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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Enzymic synthesis and biological evaluation of injectable glutathione-everolimus

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Abstract An enzymic synthesis of glutathione-everolimus is reported. This process has been optimized and scaled up with high reproducibility and yields, which will facilitate the development of such conjugate. The stability of the conjugate supported that this prodrug can be prepared into lyophilized solid, which is to be reconstituted with 0.9% sodium chloride for injection before intravenous infusion. And the results of species-related drug release experiment displayed that the performance of the conjugate in human plasma, rat and monkey was similar. Moreover, the in vivo efficacy of glutathione-everolimus in the treatment of renal cell carcinoma was investigated in detail. The conjugate was proved to be an effective, safe and well-tolerated injectable prodrug in the treatment of renal cell carcinoma. The results indicated that three times injection with a high dosage in 1 week can achieve much better in vivo efficacy, and no obvious toxic response was observed.

Keywords Everolimus · Glutathione · Renal cell carcinoma · Prodrug · Drug delivery · mTOR

Introduction

Renal cell carcinoma (RCC) is the most common primary malignant tumor of the kidney (Eble et al. 2004), accounting for approximately 85% of kidney carcinomas (Arai and Kanai 2010). And the incidence of RCC is still increasing at a rate of 2–3% per decade in most countries (Shirotake et al. 2016). Everolimus and temsirolimus (Fig. 1), two potent mammalian target of rapamycin (mTOR) inhibitors, were approved by the FDA for the treatment of advanced RCC (Sherman et al. 2015). Observational retrospective data suggested that temsirolimus, as an weekly and intravenously administered agent, exhibited lots of advantages compared to oral everolimus, including financial considerations, assurance of patient compliance given its intravenous administration, its toxicity profile, patient performance status, and patient or physician preference (Stenner-Liewen et al. 2013; Mackenzie et al. 2011; Weikert et al. 2013; Alasker et al. 2013; Patel et al. 2012). For those reasons, injectable temsirolimus was widely used as a succedaneum of oral everolimus in the clinical therapy for advanced cancer patients. However, more than eight species of excipient for assisting solubilization, including ethanol and castor oil, are used in the formulation of temsirolimus, which lead to much adverse reactions (Coiffier 2013; Farag et al. 2009).

Up to now, no injectable everolimus-based drug has been developed in the market because of its poor water solubility. Although well-tolerated profile, efficacy and safety of everolimus in treating advanced RCC have been proved in clinical trials (Motzer et al. 2010), there are limited for oral

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administration when treating patients with dysphagia induced by dental ulcer and mucous irritation, which are the most common side effects of long-term intake of everolimus (Motzer et al. 2010). In addition, daily treatment is thought to limit day-to-day variability in exposure attributable to its low bioavailability (Crowe et al. 1999) and variable absorption (Kirchner et al. 2004; Kovarik et al. 2002), which lead to treatment failure and short survival.

Thus, an injectable everolimus-based drug is required for enhancement of advanced patient compliance and improvement in therapeutic efficacy.

Some approaches to circumvent the problem of water solubility of water insoluble agents have been to conjugate the drug directly to a water-soluble polymer such as hydroxypropyl methacrylate (Tomalova et al. 2016), polyethyleneglycol (PEG) (Zhao et al. 2008), and cyclodextrins polymer (CDP) (Ryan 2013). For example, PEGylated everolimus had been successful in solubilizing the therapeutic agent (Zhu et al. 2002). An alternative solution to drug delivery has been offered in our previous work, in which an endogenous tripeptide, glutathione, was used (Wang et al. 2016, 2017). The reported conjugate, glutathione-everolimus, showed good water solubility and better in vivo efficacy (Wang et al. 2017). As a part of our ongoing researches towards the development of this conjugate, in this paper, we report an enzymic synthesis of glutathione-everolimus and evaluation of the injectable glutathione-everolimus in the treatment of human renal cell carcinoma in detail.

Results and discussion

Synthesis

In our previous work, iodoacetate of everolimus was used as an intermediate in the synthesis of glutathione-everolimus. As we known, α-iodoaliphatic acid and its ester are instable and easily decomposed, which led to low yield in the second step of the reported synthetic route (Wang et al. 2017). As an attempt to improve the synthetic method, vinyl chloroacetate was used in the synthesis of intermediate **1** by enzymic synthesis with immobilized lipase as a catalyst (Scheme 1). Unfortunately, glutathione

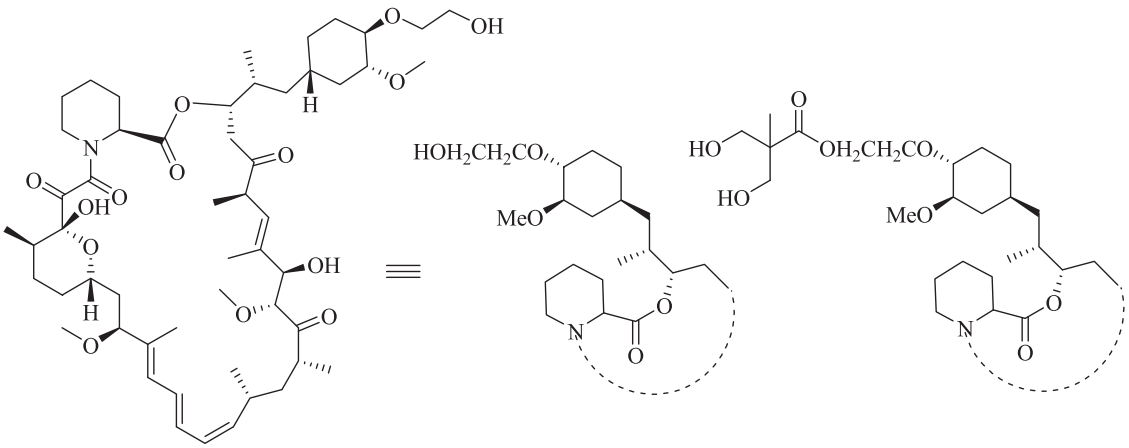
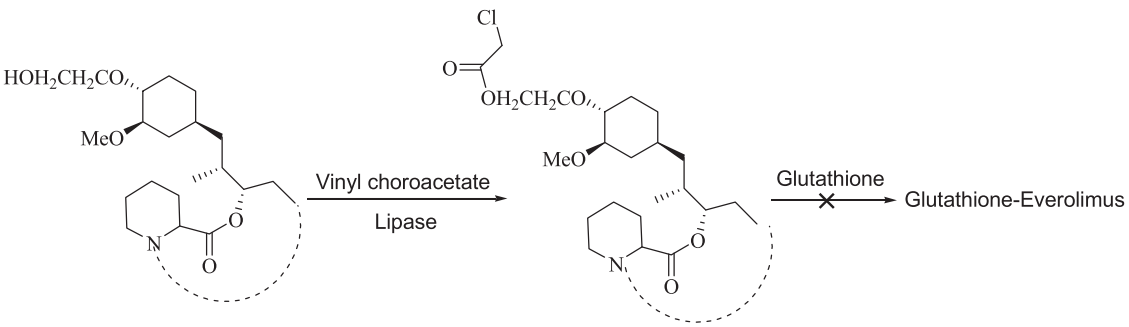
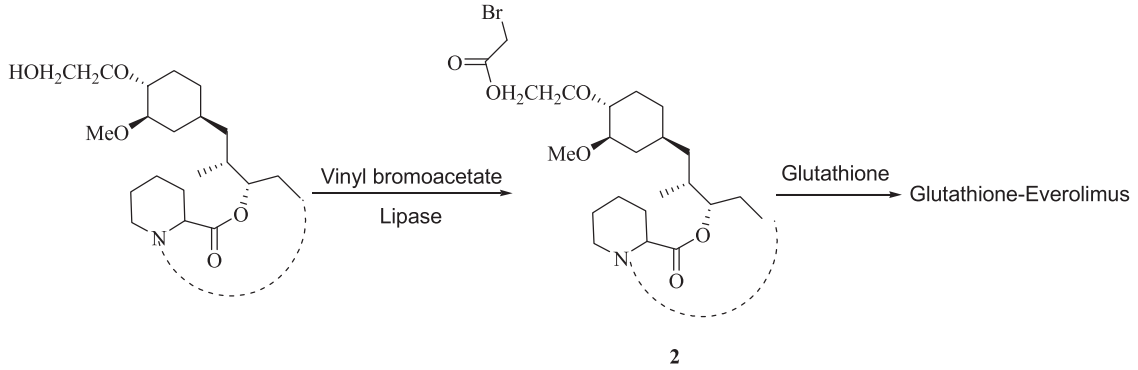


Fig. 1 Structures of everolimus and tesirolimus



Scheme 1 The enzymic synthesis of glutathione-everolimus with vinyl choroacetate



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Scheme 2 The enzymic synthesis of glutathione-everolimus with vinyl bromoacetate

Table 1 Stability of glutathione-everolimus at room temperature

Solution	Time of detection (h)	Remaining of the conjugate (%)
Saline (0.9% NaCl)	0	99.1
	24	99.1
Water for injection	0	99.1
	24	99.1

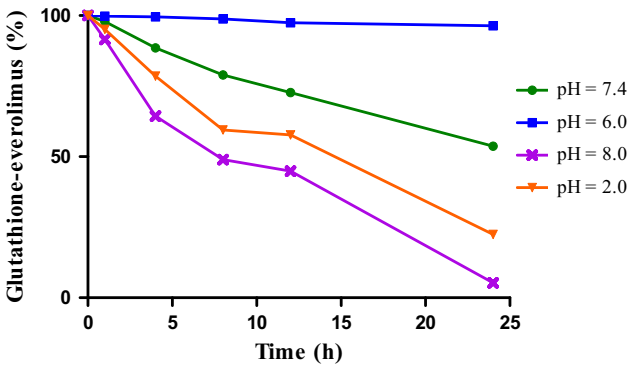

Fig. 2 The stability of glutathione-everolimus in different pH PBS solutions

was hardly reacted with the resulting intermediate **1** because of the weak leaving activity of the chlorine atom.

As an alternative, vinyl bromoacetate was used in the synthesis of intermediate **2** by enzymic synthesis (Scheme 2). This step was almost quantitative without any purification by filtering the immobilized lipase and evaporating the excessive low-boiling vinyl bromoacetate and other by-product. The more active bromine atom of intermediate **2** was easily reacted with the thiol group of glutathione to give the target conjugate, which was purified by reversed phase high performance liquid chromatography (RP-HPLC) with 83% yield. By contrast, the chemical synthesis just obtained about 42% total yield (Wang et al. 2017). This enzymic synthesis approach has been successfully optimized and scaled up to hundreds of grams per batch on a repetitive base.

pH stability study

In the preformulation research, we investigated the stability of glutathione-everolimus in different pH solutions. The results showed that it was stable under neutral conditions in solution. For example, the conjugate was very stable without any degradation over 24 h at room temperature in clinically relevant solutions (Table 1). Therefore, glutathione-everolimus can be dissolved in WFI (water for injection) and prepared into lyophilized solid, which is to be reconstituted with 0.9% sodium chloride for injection before intravenous infusion.

Table 2 pH stability studies for the conjugate at 37 °C

pH	2.0	6.0	7.4	8.0
$t_{1/2}$ (h)	12.2	429.8	26.3	8.1

Since the linkage between the acetyl spacer and everolimus is an ester bond, besides, everolimus is a macrolide, the conjugate would be easily hydrolyzed under basic conditions. As predicted, the degradation of the conjugate increased as the pH was increased above 6.0 in phosphate buffers at 37 °C (Fig. 2; Table 2). But the conjugate was relative stable at pH 6.0 with a half-life of more than 429 h. The degradation under basic condition, such as at pH 8.0, took place quickly with a half-life of only 8.1 h. Meanwhile, it was also hydrolized in highly acidic condition, and the half-life of the conjugate at pH 2.0 was about 12.2 h. The pH-dependent study clearly showed that everolimus could not be released from the conjugate in the pH 7.4 PBS solution at 37 °C with a half-life of about 26.3 h for degradation. However, release of everolimus from the conjugate in rat (Wang et al. 2017) indicated that enzymes

in vivo played an important role in this process. These enzymes could be either esterases or aminopeptidases (Yeo et al. 1998).

Species-related drug release experiment

In our previous work, the release of everolimus from the conjugate in rat was investigated, which showed that this releasing process took about 2 h (Wang et al. 2017). To evaluate this process in different species, we studied the drug release of the conjugate in human plasma and cynomolgus monkey.

As Fig. 3. displayed, everolimus was gradually eliminated in human plasma, which might be owing to in vitro metabolism or instability. The conjugate can be converted to everolimus in human plasma, and this process took more than 5 h in vitro. Meanwhile, the released everolimus from the conjugate was also continuously eliminated in human plasma, which led to the curve declined at the end point.

The drug release experiment of the conjugate was also studied in a cynomolgus monkey. As the results shown in Fig. 4, the conjugate can quickly release everolimus in cynomolgus monkey in a short time. For example, the concentration of the released everolimus was about 4600 ng/mL at the fifth minute, which was slightly higher than that of the remaining conjugate. And this slightly higher phenomenon was persistent throughout the whole process. We believe that this was owing to the in vivo metabolism of the released everolimus is slower than the releasing of the conjugate. Therefore, this releasing process only spent approximately 4 h, but the released everolimus from the conjugate can persist acting in monkey body for more than 8 h. The results also indicated that the release of everolimus from the conjugate in monkey took place more slowly than that in rat, but slightly faster than that in human plasma.

In brief, the results of the species-related drug release experiment suggested that glutathione-everolimus can release its parent compound, everolimus, in rat, monkey, and human, which indicated that the prodrug might create a similar effect in all the three species.

Acute toxicity

Glutathione-everolimus was given intravenously to mice at different doses to evaluate its acute toxicity and the maximum tolerable doses. Mice administered glutathione-everolimus at a single dose of 70 mg/kg (equivalent dose of everolimus) were still alive after 1 week without any toxic response. And no visible signs of toxicity and weight loss were observed when glutathione-everolimus was given at a dose of 45 mg/kg three times a week to the mice for 1 month. Therefore, glutathione-everolimus appeared to be low toxic and good tolerance.

In vivo evaluation

Based on our previous work, a high-dose injection of glutathione-everolimus once a week led to effective tumor inhibition. To evaluate the influence of the administration frequency and dosage to the treatment effect of glutathione-everolimus, a more detailed experiment was carried out. A

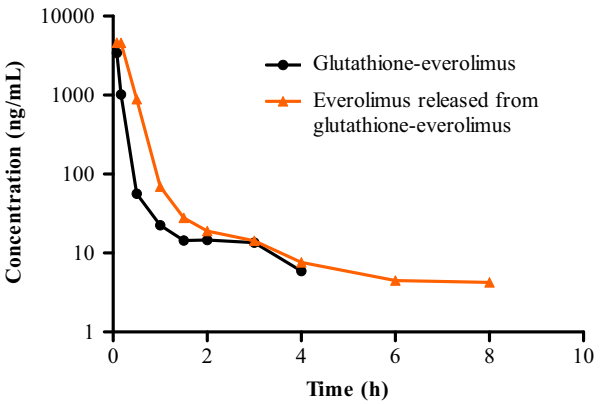

Fig. 4 The concentration–time curve of the injected glutathione-everolimus (1 mg/kg) in cynomolgus monkey body

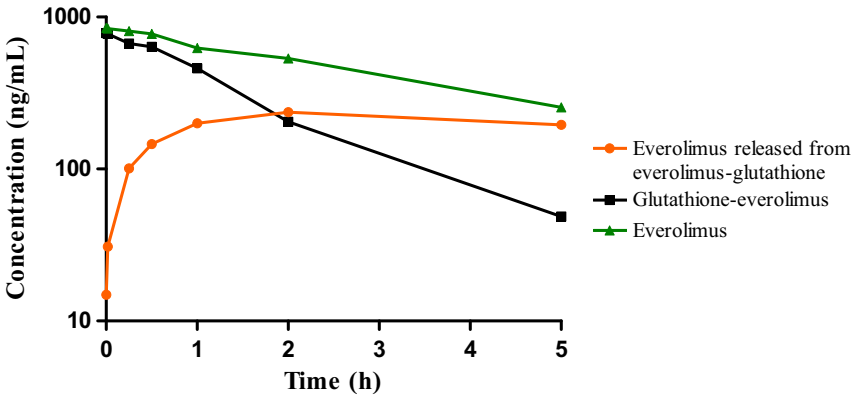

Fig. 3 Release of everolimus from glutathione-everolimus in human plasma

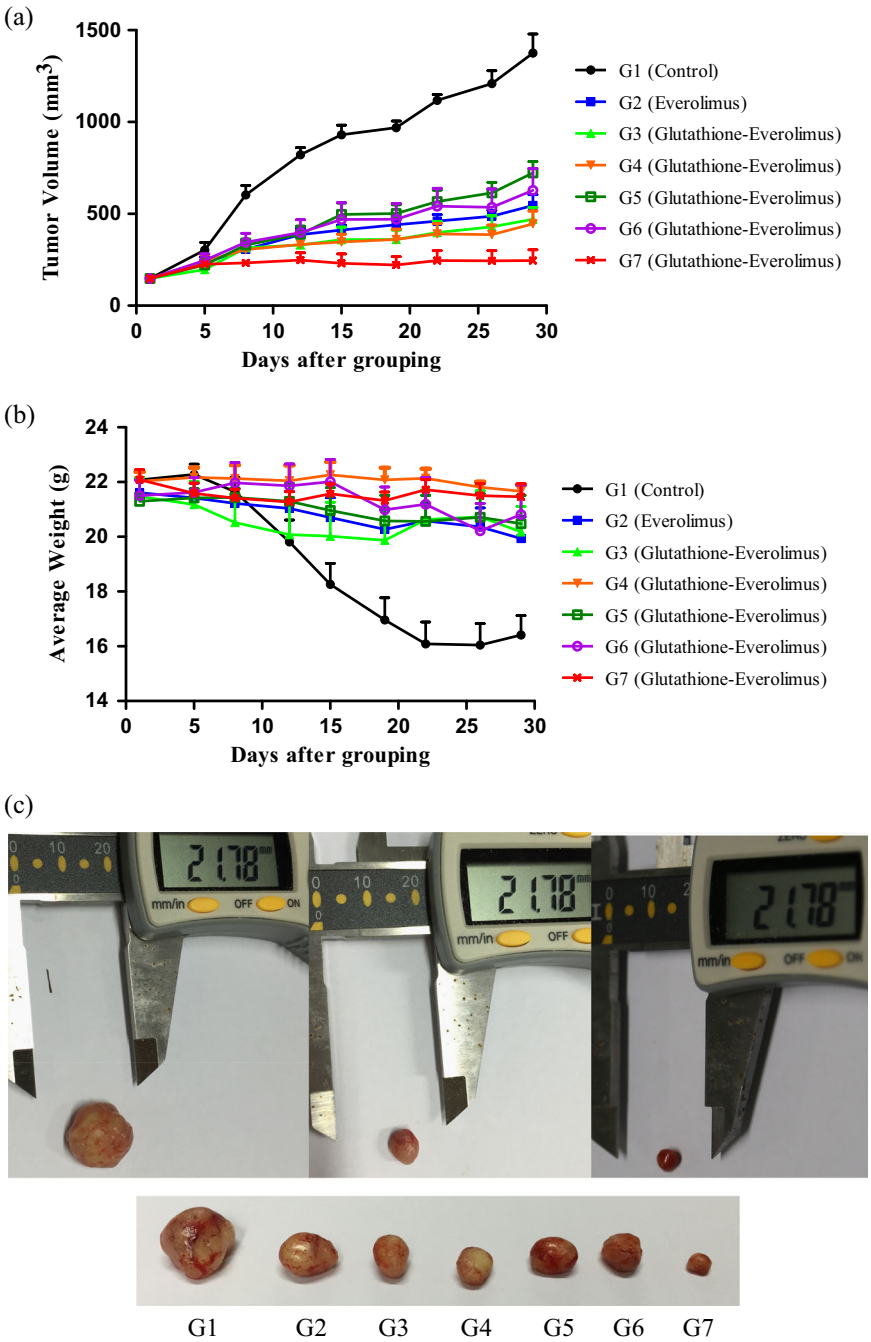

Fig. 5 **a** The tumor volume changes over 29 days of administration; **b** The body weight changes of the mice over 29 days of administration; **c** The representative photos of the tumors stripped from the sacrificed mice

summary of the preliminary results is given in Fig. 5 and Table 3.

As the results indicated that tumors were effectively inhibited in all the treated groups. The tumor inhibition of oral glutathione-everolimus was 74.8%, which was very close to that of oral everolimus (tumor inhibition: 71.7%), meaning that the in vivo activity of the oral prodrug was equivalent to that of oral everolimus. However, injected glutathione-everolimus groups gave much different results. The groups (G5 and G6) administrated intravenously once a week exhibited effective inhibition to tumors (Inhibition rate ≥ 60.0%) but with relative low inhibition rate, 60.8 and 67.4%, respectively, which was accordance with the results in our previous work (Wang et al. 2017). Meanwhile, dose escalation can not obviously improve the curative effect (G5 vs. G6). However, increasing administration frenquency gave better results (G6 vs. G7). The tumor growth was totally inhibited in group 7 (G7) with 86.6% inhibition rate, when mice were administrated three times a week by intravenous injection. Lower dosage with 10 mg/kg three times a week also achieved good tumor inhibition, which was 78.5%. Therefore, administration by intravenous

Table 3 In vivo activity of everolimus and glutathione-everolimus against renal cell carcinoma OS-RC-2

Group	Dose (mg/kg)[a]	Administration frequency	Average tumor weight (g) ± SEM	Tumor inhibition[b]	Body weight changes (%)[c]	*P* value [d]
G1	–	–	0.795 ± 0.127	–	−25.7	–
G2	5	qd, ig	0.225 ± 0.057	71.7%	−7.7	<0.001
G3	5	qd, ig	0.200 ± 0.071	74.8%	−6.0	<0.001
G4	10	3 times/qw, iv	0.171 ± 0.066	78.5%	−1.7	<0.001
G5	25	qw, iv	0.312 ± 0.087	60.8%	−3.9	<0.001
G6	35	qw, iv	0.258 ± 0.130	67.4%	−3.2	<0.001
G7	35	3 times/qw, iv	0.106 ± 0.073	86.6%	−2.8	<0.001

[a] Equivalent dose of everolimus

[b] Tumor inhibition = (1 − average tumor weight of treated group/average tumor weight of negative control group) × 100

[c] Body weight changes = (average body weight of mice at the twenty-ninth day/average body weight of mice at first day − 1) × 100

[d] Be relative to the negative control group for average tumor weight

Table 4 The liver coefficient of the sacrificed mice

Group	G1	G2	G3	G4	G5	G6	G7
Liver coefficient	0.059	0.052	0.052	0.054	0.055	0.058	0.057
$\bar{x}$ ± SD	±0.006	±0.003	±0.002	±0.003	±0.005	±0.004	±0.002
P value[a]	–	0.0410	0.0269	0.2054	0.2582	0.7598	0.4626

[a] Be relative to the negative control for liver coefficient

injection once a week afforded improved therapy compliance in clinical use for advanced cancer patients, while administration by intravenous injection frequently, such as three times a week, obtained excellent tumor growth inhibition.

There was no obvious body weight loss in all the treated mice. But mice in the oral groups exhibited slightly higher body weight loss, 7.7% for group 2 (G2) and 6.0% for groups 3 (G3), respectively, when compared with those in the injection groups. The body weight loss indirectly showed that the relative more toxic reaction in oral groups. As we previously reported, the average body weight of the mice decreased remarkably by 25.7% in the negative group (G1), which might be caused by the rapid growing tumor and nutrition deficiency. No mice died throughout the study.

In order to further investigate the hepatic toxicity of glutathione-everolimus, the liver coefficients of the mice with long-term intake of glutathione-everolimus or everolimus were calculated after mice humanely sacrificed. As the results suggested (Table 4), compared with the negative group (G1), the liver coefficients showed no significant difference ($P > 0.05$) for intravenous injection groups (G4, G5, G6, and G7), whereas, they exhibited significant difference ($P < 0.05$) for oral groups (G2 and G3). This toxic response for oral groups might be partially attributed to frequent administration and drug metabolism in liver, while the less frequence by injection led to no obvious hepatic toxicity for the mice. Thus, less frequent injection of glutathione-everolimus, such as three times a week, might decrease toxicity and increase therapeutic index.

Conclusion

Glutathione is well-known for its multiple function in lots of important physiological process. Using the endogenous tripeptide as a new method for drug delivery, we successfully developed an injectable novel glutathione-drug conjugate with good water solubility. Glutathione-everolimus is proved to be an effective, safe and well-tolerated injectable prodrug in the treatment of renal cell carcinoma. The results indicated that three times injection with a high dosage in 1 week can achieve much better in vivo efficacy, and no obvious toxic response was observed. The results of species-related drug release experiment displayed that the performance of the conjugate in human plasma, rat, and monkey was similar. Moreover, one synthetic process has been optimized and scaled up with high reproducibility and yields, which will facilitate the development of such conjugate. The stability of the conjugate supported that this prodrug can be prepared into lyophilized solid, which is to be reconstituted with 0.9% sodium chloride for injection before intravenous infusion. As our continuous preclinical researches, study of the in vivo efficacy of glutathione-everolimus on other tumor models are also ongoing.

Experimental

General procedures

Everolimus, obtained by semisynthesis, was supplied by Hisunpharm. Other reagents and solvents were obtained from commercial suppliers without further purification. All the reactions were monitored by HPLC, which was performed on Angilent apparatus. Nuclear magnetic resonance (NMR) spectra were collected on a Bruker DPX 400 NMR spectrometer with tetramethylsilane as an internal reference. High-resolution mass spectra were obtained on a Waters Micromass Q-Tof MicroTM instrument using the electrospray ionization (ESI) technique. All mice were purchased from Weitong Lihua (Beijing, China). Mice were housed in standard laboratory conditions (25 ± 2 °C, 40–70% relative humidity, and a 12-h light-dark cycle) in a barrier facility with laminar flow cabinets. A cynomolgus monkey was purchased from Academy of military medical sciences (Beijing, China).

Preparation of compounds

Everolimus (1.0 g, 1.04 mmol) and vinyl chloroacetate (0.188 g, 1.56 mmol) were dissolved in dichloromethane (10 mL). Then the immobilized lipase (0.5 g) was added to the mixture, which was stirred for about 4 h at room temperature. After reaction finished, the immobilized lipase was filtered with a funnel and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (n-hexane: acetone = 5:1 to 3:1) to obtain compound **1** (0.958 g, 0.93 mmol) as a white powder. Mp 70.8–71.3 °C; $[\alpha]_D^{20}$ −129.6° (*c* 1.0, MeOH); infrared (IR) (KBr) ν_{max} 3425, 2932, 2871, 1721, 1643 cm^{-1}; ^1H NMR (dimethyl sulphoxide (DMSO)-d_6, 400 MHz): $\delta = 6.46$ (1H, d, $J = 13.6$ Hz), 6.40 (1H, d, $J = 11.2$ Hz), 6.23 (1H, t, $J = 13.4$ Hz), 6.12 (1H, d, $J = 10.6$ Hz), 5.49–5.43 (1H, m), 5.10 (1H, d, $J = 9.2$ Hz), 4.94 (1H, br s), 4.37 (2H, br s), 4.26–4.20 (2H, m), 4.01–3.96 (2H, m), 3.72 (2H, br s), 3.64 (1H, d, $J = 11.0$ Hz), 3.42 (2H, d, $J = 11.0$ Hz), 3.15 (3H, br s), 3.05 (3H, br s), 3.01–3.05 (2H, m), 2.71–2.75 (2H, m), 2.49–2.48 (1H, m), 2.35–2.38 (2H, m), 2.22–2.25 (2H, m), 2.11 (2H, br s), 1.99–2.02 (2H, m), 1.85–1.91 (3H, m), 1.74 (2H, br s), 1.63 (6H, br s), 1.55–1.60 (6H, m), 1.35–1.40 (2H, m), 1.20–1.25 (4H, m), 1.14 (4H, br s), 0.97–1.04 (6H, m), 0.83–0.86 (6H, m), 0.73–0.78 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): $\delta = 210.9$, 208.7, 207.9, 198.9, 169.6, 169.0, 167.7, 167.4, 139.7, 138.3, 137.6, 132.8, 130.9, 127.4, 125.4, 99.4, 86.0, 83.0, 82.9, 82.7, 76.2, 74.0, 68.9, 67.4, 66.6, 65.8, 57.5, 57.4, 55.9, 55.3, 51.2, 45.6, 43.9, 41.9, 41.5, 38.7, 36.4, 35.6, 35.2, 33.8, 32.7, 32.5, 31.3, 30.2, 30.0, 26.9, 26.7, 24.9, 22.1, 20.8, 16.0, 15.1, 13.8, 13.8, 10.9; HRESIMS *m/z* (pos) 1056.5414 $[M + Na]^+$ (calcd. for $C_{55}H_{84}ClNO_{15}$ 1033.5529).

Everolimus (1.0 g, 1.04 mmol) and vinyl bromoacetate (0.257 g, 1.56 mmol) were dissolved in dichloromethane (10 mL). Then the immobilized lipase (0.5 g) was added to the mixture, which was stirred for about 4 h at room temperature. After reaction finished, the immobilized lipase was filtered with a funnel and the filtrate was concentrated in vacuo to obtain compound **2** (1.12 g, 1.04 mmol) as a white fluffy solid. Mp 64.6–66.3 °C; $[\alpha]_D^{20}$ −121.5° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3443, 2931, 2870, 1722, 1643 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): $\delta = 6.48$ (1H, s), 6.37–6.44 (1H, m), 6.19–6.25 (1H, m), 6.13 (2H, d, $J = 10.8$ Hz), 5.49 (1H, q, $J = 24.1$ Hz), 5.25–5.27 (1H, m), 5.10 (1H, d, $J = 10.2$ Hz), 4.93–4.96 (2H, m), 4.27 (2H, br s), 4.15 (2H, s), 4.01 (2H, br s), 3.96 (1H, d, $J = 4.12$ Hz), 3.70–3.72 (2H, m), 3.64 (1H, d, $J = 12.2$ Hz), 3.42–3.45 (3H, m), 3.24–3.28 (1H, m), 3.15 (3H, s), 3.09–3.12 (1H, m), 3.05 (3H, s), 2.98 (1H, s), 2.75 (1H, d, $J = 17.1$ Hz), 2.34–2.40 (2H, m), 1.98–2.22 (1H, m), 2.12 (1H, d, $J = 12.7$ Hz), 2.00–2.02 (1H, m), 1.85–1.92 (1H, m), 1.82–1.85 (2H, m), 1.74 (2H, s), 1.68–1.70 (2H, m), 1.60–1.63 (4H, m), 1.46–1.54 (4H, m), 1.37–1.43 (2H, m), 1.24–1.29 (6H, m), 1.14–1.18 (2H, m), 1.01–1.06 (3H, m), 0.94–0.99 (4H, m), 0.86–0.87 (5H, m), 0.81–0.84 (3H, m), 0.78 (2H, d, $J = 6.4$ Hz), 0.74 (3H, d, $J = 6.4$ Hz); ^{13}C NMR (DMSO-d_6, 100 MHz): $\delta = 210.9$, 207.9, 199.3, 169.6, 167.7, 167.6, 167.4, 139.7, 138.3, 137.6, 132.8, 130.9, 127.4, 125.1, 99.4, 86.0, 83.0, 82.7, 68.9, 76.2, 74.0, 67.4, 66.6, 65.9, 57.5, 57.5, 57.4, 56.3, 51.2, 45.6, 43.9, 36.4, 35.6, 35.2, 33.8, 32.7, 31.7, 31.3, 30.2, 30.1, 30.0, 28.8, 27.5, 26.9, 26.7, 24.9, 22.5, 22.1, 20.8, 16.0, 16.0, 15.1, 14.4, 13.8, 10.9; HRESIMS *m/z* (pos) 1100.4927 $[M + Na]^+$ (calcd. for $C_{55}H_{84}BrNO_{15}$ 1077.5024).

Compound **2** (0.90 g, 0.83 mmol) and glutathione (0.51 g, 1.66 mmol) were added to a mixed solution of DMF/EtOH/H_2O (1:2:2, 10 mL) to obtain a clear solution. K_2CO_3 (0.23 g, 1.66 mmol) was added subsequently to the solution, and the obtained mixture was stirred for about 24 h. Then, the reacted solution was concentrated in vacuo. The obtained residue was purified by RP-HPLC to give glutathione-everolimus (0.713 g, 0.69 mmol) as a white powder.

pH stability study

To evaluate the stability of the conjugate in different pH conditions, solutions of glutathione-everolimus (2 mg/mL) were diluted into phosphate buffered saline (PBS), which were adjusted to pH 2.0, 6.0, 7.4, and 8.0, respectively, and incubated at 37 °C. The solutions were removed at different time points and analyzed by HPLC, detecting disappearance of the conjugate. Stability profile graph was generated by

plotting the percentage of remaining starting material over a time course. The percentage was calculated on the basis of the ratio of the peak area of the sample at 0, 1, 4, 8, 12, and 24 h vs. the initial area peak.

Drug release experiment

In human plasma

Ten microliter 0.1 mmol/L solution of glutathione-everolimus was added to 990 μL human plasma. The two solutions were mixed thoroughly, and then incubated at 37 °C. Fifty microliter of the mixture was removed from the mixed solution at predetermined time intervals of 0, 1, 15, 30 min, 1, 2, and 5 h, respectively. One hundered and fifty microliter acetonitrile was added immediately to the removed solution to quench the reaction, which was then analyzed with HPLC to determine the remainder of the conjugate and the released everolimus in the solution.

In cynomolgus monkey

The conjugate, dissolved in normal saline, was given to a 5–6 years old monkey with a dose of 1 mg/kg by intravenous injection. One milliliter blood was taken from the forelimbs of the monkey at predetermined time intervals of 5, 10, 30 min, 1, 1.5, 2, 3, 6, 8, and 24 h, respectively. Centrifugation was used to remove the red blood cells. Then, 50 μL of the obtained liquid supernatant was sampled, and 150 μL acetonitrile was subsequently added to the sample. Centrifugation was used once again to remove the denatured protein in the solution. Afterwards, the remainder of the conjugate and the released everolimus in the solution were analyzed by HPLC.

Evaluation of in vivo efficacy

Human renal cell carcinoma OS-RC-2 tumors were established by implanting a 4–5 mm^3 tissue fragment of OS-RC-2 tumor collected from donor mice into the right axillary flank of recipient nude mice. When tumors reached an average volume of about 148 mm^3, mice were divided into seven groups. Normal saline was given to mice by means of intragastric administration daily for negative control (G1). Mice administered everolimus orally everyday at a dose of 5 mg/kg as the positive control (G2). As a parallel test, 5 mg/kg glutathione-everolimus was administered orally to the mice daily (G3). To investigate the influence of different administration dosage and frequency on the therapeutic effect, mice were given glutathione-everolimus intravenously at a dose of 10 mg/kg three times a week (G4), 25 mg/kg once a week (G5), 35 mg/kg once a week (G6), or 35 mg/kg three times a week (G7), respectively. Mouse weight and tumor sizes were measured every 3 or 4 days throughout the study. Finally, tumors were retrieved and weighed after 29 days administration, then, the tumor inhibition was calculated. The liver of the human sacrificed mice was taken out and weighed. The liver coefficient was calculated using the following formula: the liver coefficient = the average weight of the liver/the average body weight of the mice.

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Compliance with ethical standards

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

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