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Regioselective synthesis and initial evaluation of a folate receptor targeted rhaponticin prodrug

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Abstract

To improve the therapeutic effect of rhaponticin (RHA), a folate receptor (FR) targeted RHA prodrug was designed and regioselectively synthesized by utilizing a hydrophilic peptide spacer linked to folic acid (FA) *via* a releasable disulfide linker. A series of biological evaluation was investigated *in vitro* and *in vivo*. The positive results of biological investigations warrant further preclinical study before this novel targeted chemotherapeutic is considered for clinical investigation.

Keywords: Rhaponticin (RHA); Targeted therapeutics; Folate; Prodrug; Anticancer

Rhaponticin (RHA), a major representative of the stilbene glucoside compounds, exists widely in medicinal plant of *Rheum L.* such as *Rheum officinale*, *Rheum undulatum*, and *Rheum palmatum* [1]. Previous studies revealed that RHA showed potent antitumor and antitumor-promoting, antithrombotic, and antioxidant activities [2]. However, as an anticancer drug, it kills healthy cells along with cancerous ones. As a consequence, severe side effects were observed, and therapeutic doses become limited due to the risk of severe toxicity. In addition, RHA was rapidly eliminated from the systemic circulation [3]. Considering these problems, there is an urgent need to design it into novel anticancer prodrugs that concentrate their lethal activities specifically in cancer tissues. Receptor-specific targeting, as one approach, can potentially satisfy the selective delivery criteria for toxic agents to pathologic cells. The folate receptor (FR) has been considered as a very attractive molecular target for tumor selective drug delivery, since it is over-expressed on a wide variety of tumor cells, but highly restricted in most normal human tissues [4,5]. Moreover, the vitamin folic acid (FA) binds to FR with high affinity ($K_d = 10^{-10}$ mol/L) [6].

In this paper, we report the design and regioselective synthesis of a FA–RHA prodrug **1** (Scheme 1). As indicated in Scheme 1, conjugate **1** can be assembled by tethering the corresponding thiol-containing FA-spacer **2** to the thiol-reactive derivative of RHA **3**. As has been reported before [7], the disulfide bond is important for drug delivery application since reduction-mediated release of the drug cargo from a disulfide linked FA-conjugate efficiently occurs within the endosomes of cancer cells.

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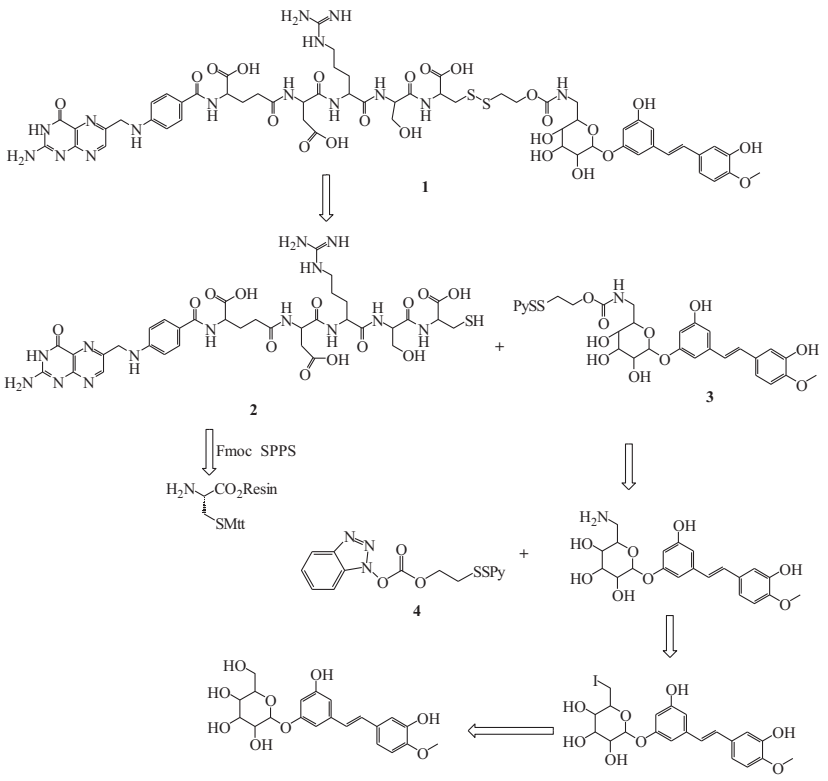
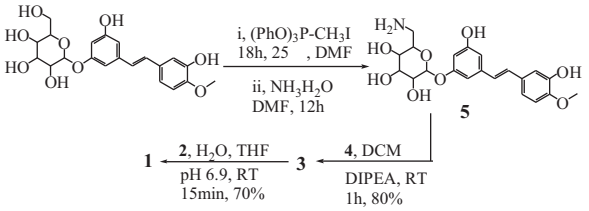


Scheme 1. The retrosynthetic analysis of the FR targeted RHA prodrug (conjugate **1**).

The peptide-based derivative FA-spacer **2** was designed as a novel bifunctional molecular spacer unit containing both acidic (Asp) and basic (Arg) amino acids to provide the best water-solubility of the FA–RHA conjugates under physiological conditions. Pteroic acid was to serve as *N*-terminus, whereas the thiol group of cysteine was to serve as the attachment site for the cleavable linker. These concepts allowed for the assembly of the spacer unit using standard fluorenylmethyloxycarbonyl-based solid phase peptide synthesis (Fmoc SPPS) as described in previous reports [7,8]. Activated carbonate **4** served as an important heterobifunctional crosslinker for the releasable drug conjugate synthesis [9], and can be reacted under mild conditions with many *N*-and *O*-nucleophiles according to Vlahov [7]. Thiol-reactive derivative of RHA **3** was prepared in three steps from RHA which was isolated by our laboratory. Briefly, as shown in Scheme 2, RHA was treated with $(PhO)_3P\text{-}CH_3I$ in DMF, followed by ammonolysis in an autoclave to yield amido-reactive derivative of RHA **5**. Compound **5** was treated with activated carbonate **4** and diisopropylethylamine (DIPEA) in DCM to yield **3**, followed by chromatographic purification on silica gel, **3** was isolated in 80% yield. Treatment of a suspension of FA-spacer **2** in PBS under argon with $NaHCO_3$ resulted in a clear yellow solution at pH $>$ 6.5. This mixture was added at once under vigorous stirring to a solution of **3** in THF. The HPLC purification [7] gave pure final conjugate **1**. ^{1}H NMR, ^{13}C NMR and HRMS signals were in agreement with the expected structure [10].

Treatment of conjugate **1** with a reducing agent demonstrated the release of free RHA. Briefly, a 50 mmol/L solution of conjugate **1** in PBS (pH 7.4) was treated with 2 mmol/L L-glutathione (GSH) at 37 °C. The HPLC-MS profile (UV detection at 324 nm) (Fig. 1) showed cleavage of the disulfide bond with concomitant release of RHA derivative **3**. Subsequently, **3** fragments resolved into free RHA [ESI $(M+H)^+$: 421], thus demonstrating self-immolative nature of the linker system [11]. Efficient liberation of parental drug upon exposure to GSH was encouraging, indicating that the release of RHA should proceed spontaneously following disulfide bond reduction within the endosomes.

In this study, HeLa cells were used to investigate the cytotoxicity of conjugates **1** in comparison with the parental RHA by utilizing the MTT proliferation assay [12]. HeLa cells were incubated at 37 °C for 48 h with increasing concentration of conjugate **1** or parental RHA in the presence or absence of 0.1 mmol/L FA (as a competitor). As shown in Fig. 2, it was obvious that the cell viability decreased with increasing of drug concentration. It was also showed that conjugate **1** were much more toxic against HeLa cells with an IC_{50} of about 9.7 nmol/L against 34 nmol/L for parental RHA. While in the presence of excess free FA, the activity of conjugate **1** was effectively blocked but kept



Scheme 2. The preparation of conjugate **1** from RHA.

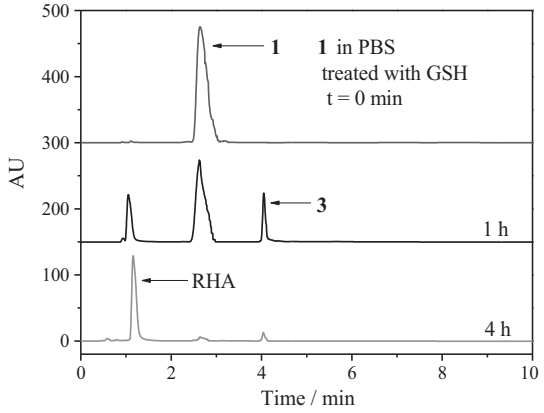


Fig. 1. Conjugate **1** in PBS buffer treated with GSH at 37 °C.

almost the same for parental RHA. These results indicated from one certain aspect that the observed activity of conjugate **1** was folate-mediated but the activity of RHA had no relationship with FA.

In this study, human serum albumin was utilized to evaluate the *in vitro* protein binding properties of conjugate **1**. As a result, when the concentration of conjugate **1** was 8.0 μmol/L, the binding rate achieved maximum 79.3 ± 2.5%. The stability of conjugate **1** and parental RHA in human serum and the blood clearance were also investigated. Fig. 3 revealed that conjugate **1** decreased with time in the serum matrix with an estimated $t_{1/2}$ of ~10 h against ~3.5 h for parental RHA. While as shown in Fig. 4, after intravenous injection to mice (Balb/c mice, treated with FR-positive KB cells), compound **1** was rapidly removed from systemic circulation. Taken together, the data in Figs. 2–4 suggested that conjugate **1** is possible to reach an FR-positive tumor in an intact form within minutes following an intravenous injection, and the further research is ongoing.

In this study, a FR targeted RHA prodrug was successfully regioselectively synthesized by utilizing a hydrophilic peptide spacer linked to FA *via* a releasable disulfide linker. Following positive *in vitro* and *in vivo* results and toxicological evaluation, compound **1** was selected as a candidate for further preclinical or clinical development and the further research is ongoing.

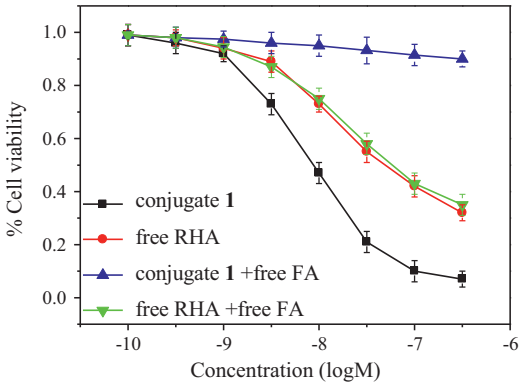


Fig. 2. Viability of HeLa cells after exposure to conjugate **1** and parental RHA in the presence or absence of FA (as a competitor) at 37 °C.

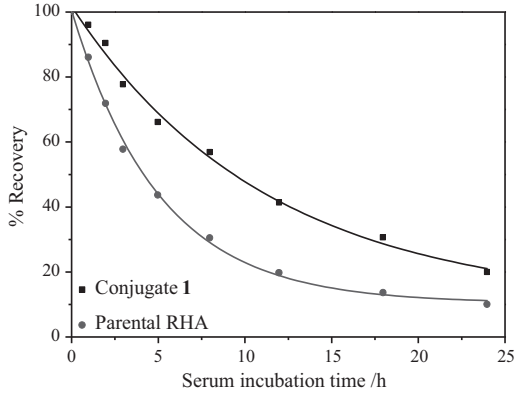


Fig. 3. Serum stability of conjugate **1** in human serum at 37 °C ($n = 3$ with %RSD < 2%).

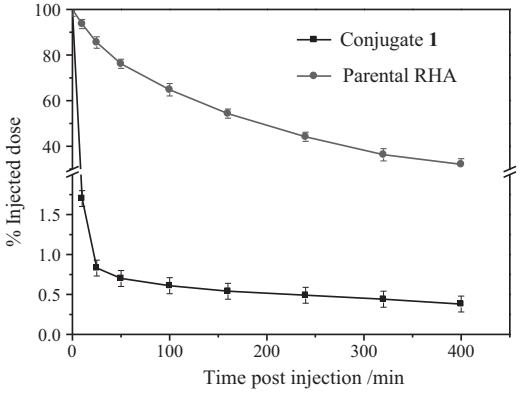


Fig. 4. Blood clearance of conjugate **1** following an 1800 nmol/kg intravenous injection dose to treated Balb/c mice ($n = 3$).

Acknowledgment

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[10] Spectra data for compound **1**: ^{1}H NMR (300 MHz, D_2O): δ 8.67 (s, 1H), 7.93–8.23 (m, 6H), 7.35 (d, 2H, J = 7.8 Hz), 6.96 (d, 2H, J = 6.78 Hz), 6.88 (d, 1H, J = 8.31 Hz), 6.75 (s, 1H), 6.64 (d, 1H, J = 7.58 Hz), 6.51 (d, 2H, J = 8.7 Hz), 6.45 (s, 2H), 6.13 (s, 1H), 5.88 (d, 1H, J = 5.69 Hz), 4.8–4.82 (m, 3H), 4.47 (t, 2H, J = 5.37 Hz), 4.35 (t, 2H, J = 7.21 Hz), 4.30 (s, 2H), 4.25 (m, 1H), 4.16 (s, 1H), 3.94 (d, 2H, J = 6.28 Hz), 3.85 (t, 1H, J = 5.83 Hz), 3.73 (s, 3H), 3.43 (d, 2H, J = 5.90 Hz), 3.17 (d, 2H, J = 7.86 Hz), 3.10 (d, 2H, J = 7.83 Hz), 3.02 (m, 3H), 2.93 (t, 2H, J = 7.21 Hz), 2.88 (d, 2H, J = 7.03 Hz), 2.72 (m, 3H), 2.63 (m, 2H), 2.41 (m, 4H), 2.00–2.30 (m, 10H), 1.69 (m, 4H); ^{13}C NMR (300 MHz, D_2O): δ 176.8, 175.2, 174.1, 167.3, 166.3, 161.7, 160.2, 158.8, 153.9, 150.7, 149.5, 148.4, 146.6, 142.8, 138.2, 136.7, 130.0, 127.9, 124.8, 121.9, 119.8, 115.4, 114.3, 111.2, 105.3, 104.1, 100.6, 98.9, 71.4, 69.5, 68.7, 67.3, 65.0, 63.8, 58.6, 56.5, 54.9, 50.4, 44.1, 39.8, 37.6, 36.0, 34.4, 29.2, 28.2, 26.9, 25.7; HRMS (ESI): $(M+Na)^+$ calcd. for $C_{59}H_{73}N_{15}O_{23}S_2Na$ 1446.4343, found 1446.4348.
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