Z-GP Modification to CI-994, a Histone Deacetylase Inhibitor and the Application in Targeting Carcinoma Chemotherapy

Yuanyuan Deng1, Peipei Gao2, Cuiping Guo1, Long Wu1, Jun Xu1, Heru Chen3 and Shao-hui Cai*1*

1Department of Clinical Pharmacology, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China
2Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China
3Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, Guangzhou 510632, P. R. China

Abstract

An adduct, namely (S)-4-acetamido-N-(2-(N-benzyloxybenzyl)-glycylprolyl)aminophenyl)benzamide (Z-GP-CI-994) has been designed and synthesized. All the evidences disclose that Z-GP-CI-994 is not the substrate of fibroblast activation protein-α (FAPα). However, the adduct is the substrate of another unknown enzyme which is ubiquitous in tumor tissue. The cytotoxicity of the adduct against HepG2, A549 and NIH3T3 cell lines is apparently decreased when compared to that of the parent compound (CI-994). Additionally, the inhibition rate of Z-GP-CI-994 on histone deacetylase is significantly lower than that of CI-994. All the results suggest preliminarily that Z-GP-CI-994 is promising to achieve enzyme-targeting delivery and to reduce systemic toxicity.

Keywords: Prodrug; HDAC inhibitor; Chemotherapy; FAPα

Introduction

Chemotherapy for the treatment of cancer was introduced into the clinic more than sixty years ago. Although this form of therapy has been successful for the treatment of some tumors such as testicular cancer and certain leukemias, however, the success suffers from systemic toxicity due to a lack of specificity, rapid drug metabolism, and both intrinsic and acquired drug resistance [1,2]. One effective strategy to solve (or partially solve) this problem is to make chemotherapeutic drugs specifically target only neoplastic cells in order to decrease tumor burden by inducing cytotoxic and/or cytostatic effects with minimal “collateral damage” to normal cells. So far, there are a number of anticancer prodrugs have been reported [2-4]. Ideally, the design of anticancer prodrugs should be selectively activated by some specific enzymes or metabolic pathways which are (is) only present or predominantly present in targeted tumor site [5-7].

Recently, we are concerning about the application of fibroblast activation protein-α (FAPα) [8-10] as a targeting enzyme in prodrug design. Investigations have disclosed that FAPα is highly restricted to tumor-associated fibroblast (TAF) in over 90% of common human epithelial carcinomas, but is not detected in normal adult tissues except tissues of healing wound [10-13]. This enzyme is a type II transmembrane serine protease belonging to the post prolyl peptidase family with essential functions to regulate the growth and development of tumor [12,13]. Compared with dipeptidyl peptidase IV (DPPIV), which is one of its homologous family members, FAPα exclusively exhibits endopeptidase activity toward N-blocked peptide substrates [14]. And more specifically, Aertgeerts et al. substantiated that FAPα, but not DPPIV, could hydrolyze the N-terminal benzyloxy carbonyl (Cbz) blocked peptides, such as Z-GP-AMC [15]. Considering the highly selective expression and the unique proteolytic activity of FAPα in tumor tissue, Huang et al. constructed a Z-GP- doxorubicin adduct (Z-GP-Dox) and proved the desired targeting effect in 4T1 tumorbearing model [16]. However, whether if FAPα is able to hydrolyze universally any Z-GP adducts remains unknown.

CI-994, also named Tacedinaline (Figure 1), is a potent member of the benamidate class of histone deacetylase (HDAC) inhibitors which demonstrates significant anticancer activity against a broad spectrum of murine, rat and human tumor models [17-19]. Currently this compound is progressing through clinical trials in combination with other standard anticancer agents such as carboplatin, paclitaxel [20], capcitabine [21] or gemcitabine [22]. But it induces adverse effects including thrombocytopenia, anemia and neutropenia [23]. Therefore, we are quite interested in applying the above-mentioned strategy, that is, the Z-GP modification to CI-994 to construct Z-GP-CI-994 adduct (Figure 1), in the purpose of decreasing these side effects.

Experimental

Materials and procedures

Chemicals and materials: p-Aminobenzoic acid and benzene-1,2-diamine were purchased from Shanghai Jinghunch Ltd., China; N-benzyloxy carbonylglycylglycylproline (Z-GP-OH) was from GL Biochem (Shanghai) Ltd (China); Silica gel for column chromatography was purchased from Qingsdao Marine Chemicals Inc.(China); Epigenase HDAC activity/inhibition direct assay kit was from Epigenek Group Inc (USA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and DMSO were purchased from Sigma; Recombinant human FAPα (rhFAPα) was purchased from R&D Systems (USA). The buffers used were vacuum-filtered through 0.2-μm membrane. Cell culture media, supplements and fetal bovine serum (FBS) were

Figure 1: Chemical structures of CI-994 and Z-GP-CI-994.

*Corresponding authors: Heru Chen, Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China, E-mail: thrchen@jnu.edu.cn
Shao-hui Cai, Department of Clinical Pharmacology, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China, E-mail: csh6688@sina.com

Received April 25, 2013; Accepted May 24, 2013; Published May 27, 2013


Copyright: © 2013 Deng Y, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
purchased from Gibco (Invitrogen, USA). All culture flasks were obtained from Corning (USA). All other chemicals used were reagent grade or higher grade and obtained from Sigma-Aldrich or Merck, and were used without further purification.

Human hepatocarcinoma cells (HepG2), human lung cancer cells (A549), murine fibroblast cells (NIH3T3) and human embryonic kidney 293 cells (HEK293) were obtained from China Center for Type Culture Collection (Shanghai, China). HEK293/FAPα+ cells, 4T1/FAPα− cells and NIH3T3/FAPα− cells were establishment and maintained by our own laboratory.

Female C57BL/6 mice were purchased from Animal Experimental Center of Guangdong Province (Guangzhou, China). MT/ret transgenic 304/B6 male seed mice were presented by the Immunology Center of Guangdong Province (Guangzhou, China). MT/ret 304/B6 male seed mice were backcrossed twice with C57BL/6 mice. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals.

General procedures: NMR spectra were recorded on Bruker AV-300 (Bruker Biospin, Swiss). Tetramethylsilane (TMS) was used as internal standard. ESI-MS were recorded on Finnigan LCQ Advantage MAX mass spectrometer. HPLC for preparation was performed on a LC-100 liquid chromatograph equipped with a tunable LC-100 UV detector (Shanghai Wufeng Inc., China) and HPLC for enzymatic cleavage of Z-GP-CI-994 and Z-GP-Dox was performed with Agilent 1200 series liquid chromatograph equipped with an Agilent 1200 Series UV detector (Agilent Technologies, USA). SynergyMx Multi-Mode Microplate Reader (Bio-Tek, USA) was used for measure the fluorescence. Pre-coated thin-layer chromatography (TLC) plates (Institute of Yantai Chemical Industry, China) were used for tracing. Spots on TLC plates were detected by either a ZF-7A portable UV detector or spraying KMnO4 detector or spraying KMnO4 (Institute of Yantai Chemical Industry, China) were used for tracing.

Synthesis of Z-GP-CI-994

**Synthesis of 4-acetamidobenzoic acid:** To a solution of acetic anhydride 45 ml (0.47 mol) was added p-amino benzoic acid 6.86 g (0.05 mol). After completely mixing, the solution was allowed to warm up to 70°C. The reaction was lasted for 4 hrs with stirring. Then the mixture was cooled down to room temperature. Crude residues were collected by filtration and purified by crystallization in hot water resulting in 4-acetamidobenzoic acid in white solid 8.27 g (92.3%). 1H NMR (300 MHz, DMSO-d6): δ: 10.23 (s, 1H, NH), 9.13 (s, 1H, NH), 7.21 (m, 2H), 7.09 (m, 2H), 6.95 (s, 1H), 7.69 (d, J = 8.87 Hz, 1H, Ar-H). ESI-MS (m/z): calcd for C15H15N3O2+: 249.1. 141.8, 136.3, 130.6, 128.7, 128.6, 128.4, 128.0, 127.6, 126.7, 126.0, 125.5, 125.3, 119.2, 66.6, 46.4, 43.3, 29.2, 24.5, 24.3; ESI-MS (m/z): calcd for C15H15N3O2+: 251.2.

**Synthesis of 4-acetamido-N-(2-aminophenyl)benzamide (CI-994):** To a solution of 4-acetamido benzonic acid 3.58 g (0.02 mol) was dissolved in 20 ml of anhydrous THF. The solution was cooled down to 0°C with ice-batch. Then triethylamine 5.06 g (0.05 mol) and ethyl chloroformate 3.26 g (0.03 mol) were added respectively. The mixed solution was warmed up to room temperature and kept stirring for 1 hr. This solution was then added dropwisely via syringe. The mixture was stirred at r.t. for 20 hrs. Afterwars, DMF was removed by lyophilization. The residues were then submitted to RP-HPLC (Column: Cosmosil C18, 20×250 mm; λ: 254 nm; Eluant: 55% methanol in water) for purification led to Z-GP-CI-994 in white solid 120.0 mg (53.8%). Purity: 96.2%; 1H NMR (300 MHz, CDCl3): δ: 9.35 (s, 1H), 9.24 (s, 1H), 8.50 (s, 1H), 7.80 (d, J = 8.6 Hz, 2H), 7.50 (d, J = 8.4 Hz, 2H), 7.44 (m, 2H), 7.27 (m, 3H), 7.21 (m, 2H), 7.09 (m, 2H), 5.33 (t, J = 4.4 Hz, 1H), 4.90 (m, 2H), 4.40 (m, 1H), 3.77 (m, 2H), 3.32 (m, 2H), 2.04 (m, 3H), 1.95 (s, 3H), 1.88 (m, 1H); 13C NMR (75 MHz, CDCl3): δ: 170.8, 169.3, 168.5, 166.3, 156.4, 141.8, 136.3, 130.6, 130.8, 128.7, 128.6, 128.4, 128.0, 127.6, 126.7, 126.0, 125.5, 125.3, 119.2, 66.6, 46.4, 43.3, 29.2, 24.5, 24.3; ESI-MS (m/z): calcd for C15H15N3O2+: 252.2. 558.22, found: 558.3; HRMS (m/z): calcd for C15H15N3O2+: Na+: 580.21665, found: 580.21699.

**Methods and assays**

**Stability investigation:** Z-GP-CI-994 was incubated in 0.01 mol/L phosphate buffer (pH 7.4) solution, RPMI 1640 culture medium and Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum at 37°C for 72 hrs respectively. Reverse-phase high performance liquid chromatography (RP-HPLC) was performed on a Cosmosil column (C18, 5 Å, 4.6×250 mm) using Agilent 1200 series HPLC system to monitor the changing of Z-GP-CI-994 at each sheduled time points. The eluent used was 55% methanol containing pure water (flow rate: 1.0 ml/min; λ: 254 nm). The experiments were repeated at least 3 times and compared with the control experiment.

**Cell culture:** HepG2, A549 and 4T1/FAPα+ were cultured in RPMI 1640 culture medium and HEK293, HEK293/FAPα−, NIH3T3, NIH3T3/FAPα− were maintained in Dulbecco’s modified Eagle’s medium (DMEM) culture medium at 37°C with 5% CO2. All growth media used were supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin. Culture medium was routinely changed every 2 days. For subculture, cells were released from the flasks by treatment with 0.25% trypsin/EDTA. For experiments, cells at the logarithmic grow phase were used.

**Measurement of cytotoxicity in vitro:** Cell viability were estimated using methyl thiazolyl tetrazolium (MTT) (sigma, USA) assay. HepG2, A549, NIH3T3 and HEK293 was seeded in a 96-well plate respectively at a cell density of 4,000 cells per well. After overnight growth, Z-GP-CI-994 and CI-994 were added to the culture media at various concentrations (0.01~30 μM) for 48 hrs and 72 hrs respectively. Cytotoxicity was assayed by 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl tetrazolium bromide (MTT) dye uptake. The cells were incubated at 37°C for 4 h with MTT at 10% of culture volume. After incubation...
period, cultures were removed from incubator and the resulted formazan was dissolved in 150 μL of dimethyl sulfoxide (DMSO). Then, the plates were gently shaken to solubilize the formed formazan. The absorbance was measured at 490 nm using an ELISA reader (Bio-Rad Laboratories Inc., USA). The effect of Z-GP-CI-994 or CI-994 on growth inhibition was assessed as percent cell viability where DMSO-treated cells were taken as 100% viability. The concentrations of DMSO used at all the experiments were guaranteed no discernable effect on cells viability.

**HDAC activity assay:** The experiments were carried out following the direct assay kit by manufacturer’s procedures of Epigenase HDAC activity/inhibition. Z-GP-CI-994 and CI-994 at various concentrations (1.0−5.0 μM) was incubated with HDAC standard solution and substrate. After antibody binding and signal enhancing, the fluorescence was read at 530/490 nm within 2 to 10 min on the fluorescence microplate reader. HDAC inhibition percentage was calculated using the following formulas:

\[
\text{Inhibition} \% = \left(1 - \frac{\text{Inhibitor Sample RFU} - \text{Blank RFU}}{\text{No Inhibitor Sample RFU} - \text{Blank RFU}} \right) \times 100\%
\]

**Enzymatic hydrolysis:** HEK293/FAPα+ cells, 4T1/FAPα+ cells, NIH3T3/FAPα+ cells, and corresponding FAPα− cells were seeded in a 12-well plate at a cell density of 2−3×10⁵ cells per well respectively. After overnight growth, culture medium was changed to fresh medium contained the prodrug Z-GP-CI-994 (30 μM) and incubated for scheduled time. The culture media without cells incubated with the compound was set as control. In the mean time, Z-GP-CI-994 was incubated with 0.25% trypsin/EDTA or culture medium without cells in order to exclude non-specific hydrolysis. Then each culture supernatants was collected and transferred to tubes containing 5 mL of PBS buffer medium; while only less than 0.1% and 1% cleavage of the adduct were observed in PRMI 1640 and DMEM culture media over a period of 72 hrs. As expected, Z-GP-CI-994 is quite stable in the evolution of each solutions was analysed by HPLC with UV detection over a period of 72 hrs. As expected, Z-GP-CI-994 is quite stable in the course of these experiments, no detectable decomposition was observed in PBS buffer medium; while only less than 0.1% and 1% cleavage of the adduct were observed in RPMI 1640 and DMEM culture media respectively (Figure 2).

**Interaction of Z-GP-CI-994 with cells and enzymes**

**Anti-proliferative activity:** To validate the assumption that the cytotoxicity of CI-994 might be reduced through the modification of Z-GP. The cytotoxicity of both CI-994 and Z-GP-CI-994 against HepG2, A549 and NIH3T3 using MTT assay were assessed respectively. As depicted in Figure 3, Z-GP-CI-994 is quite stable in the presence of both HepG2, A549 and NIH3T3 cells. After 48-hours treatment, the prodrug did not exhibit any anti-proliferative activity on HepG2 cells; whereas CI-994 displayed potent cytotoxicity against HepG2 cells with IC₅₀ value of 14 μM. There was a similar decreased cytotoxicity against A549 cells. It was found that even at a concentration up to 30 μM, the prodrug killed only about 29% of the cells, however CI-994 at the same concentration killed about 73% of the cells.

**Statistics:** All results are reported as means ± SEM over 3–5 experiments. Differences between groups were analyzed using ANOVA, followed by Dunnett’s multi-comparison test with PASW Software (SPSS Inc., Chicago, IL, USA). P-value less than 0.05 were considered statistically significant.

**Results and Discussions**

**Chemistry and stability**

**Chemistry:** The synthesis of Z-GP-CI-994, which was named (5)-4-acetamido- N-(2-((N-benzoylcarbonylglycylglycyl)aminophenyl) benzamide is outlined in Scheme 1. First, p-aminobenzoic acid was turned into 4-acetamidobenzoic acid at 70°C using acetic anhydride, which played dual roles as reagent and solvent. The yield was 92.3%. Afterwards, 4-acetamidobenzoic acid reacted with benzene-1,2-diamine applied ethyl chloroformate as coupling reagent. In order to avoid the formation of diacetylation of benzene-1,2-diamine, the activated intermediate of 4-acetamidobenzoic acid should be added dropwisely to the solution of excess benzene-1,2-diamine. After work up, CI-994 was obtained in mild yield, which was 51.5%. At last, coupling of CI-994 with N-benzoylcarbonylglycylproline (Z-GP-OH) led to the final product, that is, the Z-GP-CI-994. The yield was moderate, which was 53.8%.

**Stability:** The stability of Z-GP-CI-994 was evaluated by incubating this compound in either 0.01 mol/L phosphate buffer (pH 7.4) or cell culture media supplemented with 10% fetal bovine serum at 37°C. The results of each solutions was analysed by HPLC with UV detection over a period of 72 hrs. As expected, Z-GP-CI-994 is quite stable in the course of these experiments, no detectable decomposition was observed in PBS buffer medium; while only less than 0.1% and 1% cleavage of the adduct were observed in RPMI 1640 and DMEM culture media respectively (Figure 2).

**Interaction of Z-GP-CI-994 with cells and enzymes**

**Anti-proliferative activity:** To validate the assumption that the cytotoxicity of CI-994 might be reduced through the modification of Z-GP. The cytotoxicity of both CI-994 and Z-GP-CI-994 against HepG2, A549 and NIH3T3 using MTT assay were assessed respectively. As depicted in Figure 3, Z-GP-CI-994 exhibited extremely low cytotoxicity against HepG2, A549 and NIH3T3 cells. After 48-hours treatment, the prodrug did not exhibit any anti-proliferative activity on HepG2 cells; whereas CI-994 displayed potent cytotoxicity against HepG2 cells with IC₅₀ value of 14 μM. There was a similar decreased cytotoxicity against A549 cells. It was found that even at a concentration up to 30 μM, the prodrug killed only about 29% of the cells, however CI-994 at the same concentration killed about 73% of the cells.
Additionally, the cytotoxicity of Z-GP-CI-994 on normal cells is likewise necessary to determine. The murine fibroblast cells NIH3T3 was used as representative normal cell in the current study. As shown in Figure 3C, when the concentration of Z-GP-CI-994 comes to 30 μM, the cell viability was still higher than 90%; while in the case of CI-994, the cell viability was only 58%. This evidence demonstrated that modification with Z-GP does decrease the cytotoxicity of CI-994.

**HDAC activity assay:** It is well known that CI-994 is a potent member of the benzamide class of HDAC inhibitors. Therefore, whether the Z-GP modification blocks the active site of CI-994 is a key problem needed to make clear. The inhibition rates of Z-GP-CI-994 and CI-994 on HDAC were measured at the current investigation. As illustrated in Table 1 and Figure 4, the inhibitory effect of Z-GP-CI-994 on HDAC was significantly lower when compared to that of CI-994 at the same concentration. When Z-GP-CI-994 was at a high concentration up to 5 μM, the inhibition rate on HDAC was only 37.43% ± 3.52%; whereas CI-994 at the same concentration showed 78.04% inhibition rate. This evidence supports that Z-GP modification does block the active site of CI-994 to a great extent.

**Enzymatic hydrolysis:** Enzymatic hydrolysis of the prodrug was first conducted in the FAPα-positive cell lines. In order to identify the specificity of FAPα, corresponding non-FAPα-positive cell lines were concomitantly tested. As shown in Figure 5A to 5C, after incubating with HEK293 and HEK293/FAPα+ cells, 4T1 and 4T1/FAPα+ cells, NIH3T3 and NIH3T3/FAPα+ cells, unexpectedly, it was found that Z-GP-CI-994 could be cleaved effectively by both FAPα-positive and FAPα-negative cell lines. More surprisingly, the hydrolytic rates of both FAPα positive and negative cell lines displayed no significant difference. These evidences implied that Z-GP-CI-994 was cleaved by another enzyme but not FAPα.

To confirm further whether Z-GP-CI-994 could be cleaved by FAPα or not, a separated experiment of enzymatic hydrolysis was carried out. 50 μM of the conjugates were incubated with rhFAPα (2 μg/mL)
at 37°C. As shown in Figure 6A, Z-GP-CI-994 kept intact during the course of incubation. Obviously, rhFAPα is not able to cleave Z-GP-CI-994 to CI-994. However, as a comparison, Z-GP-Dox(16) could be effectively cleaved by FAPα (Figure 6B), which confirmed that the enzyme work properly. From the view point of chemical structure, doxorubicin is more complex than CI-994. Therefore the reason why the Z-GP adduct of CI-994 cannot be hydrolyzed by FAPα shall not be ascribed to steric hindrance caused by molecular complexity [24]. This evidence discloses that FAPα is not able to hydrolyze any Z-GP adducts universally.

**Targeting characteristics of Z-GP-CI-994 in tumor homogenate:**
One of the most critical purposes to design antitumor prodrugs is to improve the targeting effect of cytotoxic agents and thereby to reduce their systemic side effects. It is imperative to investigate whether the conjugate could be effectually hydrolyzed in the tumor tissue. In the current study, tumor homogenate of MT/ret 304/B6 mice with whole-body palpable tumors was used for this purpose. As illustrated in Figure 7A, Z-GP-CI-994 (30 μM) was cleaved to CI-994 completely, in the meantime it resulted in another unknown metabolite which presented a peak at 2.9 min after 0.5 h of incubation with tumor homogenate. With the procedure going on, peak area of CI-994 decreased gradually while the area of peak at 2.9 min increased concomitantly.

As a comparison, CI-994 was incubated with the same kind of tumor homogenate. It was found that the peak area of CI-994 gradually dwindled and completely disappeared after 24-hours incubation; while a new distinct peak was observed at 2.9 min and rose along with the falling of CI-994 peak (Figure 7B). These evidences substantiated that Z-GP-CI-994 is rapidly cleaved by tumor tissue and has a similar metabolic behavior to CI-994 in tumor tissue. In other word, Z-GP-CI-994 is hydrolyzed by other unknown enzyme which is ubiquitous in tumor tissue apart FAPα to release CI-994 and plays the similar role as CI-994.

**Conclusions**
In summary, a novel concise synthesis of Z-GP-CI-994 has been successfully developed. Although the purpose of Z-GP modification to CI-994 had been targeted to FAPα, the evidence discloses that Z-GP-CI-994 is not the substrate of FAPα. This is the first Z-GP adduct reported that cannot be cleaved by FAPα. Interestingly, Z-GP-CI-994 can be hydrolyzed by another unknown enzyme which is ubiquitous in tumor tissue. The cytotoxicity of Z-GP-CI-994 against either tumor cell lines including HepG2 cells and A549 cells or normal cell lines NIH3T3 cells is apparently decreased when compared to that of CI-994. The inhibition rate to HDAC is lowered down when CI-994 is modified with Z-GP. All the evidences come to support that Z-GP-CI-994 is promising to achieve targeted delivery as well as to reduce the side effects of CI-994. However, the comprehensive action mechanism of Z-GP-CI-994 needs to be further investigated.
Figure 6: Enzymatic cleavage of Z-GP-CI-994 (A) and Z-GP-Dox (B). Prodrug concentration: 50 μM; Enzyme concentration: 2 μg/mL.

Figure 7: Chromatograms of incubation studies of Z-GP-CI-994 (A) and CI-994 (B) with mouse tumor homogenate at 37°C for 24 h. The concentration of compounds was 30 μM.

Acknowledgements
This research was financially supported by National Natural Science Foundation of China (Nos. 81172382 and 30973565) and the Guangdong Provincial Project of Science and Technology (No. 2010A030100006).

References


