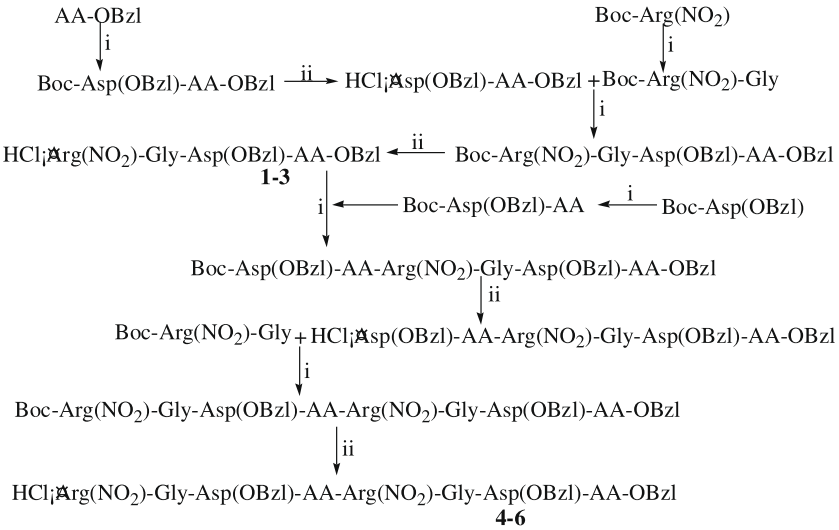
To **8** HCl·Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl [**1**–**3**, AA = Ser(Bzl), Val and Phe] were introduced and the corresponding estradiol-3-Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl **11**, **12** and **13** [AA = Ser(Bzl), Val and Phe] were obtained in 53%, 37% and 61% yield, respectively. Removing all protective groups by catalytic hydrogenation three reference conjugates estradiol-3-Arg-Gly-Asp-AA (estradiol-3S, AA = Ser; estradiol-3V, AA = Val and estradiol-3F AA = Phe) were obtained in 48–91% yield.

Estradiol was treated with succinic anhydride and 17-β-carboxyethylcarbonyl-oxyestradiol (**10**) was offered in 95% yield. Coupling **10** with **1**, **2** and **3** estradiol-17β-Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl **17**, **18** and **19** [AA = Ser(Bzl), Val and Phe] were obtained in 48%, 45% and 42% yield, respectively. Removing all protective groups by catalytic hydrogenation another three reference conjugates estradiol-17-Arg-Gly-Asp-AA (estradiol-17S: AA = Ser; estradiol-17V: AA = Val; estradiol-17F: AA = Phe) were obtained in 79–86% yield. The procedure, synthetic data, and the chemical physical data of all estradiol-3,17-di(Arg-Gly-Asp-AA) conjugates are given in a SI file.

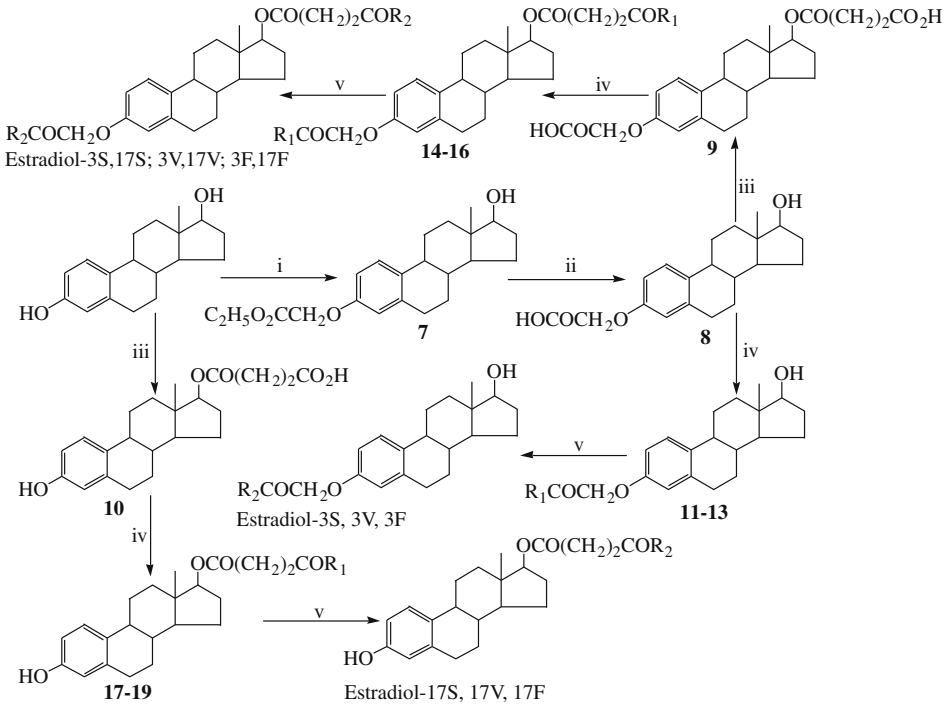
2.3. Preparing novel estrone-17–RGD octrapeptide conjugates

The preparation of novel estrone-17–RGD octrapeptide conjugates is shown with Scheme 3. The 3-hydroxyl group of estrone was first alkylated by ethyl bromoacetate to provide ethyl estrone-3-oxylacetate in 94% yield. By saponification ethyl estrone-3-oxylacetate was converted to estrone-3-oxylacetic acid (**20**, 96% yield), to which HCl·Arg(NO_2)-Gly-Asp(OBzl)-AA-Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl (**4**–**6**) were introduced to form estrone-3-Arg(NO_2)-Gly-Asp-(OBzl)-AA-Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl **24**, **25** and **26** [AA = Ser(Bzl), Val and Phe] in 71%, 82% and 80% yield, respectively. Removing all protective groups 3 novel estrone-3-Arg-Gly-Asp-AA-Arg-Gly-Asp-AA (estrone-3SS: AA = Ser; estrone-3VV: AA = Val; estrone-3FF: AA = Phe) were provided in 41–71% yield.

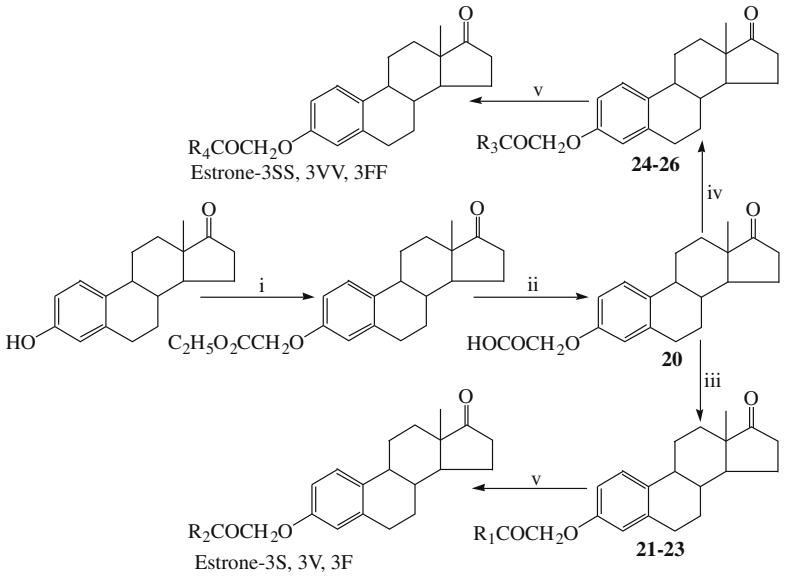
To **20** HCl·Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl [**1**-**3**, AA = Ser(Bzl), Val and Phe] were introduced to form the corresponding estrone-3-Arg(NO_2)-Gly-Asp(OBzl)-AA-OBzl **21**, **22** and **23** [AA = Ser(Bzl), Val and Phe] in 62%, 68% and 61% yield, respectively. Removing all protective groups from **21–26** by catalytic hydrogenation 3 ref-



Scheme 1. Preparation of RGD tetrapeptides and octapeptides, in **1** and **4** AA = Ser(Bzl), **2** and **5** AA = Val, **3** and **6** AA = Phe; (i) DCC, HOBt and NMM; (ii) hydrogen chloride in ethyl acetate (4 N).



Scheme 2. Route for preparing estradiol–RGD conjugates, in **11** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl, in **12** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl, in **13** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl; in Estradiol-3S R_2 = -Arg-Gly-Asp-Ser, in Estradiol-3V R_2 = -Arg-Gly-Asp-Val, in Estradiol-3F R_2 = -Arg-Gly-Asp-Phe; in **14** $R_1 = R_2$ = -Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl, in **15** $R_1 = R_2$ = -Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl, in **16** $R_1 = R_2$ = -Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl; in Estradiol-3S,17S $R_1 = R_2$ = -Arg-Gly-Asp-Ser, in Estradiol-3V,17V $R_1 = R_2$ = -Arg-Gly-Asp-Val, in Estradiol-3F,17F $R_1 = R_2$ = -Arg-Gly-Asp-Phe; in **17** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl, in **18** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl, in **19** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl; in Estradiol-17S R_1 = -Arg-Gly-Asp-Ser, in Estradiol-17V R_1 = -Arg-Gly-Asp-Val, in Estradiol-17F R_1 = -Arg-Gly-Asp-Phe; (i) $BrCH_2CO_2C_2H_5$ and $NaOC_2H_5$; (ii) NaOH in methanol; (iii) DCC, HOBt, NMM and **4**–**6**; (iv) DCC, HOBt, NMM and **1**–**3**; (v) Pt/H_2.



Scheme 3. Route for preparing Estrone–RGD conjugates, in **21** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl, in **22** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl, in **23** R_1 = -Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl; in Estrone-3S R_2 = -Arg-Gly-Asp-Ser, in Estrone-3V R_2 = -Arg-Gly-Asp-Val, in Estrone-3F R_2 = -Arg-Gly-Asp-Phe; in **24** R_3 = -Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-Arg(NO_2)-Gly-Asp(OBzl)-Ser(Bzl)-OBzl, in **25** R_3 = -Arg(NO_2)-Gly-Asp(OBzl)-Val-Arg(NO_2)-Gly-Asp(OBzl)-Val-OBzl, in **26** R_3 = -Arg(NO_2)-Gly-Asp(OBzl)-Phe-Arg(NO_2)-Gly-Asp(OBzl)-Phe-OBzl; in Estrone-3SS R_4 = -Arg-Gly-Asp-Ser-Arg-Gly-Asp-Ser, in Estrone-3VV R_4 = -Arg-Gly-Asp-Val-Arg-Gly-Asp-Val, in Estrone-3FF R_4 = -Arg-Gly-Asp-Phe-Arg-Gly-Asp-Phe; (i) $BrCH_2CO_2C_2H_5$ and $NaOC_2H_5$; (ii) NaOH in methanol; (iii) DCC, HOBt, NMM and **1**–**3**; (iv) DCC, HOBt, NMM and **4**–**6**; (v) Pt/H_2.

erence conjugates, estrone-3-Arg-Gly-Asp-AA (estrone-3S: AA = Ser; estrone-3V: AA = Val; estrone-3F: AA = Phe), were provided in 57–76% yield. The procedure, synthetic data, and chemical physical data of all estrone-17–RGD octrapeptide conjugates are given in a SI file.

2.4. Serum calcium and ALP of mice receiving estrogen–RGD conjugates

The influence of estrogen–RGD peptide conjugates on the bone loss may be embodied in serum calcium, phosphorous and ALP lev-

Table 1
Calcium, phosphorous and ALP in serum of mice receiving conjugates

Group[a]	Calcium	Phosphorous	ALP
OVX	3.39 ± 0.15	3.18 ± 0.28	28.78 ± 2.74
Sham	3.30 ± 0.11	2.82 ± 0.29	22.97 ± 1.71
Estradiol	3.34 ± 0.24	3.01 ± 0.27	27.22 ± 2.92
Estrone	3.35 ± 0.26	3.13 ± 0.43	27.97 ± 2.86
Estradiol-3S	3.33 ± 0.20	2.98 ± 0.20	26.62 ± 2.12[c]
Estradiol-3V	3.32 ± 0.19	2.99 ± 0.23	26.01 ± 2.71[c]
Estradiol-3F	3.34 ± 0.10	3.19 ± 0.61	25.42 ± 1.35[b]
Estradiol-17S	3.35 ± 0.17	3.01 ± 0.27	23.93 ± 2.90[b]
Estradiol-17V	3.36 ± 0.19	3.04 ± 0.12	22.96 ± 2.96[d]
Estradiol-17F	3.34 ± 0.25	3.01 ± 0.28	23.18 ± 2.79[d]
Estradiol-3S,17S	3.22 ± 0.16[c]	2.99 ± 0.29	19.61 ± 5.98[e]
Estradiol-3V,17V	3.17 ± 0.29[c]	2.84 ± 0.20[b]	19.94 ± 2.77[e]
Estradiol-3F,17F	3.19 ± 0.19[c]	3.09 ± 0.32	15.80 ± 2.63[e]
Estrone-3S	3.33 ± 0.29	2.98 ± 0.20	16.62 ± 4.12[e]
Estrone-3V	3.33 ± 0.12	2.96 ± 0.26	17.42 ± 2.17[e]
Estrone-3F	3.44 ± 0.22	3.17 ± 0.34	16.42 ± 2.78[e]
Estrone-3SS	3.08 ± 0.16[b]	2.96 ± 0.13[c]	13.80 ± 2.57[e]
Estrone-3VV	3.00 ± 0.14[b]	3.22 ± 0.21	13.27 ± 1.55[e]
Estrone-3FF	3.15 ± 0.19[c]	3.01 ± 0.40	15.06 ± 2.95[e]

In calcium content column: [b]Compared with ovariotomy $P < 0.01$, with estradiol and estrone $P < 0.05$. [c]Compared with ovariotomy $P < 0.05$.

In phosphorous content column: [b]Compared with ovariotomy $P < 0.01$. [c]Compared with ovariotomy $P < 0.05$.

In ALP column: [b]Compared with ovariotomy $P < 0.01$; [c]Compared with ovariotomy $P < 0.05$. [d]Compared with ovariotomy $P < 0.01$, with estradiol and estrone $P < 0.05$. [e]Compared with ovariotomy, estradiol and estrone $P < 0.01$.

[a] Dosage = 110.3 nmol/kg; $n = 12$; serum calcium and phosphorous are represented as X ± SD mM; Serum ALP is represented as X ± SD U/L king

els. To explore the effect of oral estrogen–RGD peptide conjugates on serum calcium, phosphorous and ALP levels, mice were orally administrated 110.3 nmol/kg of the novel conjugates estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF, and 110.3 nmol/kg of reference conjugates (estradiol-3S, estradiol-3V, estradiol-3F, estradiol-17S, estradiol-17V, estradiol-17F, estrone-3S, estrone-3V and estrone-3F), 110.3 nmol/kg of estradiol or estrone (estrogen control) and aqueous solution of 0.5% carboxymethylcellulose (vehicle control) following the procedure of item 4.6. The data are listed in Table 1, which indicate that the serum calcium content and ALP value of the mice of vehicle control, estrogen control and reference conjugates are significantly higher than those of the mice receiving estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF. This comparison indicates that introducing RGD tetrapeptides into both the 3- and 17-positions of estradiol and introducing RGD octapeptides into the 3-position of estrone leads to the inhibition of the bone loss of ovariectomized mice, therefore the conjugation could be an effective strategy to increase the oral activity of estrogen for ovariectomized mice. The procedure of the assay is given in a SI file.

2.5. Weights of femurs and femur ashes of mice receiving estrogen–RGD conjugates

The influence of estrogen–RGD peptide conjugates on the form and loss of bone may be directly embodied in the weights of femurs and femur ashes of mice. To explore the effect of estrogen-RGD conjugates on the weights of dry femurs and femur ashes, mice were orally administrated 110.3 nmol/kg of the novel conjugates estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF, 110.3 nmol/kg of reference conjugates, 110.3 nmol/kg of estrogen, and vehicle following the procedure of item 4.7. The data are listed in Table 2, which indicate that the weights of both the femur and femur ash of the mice receiving vehicle and estrogen controls are significantly lower than that of the mice receiving estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF. The comparison does imply that introducing RGD tetrapeptides into both the 3- and 17-positions of estradiol and introducing RGD octapeptides into the 3-position of estrone could be an effective strategy to increase the oral activity of inhibiting bone loss and/or enhancing bone forming for ovariectomized mice. The procedure of the assay is given in a SI file.

Table 2
Weights of dry femurs and femur ashes of mice receiving conjugates

Group[a]	Dry femur	Femur ash	Femur length (cm)
OVX	51.77 ± 2.50	31.57 ± 2.11	1.64 ± 0.24
Sham	53.77 ± 2.04	34.11 ± 2.66	1.64 ± 0.21
Estradiol	52.35 ± 2.22	32.22 ± 2.02	1.64 ± 0.28
Estrone	53.03 ± 2.32	32.08 ± 2.23	16.45 ± 0.42
Estradiol-3S	53.89 ± 2.20[c]	34.08 ± 2.24[e]	1.65 ± 0.20
Estradiol-3V	53.97 ± 2.31[c]	34.29 ± 2.22[e]	1.67 ± 0.27
Estradiol-3F	53.87 ± 2.15[c]	33.99 ± 2.12[e]	1.67 ± 0.20
Estradiol-17S	53.98 ± 2.08[c]	34.17 ± 2.23[e]	1.66 ± 0.28
Estradiol-17V	54.01 ± 2.27[c]	34.70 ± 2.14[d]	1.67 ± 0.24
Estradiol-17F	54.00 ± 2.15[c]	34.87 ± 2.04[d]	16.40 ± 0.26
Estradiol-3S,17S	55.22 ± 2.02[b]	35.60 ± 2.26[d]	16.39 ± 0.52
Estradiol-3V,17V	56.46 ± 2.03[b]	35.75 ± 2.27[d]	16.56 ± 0.49
Estradiol-3F,17F	55.46 ± 2.01[b]	35.22 ± 2.23[d]	16.18 ± 0.37
Estrone-3S	54.29 ± 2.20[c]	34.38 ± 2.94[e]	16.46 ± 0.50
Estrone-3V	54.12 ± 1.62[c]	33.99 ± 1.59[e]	16.60 ± 0.25
Estrone-3F	53.99 ± 2.56[c]	33.85 ± 2.01[e]	16.47 ± 0.34
Estrone-3SS	55.90 ± 2.49[b]	35.78 ± 2.31[d]	16.73 ± 0.42
Estrone-3VV	55.39 ± 2.65[b]	35.29 ± 2.28[d]	16.55 ± 0.41
Estrone-3FF	55.26 ± 2.22[b]	35.24 ± 2.22[d]	16.41 ± 0.24

[a] Dosage = 110.3 nmol/kg, $n = 12$, weights of dry femurs and femur ashes are represented as X ± SD mg, femur length is represented as X ± SD cm.

[b] Compared to ovariotomy, estradiol and estrone $P < 0.01$.

[c] Compared to ovariotomy $P < 0.05$.

[d] Compared to ovariotomy $P < 0.01$, to estradiol and estrone $P < 0.05$.

[e] Compared to ovariotomy $P < 0.05$.

2.6. Femur calcium, phosphorous and mineral of mice receiving estrogen–RGD conjugates

The influence of estrogen–RGD peptide conjugates on the bone forming and bone loss may be also embodied in femur calcium, phosphorous and mineral levels. To further explore the effect of estrogen–RGD conjugates on femur calcium, mice were orally administrated 110.3 nmol/kg of novel conjugates estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF, 110.3 nmol/kg of reference conjugates, 110.3 nmol/kg of estrogen, and vehicle following the procedure of item 4.7. The data are listed in Table 3, which indicate that femur calcium and phosphorous of the mice receiving vehicle and estrogen are significantly lower than those of the mice receiving estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF. The comparison again implies that introducing RGD tetrapeptides into both the 3- and 17-positions of estradiol and introducing RGD octapeptides into the 3-position of estrone could be an effective strategy to increase the oral activity of inhibiting bone loss and/or enhancing bone forming for ovariectomized mice. The procedure of the assay is given in a SI file.

2.7. Side effect of mice receiving estrogen–RGD conjugates

Dose-related side effects commonly occurred in long term ERT or HRT are endometrial hyperplasia and the thromboembolic events. To estimate the endometrial hyperplasia and the thromboembolic risks of oral administration of novel conjugates, mice were orally administrated 110.3 nmol/kg of estradiol-3S,17S, estradiol-3V,17V, estradiol-3F,17F, estrone-3SS, estrone-3VV and estrone-3FF, 110.3 nmol/kg of reference conjugates, 110.3 nmol/kg of estrogen, and vehicle control following the procedure of item 4.8. The weights of organ and body of the mice explored that novel conju-

Table 3
Femur calcium, phosphorous and mineral of mice receiving conjugates

Group[a]	Calcium	Phosphorous	Mineral
OVX	54.45 ± 1.35	19.31 ± 1.16	0.60 ± 0.01
Sham	62.08 ± 2.40	20.16 ± 0.19	0.63 ± 0.02
Estradiol	57.28 ± 1.87	19.81 ± 0.21	0.61 ± 0.02
Estrone	56.20 ± 2.56	19.11 ± 0.34	0.61 ± 0.03
Estradiol-3S	58.76 ± 2.13[b]	20.24 ± 0.04[f]	0.60 ± 0.01
Estradiol-3V	57.84 ± 1.76[c]	20.24 ± 0.42[f]	0.61 ± 0.02
Estradiol-3F	57.42 ± 1.04[c]	20.09 ± 0.19[f]	0.60 ± 0.03
Estradiol-17S	59.14 ± 1.22[b]	20.37 ± 0.12[f]	0.60 ± 0.01
Estradiol-17V	57.49 ± 1.98[c]	20.14 ± 0.28[f]	0.61 ± 0.03
Estradiol-17F	58.06 ± 2.17[c]	20.56 ± 0.26[f]	0.61 ± 0.02
Estradiol-3S,17S	61.05 ± 1.79[b]	20.35 ± 0.32[f]	0.62 ± 0.01[g]
Estradiol-3V,17V	61.56 ± 2.05[b]	20.94 ± 0.10[e]	0.62 ± 0.01[g]
Estradiol-3F,17F	61.17 ± 2.09[b]	21.21 ± 0.29[e]	0.61 ± 0.01[h]
Estrone-3S	57.76 ± 2.13[c]	20.44 ± 0.04[e]	0.60 ± 0.02
Estrone-3V	57.24 ± 1.71[c]	20.08 ± 0.24[f]	0.61 ± 0.02
Estrone-3F	56.96 ± 2.94[d]	20.32 ± 0.66[f]	0.59 ± 0.06
Estrone-3SS	59.95 ± 1.95[b]	21.76 ± 0.22[e]	0.62 ± 0.02[g]
Estrone-3VV	60.50 ± 2.12[b]	21.84 ± 0.23[e]	0.62 ± 0.01[g]
Estrone-3FF	59.92 ± 2.04[b]	20.33 ± 0.68[f]	0.61 ± 0.01[h]

[a] Dosage = 110.3 nmol/kg, n = 12, femurs calcium, phosphorous and mineral are represented as X ± SD%.
[b] Compared to ovariotomy, estradiol and estrone $P < 0.01$.
[c] Compared to ovariotomy $P < 0.01$.
[d] Compared with ovariotomy $P < 0.05$.
[e] Compared to ovariotomy, estradiol and estrone $P < 0.01$.
[f] Compared to ovariotomy $P < 0.05$, with estradiol and estrone $P < 0.01$.
[g] Compared with ovariotomy $P < 0.01$.
[h] Compared with ovariotomy $P < 0.05$.

gates had no effect on the weights of the spleen, lung, kidney, liver and body of the mice.

The bleeding time is listed in Table 4, which indicate that the bleeding time of the mice receiving vehicle and estrogen–RGD conjugates is significantly longer than that of the mice receiving estradiol and estrone, which suggests that thromboembolic risk of estradiol and estrone is avoided by use of novel conjugates. The weights of the uterine are also listed in Table 4. The data indicate that uterine weights of the mice receiving vehicle and the novel conjugates are significantly lower than that of the mice receiving estradiol and estrone, which suggests that the endometrial hyperplasia induced by estradiol and estrone is avoided by the novel

Table 4
Bleeding time and uterine weights of mice receiving conjugates

Group[a]	Bleeding time after last administration		Uterine weight
	30 m	90 min	
OVX	542 ± 50	548 ± 49	0.081 ± 0.022
Sham	562 ± 53	568 ± 59	0.086 ± 0.026
Estradiol	417 ± 57[b]	441 ± 43[b]	0.108 ± 0.021[c]
Estrone	411 ± 51[b]	433 ± 40[b]	0.114 ± 0.015[b]
Estradiol-3S	582 ± 50	577 ± 52	0.064 ± 0.015[d]
Estradiol-3V	569 ± 54	577 ± 52	0.064 ± 0.011[d]
Estradiol-3F	557 ± 45	560 ± 51	0.067 ± 0.021
Estradiol-17S	575 ± 51	561 ± 54	0.063 ± 0.015[d]
Estradiol-17V	551 ± 48	560 ± 51	0.064 ± 0.013[d]
Estradiol-17F	576 ± 59	568 ± 61	0.060 ± 0.028
Estradiol-3S,17S	580 ± 52	570 ± 52	0.050 ± 0.013[c]
Estradiol-3V,17V	591 ± 86	532 ± 20	0.059 ± 0.019[d]
Estradiol-3F,17F	522 ± 38	524 ± 27	0.050 ± 0.010[c]
Estrone-3S	579 ± 52	568 ± 44	0.069 ± 0.035
Estrone-3V	563 ± 40	571 ± 57	0.066 ± 0.004[d]
Estrone-3F	581 ± 49	576 ± 55	0.060 ± 0.007[c]
Estrone-3SS	588 ± 71	516 ± 39	0.058 ± 0.031[d]
Estrone-3VV	582 ± 67	514 ± 58	0.059 ± 0.012[c]
Estrone-3FF	579 ± 46	574 ± 49	0.051 ± 0.038[d]

[a] Dosage = 110.3 nmol/kg, n = 12; tail bleeding time is represented as X ± SD s and uterine weight is represented as X ± SD g.
[b] Compared to ovariotomy $P < 0.01$.
[c] Compared to ovariotomy $P < 0.01$.
[d] Compared to ovariotomy $P < 0.05$.

conjugates. The comparison implies that introducing RGD tetrapeptides into both the 3- and 17-positions of estradiol and introducing RGD octapeptides into the 3-position of estrone could be an effective strategy to limit the side effect of estrogen in ERT. The procedure of the assay is given in a SI file.

2.8. 3D QSAR analyses of estrogen–RGD conjugates

To reveal the dependence of increasing oral anti-osteoporosis activity and decreasing side effect of estrogen–RGD conjugates on their structure the weight of the femur and femur ash was selected as the anti-osteoporosis activity, bleeding time and uterine weight stand for side effect, and QSAR module of Cerius^2 was followed according to a standard procedure to establish the equations to correlate the weight of the femur and femur ash, the bleeding time and uterine weight with the structures of estrogen–RGD conjugates.

2.8.1. Alignment of estrogen and estrogen–RGD conjugates

To establish valid 3D-QSAR models a proper alignment procedure of estrogen and estrogen–RGD conjugates was performed using the target model align strategy in the align module within Cerius^2. With an assumption that each structure of estrogen and estrogen–RGD conjugates binds the same site of the receptor and exhibits activity, they were aligned in a pharmacological active orientation. To obtain a consistent alignment the estrogen ring, the common pharmacophore, was selected as the template for superposing estrogen–RGD conjugates. The method used for performing the alignment was Maximum Common Subgraph (MCS).[29] MCS looks at molecules as points and lines, and uses the techniques out of graph theory to identify the patterns. Then MCS finds the largest subset of atoms in estrogen ring that shared by estrogen–RGD conjugates. This subset was used for the alignment. A rigid fit of atom pairings was performed to superimpose each structure onto the target model estrogen ring. The stereoview of aligned estrogen and estrogen–RGD conjugates used for molecular field generation is shown in Figure 1. The alignment stereoview explores that to superimpose onto estrogen ring the RGD peptide chain of each structure has to take individual conformation. This chain conformation of individual RGD-peptide will affect on the anti-osteoporosis activity and side effects.

2.8.2. QSAR module of Cerius^2 based MFA of estrogen and conjugates

After energy-minimization using MMFF94 (Merck Molecular Force Field), Molecular Field Analysis (MFA) was performed for estrogen and estrogen–RGD conjugates using the QSAR module of Cerius^2.[30] A five-step-procedure consisted of conformer genera-

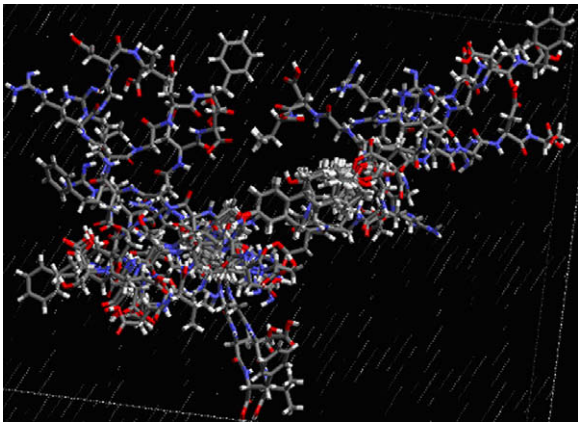


Figure 1. Alignment stereoview of estrogen and estrogen–RGD peptide conjugates used for molecular field generation.

tion, energy minimization, atom match and molecular alignment, preference setting, and regression analysis was automatically practiced in MFA. Electrostatic and steric fields of the molecules were created by use of proton and methyl groups as probes, respectively. These fields were sampled at each point of a regularly spaced grid of 1 Å. An energy cutoff of ±30.0 kcal/mol was set for both electrostatic and steric fields. Total grid points generated were 10,340. Though spatial and structural descriptors such as dipole moment, polarizability, radius of gyration, number of rotatable bonds, molecular volume, principal moment of inertia, AlogP98, number of hydrogen bond donors and acceptors, and molar refractivity were also considered, only the highest variance holder proton, methyl and hydroxyl descriptors were used. Regression analysis was carried out using Genetic Partial Least Squares (G/PLS) method consisting of 50,000 generations with a population size of 100. The number of components was set to 5. Cross-validation was performed with the leave-one-out procedure. PLS analysis was scaled with all variables normalized to a variance of 1.0.

The regions where the variations of steric or electrostatic features of estrogen and the conjugates in the training set lead to the increase or decrease of femur weight were specified. Proton descriptor with positive coefficient indicates a region favorable for electropositive group, while negative coefficient indicates electronegative group required at this position. Methyl descriptor with positive coefficient indicates a region favorable for large group, while negative coefficient indicates small group required at this position. Hydroxyl descriptor with positive coefficient indicates a region favorable for the group to form hydrogen bond, while negative coefficient indicates the group to form hydrogen bond is not required at this position.

2.8.2.1. MFA model for femur weights of mice receiving estrogen and conjugates. MFA model for the femur weights of the mice receiving estrogen or conjugates in terms of the descriptors proton, methyl and hydroxyl groups is aligned as Figure 2 and expressed by Eq. 1. The correlation of femur weights tested on the ovariectomized mouse model and femur weights calculated from Eq. 1 is shown in Figure 3.

$$\begin{aligned}\text{Femur weight} = {} & 53.59 + 0.067(H^+/5867) + 0.0017(CH_3/4110) \\ & - 0.0095(CH_3/5371) + 0.035(CH_3/6984) \\ & + 0.0082(CH_3/7040) + 0.0041(CH_3/8295) \\ & + 0.10(HO^-/2193) + 0.12(HO^-/4227) \\ & - 0.044(HO^-/5313) - 0.14(HO^-/7815) \\ & + 0.0086(HO^-/7875)0.011(HO^-/9071) \qquad (1)\end{aligned}$$

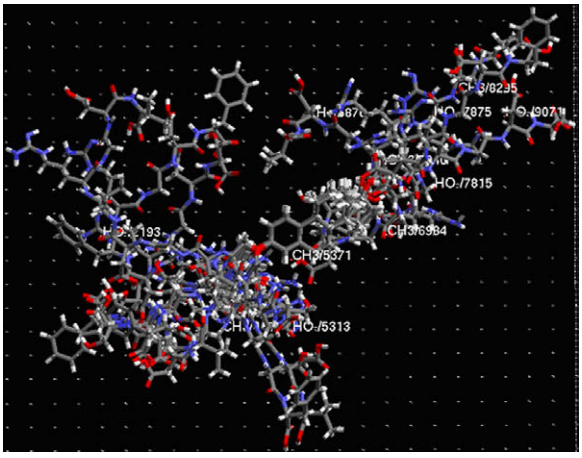


Figure 2. Alignment stereoview of estrogen and estrogen–RGD peptide conjugates affecting on the femur weight of mice.

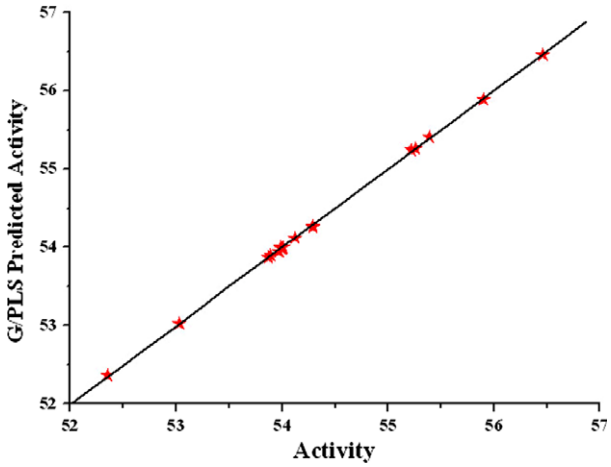


Figure 3. Graph of tested femur weight against predicted femur weight of mice receiving estrogen and estrogen–RGD peptide conjugates.

In Eq. 1 the data points (n), correlation coefficient (r) and square correlation coefficient (r^2) were 17, 0.998 and 0.989, respectively. In Eq. 1 one term, $H^+/5867$, has positive coefficient, which means that at this position electron-withdrawing group increases femur weight, four terms, $CH_3/4110$, $CH_3/6984$, $CH_3/7040$ and $CH_3/8295$, have positive coefficient, which means that at these positions large group increases femur weight, one term, $CH_3/5371$, with negative coefficient, which means that at this position small group decreases femur weight, four terms, $HO^-/2193$, $HO^-/4227$, $HO^-/7875$ and $HO^-/9071$, have positive coefficient, which means that at these positions hydrogen bond forming group increases femur weight, and two terms, $HO^-/5313$ and $HO^-/7815$, have negative coefficient, which means that at these positions hydrogen bond forming group decreases femur weight. According to the value of term coefficient of the equation if a conjugate has its electron-withdrawing group near position $H^+/5867$, hydrogen bond forming group near positions $HO^-/2193$ and $HO^-/4227$, as well as bulk group near position $CH_3/7040$ it possess high anti-osteoporosis activity.

According to Eq. 1 a favorable estrogen–RGD conjugate inhibiting femur loss should have large group near $CH_3/6984$–8295 region and/or electron-withdrawing group near $H^+/5867$ region and/or no hydrogen bond forming group near $HO^-/2193$–4227 region. To explain this requirement representatives Estradiol-3v, Estradiol-17v, and Estradiol-3v,17v are given in Figure 4a, as well as Estrone-3v and Estrone-3vv are given in Figure 4b. Besides the same electrostatic and environments as estradiol ring, Estradiol-3v has a hydrogen bond forming group near $HO^-/5313$ and results in moderate increase of femur weight, Estradiol-17v has small group near $CH_3/7040$ and electron-withdrawing group near $H^+/5867$ thus totally results in moderate increase of femur weight, while Estradiol-3v,17v has electron-withdrawing group near $H^+/5867$, hydrogen bond forming group near $HO^-/5315$ and peptide chain over $CH_3/7040$ and thus results in high increase of femur weight. Besides the same electrostatic and environments as estrone ring, Estrone-3v has hydrogen bond forming group near $HO^-/5315$, peptide chain over $CH_3/5313$ and a small group near $CH_3/4110$ and thus totally results in low femur weight, Estrone-3vv has hydrogen bond forming group near $HO^-/2193$ and thus resulting in high femur weight.

2.8.2.2. MFA model for the weight of femur ash of mice receiving estrogen and conjugates. MFA model for the weight of the femur ash of the mice receiving estrogen and conjugates in terms of the descriptors proton, methyl and hydroxyl groups is

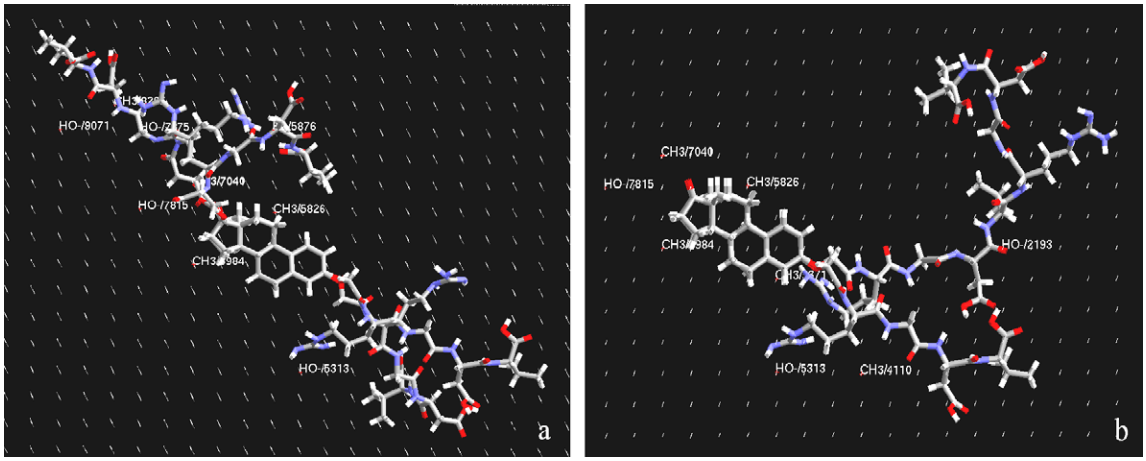


Figure 4. Electrostatic and environments of Estradiol-3v, Estradiol-17v, and Estradiol-3v,17v (a) and Estrone-3v and Estrone-3vv (b) within the grid with 3D points of Eq. 1.

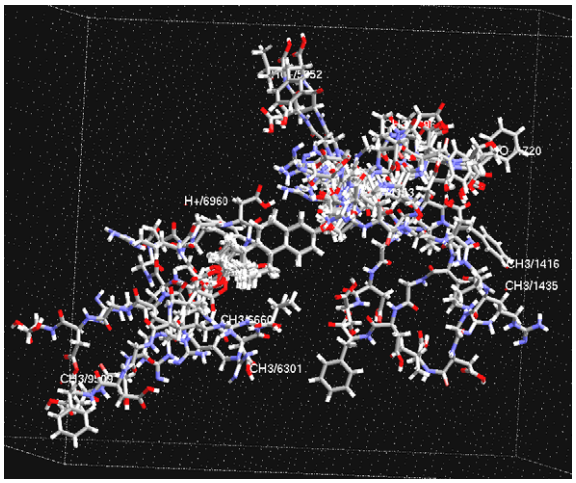


Figure 5. Alignment stereoview of estrogen and estrogen–RGD peptide conjugates affecting on the femur ash weight.

aligned as Figure 5 and expressed by Eq. 2. The correlation of tested weights of the femur ash and calculated weights is explained by Figure 6.

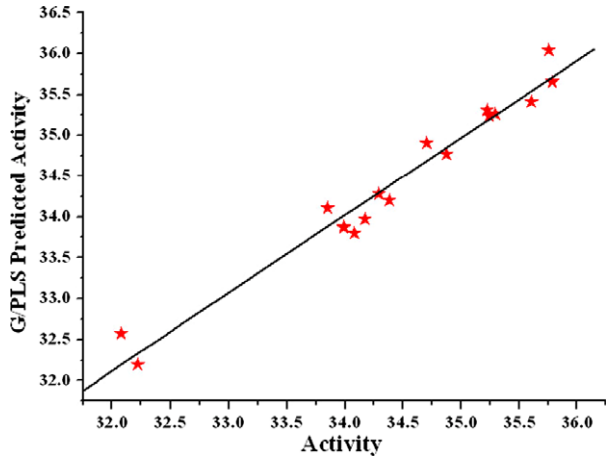


Figure 6. Graph of tested femur ash weight against predicted femur ash weight of mice receiving estrogen and estrogen–RGD peptide conjugates.

$$\begin{aligned}\text{Femur ash weight} = {} & 32.23 + 0.043(H^+/6960) + 0.022(CH_3/1416) \\ & + 0.035(CH_3/1435) + 0.046(CH_3/3696) \\ & + 0.005(CH_3/6301) + 0.045(CH_3/6660) \\ & + 0.39(CH_3/9509) - 0.009(HO^-/1720) \\ & + 0.071(HO^-/4153) + 0.046(HO^-/4548) \\ & + 0.0062(HO^-/5652) \end{aligned} \quad (2)$$

In Eq. 2 the data points (n), correlation coefficient (r) and square correlation coefficient (r^2) were 17, 0.998 and 0.990, respectively. In Eq. 2 one term, $H^+/6960$, has positive coefficient, which means that at this position an electron-withdrawing group increases the weight of the femur ash, six terms, $CH_3/1416$, $CH_3/1435$, $CH_3/3696$, $CH_3/6301$, $CH_3/6660$ and $CH_3/9509$, have positive coefficient, which means that at these positions large group increases the weight of the femur ash, three terms, $HO^-/4153$, $HO^-/4548$ and $HO^-/5652$, have positive coefficient, which means that at these positions hydrogen bond forming group increases the weight of femur ash, and one term, $HO^-/1720$, has negative coefficient, which means that at this position hydrogen bond forming group decreases the weight of the femur ash. According to the value of term coefficient of the equation if a conjugate has its electron-withdrawing group near $H^+/6960$ and/or hydrogen bond forming group near $HO^-/4153$–5652 and/or bulk group near $CH_3/6660$–9509 it will possess high anti-osteoporosis activity.

To explain this requirement representatives Estradiol-3F, Estradiol-17F, and Estradiol-3F,17F are given in Figure 7a, as well as Estrone-3F and Estrone-3FF are given in Figure 7b. Besides the same electrostatic and environments as estradiol ring Estradiol-3F has no hydrogen bond forming group near $HO^-/5652$ and thus results in no increase of the weight of femur ash, Estradiol-17F has a bulk group near $CH_3/9509$ and small group near $CH_3/6660$ and totally results in no increase of the weight of femur ash, while Estradiol-3F,17F has an electron-releasing group near $H^+/6960$, an electron-withdrawing group near $HO^-/1720$ and peptide chain over $CH_3/3696$ and thus results in high weight of femur ash. Estrone-3F has hydrogen bond forming groups near $HO^-/1720$, $HO^-/4153$ and $HO^-/4548$, thus totally results in moderate weight of femur ash, while Estrone-3FF has hydrogen bond forming group near $HO^-/4153$ and bulk groups near $CH_3/1416$, $CH_3/1435$, $CH_3/6301$ and $CH_3/6660$, thus results in high weight of femur ash.

2.8.2.3. MFA model for bleeding time after mice receiving estrogen and conjugates. The MFA model for bleeding time 90 min after the mice receiving estrogen and conjugates in terms of the descriptors proton, methyl and hydroxyl groups is aligned

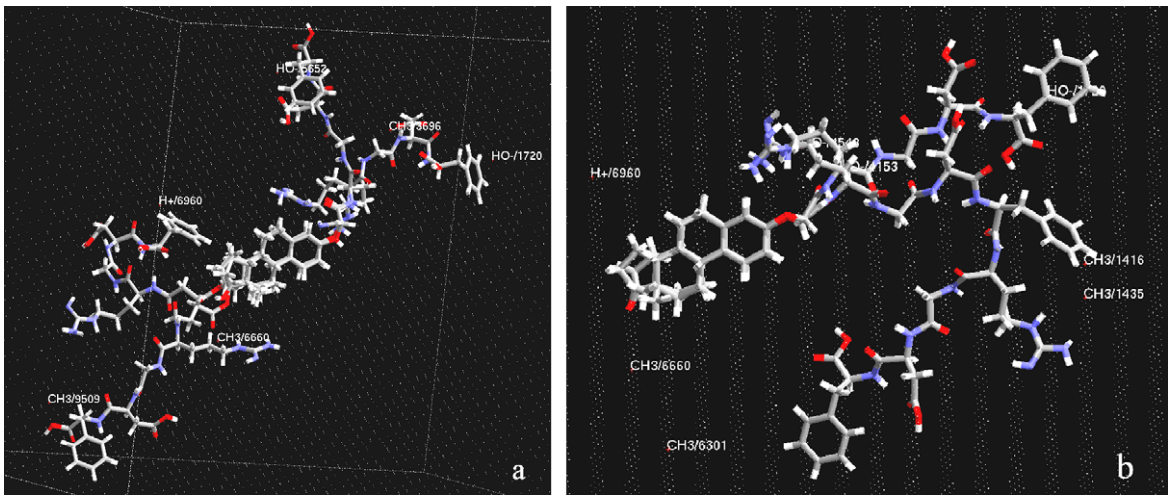


Figure 7. Electrostatic and environments of Estradiol-3F, Estradiol-17F, and Estradiol-3F,17F (a) and Estrone-3F and Estrone-3FF (b) within the grid with 3D points of Eq. 2.

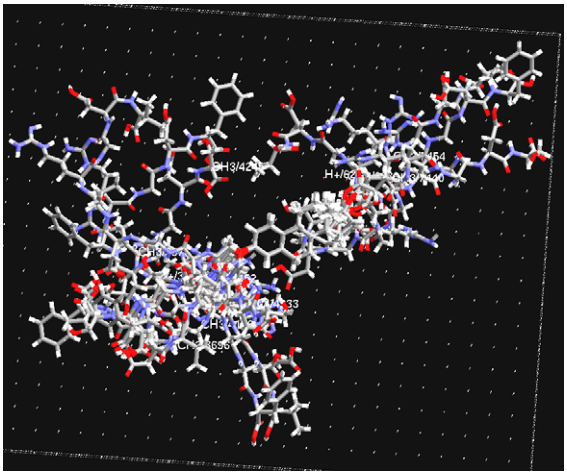


Figure 8. Alignment stereoview of estrogen and estrogen–RGD peptide conjugates affecting on the bleeding time at 90 min after the mice were administratered.

as Figure 8 and expressed by Eq. 3. The correlation of the bleeding time tested on the ovariectomized mouse model and the bleeding time calculated from Eq. 3 is explained by Figure 9.

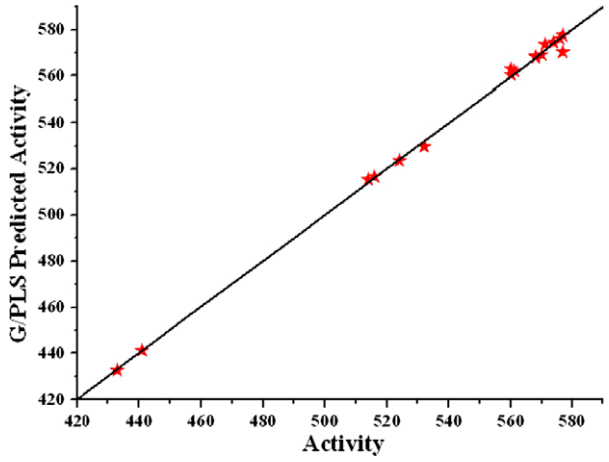


Figure 9. Graph of tested bleeding time against predicted bleeding time of mice receiving estrogen and estrogen–RGD peptide conjugates.

$$\begin{aligned}\text{Bleeding time} = {} & 404.62 + 0.51(H^+/3351) - 1.33(H^+/6241) \\ & - 1.3(CH_3/2976) - 0.74(CH_3/3696) \\ & + 5.22(CH_3/4152) - 1.2(CH_3/4254) \\ & - 0.19(CH_3/6636) + 5.46(CH_3/7440) \\ & - 0.018(CH_3/7454) - 0.21(HO^-/4531) \\ & - 0.037(HO^-/4933) \end{aligned} \quad (3)$$

In Eq. 3 the data points (n), correlation coefficient (r) and square correlation coefficient (r^2) were 17, 0.977 and 0.994, respectively. In Eq. 3 one term, $H^+/3351$, has positive coefficient, which means that at this position electron-withdrawing group increases bleeding time, one term, $H^+/6241$, has negative coefficient, which means that at this position electron-releasing group increases bleeding time, two terms, $CH_3/4152$ and $CH_3/7440$, have positive coefficient, which means that at these positions bulk group increases bleeding time, five terms, $CH_3/2976$, $CH_3/3696$, $CH_3/4254$, $CH_3/6636$ and $CH_3/7454$ have negative coefficient, which means that at these positions small group increases bleeding time, and two terms, $HO^-/4531$ and $HO^-/4933$, have negative coefficient, which means that at these positions hydrogen bond forming group increases bleeding time. According to the value of term coefficient of the equation if a conjugate has its electron-releasing group near $H^+/6241$ and/or small group near $CH_3/4152$ and $CH_3/7440$ it will possess low thromboembolic risk.

To explain this requirement representatives Estradiol-3S, Estradiol-17S, and Estradiol-3S,17S are given in Figure 10a, as well as Estrone-3F and Estrone-3FF are given in Figure 10b. Besides the same electrostatic and environments as estradiol ring, Estradiol-3S has small groups near $CH_3/3696$ and $CH_3/4252$ and thus results in lengthening bleeding time, Estradiol-17S has bulk group near $CH_3/7440$ and resulting in lengthening bleeding time, while Estradiol-3S,17S has electron-releasing group near $H^+/3351$, small groups near $CH_3/7454$, $CH_3/6636$ and $CH_3/3696$, as well as bulk group near $CH_3/4152$ thus results in significant length of bleeding time. Estrone-3F has small group near $CH_3/2976$ and thus results in lengthening of the bleeding time, while Estrone-3FF has small group near $CH_3/2976$, peptide chain over $CH_3/4152$, electron-withdrawing groups near $H^+/3351$ and thus results in low thromboembolic risk.

2.8.2.4. MFA model for uterine weight of mice receiving estrogen and conjugates. The MFA model for uterine weight of the mice receiving estrogen and conjugates in terms of the descriptors proton, methyl and hydroxyl groups is aligned as Figure 11 and

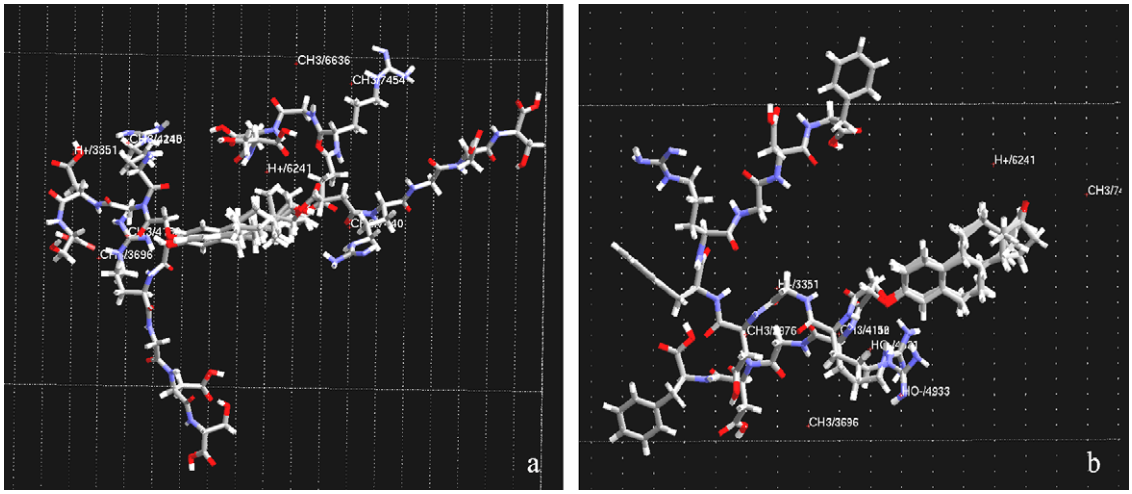


Figure 10. Electrostatic and environments of Estradiol-3S, Estradiol-17S, and Estradiol-3S,17S (a) as well as Estrone-3F and Estrone-3FF (b) within the grid with 3D points of Eq. 3.

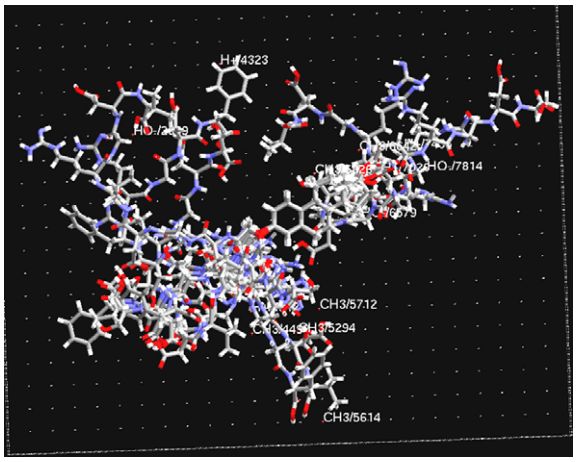


Figure 11. Alignment stereoview of estrogen and estrogen–RGD peptide conjugates affecting on uterine weight.

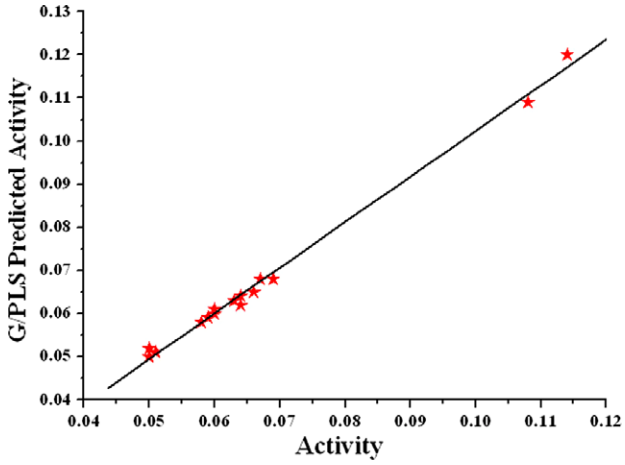


Figure 12. Graph of tested uterine weight against predicted uterine weight of mice receiving estrogen and estrogen–RGD peptide conjugates.

expressed by Eq. 4. The correlation of uterine weights tested on the ovariectomized mouse model and uterine weights calculated from Eq. 4 is explained by Figure 12.

$$\begin{aligned}\text{Uterine weight} = {} & 0.058 + 0.000635(H^+/4323) \\ & + 0.000372(H^+/4512) + 0.000653(H^+/7020) \\ & + 0.000111(CH_3/5294) + 0.00001(CH_3/5614) \\ & + 0.00097(CH_3/5712) + 0.0021(CH_3/5826) \\ & - 0.0016(CH_3/6642) - 0.000374(HO^-/2669) \\ & - 0.0017(HO^-/6579) - 0.000374(HO^-/7814) \qquad (4)\end{aligned}$$

In Eq. 4 the data points (n), correlation coefficient (r), square correlation and coefficient (r^2) were 17, 0.990 and 0.995, respectively. In Eq. 4 three terms, $H^+/4323$, $H^+/4512$ and $H^+/7020$, have positive coefficient, which means that at these positions electron-withdrawing group increases uterine weight, four terms, $CH_3/5294$, $CH_3/5614$, $CH_3/5712$ and $CH_3/5826$, have positive coefficient, which means that at these positions bulk group increases uterine weight, one term, $CH_3/6642$, has negative coefficient, which means that at this position small group increases uterine weight, three terms, $HO^-/2669$, $HO^-/6579$ and $HO^-/7814$, have negative coefficient, which means that at these positions hydrogen bond forming group increases uterine weight. According to the value of term coefficient of equation if a conjugate has its electron-releasing group near $H^+/4323$–7020 and/or small group near $CH_3/5294$–6642 it will possess low risk of endometrial hyperplasia.

To explain this requirement representatives Estradiol-3S, Estradiol-17S, and Estradiol-3S,17S are given in Figure 13a, as well as Estrone-3F and Estrone-3FF are given in Figure 13b. Besides the same electrostatic and environments as estradiol ring, Estradiol-3S has electron-withdrawing and releasing groups near $H^+/4512$ and thus results in no increase of uterine weight, Estradiol-17S has electron-withdrawing and releasing groups near $H^+/7020$ and hydrogen bond forming group near $HO^-/6579$ and thus results in the decrease of uterine weight, while Estradiol-3S,17S has electron-withdrawing and releasing groups near $H^+/7020$ and peptide chain over $CH_3/6642$ and thus results in low uterine weight. Besides the same electrostatic and environments as estrone, Estrone-3F has electron-releasing group near $H^+/4512$ and thus resulting in decrease of uterine weight, while Estrone-3FF has electron-releasing group near $H^+/4323$ and hydrogen bond forming group near $HO^-/2669$ thus results in significant decrease of uterine weight.

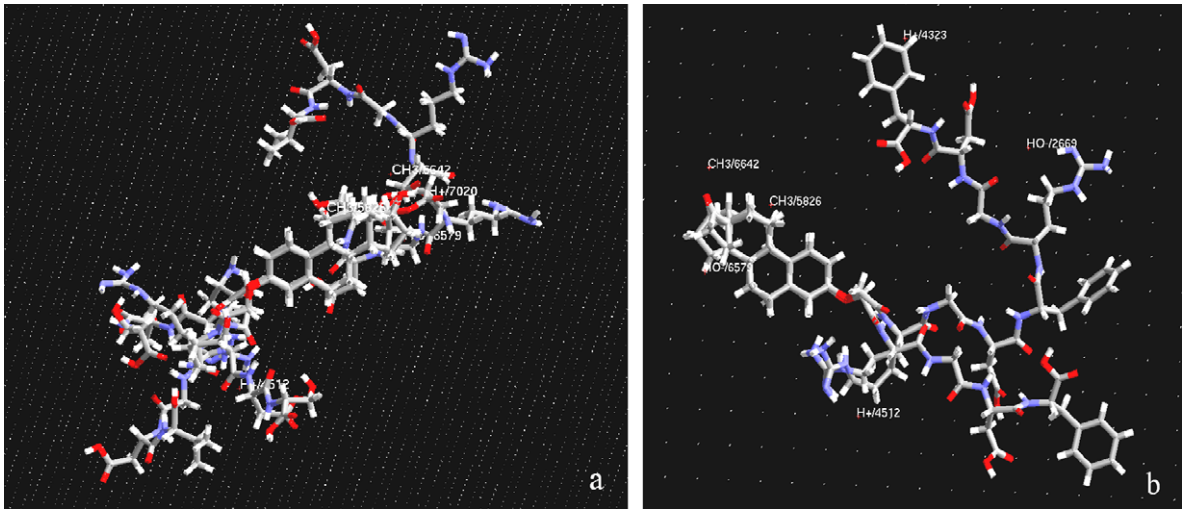


Figure 13. Electrostatic and environments of Estradiol-3S, Estradiol-17S, and Estradiol-3S,17S (a) and Estrone-3F and Estrone-3FF (b) within the grid with 3D points of Eq. 4.

3. Conclusions

Osteoporosis has being crushing pressure for our society. The modifications of estradiol and estrone aimed at the improvement of ERT or HRT are clinically important. The observations provided here explored that introducing RGD-tetrapeptide into both 3-position and 17-position of estradiol, as well as introducing RGD-octapeptide into 3-position of estrone did result in the conjugate with high oral anti-osteoporosis activity and lower risk of endometrial hyperplasia and thromboembolic events. With the helps of the 3D QSAR analysis the conjugate met the requirements related to high anti-osteoporosis activity and/or low side effect could be designed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.03.057.

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