



# Introduction of Z-GP scaffold into procarbazine reduces spermatotoxicity and myelosuppression

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## ABSTRACT

Incorporation of carbobenzyoxy-glycylprolyl (Z-GP) to either  $\alpha$  or  $\beta$  position of the hydrazine moiety in procarbazine (Pcb) has been carried on in 5-steps process. The overall yield was 32.7%. The new entity Z-GP-Pcb was confirmed targeting to fibroblast activation protein- $\alpha$  (FAP $\alpha$ ). Z-GP-Pcb may be hydrolyzed by either isolated rhFAP $\alpha$  or tumor homogenate. It was shown far less cytotoxicity against NCI-H460 cell line than Pcb. Z-GP-Pcb was displayed the potency to reduce spermatotoxicity in H22-bearing mice. The mechanism may be ascribed to the blockade of dehydrogenation by  $\alpha$ -glycerolphosphate dehydrogenase. This candidate was further proved equal antitumor activity to Pcb. However, the introduction of Z-GP scaffold decreased myelosuppression. All the evidences support that Z-GP-Pcb is a better antitumor agent than Pcb.

## 1. Introduction

Procarbazine (Pcb), chemically named as *N*-isopropyl- $\alpha$ -(2-methylhydrazino) *p*-toluamide hydrochloride, is an alkylating anticancer drug. It has been widely used as an effective anticancer agent in the treatment of Hodgkin's disease [1]. It is also administered as a component of a chemotherapeutic cocktail in the treatment of melanoma, bronchogenic carcinoma, and central nervous system (CNS) malignancies [2,3]. However, there are very common adverse effects of procarbazine in combination chemotherapy including loss of appetite, nausea, and vomiting, especially reproductive effects such as reduction in sperm count and ovarian failure [4]. Another major toxic effect for Pcb in humans is myelosuppression [5]. Generally, the toxic side effects of cytotoxic agents are primarily attributable to the lack of selectivity toward tumor. Hence, the targeted delivery of antitumor agents is an effective strategy in the tumor therapy [6–8].

Nowadays, it is clear that fibroblast activation protein- $\alpha$  (FAP $\alpha$ ) is a tumor-associated antigen uniquely expressed by reactive stromal fibroblasts in the majority of human epithelial tumors [9]. Studies have indicated that FAP $\alpha$  is highly expressed in over 90% of common human epithelial carcinomas and regulates the growth and development of

tumor, but is not detected in normal adult tissues except tissues of healing wound [10,11]. Therefore, FAP $\alpha$  has been regarded as a tumor-associated antigen.

As we know, FAP $\alpha$  possesses both post-prolyl peptidase and endopeptidase activities, which has the capability of cleaving *N*-terminal post-prolyl aminos such as Ala-Pro-7-amino-4-trifluoro-methylcoumarin (AFC), Gly-Pro-AFC (GP-AFC), and Lys-Pro-AFC [12–14]. Moreover, FAP $\alpha$  can specifically hydrolyze the *N*-terminal benzyloxycarbonyl (Z)-blocked peptides, such as substrates Z-GP-AMC and FAP $\alpha$ -targeting prodrug of Dox (FTPD) [15,16], where FTPD were shown without obvious cardiotoxic effect in 4T1 tumor-bearing mice.

Of particular note, on the basis of the highly selective expression and the unique proteolysis activity, FAP $\alpha$ -based prodrug strategy is promising to achieve targeted delivery of antitumor agents [17]. By applying this strategy, Chen and Ye's groups developed Z-GP-DAVLBH prodrug, where DAVLGH is the desacetylvincristine monohydrate, and proved that Z-GP-DAVLBH selectively destroys the cytoskeleton of FAP-expressing tumor pericytes, disrupting blood vessels both within the core and around the periphery of tumors [18].

Concerning about the toxicities of Pcb in clinic, we believe that targeting FAP $\alpha$  may probably an effective strategy for solving this

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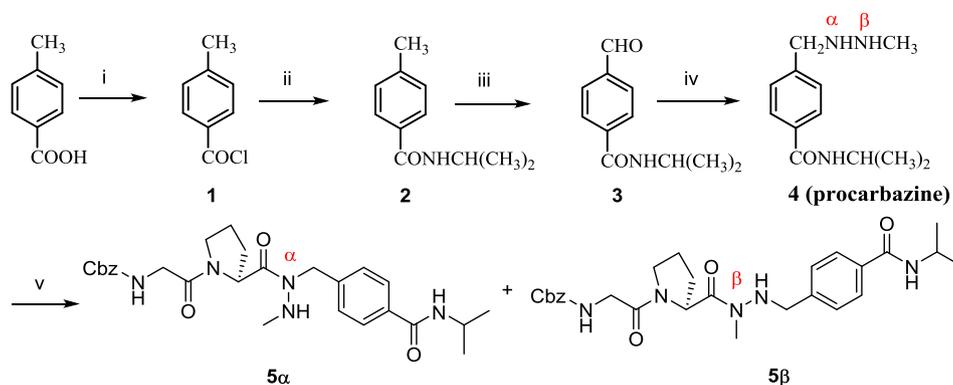
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**Scheme 1.** Synthesis of Z-GP-Pcb. Conditions and Agents: (i)  $\text{SOCl}_2$ , 80 °C, 3 h; (ii)  $(\text{CH}_3)_2\text{CHNH}_2$ , 30 °C → 40 °C, 30 min; (iii)  $\text{Ce}(\text{NH}_4)_2(\text{NO}_3)_6/\text{HNO}_3$ , 100 °C, 24 h; (iv)  $\text{CH}_3\text{NHNH}_2\cdot\text{HCl}/\text{NaCNBH}_3/\text{Et}_3\text{N}$ , EtOH, 0 °C → r.t., overnight; (v) Z-GP-OH, HATU/HOBt/DIPEA, DMF.

problem. Therefore, in the current study, we designed and synthesized the Z-GP-Pcb prodrug. The biology of Z-GP-Pcb and its antitumor properties will also be explored herewith.

## 2. Chemistry

The synthesis of Z-GP-Pcb (**5a/5b**) was started from *p*-methylbenzoic acid. As outlined in **Scheme 1**, *p*-methylbenzoic acid was turned into *p*-methylbenzoyl chloride (**1**) by  $\text{SOCl}_2$  in high yield. Then it reacted with excess isopropyl amine resulted in *N*-isopropyl-4-methylbenzamide (**2**) in a yield of 88.9%. The key step in **Scheme 1** is the transformation of 4-methyl of compound **2** into 4-formyl. We chose  $\text{Ce}(\text{NH}_4)_2(\text{NO}_3)_6$  (CAN) as oxidant. It selectively oxidized methyl to formyl under 3.5 mol/L  $\text{HNO}_3$  medium at 100 °C with a yield of 72.0%. Other oxidants such as  $\text{SeO}_2$ ,  $\text{KBrO}_3$ ,  $\text{CrO}_3$  have ever been tried, but did not work. The choice of medium is very important for this reaction. CAN in ice vinegar atmosphere at 100 °C achieved only 35% yield. When the reaction temperature was increased, it led to more side products.

Reductive amination was used to change compound **3** into procarbazine (**4**) with  $\text{NaCNBH}_3$  as reductant. The yield was 74.0%. Binding of carbobenzoxyglycyl proline (Z-GP-OH) with Pcb was obtained smoothly by applying TBTU/HOBt/DIPEA as coupling reagent. The Z-GP scaffold may bind at either position  $\alpha$  or  $\beta$  of Pcb resulted in mixed products **5a** and **5b**. The yield was 69.2%. The overall yield of this 5-steps procedure was 32.7%.

Based on the structure of **5a** and **5b**, we know they are position isomers and hard to separate. However, when **5a** and **5b** are hydrolyzed by FAP $\alpha$  respectively, the products are the same, where they are the parent compound Pcb, and the dipeptide fragment Z-GP-OH. Therefore, we did not ever try to separate **5a** and **5b**. In the current research, both isomers and their mixture are regarded as Z-GP-Pcb.

## 3. Results and discussion

### 3.1. Release of Pcb from Z-GP-Pcb by FAP $\alpha$

To confirm whether and how the Z-GP-Pcb conjugate was cleaved by FAP $\alpha$ , enzymolysis experiment was carried on. As shown in **Fig. 1A**, when 30  $\mu\text{M}$  of Z-GP-Pcb were incubated with 2  $\mu\text{g}/\text{mL}$  rhFAP $\alpha$  at 37 °C for 24 h, 60.4  $\pm$  6.1% of Z-GP-Pcb was hydrolyzed to Pcb. When the enzyme concentration increased to 5  $\mu\text{g}/\text{mL}$ , the cleavage rate of Z-GP-Pcb reached 82.2  $\pm$  8.3%. Of no doubt, the release of Pcb was dramatically FAP $\alpha$ -dependent. As expected, this result demonstrated that Z-GP-Pcb may effectively be cleaved by isolated FAP $\alpha$  *in vitro*.

Next, another equally imperative problem needs to be make clear is that whether Z-GP-Pcb is effectively hydrolyzed in the FAP $\alpha$ -positive tumor tissue. As reported, in a murine liver tumor model, it was found that FAP $\alpha$  was positively expressed in liver tumor tissue, which

promoted tumor growth [19]. Therefore, in the current study, tumor tissue was obtained from H22 tumor-bearing model mice and cut into small pieces. This homogenate was used to check whether if it can hydrolyze Z-GP-Pcb. As shown in **Fig. 2B**, after 16 h incubation of the conjugates (50  $\mu\text{M}$ ) in H22 tumor homogenate, approximately 76.3  $\pm$  6.3% was cleaved to release Pcb. The enzymolysis was almost completed after 24 h incubation at 37 °C. This evidence suggested that Pcb may efficaciously be released from Z-GP-Pcb in the FAP $\alpha$ -positive tumor tissue.

### 3.2. Z-GP-Pcb reduced cytotoxicity against NCI-H460 cell line

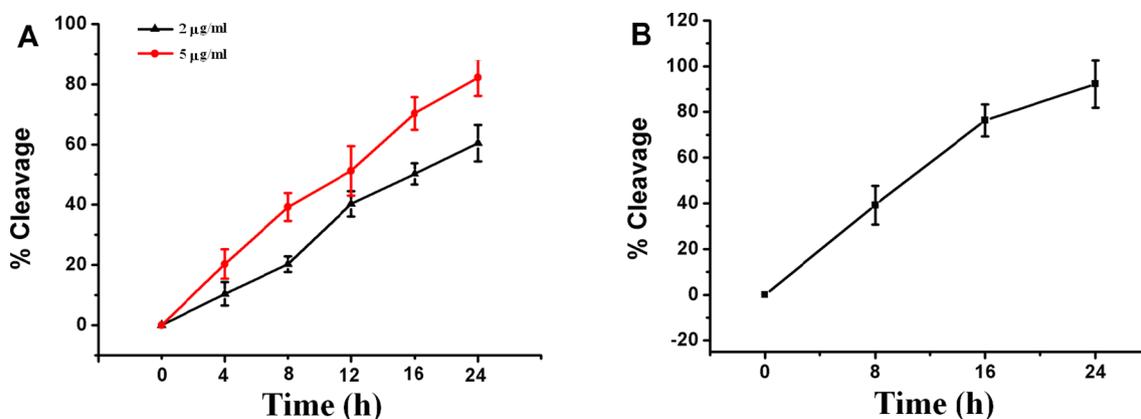
One of the purposes for introduction of Z-GP scaffold is to reduce the toxicity of Pcb. As we know, Pcb is an alkylation agent in the treatment of cancer. Its anticancer activity is based on the production of methyl radical or other radicals during the microsomal metabolism of Pcb, where Pcb is firstly metabolized into azoprocarbazine or hydrazone [20,21]. Azoxyprocarbazine is another metabolites of Pcb, it has been shown to be more effective against murine L1210 than either Pcb or azoprocarbazine [22].

Clearly, Pcb itself is not an active cytotoxic anticancer agent. This was confirmed by our experiment. As indicated in **Fig. 2**, the  $\text{IC}_{50}$  value of Pcb against NCI-H460 cell line, a kind of non-small cell lung cancer cells, was 27.2  $\pm$  3.5  $\mu\text{M}$ . Quite interestingly, introduction of Z-GP scaffold to Pcb dramatically decreased the cytotoxicity against NCI-H460 cell line. When Z-GP was removed by rhFAP $\alpha$ , the zymolytic homogenate of Z-GP-Pcb showed almost the same cytotoxicity as that of Pcb (**Fig. 2B**). This evidence implicates that the hydrazine moiety in Pcb contributes to cytotoxicity. When either position  $\alpha$  or  $\beta$  of hydrazine is blocked by Z-GP, the cytotoxicity against NCI-H460 cells will enormously be reduced.

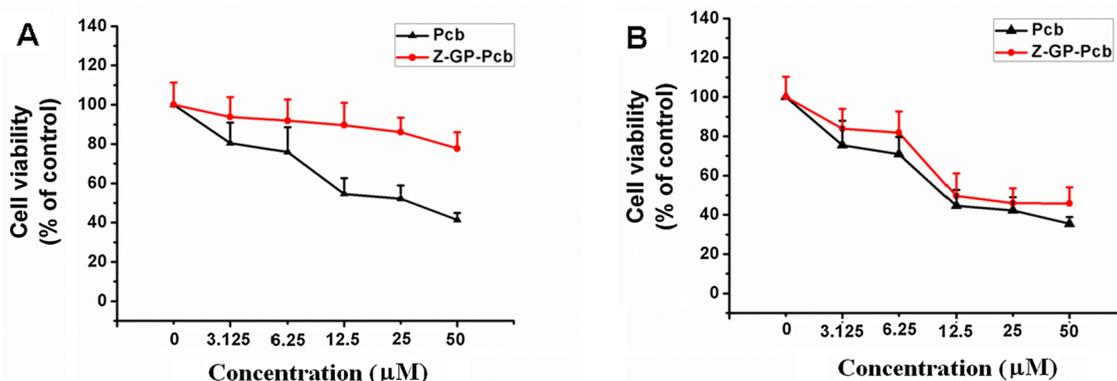
### 3.3. Z-GP-Pcb caused less spermatotoxicity than Pcb

As we know, irreversible azoospermia in human is an unfortunate side effect of Pcb in combination chemotherapy [23]. It has been disclosed by Horstman, *ect.*, that the spermatotoxicity of Pcb is caused by an alkylating agent that is produced through the sequential formation of azoprocarbazine and the azoxyprocarbazine isomers, without the intervention of the methyl radical [24]. They also found that when Pcb was co-administered with equimolar doses of antioxidants such as *N*-acetylcysteine or ascorbate, spermatotoxicity was decreased by 13–17%.

Excitingly, as shown in **Table 1**, *i.p.* administration of Pcb in Kunming mice for 18 days caused an enormously decrease of testicular weight in a dose-dependent manner. However, *i.p.* administration of Z-GP-Pcb almost did not reduce testicular weight even at high dose (150 mM).



**Fig. 1.** Enzymolysis of Z-GP-Pcb by FAP $\alpha$ . (A) Release of Pcb from the prodrug Z-GP-Pcb by isolated FAP $\alpha$ . 30  $\mu$ M of Z-GP-Pcb was incubated with 5  $\mu$ g/mL, and 2  $\mu$ g/mL rhFAP $\alpha$  at 37 °C, respectively. 10  $\mu$ L of sample solution was submitted to RP-HPLC to analyze the concentrations of Z-GP-Pcb and Pcb at time points of 0, 4, 8, 12, 16, and 24 h, respectively; (B) Release of Pcb from Z-GP-Pcb by H22 tumor homogenate. 50  $\mu$ M of Z-GP-Pcb was incubated with H22 tumor homogenate at 37 °C. 10  $\mu$ L of sample solution was submitted to RP-HPLC to analyze the concentrations of Z-GP-Pcb and Pcb at time points of 0, 8, 16, and 24 h, respectively. Each experiment was repeated 3 times. The results were expressed as mean  $\pm$  standard error of the mean (SD).



**Fig. 2.** Cytotoxicities of Pcb and Z-GP-Pcb against NCI-H460 cancer cell line. (A) Cytotoxicity comparison of Pcb and Z-GP-Pcb. NCI-H460 cells were treated with different concentration of Pcb and Z-GP-Pcb for 48 h, respectively, where the concentrations of tested compounds were 0, 3.125, 6.25, 12.5, 25, and 50  $\mu$ M. Cell viability was determined by MTT method; (B). Cytotoxicity comparison of Pcb and the zymolytic homogenate of Z-GP-Pcb. 0.25 mM of Z-GP-Pcb was incubated with 5  $\mu$ g/mL rhFAP $\alpha$  at 37 °C for 24 h. Afterwards, 40, 20, 10, 5, and 2.5  $\mu$ L of supernatant was transferred to 200  $\mu$ L wells plated with NCI-H460 cells at a density of  $1 \times 10^4$  cells/well, respectively. Fresh culture medium was added to make each wells at a total volume of 200  $\mu$ L. The plate was then incubated at 37 °C for 48 h. Cell viability was determined by MTT method. Each experiment was repeated 3 times. The results were expressed as mean  $\pm$  standard error of the mean (SD).

**Table 1**  
Effects of Pcb and Z-GP-Pcb on weight of testis in Kunming mice.

Compound	Dose (mM)	Testicular weight (g)	% of control
Vehicle	–	0.112 $\pm$ 0.025	–
PCB	75.00	0.079 $\pm$ 0.017 <sup>#</sup>	70.54
PCB	150.00	0.050 $\pm$ 0.012 <sup>#</sup>	44.64
ZGP-PCB	75.00	0.116 $\pm$ 0.017 <sup>*</sup>	103.57
ZGP-PCB	150.00	0.111 $\pm$ 0.021 <sup>*</sup>	99.11

“–” means no data; Values are expressed as mean  $\pm$  SD (n = 6). Significance was determined using the Student's *t*-test.

<sup>#</sup> *P* < 0.05 vs. vehicle group.

<sup>\*</sup> *P* < 0.05 vs. Pcb group.

Similarly, Pcb produced significantly greater inhibition of sperm count than Z-GP-Pcb at the same dose as demonstrated in Table 2. E.g., at a dose of 150 mM, Pcb caused 52.73% decrease of sperm count; while Z-GP-Pcb caused only 35.27% reduction of sperm count. Evidently, Pcb was significantly more spermatotoxic than Z-GP-Pcb.

As identified in Fig. 3, the reason why the incorporation of Z-GP into Pcb may reduce spermatotoxicity is that the binding of this scaffold to either  $\alpha$  or  $\beta$  position of hydrazine moiety in Pcb hinders the formation of azoprocabazine and the azoxyprocabazine isomers, which are very important intermediates for the generation of spermatotoxicity. As

**Table 2**  
Effect of Pcb and Z-GP-Pcb on spermatogenesis in Kunming mice.

Compound	dose (mM)	Sperm count ( $\times 10^7/0.1$ g epididymis)	% of control
Vehicle	–	2.75 $\pm$ 0.63	–
PCB	75.00	1.59 $\pm$ 0.16 <sup>#</sup>	57.82
PCB	150.00	1.30 $\pm$ 0.19 <sup>#</sup>	47.27
Z-GP-PCB	75.00	1.83 $\pm$ 0.15 <sup>#,*</sup>	66.55
Z-GP-PCB	150.00	1.78 $\pm$ 0.18 <sup>#,*</sup>	64.73

“–” means no data; Values are expressed as mean  $\pm$  SD (n = 6). Significance was determined using the Student's *t*-test.

<sup>#</sup> *P* < 0.05 vs. vehicle group.

<sup>\*</sup> *P* < 0.05 vs. Pcb group.

reported,  $\alpha$ -glycerolphosphate dehydrogenase is a key enzyme involved in the formation of azo- metabolites [25]. Some antioxidants such as *N*-acetylcysteine or ascorbate may inhibit the activity of the enzyme, therefore cut down the formation of azocompounds. This is another way to decrease spermatotoxicity of Pcb which has already been disclosed by Horstman, *ect.* [24].

It was also observed in Table 2 that Z-GP-Pcb was still somewhat spermatotoxic despite the assertion of Z-GP that azoprocabazine and azoxyprocabazine formation was hindered. This is reasonable. Although Z-GP-Pcb had a greater distribution in tumor tissues than that in normal

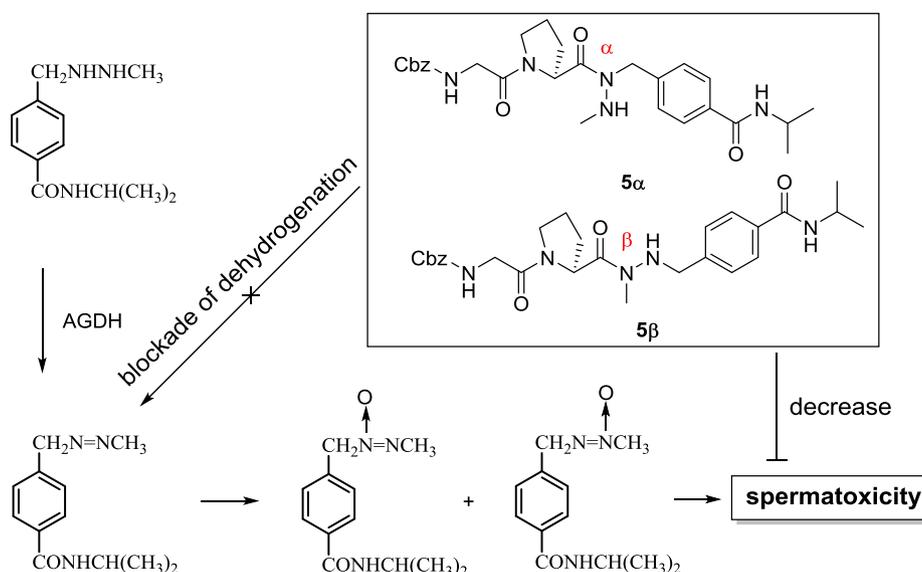


Fig. 3. Putative mechanism of spermatotoxicity reduction by Z-GP-Pcb. AGDH:  $\alpha$ -glycerol- phosphate dehydrogenase.

tissues, it did exit in normal tissues including testis. The Z-GP scaffold may be removed by less-expressed FAP $\alpha$  in the testis, and finally resulted in spermatotoxic azoprocarbazine and azoxyprocarbazine. By the way, as far as we know, there is no precedent report for suppression of azo-intermediate formation by Z-GP motif.

### 3.4. Z-GP-Pcb showed identical tumor inhibition and less myelosuppression toxicity than Pcb

To evaluate the *in vivo* antitumor activity of Z-GP-Pcb, a tumor mice model bearing H22 mice liver cancer cells was set up. As shown in Fig. 4 and Table 3, Z-GP-Pcb ( $\text{C}_{27}\text{H}_{35}\text{N}_5\text{O}_5$ , MW: 509.61) showed almost the same tumor inhibition rate as the parent compound Pcb ( $\text{C}_{12}\text{H}_{19}\text{N}_3\text{O}\cdot\text{HCl}$ , MW: 257.76) when i.p. administration at the same equimolar dose. E.g. i.p. administration of 40 mg/kg Z-GP-Pcb reduced 69.81% solid tumor weight; this was almost the same effect with i.p. administration of 20 mg/kg Pcb. It can be seen that the tumor inhibition was dose-dependent.

It was of notice that i.p. administration of Z-GP-Pcb at both doses did not cause significant loss of body weight, few effects on spleen index and thymus index of tumor-bearing mice; while Pcb decreased spleen index. The thymus index and spleen index are closely related to the immune status inside the life body. It seemed to us that usage of Z-GP-Pcb had less impact on immune system than that of Pcb.

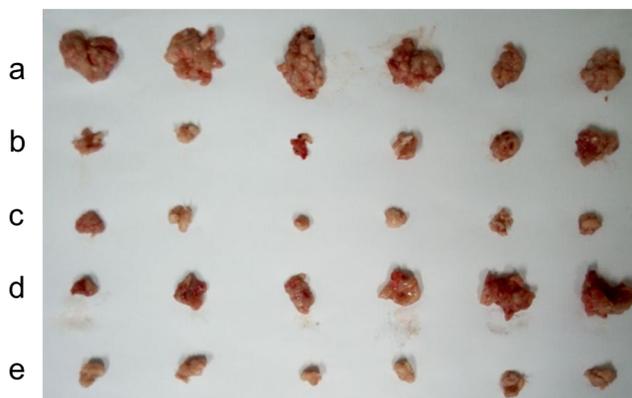


Fig. 4. Anticancer activity of the Pcb/Z-GP-Pcb on H22-bearing mice. a: Vehicle; b: 10 mg/kg Pcb; c: 20 mg/kg Pcb; d: 20 mg/kg Z-GP-Pcb; e: 40 mg/kg Z-GP-Pcb.

On the other hand, it was found in Table 4 that Pcb-treated groups significantly lowered both levels of WBC and PLT ( $P < 0.05$  or  $P < 0.01$  vs. vehicle group), where WBC means white blood cell, and PLT means platelet haematoblast. However, i.p. administration of Z-GP-Pcb caused less impact on WBC and PLT, especially PLT. We could see from Table 4 that treatment with Z-GP-Pcb at either high or low dose did not have impact on PLT level.

As we know, myelosuppression is the decrease in production of cells responsible for providing immunity (leukocytes), carrying oxygen (erythrocytes), and/or those responsible for normal blood clotting (thrombocytes). This bone marrow suppression is a serious side effect of chemotherapy and certain drugs affecting the immune system such as azathioprine [26]. The risk is especially high in cytotoxic chemotherapy for leukemia [27]. Although Pcb is not a cytotoxic anticancer agent, it has been reported with myelosuppression [5]. This was confirmed by our experiment characterized by the decrease of WBC and PLT levels. Interestingly, Z-GP-Pcb may reduce the risk of myelosuppression.

By the way, the levels of RBC (red blood cell) and HGB (hemoglobin) were no significant difference among all groups (Table 4).

## 4. Conclusions

In short, a 5-steps process for the synthesis of Z-GP-Pcb was developed. The overall yield of this process was 32.7%. It was disclosed that the incorporation of Z-GP to either  $\alpha$  or  $\beta$  position of hydrazine moiety in Pcb made the new entity targeting FAP $\alpha$ . It has been confirmed that Z-GP-Pcb may be hydrolyzed by either isolated rhFAP $\alpha$  or tumor homogenate. The new entity was shown far less cytotoxicity against NCI-H460 cancer cell than its parent compound Pcb. Z-GP-Pcb was demonstrated the potency to reduce spermatotoxicity in H22-bearing mice. The mechanism may be ascribed to the blockade of dehydrogenation by  $\alpha$ -glycerolphosphate dehydrogenase, which may metabolize Pcb to azoprocarbazine that is further changed into azoxyprocarbazine isomers. Azoprocarbazine and azoxyprocarbazine isomers are key intermediates that cause spermatotoxicity. It was disclosed that Z-GP-Pcb has almost the same antitumor activity as Pcb *in vivo*. However, introduction of Z-GP scaffold decreased myelosuppression. All the evidences support that Z-GP-Pcb is a better antitumor agent than Pcb.

**Table 3**

Effects of Pcb/Z-GP-Pcb on body weight, tumor weight, tumor inhibitory rate and immune organ index of H22-bearing mice.

Group	dosage (mg/kg)	Body weight (g)	Tumor weight (g)	Inhibitory rate (%)	Thymus index	Spleen index
Vehicle	–	40.94 ± 3.93	1.55 ± 0.95	–	1.95 ± 0.24	5.46 ± 1.49
Pcb	10	38.57 ± 3.06	1.02 ± 0.62*	33.93	1.83 ± 0.51	4.60 ± 1.66
Pcb	20	38.15 ± 2.15	0.40 ± 0.29**	73.91	2.16 ± 0.71	4.54 ± 0.81
Z-GP -Pcb	20	42.40 ± 2.67	1.15 ± 1.02*	25.80	2.04 ± 0.63	5.74 ± 1.17
Z-GP -Pcb	40	38.27 ± 6.34	0.47 ± 0.12**	69.81	2.14 ± 0.39	5.77 ± 2.31

“–” means no data; Values were expressed as mean ± SD (n = 6); Significance was determined using the Student's *t*-test.\* *P* < 0.05 vs vehicle group.\*\* *P* < 0.01 vs vehicle group.**Table 4**

Effects of Pcb and Z-GP-Pcb on WBC, RBC, HGB, and PLT (± s, n = 6).

Group	dosage (mg/kg)	WBC (10 <sup>9</sup> /μl)	PLT (10 <sup>3</sup> /μl)	RBC (10 <sup>6</sup> /μl)	HGB (g/dL)
Vehicle	–	5.77 ± 1.80	1507 ± 266	7.77 ± 0.76	12.37 ± 1.35
Pcb	20	3.36 ± 1.23**	1152 ± 263*	7.71 ± 0.38	12.30 ± 0.70
Pcb	10	3.54 ± 0.91**	1102 ± 454*	7.45 ± 0.87	11.95 ± 1.29
Z-GP -Pcb	40	4.65 ± 2.32	1620 ± 239##	7.57 ± 0.79	12.29 ± 0.88
Z-GP -Pcb	20	4.73 ± 1.30	1439 ± 171#	8.05 ± 0.74	12.40 ± 1.09

“–” means no data; Values were expressed as mean ± SD (n = 6); Significance was determined using the Student's *t*-test;\* *P* < 0.05 vs. vehicle group.\*\* *P* < 0.01 vs. vehicle group.# *P* < 0.05 vs. Pcb (10 mg/kg) group.## *P* < 0.01 vs. Pcb (20 mg/kg) group.

## 5. Experimental section

### 5.1. Research governance

All animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of Jinan University (20141112017) and Jiangxi University of Traditional Chinese Medicine, Institutional Animal Care and Utilization Committee, and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (7th edition, USA).

### 5.2. Materials

Chemicals including 4-methylbenzoic acid, Ce(NH<sub>4</sub>)<sub>4</sub>(NO<sub>3</sub>)<sub>6</sub>, (CH<sub>3</sub>)<sub>2</sub>CHNH<sub>2</sub>, and SOCl<sub>2</sub> were purchased from Aldrich or Adams without further purification. Carbobenzoxyglycyl proline (Z-GP-OH), 1-hydroxy-benzotriazole (HOBt), *O*-(7-aza-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), and diisopropylethylamine (DIPEA) were purchased from GL Biochem (Shanghai) Ltd., China. Silica gel for column chromatography was purchased from Qingdao Marine Chemicals Inc, China. Chromatographic grade methanol was bought from Shandong YuWang Reagent Company (China).

The NCI-H460 and murine hepatoma H22 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA).

Recombinant human FAPα (rhFAPα) was purchased from R&D Systems (Minneapolis, MN). PBS buffers used were vacuum-filtered through a 0.22 μm membrane. Cell culture media, supplements and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD). All culture flasks were obtained from Corning (Corning, NY).

Male Kunming mice were obtained from the Laboratory Animal Centre of Jiangxi University of Traditional Chinese Medicine. Mice used for either the Spermatotoxicity assays or tumor inoculation were 6–8 weeks old and weighed between 18 and 22 g.

### 5.3. Synthetic process

#### 5.3.1. General chemical experimental procedures

Nuclear Magnetic Resonance (NMR) spectra were recorded on either a Bruker AV-300 or a Bruker AV-400, or a Bruker AV-500 (Bruker Biospin, Swiss). Tetramethylsilicane (TMS) was used as an internal standard. ESI-MS were recorded on a Finnigan LCQ Advantage MAX mass spectrometer. HPLC was performed on either a LC-100 liquid chromatograph equipped with a tunable LC-100 UV detector (Shanghai Wufeng Inc., China) or an Agilent 1200 series liquid chromatograph equipped with an Agilent 1200 Series UV detector (Agilent Technologies, USA). Columns used were Cosmosil 5C<sub>18</sub> (Nacalai Tesque Inc., Japan) for general purification. Pre-coated thin-layer chromatography (TLC) plates (Institute of Yantai Chemical Industry, China) were used for TLC. Spots on TLC plates were detected by either a ZF-7A portable UV detector or spraying Bismuth potassium iodide solution followed subsequent heating. Ethanol was refluxed over Fresh magnesium ribbon for 5 h and redistilled.

#### 5.3.2. Synthesis of 4-methylbenzoyl chloride (1)

To a 25-ml flask, 4-methylbenzoic acid (273 mg, 2.0 mmol), and SOCl<sub>2</sub> (5.0 ml, 68.8 mmol) were added. The mixture was refluxed over an oil-bath at 78 °C for 2 h. Afterwards, excess SOCl<sub>2</sub> was removed by rotative evaporation under reduced pressure. TLC analysis showed the residue pure enough. It was used directly in the next reaction without further purification. Slight yellow oil 308.3 mg was obtained, yield 99.7%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 8.01 (d, *J* = 3.2 Hz, 2H), 7.43 (d, *J* = 3.2 Hz, 2H), 2.38 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 167.8, 143.6, 132.2 (2), 129.1, 130.1, 127.8 (2), 21.3. ESI-MS *m/z*: 155.1, 157.2[M + H]<sup>+</sup>.

#### 5.3.3. Synthesis of *N*-isopropyl-4-methylbenzamide (2)

4-Methylbenzoyl chloride (309.2 mg, 2.0 mmol) was dissolved in 2.0 ml dried dichloromethane (DCM) at a 25-ml flask. A solution of isopropylamine (1.0 ml, 11.7 mmol) in 2.0 ml DCM was added to the flask dropwise with stirring. The reaction temperature was maintained at 30 °C. Afterwards, the mixture was stirred for another 30 min. The reaction was lasted for 3 h at room temperature. Then the temperature

was raised to 40 °C for 30 min. With the termination of heating, the mixture was poured into 50 ml ice-water under stirring. Extraction with 20 ml DCM/ether (1:5, V/V) for 4 times. All the extractions were combined. The organic layer was then washed water, 0.1 mol/L HCl solution, water, 0.1 mol/L NaOH solution, and water, respectively. Then it was dried over anhydrous MgSO<sub>4</sub>. Excess solvents were removed by rotative evaporation under reduced pressure. The residues were purified by flash column liquid chromatography with EtOAc/petroleum ether (1:5, V/V) as eluant, led to white solid 289.8 mg, yield 88.9%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.67 (d, *J* = 3.0 Hz, 1H), 7.64 (d, *J* = 3.0 Hz, 1H), 7.21 (d, *J* = 3.0 Hz, 1H), 7.19 (d, *J* = 3.0 Hz, 1H), 4.22–4.33 (m, 1H), 2.38 (s, 3H), 1.26 (s, 3H), 1.24 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 166.6, 141.5, 132.1, 129.1, 129.1, 126.8, 126.8, 41.7, 22.8, 21.3, 21.3. ESI-MS *m/z*: 178.2[M+H]<sup>+</sup>, 200.3[M+Na]<sup>+</sup>.

#### 5.3.4. Synthesis of *N*-isopropyl-4-formylbenzamide (3)

To a 20-ml pressure-proof tube, *N*-isopropyl-4-methylbenzamide (177.2 mg, 1.0 mmol) and 2.0 ml of 3.5 mol/L HNO<sub>3</sub> solution were added, and well-distributed by ultrasonic. A solution of ceric ammonium nitrate (2.19 g, 4.0 mmol) in 8.0 ml 3.5 mol/L HNO<sub>3</sub> was added dropwise to the tube with stirring. At the end of addition, the sealed tube was put over an oil-bath at 100 °C to react for 24 h. Afterwards, the tube was cooled down and the reaction mixture was poured into 100 ml of saturated NaCl solution. Then the mixture was extracted by DCM for 3 times (3 × 100 ml). All the extractions were combined. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Excess solvents were removed by rotative evaporation under reduced pressure. The residues were purified by flash column liquid chromatography with EtOAc/petroleum ether (1:5, V/V) as eluant, led to white solid 136.2 mg, yield 71.2%. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ: 10.03 (s, 1H), 7.95 (d, *J* = 9.0 Hz, 2H), 7.58 (d, *J* = 9.0 Hz, 2H), 4.10–4.30 (m, 1H), 1.26 (d, *J* = 6.0 Hz, 3H), 1.24 (d, *J* = 6.0 Hz, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 193.5, 168.4, 130.6, 129.0, 128.1(2), 127.2(2), 43.4, 22.5(2); ESI-MS (*m/z*): 192.3[M+H]<sup>+</sup>, 224.5[M+Na]<sup>+</sup>.

#### 5.3.5. Synthesis of procarbazine (4)

To a 250-ml flask, *N*-isopropyl-4-formylbenzamide (192.3 mg, 1.0 mmol), methyl hydrazine hydrochloride (505.0 mg, 3.5 mmol), and 20 ml absolute ethanol were added. After stirred for 20 min, 1.0 ml triethylamine was added. The sealed flask was kept over an oil-bath at 60 °C to react for 6 h. Afterwards, excess solvents were removed. The residues were re-dissolved in 10.0 ml DMF, following the addition of NaCNBH<sub>3</sub> (126.0 mg, 2.0 mmol) slowly at 0 °C. The mixture was then warmed up to room temperature and kept stirring overnight. The reaction was quenched by the addition of 200 ml of saturated NaCl solution. Then the mixture was extracted by EtOAc for 5 times (5 × 40 ml). All the extractions were combined. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Excess solvents were removed by rotative evaporation under reduced pressure. The residues were purified by flash column liquid chromatography with EtOAc/petroleum ether (1:3, V/V) as eluant, led to white solid 161.9 mg, yield 74.0%, purity 97.3%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.47 (d, *J* = 7.0 Hz, 1H), 7.43 (d, *J* = 7.0 Hz, 1H), 7.31 (d, *J* = 7.0 Hz, 1H), 7.24 (d, *J* = 7.0 Hz, 1H), 3.88–3.91 (m, 1H), 3.93 (s, 2H), 2.47 (s, 3H), 1.25 (d, *J* = 6.0 Hz, 3H), 1.23 (d, *J* = 6.0 Hz, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 166.2, 144.4, 130.1, 125.8, 125.8, 125.7, 125.7, 55.2, 40.4, 33.7, 22.3, 22.3; ESI-MS (*m/z*): 222.3[M+H]<sup>+</sup>, 244.3[M+Na]<sup>+</sup>.

#### 5.3.6. Synthesis of carbobenzoxy glycylylprolyl procarbazine (Z-GP-Pcb, 5α/5β)

Z-GP-OH (612.0 mg, 2.0 mmol), HOBt (283.0 mg, 2.1 mmol), HATU (798.0 mg, 2.1 mmol), and 10.0 ml dried DCM were added to a 50-ml flask. The flask was cooled over an ice-bath. Then the addition of 0.3 ml DIPEA was followed under stirring. The mixture was stirred for 30 min to activate the carboxyl group, which was called activated body. Meanwhile, procarbazine (442.6 mg, 2.0 mmol) was dissolved in 5.0 ml

DCM with 0.5 ml DIPEA. This solution was then added to the activated body dropwise. Afterwards, the reaction mixture was warmed up to room temperature and kept stirring for 3–4 h. Then, 100 ml saturated NaCl solution was added to quench the reaction. The mixture was extracted by DCM for 5 times (5 × 60 ml). All the extractions were combined. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Excess solvents were removed by rotative evaporation under reduced pressure. The residues were purified by flash column liquid chromatography with CHCl<sub>3</sub>/methanol (10:1, V/V) as eluant, following further purification by RP-HPLC with methanol/water (50:50, V/V) as eluant, led to slight yellow sticky oil 690.1 mg, yield 69.0%, purity 98.5%. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ: 7.83–7.78 (m, 2H), 7.52–7.47 (m, 2H), 7.38–7.30 (m, 5H), 5.10 (d, *J* = 6.6 Hz, 2H), 4.29–3.96 (m, 5H), 3.57 (m, 2H), 3.22 (s, 1H), 3.17 (s, 2H), 2.05 (m, 3H), 1.87–1.66 (m, 2H), 1.26 (d, *J* = 6.7 Hz, 6H); <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD) δ: 173.58, 166.38, 165.55, 156.31, 140.79, 137.39, 134.46, 129.11, 128.77(2), 128.31, 127.75, 127.71, 127.48, 127.27(2), 65.88, 65.84, 56.59, 52.32, 48.91, 45.92, 41.43, 29.76, 24.29, 21.89 (2); ESI-MS (*m/z*): 532.5[M+Na]<sup>+</sup>; HRMS (*m/z*): calc. for [C<sub>27</sub>H<sub>35</sub>N<sub>5</sub>O<sub>5</sub> + Na]<sup>+</sup> 532.2536, found 532.2533.

### 5.4. Biological section

#### 5.4.1. Cell culture

All the cell lines were grown in specific media supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were grown in a 5% CO<sub>2</sub> humidified atmosphere in incubators maintained at 37 °C.

#### 5.4.2. MTT assay

3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect inhibition of cellular proliferation mediated by the drugs. This assay was applied to all cell lines. The process was described below: Cells in suspension were plated in 96-well plates at a density of 1 × 10<sup>4</sup> cells/well and cultured for 24 h. Then the medium was replaced with the respective medium containing drugs at different concentrations and incubated for 48 h. The final DMSO concentration in all experiments was less than 0.1% in medium. The concentration range of tested samples was 0–50 μM and two-fold serial dilutions were applied. Afterwards, 10 μL MTT solutions (5 mg/ml) were added to each well, and the plate was incubated for an additional 4 h. The absorbance of the converted dye in living cells was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad; Hercules, CA, USA) after 100 μL of DMSO added. IC<sub>50</sub> values were determined by the nonlinear multipurpose curve fitting program GraphPad Prism. All of the tests were repeated at least 3 times.

#### 5.4.3. Chromatography for detection of purity and concentration

High-performance liquid chromatography (HPLC) assay for cleavage of Z-GP-Pcb by FAPα was performed with Agilent 1200 series (Karlsruhe, Germany); UV-detection at 214/245 nm; column: ZirChrom Separations Inc. (Anoka, MN), reverse-phase C<sub>18</sub> (4.6 × 250 mm) with precolumn; conditions: flow 1.0 ml/min; mobile phase: water/methanol (40/60, V/V); time: 0–15 min; injection volume: 10 μL. All of the tests were repeated at least 3 times.

#### 5.4.4. Cleavage of Z-GP-Pcb by rhFAPα

Z-GP-Pcb was incubated in Tris-buffer (pH 7.4) with rhFAPα (2 μg/ml, and 5 μg/ml, respectively) at a final concentration of 30 μM at 37 °C for 1, 2, 4, 8, 16, and 24 h. Subsequently, the sample of each time point was collected and analyzed by RP-HPLC. All of the tests were repeated at least 3 times.

#### 5.4.5. Cleavage of Z-GP-Pcb with tumor homogenate

Tumor tissue was obtained from model mice bearing H22 tumor and cut into small pieces. Every 200 mg of tumor tissue was added to 800 μL of homogenates buffer [50 mM Tris-HCl buffer (pH 7.4)] to prepare

homogenate. Z-GP-Pcb was incubated with the tumor homogenate at a final concentration of 50  $\mu\text{M}$ . The samples were collected at set time points, processed and measured using RP-HPLC methods. Briefly, each sample (400  $\mu\text{L}$ ) was added to tube containing 4 ml of DCM/ acetonitrile (4:1, V/V) solution. The tube was vigorously shaken, and the organic layer was collected by centrifugation. After solvent evaporation, the residue was dissolved in 150  $\mu\text{L}$  of methanol, and filtered through 0.22  $\mu\text{m}$  membrane. 10  $\mu\text{L}$  of sample was submitted to RP-HPLC for analysis. All of the tests were repeated at least 3 times.

#### 5.4.6. Sperm count determination

Kunming mice (12 mice per treatment group) were treated i.p. with the following tested compounds: (a) Pcb (75 mM, 150 mM); (b) Z-GP-Pcb (75, 150 mM). Control animals received the appropriate volume of saline. Epididymal sperm counts were obtained 18 days later, as previously described [12]. Briefly, Mice were injected i.p. with each of the tested compounds in 0.9% NaCl solution, epididymides were removed and weighed 18 days later, minced finely, and suspension of spermatozoa in a 10% Ficoll and 0.5% sodium lauryl sulfate aqueous solution. Then the determination of sperm counts was carried on with a hemacytometer. All of the tests were repeated at least 3 times.

#### 5.4.7. Determination of antitumor activity in vivo

Male Kunming mice (18–22 g) were randomized into A to F groups ( $n = 10$ ). The animals were subcutaneously injected with H22 cells ( $1 \times 10^7$  cells/mouse) into the armpit of the right hind limb. Group A served as the tumor control (Vehicle group), which received sodium suspension (0.9%); While groups B was low dose Pcb group (10 mg/kg), C/high dose Pcb group (20 mg/kg), D/low dose Z-GP-Pcb group (20 mg/kg) and E/high dose Z-GP-Pcb group (40 mg/kg). Either Pcb or Z-GP-Pcb was injected into the abdominal cavity once daily for 14 days.

Twenty four hours after the last dose, blood was collected from the animals for the white blood cell (WBC) count by retro-orbital puncture under slight anesthesia (diethyl ether). Mice from each group were sacrificed under anesthesia using  $\text{CO}_2$ . The mice and the separated tumor were weighed. Antitumor activity was evaluated by tumor weighing. The tumor inhibition rate was calculated as: Tumor inhibitory rate (%) =  $(1 - \text{average tumor weighing of administration group} / \text{average tumor weighing of the tumor control group}) \times 100\%$  [13]. Simultaneously, the thymus and spleen were excised and weighed. Indices were calculated as: Thymus index =  $[\text{the thymus weight (mg)} \times 10] / \text{body weight (g)}$  and spleen index =  $[\text{the spleen weight (mg)} \times 10] / \text{body weight (g)}$ .

#### 5.4.8. Statistical analysis

All data are expressed as the mean  $\pm$  standard deviation (SD) of three independent experiments. Statistical significance was assessed using Student's *t*-test (for comparisons of two treatment groups) or one-way ANOVA (for comparisons of three or more groups). *P*-values < 0.05 were considered statistically significant.

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#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2018.11.011>.

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