Amino Acids (2013) 45:1149–1156
DOI 10.1007/s00726-013-1570-5

ORIGINAL ARTICLE

Targeted delivery of a phosphopeptide prodrug inhibits the proliferation of a human glioma cell line

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Received: 26 April 2013 / Accepted: 23 July 2013 / Published online: 2 August 2013

Abstract Peptides are ideal candidates for developing therapeutics. Polo-like kinase 1 is an important regulatory protein in the cell cycle and contains a C-terminal polo-box domain, which is the hallmark of this protein family. We developed a peptide inhibitor of polo-like kinase 1 that targets its polo-box domain. This new phosphopeptide, cRGDyK-S-S-CPLHSpT, preferentially penetrates the cancer cell membrane mediated by the integrin receptor, which is expressed at high levels by cancer cells. In the present study, using high performance liquid chromatography and mass spectroscopy, we determined the stability of cRGDyK-S-S-CPLHSpT and its cleavage by glutathione under typical conditions for cell culture. We further assessed the ability of the peptide to inhibit the proliferation of the U87MG glioma cell line. The phosphorylated peptide was stable, and the disulfide bond of cRGDyK-S-S-CPLHSpT was cleaved in 50 mM glutathione. This peptide inhibited the growth of cancer cells and changed their morphology. Therefore, we conclude that the phosphopeptide shows promise as a prodrug and has a high potential to act as an anticancer agent by inhibiting polo-like kinase 1 by binding its polo-box domain. These findings indicate the therapeutic potential of PLHSpT and peptides similarly targeted to surface receptors of cancer cells and to the functional domains of regulatory proteins.

Keywords Polo-like kinase 1 · Polo-box domain · cRGDyK-S-S-CPLHSpT · Metabolite · Prodrug · Anticancer agent

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Introduction

Peptides exhibit high bioavailability, are minimally immunogenic and can be specifically targeted to cells and tissues in vivo (Torchilin and Lukyanov 2003). Moreover, they are highly soluble and can be chemically modified. These properties make peptides ideal candidates as drugs. However, there are some limitations, such as stability or cell membrane permeability in vivo, because peptides are subject to extracellular and intracellular proteolytic cleavage (Malik et al. 2007). Therefore, recent research has attempted to enhance the intracellular accumulation and retention of peptides using drug delivery systems (Richard et al. 2003; Moore et al. 1998; Weissleder et al. 1997).

In drug delivery systems that employ cleavable linker groups, disulfide bonds are widely incorporated into peptides (Wang et al. 2011; Saito et al. 2003). Disulfide bonds readily allow conjugation of a peptide to a delivery protein and are readily cleaved in the cell by enzymes or small molecules. Glutathione (L-γ-glutamyl-L-cysteinylglycine) is the most abundant non-protein source of thiol and is active in all mammalian cells at millimolar concentrations (Meister and Anderson 1983). Thus, glutathione is an effective reducing agent in the cytoplasm, enabling the cleavage of disulfide bonds (Mok et al. 2010). A number of drug formulations with disulfide bonds have been developed for pre- or clinical trials (Jaracz et al. 2005; Kratz et al. 2008).

Polo-like kinase 1 (PLK1) regulates the G2/M transition of the cell cycle and controls bipolar spindle formation, chromosome segregation, and cytokinesis (Barr et al. 2004; Elia et al. 2003a; Garcia-Alvarez et al. 2007). Moreover, PLK1 serves as a biomarker and target for cancer therapy, because it is overexpressed in patients with cancer and by cancer cell lines (Knecht et al. 1999; Simizu and Osada 2000; Holtrich et al. 1994). Overexpression of PLK1

induces tumorigenesis in nude mice by increasing centrosome size and/or centrosome number (Holtrich et al. 1994).

The C-terminal polo-box domain (PBD) of PLK1 is the hallmark of the family of polo-like kinases (Garcia-Alvarez et al. 2007). The kinase activity of PLK1 is regulated by binding of the PBD to certain phosphopeptides through the phosphorylation-dependent targeting of PLK1 and by its subcellular localization (Lowery et al. 2005; Elia et al. 2003a). The phosphopeptides can specifically bind and dock to the PBD and regulate PLK1 activity (Liu et al. 2011). These findings have stimulated research on the development of selective and effective substrates of inhibitors of the PBD for cancer treatment (Elia et al. 2003b; Reindl et al. 2008; Lansing et al. 2007; Liu et al. 2011). One of the inhibitors, the peptide PLHSpT, binds to the PBD with high specificity and affinity (Yun et al. 2009). The interaction of PLHSpT with the PBD induces mitotic arrest by inhibiting the localization of PLK1 to centrosomes and kinetochores, and increases apoptotic cell death of cancer cells (Yun et al. 2009). However, in this study, it was necessary to microinject the cancer cells, because the phosphopeptides could not penetrate the cell membrane.

Therefore, we previously developed a new phosphopeptide cRGDyK-S-S-CPLHSpT that preferentially penetrates the cancer cell membrane because it is contains the arginyl-glycyl-aspartyl (RGD) sequence that binds to the αvβ3 integrin receptor, which is overexpressed in cancer cells (Kim et al. 2012). We created a disulfide bridge to link the integrin receptor-targeting peptide, cRGDyK, and the drug phosphopeptide, CPLHSpT, that inhibits the PBD. Therefore, we hypothesized that the cleavage of the disulfide bond would release the phosphopeptide and inhibit cell growth. We further predicted that only the cleaved peptide would exhibit this affect.

To test this hypothesis, we synthesized cleavable and non-cleavable phosphopeptides using *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), which is a bifunctional cross linker that contains a redox-responsive disulfide bond (Lin et al. 1996). The phosphopeptides would be cleaved intracellularly by glutathione. We employed high performance liquid chromatography (HPLC) and mass spectroscopy (MALDI-TOF) to identify cleaved and intact peptides after entry into cells. Here, we present evidence that the cleaved phosphopeptide inhibits the proliferation of cancer cells.

Materials and methods

Preparation of thiol-cleavable and non-cleavable peptides

The detailed procedures used here to synthesize the phosphopeptides have been published (Kim et al. 2012). Briefly, succinimidyl 3-(2-pyridyldithio)propionate (SPDP)-activated cRGDyK was prepared with protected-cRGDyK (62.5 mg, 63.5 μmol) and SPDP (25 mg, 80.0 μmol). SPDP was conjugated to the lysyl side chain of the protected-cRGDyK peptide under basic conditions (diisopropylethylamine, DIEA) for 2 h in dimethyl sulfoxide (1.5 mL) at room temperature. The mixture was purified using HPLC with a C18 column (5 μ, 4.6 × 150 mm column) and the eluants as follows: acetonitrile/0.05 % trifluoroacetic acid (TFA) (A) and H_2O/0.05 % TFA (B); 0–10 min 10 % at 10 min A, and at 10–30 min 90 % A, for 30 min at a flow rate of 2 mL/min. UV detection was at a wavelength of 230 nm. Solid-phase synthesis of phosphopeptides was carried out using a 9-fluorenylmethoxycarbonyl (Fmoc)-protected rink amide resin (0.61 mmol/g). After swelling the resin with dimethyl formamide (DMF), the Fmoc-group was deprotected with 20 % (v/v) piperidine in DMF. Deprotected amino acid residues were sequentially condensed using five equivalents of *O*-benzotriazole-*N,N,N,N′*-tetramethyl-uronium-hexafluoro-phosphate, and hydroxybenzotriazole in the presence of DIEA and DMF (3 mL) for 50 min at room temperature. Peptides were cleaved from the resin and the protecting groups removed using trifluoroacetic acid (TFA) (90 %) in the presence of scavengers (triisopropylsilane/water 5:5 v/v) for 24 h at room temperature. The mixture was collected in a BD falcon plastic tube in the presence of cold ether (35 mL) and then concentrated by centrifugation. The collected precipitate was dissolved in H_2O/0.05 % TFA solvent and purified using HPLC as described above. The peptide cRGDyK-S-S-CPLHSpT was conjugated to SPDP-activated RGDyK (37 mg, 31.3 μmol) and Fmoc-CPLHSpT (77 mg, 50.0 μmol) in DMF (1.5 mL) at pH 9–10 for 24 h. The conjugated peptides were purified using HPLC. The non-cleavable phosphopeptide was conjugated through an aspartic acid (Asp) residue between cRGDyK and CPLHSpT.

Sulfide bond cleavage and stability of the phosphopeptides

To determine the whether the disulfide linkage could be cleaved, the peptides were incubated in 50 mM glutathione with or without 10 % fetal bovine serum (FBS) or phosphate-buffered saline (PBS) for 1, 3, and 24 h, which mimics intracellular conditions. Generally, the intracellular and extracellular concentrations of glutathione are approximately 10 and 100 mM, respectively (Park et al. 2002; Miyata et al. 2004). Here, we used at least 5× higher glutathione concentrations by followed the reference (Hao et al. 2006). Aliquots were removed at each time point and filtered using a syringe filter and analyzed using HPLC and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF).

Cytotoxicity assay

The cytotoxicity of the fusion peptides was assessed using an XTT assay method (Cell Proliferation Reagent XTT, Sigma-Aldrich, USA). U87MG cells (1×10^3 cells/well) were grown for 24 h at 37 °C in 5 % CO_2 in 96-well plates with Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, USA) containing penicillin (100 U/mL), streptomycin (100 g/mL), and 10 % FBS. When the cultures greater than 80 % confluent, the medium was replaced with fresh FBS-free medium and incubated for 1 h. The cells were incubated for 24 h with a range of concentrations (20, 50, 100 nM, and 1, 10, 20, and 50 μM) of cRGDyK, PLHSpT, cRGDyK-Asp-PLHSpT, and cRGDyK-S-S-CPLHSpT). The medium was removed, FBS-free medium with XTT reagent was added to each well, and the cells were incubated at 37 °C for 4 h. Cell viability was quantified by measuring absorbance at 475 nm in an ELISA plate reader; data were processed using Soft Max Pro software (Molecular Devices Corp., USA). The experiment was repeated three times in duplicate.

Tumor cell morphology

A total of 2×10^4 cells/well of U87MG cells were grown for 24 h in 12-well plates containing DMEM and 10 % FBS; the medium was then changed to DMEM supplemented with 1 % FBS. U87MG cells were incubated for 24 h with 50, 100, 200, and 500 nM and 1, 10, and 20 μM of different cRGDyK-S-S-CPLHSpT. Cell morphology was observed using an Olympus IX81 microscope (Olympus Inc., Japan). Images were processed using Metamorph image analysis software (Molecular Devices Inc., Sunnyvale, USA).

Results and discussion

We demonstrate here that linking PLHSpT to cRGDyK via a disulfide bond enhanced its cytotoxic effects on U87MG cells. We tested the hypothesis that only PLHSpT, which is generated from cRGDyK-S-S-CPLHSpT, could inhibit cell proliferation by interacting with the PBD domain of PLK1. Therefore, we designed a targeted delivery system by conjugating PLHSpT to an RGD sequence to direct binding to the integrin receptor. This strategy circumvents the inability of PLHSpT to permeate the cell membrane and would further eliminate peptide delivery in vivo by injection. The RGD-conjugated peptide could then increase specific binding to its target site when injected in vivo. We introduced a disulfide bond between the cRGDyK and PLHSpT peptides, because this modification increases prodrug efficacy or stability in vivo. Thus, the cRGDyK-S-

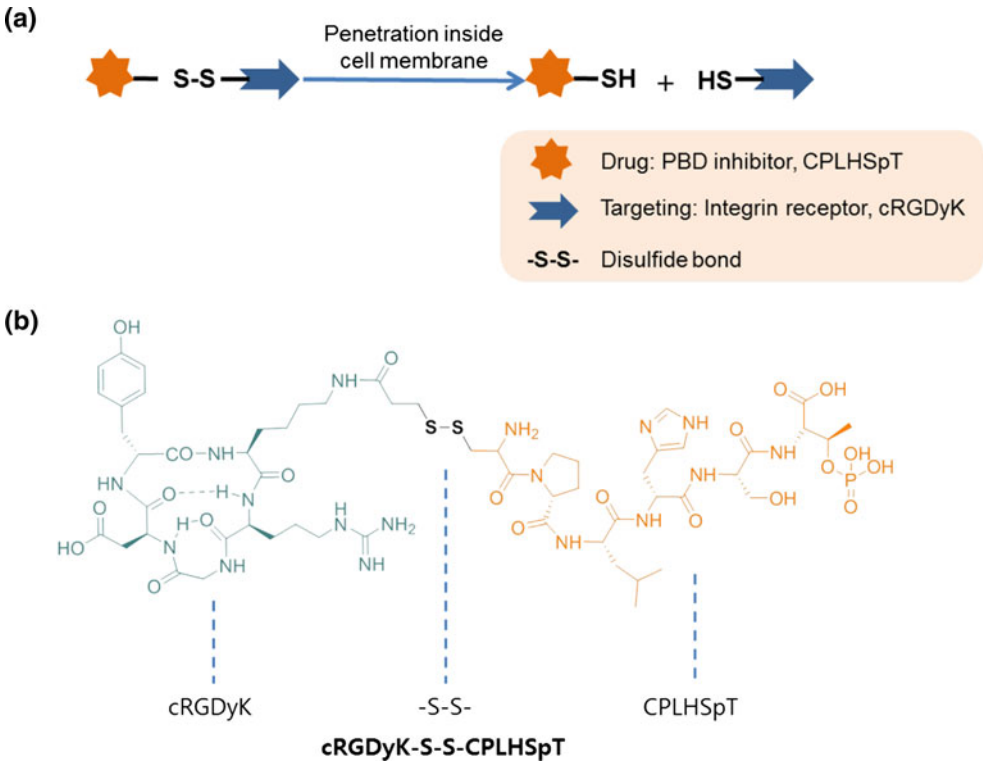

Fig. 1 Strategy for target-specific proteins with peptide prodrugs. **a** Entry of the peptide into the cell and activation by cleavage of disulfide bonds. **b** The structure of the phosphopeptide cRGDyK-S-S-CPLHSpT showing the integrin-targeting and PLK1-inhibitory domains

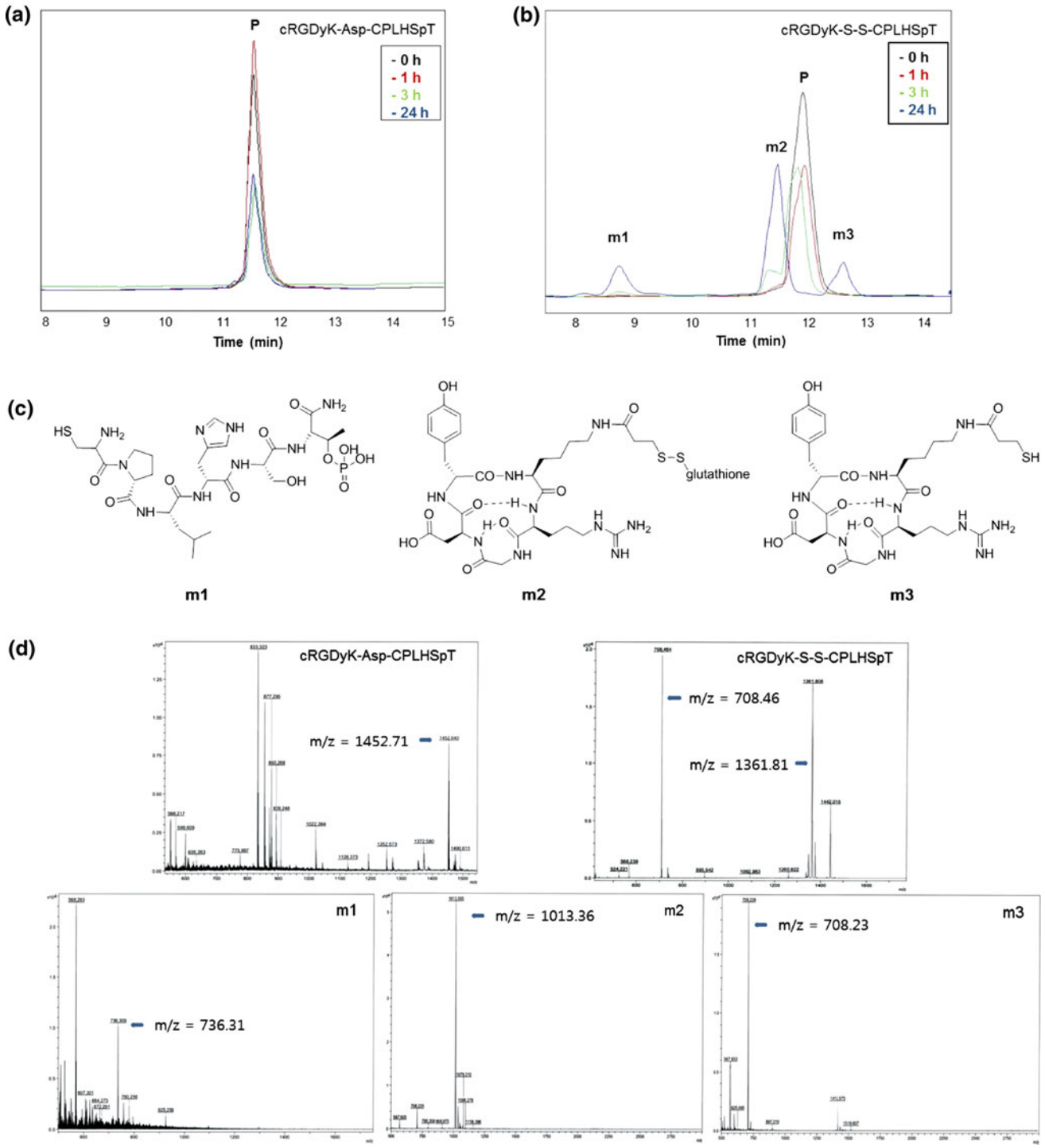

Fig. 2 HPLC profiles and cleavable and non-cleavable phosphopeptides. HPLC profiles of non-cleavable, (cRGDyK-Asp-CPLHspT) (**a**) and cleavable (cRGDyK-S-S-CPLHSpT) (**b**) phosphopeptides after incubation with 50 mM glutathione in PBS for 24 h. **c** Reaction products identified using MALDI-TOF and their predicted structures. P: cRGDyK-Asp-CPLHSpT or cRGDyK-S-S-CPLHSpT. Reaction products: m1, m2, and m3. **d** The MALDI-TOF analysis spectra of the phosphopeptides and its metabolites

S-CPLHSpT inhibited PLK1 activity in cancer cells (Kim et al. 2012). In the previous studies, we performed cell cycle analysis using flow cytometry, a PLK1 kinase inhibition assay, and cellular uptake using a fluorescence assay. cRGDyK-S-S-CPLHSpT was shown to significantly inhibit PLK1 kinase activity in tumor cells and spindle

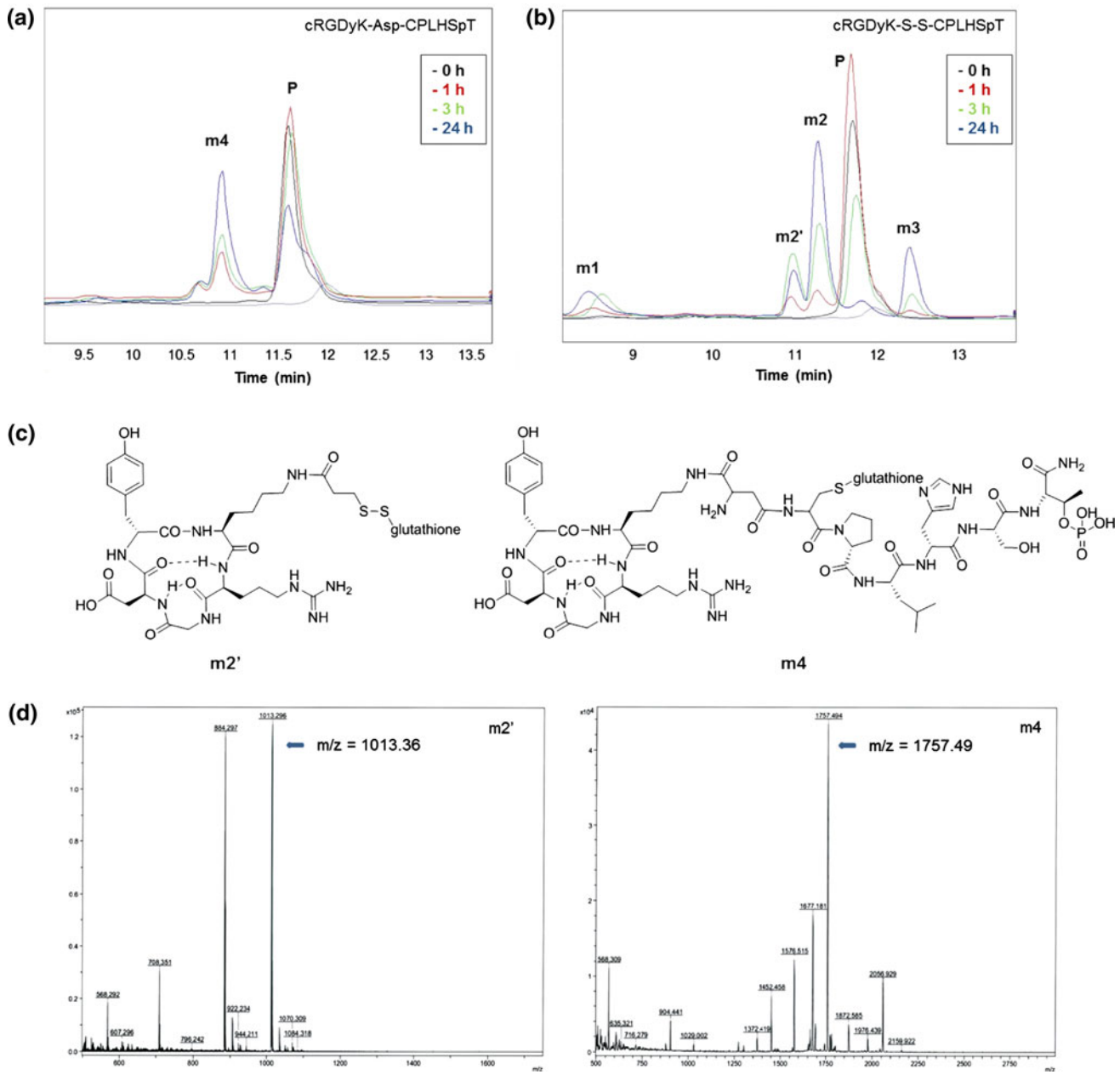

Fig. 3 HPLC profiles of reaction products of cleavable and non-cleavable phosphopeptides. Non-cleavable (cRGDyK-Asp-CPLHspT) (**a**) and cleavable (cRGDyK-S-S-CPLHSpT) (**b**) phosphopeptides incubated with 50 mM glutathione in serum for 24 h. **c** MALDI-TOF analysis and predicted metabolites structures; P: cRGDyK-Asp-CPLHSpT or cRGDyK-S-S-CPLHSpT. Reaction products: m2′ and m4. **d** The MALDI-TOF analysis spectra of the metabolites

assembly. Moreover, flow cytometry study showed that cRGDyK-S-S-CPLHSpT inhibited tumor cell proliferation by inducing apoptosis. Moreover, we evaluated an RGD blocking assay for identifying the RGD-specific binding site. Radiolabeled cRGDyK-S-S-CPLHSpT was incubation with cRGDyK (100 μg/mL), an αvβ3 integrin-positive inhibitor, to evaluate its specificity for binding the αvβ3 integrin receptor in tumor cells. The results showed that the binding was significantly reduced (50 %). From these results, we reasoned that the cleavable phosphopeptide should be effective for inhibiting tumor cell growth and attempted to demonstrate that cRGDyK-S-S-CPLHSpT acts as an intracellular prodrug.

Prodrugs act by several mechanisms that can be potentially exploited at the intracellular level, such as inducing enzyme expression, hypoxia, and pH changes, particularly in cancer cells (Denny 2001). In the present study, we tested the stability under mimicking intracellular

conditions, and the efficacy of cRGDyK-S-S-CPLHSpT as a prodrug for inhibiting the proliferation of a glioma cancer cell line. Disulfide bonds are readily cleaved at low pH or by enzymes in cancer cells, which distinguish them from normal cells (Reents et al. 2002; Saito et al. 2003). Therefore, we predicted that this strategy would facilitate the delivery of PLHSpT to its target, the PBD of PLK1, thereby inhibiting cell proliferation and inducing cell death. Figure 1a illustrates the strategy for introducing cRGDyK-S-S-CPLHSpT into cancer cells, and Fig. 1b shows the structure of the peptide and its cleavage site.

To study the stability of the phosphopeptides, we determined their metabolites with or without disulfide bond-containing phosphopeptides using HPLC and MALDI-TOF spectroscopy. Figures 2 and 3 show the results of the analysis of cleavable (cRGDyK-S-S-CPLHSpT) and non-cleavable (cRGDyK-Asp-CPLHSpT) phosphopeptides after incubation with glutathione (see "Materials and methods"). Both cRGDyK-S-S-CPLHSpT and cRGDyK-Asp-CPLHSpT were detected at 11.8 and 11.7 min, respectively (Fig. 2a, b). The non-cleavable phosphopeptide was stable for 24 h in PBS, which demonstrates that the phosphate group of cRGDyK-Asp-CPLHSpT was not hydrolyzed. Therefore, we reasoned that the phosphate group in another peptide, cRGDyK-S-S-CPLHSpT, should also be stable under these conditions (Fig. 2a). Analysis of the reaction products generated by incubating cRGDyK-S-S-CPLHSpT with glutathione for 24 h revealed three distinct species labeled m1, m2, and m3 in the HPLC profile (Fig. 2b). We identified the metabolites using MALDI-TOF (Fig. 2d). The reaction product m1 was detected as a small peak at 8.8 min and represents CPLHSpT produced by cleavage of the starting peptide. The m2 peak was larger (11.2 min) and represents cRGDyK-S-glutathione. The small m3 peak (12.5 min) represents the reduced peptide, cRGDyK-SH (Fig. 2c). The products of the reaction of glutathione with cRGDyK-S-S-CPLHSpT were determined after 3-h incubation, indicating that the disulfide bond was cleaved under conditions characteristic of the intracellular milieu.

We also determined whether the phosphopeptides retained their stability in serum. Incubating the non-cleavable phosphopeptide with glutathione generated another product, m4 (*M/Z*: 1757.49), which represents the oxidized form of cRGDyK-S-S-CPLHSpT (Fig. 3a, c, and d). When cRGDyK-S-S-CPLHSpT was incubated with serum, the HPLC elution profile was the same as incubation with PBS with the exception of peak m2′ (11.1 min, *M/Z*: 1013.30) that might represent the stereoisomer of cRGDyK-S–S-glutathione. HPLC analysis revealed that the phosphopeptide was stable and remained phosphorylated under intracellular conditions (Fig. 2a). From these results, we expected that cRGDyK-S-S-CPLHSpT would remain phosphorylated after 24 h. Moreover, cRGDyK-S-S-CPLHSpT was gradually cleaved so that the starting peptide was no longer present after a 24 h incubation. Therefore, this result indicates that cRGDyK-S-S-CPLHSpT was not dephosphorylated after cleavage of the disulfide bond. This would prevent the RGD moiety from sterically interfering with the binding of CPLHSpT to the PBD of PLK1 and inhibit the growth of cancer cells. Therefore, we reasoned that only cRGDyK-S-S-CPLHSpT could inhibit cancer cell proliferation.

To clarify whether the inhibitory effect of cRGDyK-S-S-CPLHSpT was mediated through disulfide bond cleavage, we determined the effect of cRGDyK-S-S-CPLHSpT on cell proliferation and morphology. The human glioma cell line (U87MG) expresses high levels of the integrin receptor and PLK-1 (Dietzmann et al. 2001; Chen et al. 2009). Figure 4 shows that cRGDyK, PLHSpT, and the non-cleavable peptide cRGDyK-Asp-CPLHSpT did not inhibit cell proliferation. The failure of PLHSpT to inhibit growth can be explained by its inability to penetrate the cell membrane (Yun et al. 2009). Although we expected that cRGDyK-Asp-CPLHSpT would be internalized through receptor-mediated endocytosis, it would not be cleaved and interact with the PBD. In contrast, cRGDyK-S-S-CPLHSpT inhibited cell viability as a function of its concentration as follows: 7, 42, 54, 58, and 61 % inhibition at concentrations of 0.1, 1, 10, 20, and 50 μM, respectively. Notably, the survival rate of U87MG cells was <50 % when treated with cRGDyK-S-S-CPLHSpT at concentrations greater than 10 μM (Fig. 4). Replacing the disulfide bond with aspartic acid (Asp) did not affect cell survival, indicating that cleavage of the disulfide bond released PLHSpT, which inhibited cell proliferation.

We found that the morphology of the U87MG cells changed after treatment with the peptides (Fig. 5). This

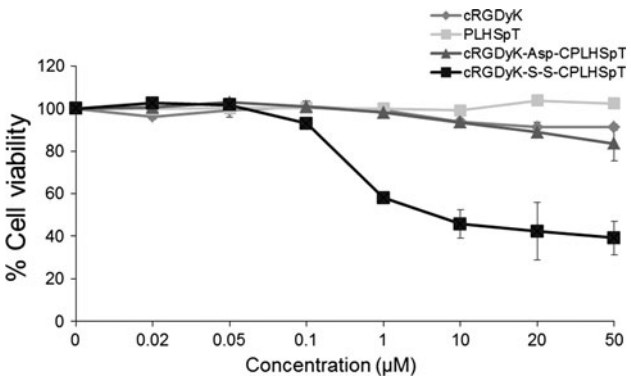

Fig. 4 The viability of U87MG cells incubated with cRGDyK-S-S-CPLHSpT was determined using the XTT assay. The cells were grown for 24 h with various concentrations (0.02, 0.05, 0.1, 1, 10, 20, and 50 μM) of fusion peptides. The experiment was repeated three times

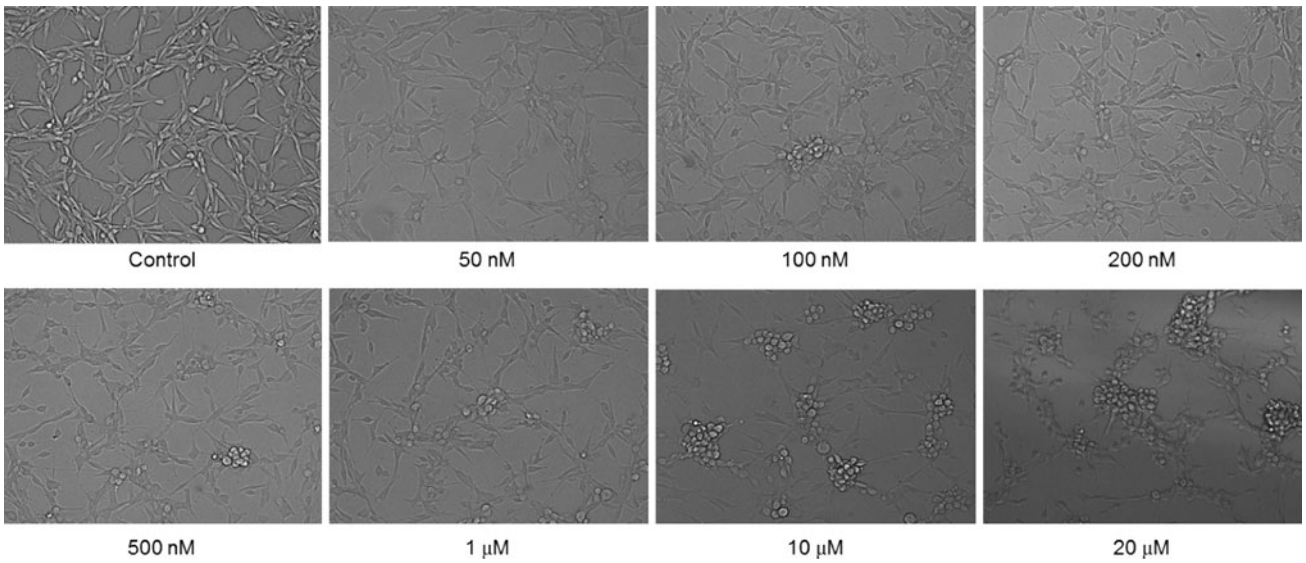

Fig. 5 Effect of peptides on the morphology of U87MG cells. U87MG cells were incubated for 24 h with the indicated concentrations of cRGDyK-S-S-CPLHSpT

effect was dependent on the dose of cRGDyK-S-S-CPLHSpT. In particular, the morphology of U87MG cells was round in the presence of at least 10 μM peptide. In contrast, cRGDyK and PLHSpT did not affect morphology (data not shown here).

Conclusions

The phosphopeptide cRGDyK-S-S-CPLHSpT was prepared by linking a target-specific peptide to CPLHSpT peptide, which is an inhibitor of PBD. The inhibitory peptide is generated from the prodrug via cleavage of its disulfide bond. Using HPLC and mass spectrometry, we showed that the peptides were stable in PBS and serum under intracellular condition. The disulfide linker of cRGDyK-S-S-CPLHSpT was cleaved by glutathione, an intracellular reducing agent. We demonstrate that cRGDyK-S-S-CPLHSpT potently inhibited the proliferation of U87MG cells. Therefore, our strategy for designing phosphopeptides that penetrate the cell membrane by binding the integrin receptor and target proteins required for cell proliferation, such as PLK1, shows promise as prodrugs for inhibiting the growth of cancer cells.

Acknowledgments This research was supported in part by a grant from the Korea Basic Science Institute (D33404) and by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2012-0006388).

Conflict of interest The authors declare that they have no competing financial interests.

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