Does ligand–receptor mediated competitive effect or penetrating effect of iRGD peptide when co-administration with iRGD-modified SSL?

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Abstract
Ligand-mediated targeting of anticancer therapeutic agents is a useful strategy for improving anti-tumor efficacy. It has been reported that co-administration of a tumor-penetrating peptide iRGD (CRGDK/RGPD/EC) enhances the efficacy of anticancer drugs. Here, we designed an experiment involving co-administration of iRGD-SSL-DOX with free iRGD to B16-F10 tumor bearing mice to examine the action of free iRGD. We also designed an experiment to investigate the location of iRGD-modified SSL when co-administered with free iRGD or free RGD to B16-F10 tumor bearing nude mice. Considering the sequence of iRGD, we selected the GPDC, RGD and CRGDK as targeting ligands to investigate the targeting effect of these peptides compared with iRGD on B16-F10 and MCF-7 cells, with or without enzymatic degradation. Finally, we selected free RGD, free CRGDK and free iRGD as ligand to investigate the inhibitory effect on RGD-, CRGDK- or iRGD-modified SSL on B16-F10 or MCF-7 cells. Our results indicated that iRGD targeting to tumor cells was ligand–receptor mediated involving RGD to $\alpha_v$-integrin receptor and CRGDK to NRP-1 receptor. Being competitive effect, the administration of free iRGD would not be able to further enhance the anti-tumor activity of iRGD-modified SSL. There is no need to co-administrate of free iRGD with the iRGD-modified nanoparticles for further therapeutic benefit.

Introduction
Ligand-mediated targeting of anticancer therapeutic agents is a useful strategy for improving anti-tumor efficacy [1]. This targeting effect relies on ligand binding to receptors which are either uniquely expressed or overexpressed on the target cells. Now, many tumor-homing peptides and tumor-penetrating peptides have been used as ligands to deliver anticancer drug delivery systems specifically to the tumor site [2,3].

iRGD (CRGDK/RGPD/EC), a tumor-homing and tumor-penetrating peptide, has been reported [4]. The mechanism by which iRGD homes on the tumor involves iRGD being able to target tumors by initially binding to $\alpha_v$-integrin and then undergoing proteolytic cleavage in the tumor to produce CRGDK/R which has an affinity for neuropilin-1 (NRP-1) resulting in tissue penetration [4]. iRGD-modified nanocarriers [5–10] and iRGD-conjugated drug or factor [11–13] have been reported to improve the anti-tumor activity. More interestingly, it has been reported that co-administration of free iRGD can enhance the efficacy of anti-cancer drugs including those with a low-molecular weight (doxorubicin), nanoparticles (nab-paclitaxel and doxorubicin liposomes) and monoclonal antibodies (trastuzumab) [14]. This effect has been also observed in gemcitabine co-administered with free iRGD peptide [15], in paclitaxel-loaded MT1-AF7p-conjugated nanoparticles co-administered with free iRGD [16] and in methoxypoly(ethylene glycol)-block-poly(1-glutamic acid)-loaded cisplatin combined with free iRGD [17]. These exciting results suggest that iRGD can be used as a penetrating agent for improving anti-tumor activity when co-administered with anti-cancer drugs or anti-cancer drug delivery systems.

We have demonstrated the anti-tumor activity of doxorubicin-loaded iRGD-modified liposomes (iRGD-SSL-DOX) on B16-F10 melanoma cells in a previous report [7], and, regarding the effect of iRGD, we want to know if the anti-tumor activity of iRGD-SSL-DOX co-administered with free iRGD could be further enhanced.

Materials and methods
Materials
1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene-glycol)-2000 (DSPE-PEG-MAL) and 1,2-distearoyl-sn-glycero-3-phosphatidylethanol-amine-N-[methoxy (polyethylene-glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL). N-hydroxysuccinimidyld-PEG2000-DSPE (DSPE-PEG-NHS) was provided by
Molecular Probes Inc. (Eugene, OR). Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO), purchased from Lipoid GmbH (Ludwigshafen, Germany) (Zhejiang, China). Egg phosphatidylcholine (EPC) was purchased from the Academy of Military Medical Sciences, Beijing, China. iRGD, GPDC, RGD and CRGDK peptide were synthesized by GL Bio-Chem Co, Ltd (Shanghai, China). iRGD-PEG-DPPE, GPDC-PEG-DSPE and CRGDK-PEG-DSPE were synthesized from iRGD, GPDC or CRGDK peptide and DSPE-PEG-MAL using a method previously reported by our laboratory [7]. RGD-PEG-DSPE was synthesized from RGD peptide and DSPE-PEG-NHS using a method previously reported by our laboratory [18]. Doxorubicin hydrochloride (DOX) was supplied by Hisun Pharmaceutical Co. Ltd (Zhejiang, China). iRGD (CRGDK/RGPD/EC), iRGD-SSL-DiI, RGD-SSL-DiI and SSL-DiI were prepared by the thin-film hydration method. Cell culture media, DMEM (high glucose), RPMI 1640, penicillin-streptomycin and fetal bovine serum were obtained from GIBCO Invitrogen Corp. (Carlsbad, CA). All other reagents were of analytical grade.

Cells and animals

Murine B16-F10 cells (Chinese Academy of Sciences Cells Bank, Shanghai, China) and human breast adenocarcinoma MCF-7 cells (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China) were cultivated according to the recommended instructions.

Female C57BL/6 mice weighing 20–25 g (6–8 weeks) were obtained from the Experimental Animal Center of Peking University Health Science Center (Beijing, China). Female BALB/c nude mice (initially weighing 20–25 g) were purchased from the Academy of Military Medical Sciences (Beijing, China). All care and handling of the animals were performed according to the requirements of the Institutional Authority for Laboratory Animal Care of Peking University. For preparation of the tumor-bearing model, C57BL/6 mice or BALB/c nude mice received an s.c. inoculation of 100 μl B16-F10 cell suspension (1 × 10⁶) in the right armpit.

In vivo anti-tumor activity of iRGD-SSL-DOX

When the tumor volume reached about 200 mm³, the tumor-bearing C57BL/6 mice were treated with physiological saline as control, free iRGD (4 μmol/kg), SSL-DOX, SSL-DOX co-administered with free iRGD (4 μmol/kg), iRGD-SSL-DOX, and iRGD-SSL-DOX co-administered with free iRGD (4 μmol/kg). The DOX formulations were all given via the tail vein on days 12, 16 and 20, at a dose of 3 mg/kg. Throughout the study, mice were weighed and tumors were measured with calipers twice a week. Tumor volumes were calculated from the formula: \( V = \text{length (cm)} \times \text{width (cm)} \times 0.5 \).

On day 22 after tumor inoculation, one or two mice in each group were executed, and the tumors were collected for the preparation of histological sections. TUNEL staining of the paraffin-embedded tissue sections was performed according to the standard protocols provided by the manufacturers.

The survival time was calculated from the day of B16-F10 cell inoculation (0 day) to the day of death. Kaplan–Meier survival curves were plotted for each group.

Confocal immunofluorescence microscopy study

In order to investigate the microcosmic characteristics of distribution differences of the SSL, we used DiI as a tracer and examined the location of iRGD-SSL-DiI, RGD-SSL-DiI and SSL-DiI, with or without co-administration of iRGD or RGD. When the tumor volume reached about 200 mm³, the tumor-bearing BALB/c nude mice were treated with SSL-DiI, SSL-DiI + RGD, SSL-DiI + iRGD, RGD-SSL-DiI, RGD-SSL-DiI + RGD, RGD-SSL-DiI + iRGD, iRGD-SSL-DiI, iRGD-SSL-DiI + RGD and iRGD-SSL-DiI + iRGD. The dose of all DiI formulations was 1600 ng per mouse. Free iRGD or free RGD co-administered with DiI formulations was injected at a dose of 4 μmol/kg. At 3, 6 or 12 h after administration, the mice were sacrificed, and the tumors were harvested and frozen in OCT embedding medium. Tumor sections (8 μm) were incubated with 10% BSA for 3 h at room temperature, and nuclei were counterstained with Hoechst 33258 (5 μg/ml). The sections were placed in gel/mount medium (Biomed, Foster City, CA) and observed under a confocal microscope (Leica, Germany). Finally, sections were investigated by a confocal laser scanning microscope (CLSM, Leica SP5, Germany) and were quantified with a Leica Qwin image analysis software (TUNEL, immunofluorescence).

Flow cytometry

B16-F10 cells were seeded at a density of 3 × 10⁵ cells/well in 6-well plates and incubated at 37°C for 24 h. Then, the medium was replaced with SSL-coumarin-6, GPDC-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 (the final concentration of coumarin-6 was 150 ng/ml). The plates were divided in two groups (with or without trypsin treatment). For group I, without trypsin treatment, after a 2-h incubation at 37°C, the cells were washed three times with PBS solution. For group II, with trypsin treatment, SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 (the final concentration of coumarin-6 was 150 ng/ml) was added in 50 μl trypsin solution (250 μg/ml) and incubated for 5 min at 37°C, and then the soybean inhibitor (50 μl, 30 mg/ml) was added to stop the reaction. The above solutions were then incubated with the cells for 2 h at 37°C. Finally, the cells were washed three times with PBS, and the cells in groups I and II were harvested by trypsinization, centrifuged at 1000 rpm for 5 min, resuspended in 500 μl PBS medium and examined using an FACSscan (Becton Dickinson, San Jose, CA). The coumarin-6 in the cells was excited with an argon laser (467 nm), and fluorescence was detected at 502 nm.
In another flow cytometer experiment, the MCF-7 cells were seeded at a density of $2 \times 10^5$ cells/well in 6-well plates and processed as described above using a B16-F10 cell line.

**Competition experiment**

For the competition experiment, B16-F10 cells were seeded at a density of $3 \times 10^5$ cells/well in 6-well plates and incubated at $37^\circ C$ for 24 h. After that, cells were pre-incubated with 1 mM free RGD, free CRGDK or free iRGD peptides for 30 min to saturate the receptor. Then, the medium was replaced with SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 (the final concentration of coumarin-6 was 150 ng/ml) for a 2-h incubation at $37^\circ C$. Then, the cells were washed three times with PBS. All cells were harvested by trypsinization and centrifuged at 1000 rpm for 5 min and resuspended in 500 μl PBS medium and tested using an FACScan (Becton Dickinson, San Jose, CA). The coumarin-6 in the cells was excited with an argon laser (467 nm), and fluorescence was detected at 502 nm.

In another competition experiment, MCF-7 cells were seeded at a density of $2 \times 10^5$ cells/well in 6-well plates and treated as described above using a B16-F10 cell line.

**Statistical analysis**

All data are shown as the mean ± SD. One-way analysis of variance (ANOVA) was used to determine significance among groups, after which post-hoc tests with the Bonferroni correction were used to compare differences between individual groups. Statistical significance was set at $p < 0.05$.

**Results and discussion**

In the present study, we designed a co-administration experiment involving B16-F10 tumor bearing C57BL/6 mice to examine the anti-tumor activity of iRGD-SSL-DOX when co-administered with free iRGD. As shown in Figure 1(A), the tumor growth was significantly inhibited in the iRGD-SSL-DOX and SSL-DOX treatment groups compared with the physiological saline-treated group used as a control ($p < 0.01$). iRGD-SSL-DOX significantly inhibited the growth of B16-F10 tumors compared with that in the SSL-DOX treatment group ($p < 0.01$). In addition, we observed that the anti-tumor activity of free iRGD was not significantly different from that seen in the control group ($p > 0.05$, Figure 1A).

As shown in Figure 1(A), our results showed that the anti-tumor activity of iRGD-SSL-DOX co-administered with free iRGD was similar to that of iRGD-SSL-DOX ($p > 0.05$). The median survival time of mice treated with iRGD-SSL-DOX co-administered with free iRGD (44 days) was also similar to that of mice treated with iRGD-SSL-DOX (46 days, $p > 0.05$), as shown in Figure 1(B). We also evaluated the effect of tumor cell apoptosis by TUNEL analysis staining of tumor tissue sections. As shown in Figure 2(E–G), tumors from the iRGD-SSL-DOX co-administered free iRGD-treated group exhibited similar cell apoptosis compared with the iRGD-SSL-DOX-treated group ($p > 0.05$). The data supporting these findings are shown in Figure 2(G).

In addition, similar results were also observed between SSL-DOX co-administered free iRGD group and SSL-DOX group, as shown in Figures 1 and 2.

In the light of these results, we believe that free iRGD has no effect when co-administered with iRGD-SSL-DOX or SSL-DOX. It has been reported that the plasma half-life of free iRGD is only about 8 min [19], so, if free iRGD is rapidly eliminated after intravenous administration, it will have no effect. Therefore, we designed an experiment to investigate the location of iRGD-modified SSL and RGD-modified SSL when co-administered with free iRGD or free RGD. We selected DiI as a fluorescent agent to prepare iRGD-SSL-DiI, RGD-SSL-DiI and SSL-DiI. B16-F10 tumor-bearing BALB/c nude mice were given intravenous iRGD-SSL-DiI, RGD-SSL-DiI and SSL-DiI, with or without co-administered free iRGD or free RGD. Then, 3, 6 or 12 h after administration, the mice were euthanized, and the tumor tissues were collected and cut into sections. These tumor sections were stained and observed under a confocal microscope. The obtained results indicated that the red fluorescence intensity of DiI in the tumor sections in the iRGD-SSL-DiI treatment group was stronger than that in the RGD-SSL-DiI and SSL-DiI treatment groups, confirming the targeting effect of iRGD-modified SSL (Figure 3). We observed that the fluorescence intensity of iRGD-SSL-DiI co-administered with free iRGD was significantly reduced compared with that of iRGD-SSL-DiI ($p < 0.01$). Similar results were also observed for iRGD-SSL-DiI co-administered with free RGD compared with that of iRGD-SSL-DiI ($p < 0.01$). In addition, the inhibitory effect of free iRGD on iRGD-SSL-DiI was higher than that of free RGD on iRGD-SSL-DiI. We also found that the fluorescence intensity was reduced when RGD-SSL-DiI was co-administered with free iRGD or free RGD compared with that of RGD-SSL-DiI ($p < 0.01$). Interestingly, the fluorescence intensity of SSL-DiI was not increased following co-administration of free iRGD or free RGD ($p > 0.05$). Considering these findings, we believe that co-administration of free iRGD or free RGD would not increase the cumulative effect of SSL-DiI and might even reduce the accumulation of iRGD-SSL-DiI or RGD-SSL-DiI. In the case of ligand-modified delivery systems, the targeting effect relies on ligand–receptor specific binding. If the receptor sites are occupied by free ligand, the ligand-modified delivery system will not be able to bind to these receptor sites. Therefore, the targeting effect of this ligand-modified delivery system will be reduced [20]. We believe that if free iRGD or free RGD ligand occupied the receptor sites, this would result in a reduced ligand–receptor binding effect of iRGD-modified SSL.

Considering the sequence of iRGD (CRGDK/RGD/EC), in the present research, we selected GPDC, RGD and CRGDK as targeting ligands to investigate the targeting effect of these peptides compared with iRGD. Coumarin-6 was selected as a fluorescent agent to prepare SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 and iRGD-SSL-coumarin-6. We selected both over-expressing integrin and over-expressing NRP-1 receptors B16-F10 cells and negative-expressing integrin.
receptor and over-expressing NRP-1 receptor MCF-7 cells as cell models. Our results indicated that an excellent targeting effect was observed in RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 and iRGD-SSL-coumarin-6, but not GPDC-SSL-coumarin-6, compared with SSL-coumarin-6 in B16-F10 cells [Figure 4(A1, A2)]. We also observed a targeting effect with CRGDK-SSL-coumarin-6 and iRGD-SSL-coumarin-6, but not GPDC-SSL-coumarin-6 and RGD-SSL-coumarin-6, compared with SSL-coumarin-6 in MCF-7 cells [Figure 4(B1, B2)]. This is due to the ligand–receptor interaction, involving RGD binding to αv-integrin receptor, CRGDK binding to NRP-1 receptor, iRGD binding to integrin receptor and then binding to NRP-1 receptor.

Considering to the enzymatic degradation of iRGD, we investigated the proteolytic cleavage effect on the targeting of GPDC, RGD, CRGDK and iRGD ligands (Figure 5). The targeting effect of RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 and iRGD-SSL-coumarin-6 was also observed in B16-F10 cells after enzymatic degradation [Figure 5(A1, A2)]. Also, the targeting effect of CRGDK-SSL-coumarin-6

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**Figure 1.** *In vivo* anti-tumor activity of iRGD-SSL-DOX co-administered with free iRGD. (A): Tumor growth. C57BL/6 mice were inoculated SC with B16-F10 cells and treated with physiological saline as a control, free iRGD (4 μmol/kg), SSL-DOX, SSL-DOX co-administered with free iRGD (4 μmol/kg), iRGD-SSL-DOX, iRGD-SSL-DOX co-administered with free iRGD (4 μmol/kg). The DOX formulations were given via the tail vein for all administrations on day 12, 16 and 20 at a dose of 3 mg/kg. The tumors were measured with calipers twice a week throughout the study. (B): Kaplan-Meier survival curves. Kaplan-Meier survival curves of B16-F10 tumor-bearing C57BL/6 mice treated with physiological saline as a control, free iRGD (4 μmol/kg), SSL-DOX, SSL-DOX co-administered with free iRGD (4 μmol/kg), iRGD-SSL-DOX and iRGD-SSL-DOX co-administered with free iRGD (4 μmol/kg). The DOX formulations were given via the tail vein for all administrations on day 12, 16 and 20 at a dose of 3 mg/kg. Results indicated that the iRGD-SSL-DOX (46.2 days) significantly improved the survival rate of mice compared with those treated with SSL-DOX (33.8 days, $p<0.01$) and physiological saline (27 days, $p<0.01$). The median survival time of mice treated with iRGD-SSL-DOX co-administered with free iRGD (44 days) was similar to that of mice treated with iRGD-SSL-DOX (46 days $p>0.05$). The median survival time of mice treated with SSL-DOX co-administered with free iRGD (44 days) was also similar to that of mice treated with SSL-DOX (33.8 days $p>0.05$). The median survival time of the free iRGD group (27.7 days) was not significantly different compared with the control group (27 days, $p>0.05$). **$p<0.01$ versus the physiological saline treatment group as a control; &&$p<0.01$ versus the SSL-DOX treatment group.
Figure 2. Effects of iRGD-SSL-DOX co-administered with free iRGD on the apoptosis of B16-F10 melanoma tumors. C57BL/6 mice were inoculated SC with B16-F10 cells and treated with physiological saline as a control, free iRGD (4 μmol/kg), SSL-DOX, SSL-DOX co-administered with free iRGD (4 μmol/kg), iRGD-SSL-DOX, iRGD-SSL-DOX co-administered with free iRGD (4 μmol/kg). The DOX formulations were given via the tail vein for all administrations on day 12, 16 and 20 at a dose of 3 mg/kg. On day 22 day of tumor inoculation, one or two mice in each group were executed and the tumors were collected for the preparation of histological sections. TUNEL staining of the paraffin-embedded tumors was performed according to the standard protocols provided by the manufacturers. Tumor apoptosis cells were detected by TUNEL (A–E). ((A1–A3) physiological saline as a control; (B1–B3) free iRGD; (C1–C3) SSL-DOX; (D1–D3) SSL-DOX + free iRGD; (E1–E3) iRGD-SSL-DOX; (F1–F3) iRGD-SSL-DOX + free iRGD). DNA strand breaks were labelled and nuclei were stained with Hoechst 332589. Apoptotic cells exhibited a turquoise color as a result of color merging of these two labels. The fluorescence area of each group was used for the statistical analysis of apoptosis activity (G). **p < 0.01 versus the physiological saline group as a control; &&p < 0.01 versus the SSL-DOX treatment group.
and iRGD-SSL-coumarin-6 was observed in MCF-7 cells after enzymatic degradation [Figure 5(B1, B2)]. In addition, we found that proteolytic cleavage did not affect the targeting effect intensity of GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6 and CRGDK-SSL-coumarin-6 on both B16-F10 and MCF-7 cells (Tables S1 and S2). However, the targeting effect intensity of iRGD-SSL-coumarin-6 was significantly increased after enzymatic degradation in both B16-F10 and MCF-7 cells, about a 20% increase compared with non-enzymatic degradation (Tables S1 and S2). We believe that CRGDK, produced by iRGD degradation, would further increase the targeting effect of iRGD-SSL-coumarin-6 in B16-F10 cells and, especially, in MCF-7 cells (Tables S1 and S2). This is consistent with the mechanism of iRGD by which
Figure 3. Localization of iRGD-SSL-DiI co-administered with free iRGD in B16-F10 melanoma tumor tissues. BALB/c nude mice bearing B16-F10 tumors were intravenously injected with SSL-DiI, SSL-DiI + RGD, SSL-DiI + iRGD, RGD-SSL-DiI, RGD-SSL-DiI + RGD, RGD-SSL-DiI + iRGD, iRGD-SSL-DiI, iRGD-SSL-DiI + RGD and iRGD-SSL-DiI + iRGD. The dose of all DiI formulations was 1600 ng per mouse. Free iRGD or free RGD co-administered with DiI formulations was injected at a dose of 4 μmol/kg. At 3, 6 or 12 h after administration, the mice were sacrificed, and the tumors were harvested and tumor sections were stained for examination of the red fluorescence. A1: 3 h after being given DiI formulations; B1: 6 h after being given DiI formulations; C1: 12 h after being given DiI formulations. The fluorescence area of each group was used for the statistical analysis of fluorescence activity (A2: 3 h after being given DiI formulations; B2: 6 h after being given DiI formulations; C2: 12 h after being given DiI formulations). Nuclei were counterstained with Hoechst 33258; DiI exhibited a red fluorescence. **p < 0.01 vs RGD-SSL-DiI; &&p < 0.01 vs iRGD-SSL-DiI.
Figure 3. Continued.

Figure 4. The flow cytometric measurement of coumarin-6 uptake by B16-F10 cells and MCF-7 cells. The flow cytometric measurement of coumarin-6 uptake from SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 by B16-F10 cells (A1) and MCF-7 cells (B1). The calculated fluorescence intensity of coumarin-6 uptake from SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 by B16-F10 cells (A2) and MCF-7 cells (B2). **p < 0.01 vs SSL-coumarin-6; &p < 0.01 vs RGD-SSL-coumarin-6; $$$p < 0.01$ vs CRGDK-SSL-coumarin-6.
iRGD targets tumors by initially binding to αv-integrin and then undergoing proteolytic cleavage in the tumor to produce CRGDK/R which has an affinity for NRP-1 receptor.

Next, we selected free RGD, free CRGDK or free iRGD as ligand to investigate the inhibitory effect on SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 after trypsin treatment by B16-F10 cells (A1) and MCF-7 cells (B1). The calculated fluorescence intensity of coumarin-6 uptake from SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 after trypsin treatment by B16-F10 cells (A2) and MCF-7 cells (B2). **p<0.01 vs SSL-coumarin-6; &&p<0.01 vs RGD-SSL-coumarin-6; $$p<0.01 vs CRGDK-SSL-coumarin-6.

In our previous research, we investigated the endocytosis pathway of iRGD-modified SSL [21]. Our results indicated that energy and lipid raft endocytosis was exhibited in iRGD-modified SSL. In the present research, we also investigated the endocytosis pathway of RGD- and

**Figure 5.** The flow cytometric measurement of coumarin-6 uptake by B16-F10 cells and MCF-7 cells, with trypsin treatment. The flow cytometric measurement of coumarin-6 uptake from SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 after trypsin treatment by B16-F10 cells (A1) and MCF-7 cells (B1). The calculated fluorescence intensity of coumarin-6 uptake from SSL-coumarin-6, GPDC-SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 after trypsin treatment by B16-F10 cells (A2) and MCF-7 cells (B2). **p<0.01 vs SSL-coumarin-6; &&p<0.01 vs RGD-SSL-coumarin-6; $$p<0.01 vs CRGDK-SSL-coumarin-6.
Figure 6. Competitive effect of free RGD, free CRGDK or free iRGD on SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 in B16-F10 cells. The flow cytometric measurement of coumarin-6 uptake from SSL-coumarin-6, SSL-coumarin-6+RGD, SSL-coumarin-6+CRGDK, SSL-coumarin-6+iRGD (A1); RGD-SSL-coumarin-6, RGD-SSL-coumarin-6+RGD, RGD-SSL-coumarin-6+CRGDK, RGD-SSL-coumarin-6+iRGD (B1); CRGDK-SSL-coumarin-6, CRGDK-SSL-coumarin-6+RGD, CRGDK-SSL-coumarin-6+CRGDK, CRGDK-SSL-coumarin-6+iRGD (C1); iRGD-SSL-coumarin-6, iRGD-SSL-coumarin-6+RGD, iRGD-SSL-coumarin-6+CRGDK, and iRGD-SSL-coumarin-6+iRGD (D1). The calculated fluorescence intensity of coumarin-6 uptake from SSL-coumarin-6, SSL-coumarin-6+RGD, SSL-coumarin-6+CRGDK, SSL-coumarin-6+iRGD (A2); RGD-SSL-coumarin-6, RGD-SSL-coumarin-6+RGD, RGD-SSL-coumarin-6+CRGDK, RGD-SSL-coumarin-6+iRGD (B2); CRGDK-SSL-coumarin-6, CRGDK-SSL-coumarin-6+RGD, CRGDK-SSL-coumarin-6+CRGDK, CRGDK-SSL-coumarin-6+iRGD (C2); iRGD-SSL-coumarin-6, iRGD-SSL-coumarin-6+RGD, iRGD-SSL-coumarin-6+CRGDK, and iRGD-SSL-coumarin-6+iRGD (D2). **p < 0.01 vs RGD-SSL-coumarin-6; $$p < 0.01 vs CRGDK-SSL-coumarin-6; &&p < 0.01 vs iRGD-SSL-coumarin-6.
Figure 7. Competitive effect of free RGD, free CRGDK or free iRGD on SSL-coumarin-6, RGD-SSL-coumarin-6, CRGDK-SSL-coumarin-6 or iRGD-SSL-coumarin-6 in MCF-7 cells. The flow cytometric measurement of coumarin-6 uptake from SSL-coumarin-6, SSL-coumarin-6 + RGD, SSL-coumarin-6 + CRGDK, SSL-coumarin-6 + iRGD (A1); RGD-SSL-coumarin-6, RGD-SSL-coumarin-6 + RGD, RGD-SSL-coumarin-6 + CRGDK, RGD-SSL-coumarin-6 + iRGD (B1); CRGDK-SSL-coumarin-6, CRGDK-SSL-coumarin-6 + RGD, CRGDK-SSL-coumarin-6 + CRGDK, CRGDK-SSL-coumarin-6 + iRGD (C1); iRGD-SSL-coumarin-6, iRGD-SSL-coumarin-6 + RGD, iRGD-SSL-coumarin-6 + CRGDK, and iRGD-SSL-coumarin-6 + iRGD (D1). The calculated fluorescence intensity of coumarin-6 uptake from SSL-coumarin-6, SSL-coumarin-6 + RGD, SSL-coumarin-6 + CRGDK, SSL-coumarin-6 + iRGD (A2); RGD-SSL-coumarin-6, RGD-SSL-coumarin-6 + RGD, RGD-SSL-coumarin-6 + CRGDK, RGD-SSL-coumarin-6 + iRGD (B2); CRGDK-SSL-coumarin-6, CRGDK-SSL-coumarin-6 + RGD, CRGDK-SSL-coumarin-6 + CRGDK, CRGDK-SSL-coumarin-6 + iRGD (B2); iRGD-SSL-coumarin-6, iRGD-SSL-coumarin-6 + RGD, iRGD-SSL-coumarin-6 + CRGDK, and iRGD-SSL-coumarin-6 + iRGD (D2). **p < 0.01 vs RGD-SSL-coumarin-6; $p < 0.01$ vs CRGDK-SSL-coumarin-6; &p < 0.01 vs iRGD-SSL-coumarin-6.
CRGDK-modified SSL using an endocytosis inhibition assay in B16-F10 cells. As shown in Figure S1, the rate of uptake was inhibited at 4°C compared with that at 37°C ($p < 0.01$), and was also inhibited by M-β-CD ($p < 0.01$), confirming the energy and lipid raft endocytosis of RGD- and CRGDK-modified SSL. Other inhibitors, such as chlorpromazine, amiloride and filipin, had only a weak inhibitory effect on RGD- and CRGDK-modified SSL. Accordingly, our results indicate that the endocytosis pathways of RGD, CRGDK and iRGD were similar.

It is well known that RGD is more commonly used as a targeting ligand for drug delivery systems [22–25]. Also, CRGDK has been used as a targeting ligand for modified nanocarriers [26–28]. Many iRGD-modified nanocarriers have also been reported [5–10]. Compared with the ligands of RGD and CRGDK, we suggest that iRGD could bind to both the $\alpha v$-integrin and NRP-1 receptors which are over-expressed in tumor endothelial cells or tumor cells, highlighting the potentially wide targeting applications for the iRGD ligand.

Considering our present results, we suggest that the mechanism of iRGD targeting to tumor cells takes place via a ligand–receptor-mediated targeting effect, which is by both RGD to $\alpha v$-integrin receptors and CRGDK to NRP-1 receptors, as shown in Figure 8. The triggering penetration effect of iRGD peptide when co-administrated with drug or drug delivery systems should be investigated in detail.

It has also been reported that some targeting ligands, such as hyaluronic acid, can be directly absorbed on the surface of nanocarrier systems to achieve a targeting effect [29]. In the case of reports of co-administration of free iRGD increasing the efficacy of anticancer drugs or nanoparticles, we suggest that if free iRGD is combined with an anti-cancer drug or absorbed on the surface of nanoparticles, the targeting of these complexes would be iRGD mediated. This hypothesis should be investigated in greater detail.

In fact, the characteristic of SSL, such as size, surface properties as well as the ratio of iRGD on the SSL, is also a factor which might affect this conclusion. We would consider these factors in future research. In addition, for other nanocarrier system (such as micelle or nanoparticle), this conclusion should be also further investigated.

Conclusions

Considering our present results, we suggest that iRGD targeting to tumor cells is ligand–receptor mediated, involving RGD to $\alpha v$-integrin receptor or/and CRGDK to NRP-1 receptor. The administration of free iRGD would not be able to further enhance the anti-tumor activity of iRGD-modified SSL being competitive effect.

Declaration of interest

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Supplementary material available online
Figure S1 and Tables S1–S4.