Published in final edited form as:

Bioorg Med Chem Lett. 2014 April 15; 24(8): 1928–1933. doi:10.1016/j.bmcl.2014.03.006.

Synthesis and Activity of Tumor-Homing Peptide iRGD and Histone Deacetylase Inhibitor Valproic Acid Conjugate

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Abstract

In this report, we present a concise strategy to prepare a conjugate of the tumor homing peptide iRGD and histone deacetylase inhibitor Valproic acid, VPA-GFLG-iRGD. The conjugate VPA-GFLG-iRGD and a mixture of VPA and GFLG-iRGD have shown similar cytotoxicity against DU-145 prostate cancer cells. However, the treatment of DU-145 cells with conjugate VPA-GFLG-iRGD resulted in a decreased percentage of cells in the G2 phase, whereas the exposure of a mixture of VPA and GFLG-iRGD led to an increased percentage of cells in the G2 phase. We also found that GFLG-iRGD possessed cytotoxicity at the tested concentrations.

Keywords

Histone deacetylase inhibitor; Valproic acid; Cell penetrating peptide; Cell cycle arrest

Prostate cancer is the most frequently diagnosed in men and has the second highest mortality rate in United States.¹ Although great progress has been made in the treatment of prostate cancer, the overall results are limited and unsatisfactory.² Histone deacetylases (HDACs) catalyze the removal of acetyl groups from amino terminal lysine residue in histones and the resulting ionic interaction between the positively charged histones and negatively charged DNA induces transcriptional repression through chromatin condensation.^{3–5} Histone deacetylase (HDAC) activities in prostate cancer cell lines are two to three folds higher than those in a benign prostatic hyperplasia cell line.^{6,7} HDAC isoforms are strongly expressed in a majority of prostate carcinomas.⁸ HDAC inhibitors (HDACi) have shown promise in the treatment of prostate cancer.⁹ Valproic acid (VPA), an HDACi, causes prostate cancer cell cycle arrest and xenograft tumor reduction.^{10,11} Furthermore, VPA has the potential to

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inhibit prostate cancer metastasis by inhibiting prostate cancer migration through up-regulating E-cadherin expression.¹²

Protein transduction domains (PTDs) or cell penetrating peptides (CPPs) are often attached to therapeutics to enhance the cellular uptake of drugs.^{13,14} However, most CPPs cause undesired toxicities because they cannot distinguish between normal and tumor cells. Targeted drug delivery strategies are often used to reduce therapeutics' side effects in normal cells.^{15–18} Extra targeting moieties are also attached to CPPs or CPP conjugates to improve CPPs' selectivity.^{19,20} Recently, Ruoslahti and coworkers discovered that a specific tumor homing peptide iRGD, which combines a targeting peptide and a cell penetrating peptide, can significantly improve drug uptake into specific tumors.^{21,22}

In this study, we designed, synthesized and tested a novel drug conjugate VPA-GFLG-iRGD (Fig. 1A). The structure of VPA-GFLG-iRGD is composed of three components: the cell penetrating peptide iRGD (blue), VPA (red), and the lysosomally degradable tetrapeptide (-GlyPheLeuGly-, -GFLG-) spacer (black).¹⁵ The GFLG-iRGD (Fig. 1C) was used as a control.

As shown in Scheme 1, the conjugate VPA-GFLG-iRGD was prepared by solid phase synthesis. The first step was to prepare the linear conjugate VPA-Gly-Phe-Leu-Gly-Cys(Acm)-Arg(Pbf)-Gly-Asp(OBu^t)-Lys(Boc)-Gly-Pro-Asp(OBu^t)-Cys(Acm) on the MBHA Rink amide resin.²³ The general procedure for stepwise attachment of each protected amino acid (Fmoc-Cys(Acm)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH) (468 μmol) and VPA (468 μmol) on MBHA Rink amide resin (300 mg, 156 μmol) includes two parts: (i) 20% of piperidine in dimethylformamide (DMF) was mixed with resin for 5 min to remove Fmoc protecting group; (ii) the Fmoc protected amino acid or VPA was dissolved in a mixture of 0.4 mM *N*-methyl morpholine (NMM) and DMF then incubated with the resin for 20 min at room temperature. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) was used as the coupling agent.

The second step was to cyclize the iRGD peptide via a disulfide bridge by treating the resin bound peptide with a solution of thallium trifluoroacetate (Tl(OOCCF₃)₃) in DMF.²⁴ Finally, the peptide conjugate was cleaved from the resin by using a mixture of H₂O/triisopropylsilane (TIS)/trifluoroacetic acid (TFA) = 2.5/2.5/95. Other acid labile protecting groups (Bu^t on Asp, Boc on Lys, and Pbf on Arg) were also removed during the cleavage process. Pure VPA-GFLG-iRGD was obtained after purification by semi-preparative HPLC (Agilent); 0.1% of TFA in acetonitrile and 0.1% of TFA in de-ionized water were used as the Buffer A and Buffer B, respectively.

The structure of the VPA-GFLG-iRGD conjugate was confirmed by MALDI-TOF (matrix-assisted laser desorption ionization, time-of-flight) mass spectroscopy. As shown in Fig. 2, the peaks at *m/z* 1447.69 corresponds to [M+H]⁺ of the desired product. To further check the structure of VPA-GFLG-iRGD, the product was reduced with 10 mM tris (2-carboxyethyl) phosphine (TCEP) in phosphate buffered saline (PBS) buffer to break the

cyclic disulfide bond (Scheme 2). The elution time of VPA-GFLG-iRGD and its reduced product on an HPLC analytical column (Agilent, 300SB-C18, 4.6 * 250 mm, 5 μ m) were 14.12 and 14.53 min, respectively when using a gradient solvent from 10% Buffer B to 100% Buffer B in 30 min (Buffer A and Buffer B are the same as used in purification method). The reduction of the disulfide bond produced two sulfhydryl groups, which should result in a mass 2 Da higher than the original VPA-GFLG-iRGD. As shown in Fig. 3, the peak at m/z (MALDI-TOF) 1449.56 corresponds to the reduced product $[M_1+H]^+$. Mass spectrum peaks $[M_1+Na]^+$ and $[M_1+K]^+$ at m/z 1471.54 and 1487.49 respectively support the reduced product.

The control compound GFLG-iRGD (Fig. 1C) was prepared and purified using similar methods as those used for VPA-GFLG-iRGD. The only difference was that no VPA was added during the synthesis.

Next, we assessed the cell cycle arrest of DU-145 prostate cancer cells after treatment with 1.5 mM of VPA, GFLG-iRGD, VPA-GFLG-iRGD or a mixture of VPA and GFLG-iRGD using the reported protocol.²⁵ In the cell cycle arrest experiment, the following steps were performed: (I) DU-145 cells were seeded at a density of 5×10^5 cells/well in 6-well plates for 24 h; (II) the media was replaced with 1.5 mM of VPA, GFLG-iRGD, VPA-GFLG-iRGD or a mixture of VPA and GFLG-iRGD, then incubated for another 24 h; (III) the media was removed, and the cells were washed with PBS; (IV) the cells were detached from 6-well plates, and transferred to 1.5 mL Eppendorf vials; (V) after being removed the supernatant, the cells were re-suspended in 100 μ L PBS; (VI) added 400 μ L of ice cold 100% ethanol to each vial and kept at room temperature for 2 h; (VII) added 1 mL of PBS to cells and spun for 5 min, then aspirated and washed with 1 mL PBS; (VIII) re-suspended cells in 500 μ L of propidium iodide (PI) staining solution (50 μ g/mL PI, 200 μ g/mL RNase); (IX) covered with foil and analyzed on flow cytometer. The cell cycle arrest assay was repeated four times. Representative results are shown in Fig. 4, and the average cell cycle arrest results are shown in Fig. 5. The treatment with VPA-GFLG-iRGD or a mixture of VPA and GFLG-iRGD showed a statistically significant difference in the distribution of DU-145 cells in G1 and G2 phase. The treatment with conjugate VPA-GFLG-iRGD resulted in a decreased percentage of DU-145 cells in the G2 phase, whereas the exposure of cells to a mixture of VPA and GFLG-iRGD led to an increased percentage of cells in the G2 phase. The decreased G2 phase cell population in VPA-GFLG-iRGD treated group might be attributed to the blockage of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the DU-145 cell surface by RGD.²⁶

Finally, we tested the cytotoxicity of VPA-GFLG-iRGD against DU-145 prostate cancer cells using the Cell Counting Kit-8 (CCK-8) assay.¹⁵ Free VPA, GFLG-iRGD, and free VPA plus GFLG-iRGD were used as controls. After incubation with drug or drug equivalent (0.5 mM or 1.5 mM) for 72 h, the number of viable cells was determined by measuring the absorbance at 450 nm (630 nm as the reference) after adding the diluted CCK-8 solution to the treated or control cells. The average viable cell number in untreated group was set as 100%. The viability% was calculated by dividing the viable cell number in the treated group by the average viable cell number in the untreated group. The results are expressed as (mean $\pm$ SEM) %. The results after treatment with 0.5 mM drug or drug equivalents are shown in Fig. 6A: control (99.97 ± 0.84), VPA (99.56 ± 7.46), GFLG-iRGD (70.52 ± 2.89), GFLG-

iRGD + VPA (57.87 ± 6.01), VPA-GFLG-iRGD (69.30 ± 0.30). Fig. 6B shows the results after exposure to 1.5 mM drug or drug equivalent: control (100.00 ± 2.47), VPA (79.87 ± 2.44), GFLG-iRGD (58.67 ± 3.89), GFLG-iRGD + VPA (31.22 ± 1.33), VPA-GFLG-iRGD (38.96 ± 2.76). As shown in Fig. 6B, VPA-GFLG-iRGD was significantly more toxic than either VPA or GFLG-iRGD. Although the combination of VPA and GFLG-iRGD seems to possess a slightly higher toxicity than VPA-GFLG-iRGD, the difference is not statistically significant. Interestingly, we found that GFLG-iRGD has cytotoxicity to DU-145 cells at the two concentrations used. The cytotoxicity of GFLG-iRGD may be due to the blockage of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the DU-145 cell surface by RGD.²⁷ The data were analyzed with One-Way ANOVA plus Turkey's post-hoc test by using Prism 5 GraphPad software.

In summary, we have successfully prepared a tumor cell penetrating peptide iRGD targeted HDAC inhibitor conjugate (VPA-GFLG-iRGD). The conjugate structure was confirmed by both mass spectrometry of cyclized construct and of its reduced product. This synthesis methodology will help to design other iRGD or other cyclic peptide targeted pro-drugs. The cytotoxicity of VPA toward prostate cancer cells was enhanced both by covalent attachment or mixture with iRGD. Both forms of VPA changed the stage of cell cycle arrest: the VPA-GFLG-iRGD conjugate induced the decreased DU-145 cell population in G2 phase, and the combination of VPA and GFLG-iRGD enhanced G2 cell cycle arrest in DU-145 cells. We also found that the iRGD derivative has some cytotoxicity at the tested concentrations. The exact iRGD uptake mechanism is still being evaluated.

Acknowledgments

We thank Pavla Kopečková for valuable discussions and Hamid Ghandehari for the generous gift of DU-145 cells. We acknowledge James Marvin, Chris Leukel, Chad Nelson, and Krishna Parsawar at the University of Utah core facility for assistance with flow cytometry and mass spectrometry, respectively. This work was supported in part by NIH through grant RO1 CA132831. We also acknowledge support in conjunction with grant P30 CA042014 awarded to the Huntsman Cancer Institute, University of Utah.

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Figure 1.
Chemical structure of VPA-GFLG-iRGD (**A**), VPA (**B**), and GFLG-iRGD (**C**).

Figure 2.
Mass spectrum of conjugate VPA-GFLG-iRGD.

Figure 3.
Mass spectrum of the reduced product from VPA-GFLG-iRGD.

Figure 4.

Representative flow cytometric pictures and percentages of cells in G1 and G2 phase of the DU-145 cell cycle: (A) control, (B) 1.5 mM VPA, (C) 1.5 mM GFLG-iRGD, (D) 1.5 mM VPA + 1.5 mM GFLG-iRGD, (E) 1.5 mM VPA-GFLG-iRGD.

Figure 5.

Average percentage of cells in G1 (A) and G2 (B) phase of the DU-145 cell cycle. Statistics: One Way ANOVA plus Turkey's post-hoc test ($0.001 < P < 0.01 = **$; $0.05 < P < 0.01 = *$; not significant = n.s).

Figure 6. Cytotoxicity of VPA, GFLG-iRGD, VPA-GFLG-iRGD, a mixture of VPA and GFLG-iRGD against DU-145 prostate cancer cells: (A) 0.5 mM; (B) 1.5 mM. Statistics: One Way ANOVA plus Turkey's post-hoc test ($P < 0.001 = ***$; $0.001 < P < 0.01 = **$; not significant = n.s).

Scheme 1.
 Synthesis of conjugate VPA-GFLG-iRGD.

Scheme 2.
Scheme for reduction of conjugate VPA-GFLG-iRGD.