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Synthesis and biological evaluation of a folate-targeted rhaponticin conjugate

Xuhua Liang [a,†], Yang Sun [b,†], Wenyuan Zeng [c], Lusha Liu [a], Xuan Ma [a], Yingyong Zhao [d,*], Jun Fan [a,*]

[a] School of Chemical Engineering, Northwest University, No. 229 Taibai North Road, Xi'an, Shaanxi 710069, China
[b] Department of Chemistry & Chemical Engineering, Xi'an University of Arts and Science, No. 168 Taibai South Road, Xi'an 710065, China
[c] School of Pharmaceutical Sciences, Zhengzhou University, No. 100 Kexue Avenue, Zhengzhou, Henan 450001, China
[d] Biomedicine Key Laboratory of Shaanxi Province, Northwest University, No. 229 Taibai North Road, Xi'an, Shaanxi 710069, China

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ABSTRACT

To improve the therapeutic effect of rhaponticin (RHA), a folate receptor (FR) targeted RHA conjugate was synthesized by utilizing a hydrophilic peptide spacer linked to folic acid (FA) via a releasable disulfide linker. This water-soluble conjugate was found to retain high affinity for FR-positive cells, and it produced specific, dose-responsive activity in vitro. Treatment of FRHA with a reducing agent indicated that the amino-reactive derivative of RHA would be released spontaneously following disulfide bond reduction within the endosomes. FRHA also proved to be active predominantly specific against FR-positive syngeneic and xenograft models in vivo, and possible curative activity resulted with minimal to moderate toxicity. The FRHA conjugate greatly enhanced the therapeutic effects and reduced the toxicity of RHA. In conclusion, FRHA represents a folate-targeted chemotherapeutic that can produce potent activity against established sc tumors. Hence, this report has a great significance in pharmacology and clinical medicine as well as methodology.

1. Introduction

Notwithstanding the noteworthy advances in its treatment, cancer persists as one of the main diseases threatening human health. As the principal method of cancer therapy, chemotherapy is designed to administer a drug in an intermittent fashion at its maximum tolerated dose followed by a recovery period. However, this method is plagued by toxicity resulting from the indiscriminate uptake into both normal and neoplastic tissues. Further more, the tumor tissues recovered much faster than normal ones so that tumor elimination is highly unlikely. The obvious solution to the unwanted toxicities is to somehow focus the therapeutic potency on the cancer cells. Receptor-specific targeting, as one approach, can potentially satisfy the selective delivery criteria for toxic agents to pathologic cells. Rhaponticin (RHA, Fig. 1), a major representative of the stilbene glucoside compounds, exists widely in medicinal plant of Rheum L. such as Rheum officinale, Rheum undulatum, Rheum hotaoense and Rheum palmatum.[1,2] Previously studies revealed that RHA showed potent antitumor and antitumor-promoting, antithrombotic, antioxidant and vasorelaxant activities.[3–5] 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, the time point of maximum plasma concentration (T_{max}) and the half-life of drug elimination during the terminal phase ($t_{1/2}$) were very short,[6] which means it was rapid eliminated from the systemic circulation. Considering these problems, there is an urgent need to design it into novel anticancer conjugate that concentrate their lethal activities specifically in cancer tissues and prolong their T_{max} and $t_{1/2}$.

The folate receptor (FR), a glycosylphosphatidylinositol-anchored membrane glycoprotein, has been respected 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.[7,8] Moreover, the vitamin folic acid (FA) binds FR with high affinity ($K_d = 10^{-10}$ M). After binding, FA is transported into the cell via FR-mediated endocytosis.[9] Consequently, FA can be exploited as a molecular 'Trojan horse' for the targeted delivery of covalently-attached, biologically active molecules.[10] The physiological events involved in the latter process was identical to those for the free FA.[11] Previously, there were a few reports about the synthesis and biological evaluation for FA targeted chemotherapeutic conjugates of mitomycin C (EC72 and EC118),[9,12] desacetylvinblastine monohydrazide (DAVLBH, EC140 and EC145),[10,13–15] tubulysins and their hydrazides,[16] and a novel folate-targeted dual drug conjugate EC0225.[17] Among them, the anticancer drugs were attached to FA via a water-soluble peptidic spacer and a reducible disulfide linker system or pH-sensitive hydrazone bonds. Once be administered, the conjugates targets to FR over-expressed cancer cells and release the base drug after internalization. This receptor-targeted delivery allows reduced toxicity and hence improves efficacy.

* Corresponding authors. Tel.: +86 29 88305252.
E-mail addresses: zyy@nwu.edu.cn (Y. Zhao), fanjun@nwu.edu.cn (J. Fan).
† Xuhua Liang and Yang Sun are co-first authors.

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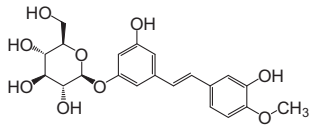


Figure 1. Chemical structure of rhaponticin (RHA).

In this paper, we report the design and regionselective synthesis of a FR targeted RHA conjugate (FRHA) (Fig. 2) by utilizing a hydrophilic peptide spacer linked to FA via a releasable disulfide linker. The drug release, protein binding rate, serum stability and blood clearance of the conjugate were determined. In vitro cytotoxicity of the FRHA conjugate were also investigated by using FR-positive cells (KB and M109 cells) and FR-negative 4T1 cells. In addition, FRHA's therapeutic activity was investigated using FR-positive and FR-negative established tumor models in vivo.

2. Chemistry

As indicated in Figure 2, disulfide-linked FRHA can be assembled by tethering the corresponding thiol-containing FA-spacer **1** to the thiol-reactive derivative of RHA **2**.

The peptide-based derivative FA-spacer **1** was designed as a novel molecular spacer unit containing a discrete number of 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 L-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.[18]

Activated carbonate **3** served as an important heterobifunctional crosslinker for the releasable drug conjugate synthesis, and can be readily prepared from 2-mercaptoethanol in two steps.[10,19] Thiol-reactive derivative of RHA **2** was prepared in three steps from RHA which was isolated by our laboratory. Briefly, as shown in Scheme 1, RHA was treated with $(PhO)_3P–CH_3I$ in DMF, followed by ammonolysis in an autoclave to yield amino-reactive derivatives of RHA **4**. Compound **4** was treated with the activated carbonate **3** and diisopropylethylamine (DIPEA) in DCM to yield **2**, followed by chromatographic purification (silica gel, 10% MeOH/$CHCl_3$ follow by 35% MeOH/$CHCl_3$), **2** was isolated.

Treatment of a suspension of FA-Spacer **1** 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 **2** in THF. The formation of the conjugate, FRHA, was monitored by analytical HPLC. Then, this product was purified by preparative HPLC (Eclipse XDB-C_{18} 4.6 × 250 mm) with PBS, pH 7.0 (solvent A) and acetonitrile (solvent B) using a gradient consisting of 10–50% B over 30 min at a flow rate of 1.5 mL/min. Acetonitrile was removed from the collected fractions in vacuo and the residue subjected to freeze-drying to give FRHA.

3. Results and discussion

3.1. Synthesis of FRHA and evaluation of base drug release

This novel FR-targetable chemotherapeutic agent was constructed using disulfide linker technology to afford molecular separation of active ingredient from FA once this conjugate enters the endosomes of FR-positive cancer cells. The mechanism of folate-targeted drug delivery has been described previously.[9,11] Briefly, cells expressing the FR protein can bind folate-drug conjugates very tightly and endocytose them inside. Then the conformation of FR changes and releases the conjugate inside the acidic milieu of the endocytic elements. Depending on the nature of the chemical linkage between them, separation of the FA and drug moieties can occur within these vesicular structures. Thus, it is possible that

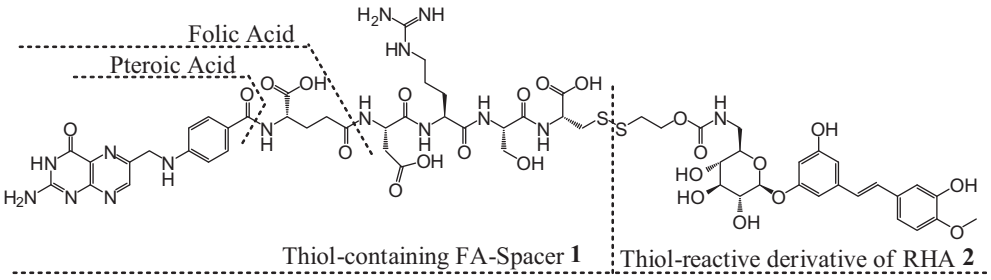
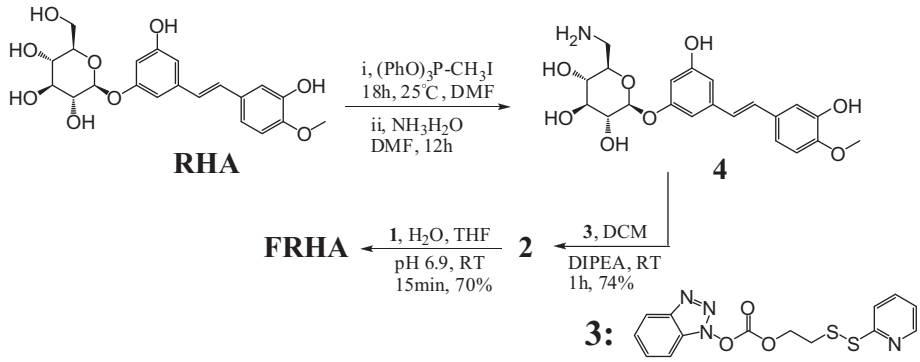


Figure 2. Chemical structure of the FR targeted RHA conjugate (FRHA).



Scheme 1. The preparation of FRHA from natural product rhaponticin (RHA).

the released drug molecules are permitted to diffuse across these compartmentalized membranes to exert activity.

The synthesis of FRHA is schematically shown in Scheme 1. A thiol-containing FA-peptide spacer **1** was coupled in near-quantitative yield to the thiol-reactive derivative of RHA **2** to produce FRHA. The peptide-based derivative FA-spacer **1** was designed as a novel bifunctional molecular spacer unit containing both acidic (Asp) and basic (Arg) amino acids to provide the best potential for water-solubility of the FA-RHA conjugates under physiological conditions. Pteroic acid (Pte) was to serve as N-terminus, whereas the thiol group of cysteine (Cys) was to serve as the attachment site for the cleavable linker. As reported before,[20] the disulfide bond is important for drug delivery applications since reduction-mediated release of the drug cargo from a disulfide linked FA-conjugate efficiently occurs within the endosomes of cancer cells. This product, FRHA, was purified by preparative HPLC to give 70% yield stored as a lyophilized powder. The structure of FRHA was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS signals.

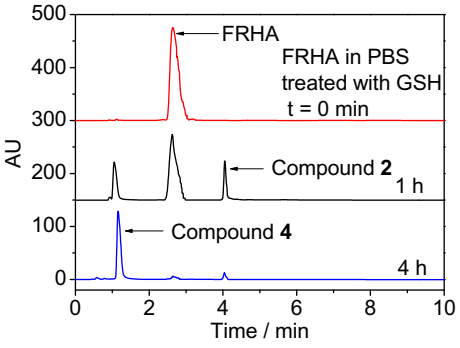


Figure 3. FRHA in PBS buffer treated with GSH at 37 °C (λ = 324 nm).

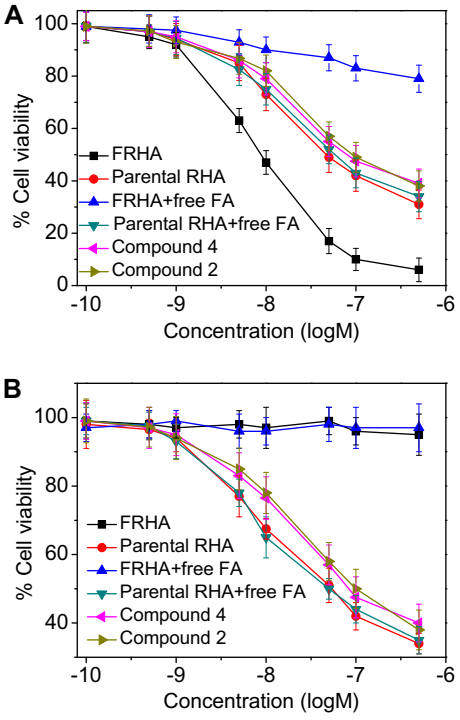


Figure 4. Viability of FR-positive KB cells (Panel A) and FR-negative 4T1 cells (Panel B) after exposure to FRHA or parental RHA or the RHA derivative (compound **2** or compound **4**) with increasing concentrations in the presence or absence of 0.01 mM FA (as a competitor) at 37 °C for 48 h.

Treatment of FRHA with a reducing agent demonstrated the releasing property. The HPLC-MS profile (UV detection at 324 nm, Fig. 3) showed cleavage of the disulfide bond ($t_{1/2}$ ~50 min) with concomitant release of RHA derivative **2**. Subsequently, **2** fragments resolved into compound **4**, thus demonstrating the self-immolative nature of the linker system.[21,22] Efficient liberation of compound **4** upon exposure to GSH was encouraging, since it indicated that the release of compound **4** should proceed spontaneously following disulfide bond reduction within the endosomes. The following in vitro cytotoxicity results demonstrated that compound **4** possessed similar antitumor activities as parental RHA, which indicated the replacement of the 5'-OH group by a 5′-amino group didn't affect the activity of RHA.

3.2. In vitro cytotoxicity

In this study, FR-positive KB cells and FR-negative 4T1 cells were used to investigate the cytotoxicity of FRHA in comparison with the parental RHA and compound **4** by MTT proliferation assay. As shown in Figure 4A, when treated with KB cells, it was obvious that the cell viability decreased with the increasing of drug concentration. It also showed that the IC_{50} value of parental RHA was about 34 nM, which was clearly a low concentration and served to demonstrate the potency of the drug. In sharp contrast, the FRHA were much more toxic against KB cells with an IC_{50} of about 9.7 nM which suggested that the FRHA conjugate greatly enhanced the therapeutic effects of RHA. As for the derivative of RHA—compound **2** and compound **4**, they possessed similar antitumor activities as parental RHA, which indicated the replacement of the 5′-OH group by a 5′-amino group didn't affect the activity of RHA. Two common controls were used to evaluate the specificity of FRHA. The first involved the blocking of FRHA's activity with the use of an excess of free FA. As shown in Figure 4A, the activity of FRHA was effectively blocked in the presence of excess free FA, indicating from one certain aspect that the observed activity was folate-mediated. The second method tested the cytotoxic effect of FRHA against an FR-negative cell line; thus as shown in Figure 4B, FRHA was not found to be toxic towards FR-negative 4T1 cells. Taken together, the data in Figure 4 indicated that FRHA's potent cell-killing activity is specific for cells that express the FR.

3.3. Protein binding rate

The pharmacokinetic and pharmacodynamic properties of a compound are profoundly affected by the extent of its binding to plasma proteins.[23] Thus, the determination of a compound's protein-binding properties can be essential during the lead prioritization phase of drug development. When the excitation wavelength was set at 377 nm, FRHA exhibited fluorescence peak around 452 nm, and the ratio of F_{377} was proportional to the concentration

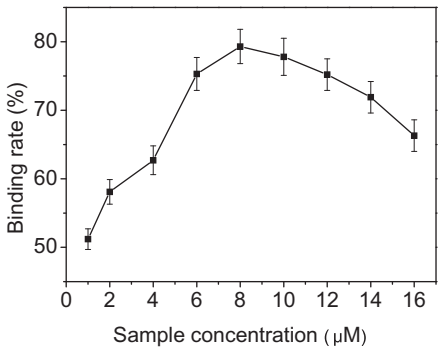


Figure 5. The binding fractions in HSA solution, n = 6. c(HSA) = 10.0 μM.

of FRHA, based on which, a simple and rapid method for determining the amount of FRHA was developed. When the concentration range of FRHA was 0.5–18.0 μM, the calibration curve was linear with the detection limits of 0.03 μM and the equation of $Y = 3.4432X - 4.8321$ ($R = 0.9994$). The result of varying concentration of FRHA bound with HSA was shown in Figure 5. When the concentration of FRHA was 8.0 μM, the binding rate of HSA achieved maximum 79.3 ± 2.5%. The results suggested that a large fraction of FRHA should be available to extravasate from the circulation to bind tumor-associated FR.[23] Satisfactory accuracy, reproducibility were realized. In comparison with HPLC method, the proposed method developed in the present study had merits of simplicity and quickness.

3.4. Serum stability and blood clearance

Since FRHA contains an intramolecular disulfide bond which could be susceptible to cleavage in a biological matrix, its stability in human serum was investigated at 37 °C. As a comparison, the parental RHA was also measured using the same method. As shown in Figure 6 (Panel A), results from an HPLC-based solid-phase extraction method revealed that FRHA decreased with time in the serum matrix with an estimated $t_{1/2}$ of ~10 h against ~3.5 h for parental RHA. The relevance of this finding was emphasized by a follow up experiment which assessed the blood clearance of FRHA. Thus, a 2 μmol/kg intravenous dose of FRHA or RHA was given to corresponding mice. As shown in Figure 6 (Panel B), FRHA was rapidly removed (with half-life <10 min) from systemic circulation after its injection to Balb/c mice treated by FR-positive M109 cells. While after injection to mice treated by FR-negative 4T1 cells or normal mice, the elimination rate was much slower. And no significant differences were found between FR-negative mice group and normal mice group. However, after injection to the three kinds of mice with parental RHA, the clearance rate slowed down much more significantly than injected with FRHA. The FRHA was mainly eliminated from the kidney when injected to mice treated with 4T1 cells and normal mice. And the clearance of parental RHA was due to the metabolism at the liver. Taken together, with the longer half-time (about 10 h) in serum matrix and short half-time (less than 10 min) after its injection to mice treated by FR-positive M109 cells (the injection to mice treated with 4T1 cells and normal mice were control groups to eliminate the effects of kidney and the metabolism at the liver), it suggested that FRHA is possible to reach an FR-positive tumor within minutes following an intravenous injection.

3.5. In vivo activity

FRHA was evaluated against established sc KB tumor-bearing Balb/c mice. Furthermore, the performance of FRHA was also compared to that of the parental drug, RHA, administered to Balb/c mice. As shown in Figure 7A, tumors in mice treated with PBS reached about 1500 mm^3 by approximately day-33 post-tumor implantation (PTI). However, when mice were treated iv with FRHA at 2 μmol/kg, following a TIW 2 week schedule, tumors in all the five treated mice quickly regressed with 5/5 cures by the end of the study. These remarkable effects also occurred in the apparent absence of weight loss (Fig. 7B), and no gross toxicity or adverse events were noted in these mice during and after therapy. In contrast, therapy with 2 μmol/kg of the unconjugated parental drug, RHA, was found to be extremely toxic to the mice; although there was evidence of emerging antitumor activity (Fig. 7A), this untargeted regimen was clearly not tolerable. Then, the performances of FRHA and of RHA were next evaluated in a

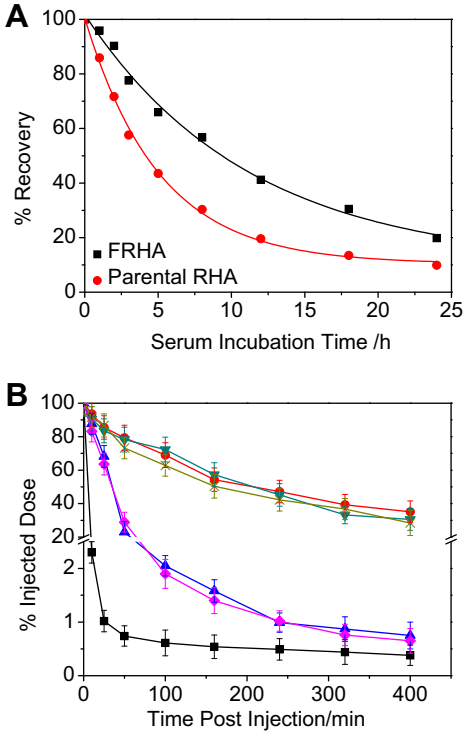


Figure 6. Serum stability and blood clearance of FRHA. Panel A, stability of FRHA in human serum at 37 °C ($n = 3$ with %RSD < 2%). Panel B, blood clearance of FRHA following a 2 μmol/kg intravenous injection dose to mice ($n = 3$), (■) FRHA to mice treated by FR-positive M109 cells; (●) Parental RHA to mice treated by FR-positive M109 cells; (▲) FRHA to mice treated by FR-negative 4T1 cells; (▼) Parental RHA to mice treated by FR-negative 4T1 cells; (◆) FRHA to normal mice; (×) Parental RHA to normal mice.

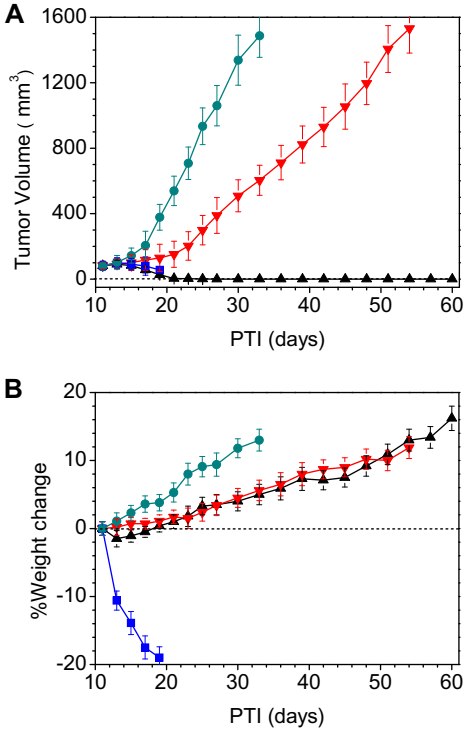


Figure 7. Activity of FRHA and RHA against the FR-positive KB xenograft tumor. In panel A, Balb/c mice bearing sc FR-positive KB tumors (when grew to 50–110 mm^3) were treated iv with (▲) 2 μmol/kg FRHA or (■) 2 μmol/kg RHA or (▼) 2 μmol/kg FRHA plus 40-fold excess FA following a TIW, 2 week schedule. Control cohorts (●) were treated with PBS. Tumor volume was assessed every 2–3 days. Changes in animal weights were shown in panel B. $n = 5$ mice per cohort.

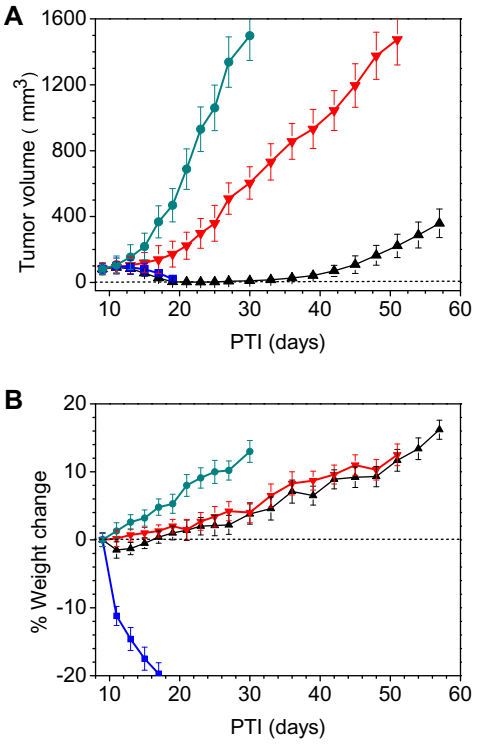


Figure 8. Activity of FRHA and RHA against the FR-positive M109 syngeneic tumor. In panel A, Balb/c mice bearing sc FR-positive M109 tumors were treated iv with (▲) 2 μmol/kg FRHA or (■) 2 μmol/kg RHA or (▼) 2 μmol/kg FRHA plus 40-fold excess FA following a TIW, 3 week schedule. Control cohorts (●) were treated with PBS. Tumor volume was assessed every 2–3 days. Changes in animal weights were shown in panel B. $n = 5$ mice per cohort.

second FR-expressing tumor, the therapeutically challenging M109 model which was found to be rather chemoresistant.[14,17,23] The M109 tumor is an FR-positive lung adenocarcinoma that is syngeneic to the Balb/c mouse strain. As shown in the Figure 8A, a TIW, 3-week regimen of FRHA was found to be highly effective against M109 tumors under conditions. In contrast, treatment with the untargeted RHA was found to inferior effect against the M109 tumor. Clearly, FRHA offered a distinct therapeutic advantage over the unconjugated drugs.

To gain insight on in vivo specificity of FRHA, two common methods were used. One method that can be used to demonstrate an agent's FR-specific activity in vivo is to co-dose with an excess of FA. As shown in Figure 7, FRHA was found to produce 5/5 CRs and cures against established sc KB tumors in Balb/c mice in a manner that did not produce weight loss. In contrast, when co-dosed with a modest excess of FA, FRHA's activity was determined to be compromised, since no CRs or cures resulted in this "competed" cohort. This outcome indicated that FRHA's activity was predominantly dependent on binding to tumor-associated FR. As a second measure for target specificity, FRHA was tested for activity against a recognized FR-negative tumor model, the murine breast carcinoma 4T1. As shown in Figure 9, despite using an established, therapeutically effective dosing regimen (2 μmol/kg, TIW, 2 weeks), 4T1 tumors grew at the same rate in both the untreated and FRHA-treated mice. Taken together with the studies in Figure 7, these results confirm that FRHA's activity in vivo is predominantly specific for FR-expressing tumors.

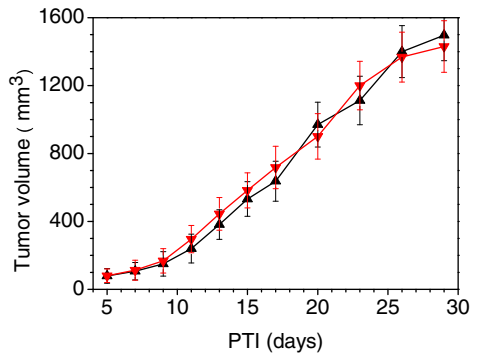


Figure 9. Antitumor activity of FRHA in a FR-negative model. 1×10^6 FR-negative 4T1 cells were inoculated sc into each Balb/c mice. Five days later, animals were treated iv with (▲) 2 μmol/kg FRHA or PBS (▼, for control cohort). Tumor volume was assessed every 2–3 days. $n = 5$ mice per cohort.

4. Conclusions

In this study, a FR targeted RHA conjugate (FRHA) was first successfully synthesized by utilizing a hydrophilic peptide spacer linked to FA via a releasable disulfide linker. Treatment of FRHA with a reducing agent indicated that the release of RHA's amino-reactive derivative—compound **4**— should proceed spontaneously following disulfide bond reduction within the endosomes. The cytotoxicity of FRHA had been determined against FR-positive KB cells and FR-negative 4T1 cells by MTT proliferation assay. Compared with the parental RHA and compound **4**, the conjugate FRHA showed higher toxic against KB cells with an IC_{50} of about 9.7 nM which suggested that the FRHA conjugate greatly enhanced the therapeutic effects of RHA. And compound **4** showed almost the same antitumor activity as the parental RHA, which indicated the replacement of the 5′-OH group by a 5′-amino group didn't affect the activity of RHA. Moreover, protein binding ability of FRHA to HSA was investigated by equilibrium dialysis and fluorescence assay, which indicated that the binding rate achieved maximum 79.3±2.5%. When treated with serum matrix, FRHA decreased with time with an estimated $t_{1/2}$ of ~10 h against ~3.5 h for parental RHA. What's more, FRHA was rapidly removed (with half-life around 10 min) from systemic circulation after its injection to mice treated by FR-positive M109 cells but much slower to those treated by FR-negative 4T1 cells or normal mice. FRHA also proved to be active against FR-positive syngeneic and xenograft models in vivo, and possible curative activity resulted with minimal to moderate toxicity. The FRHA conjugate greatly enhanced the therapeutic effects and reduced the toxicity of RHA. In conclusion, FRHA represents a folate-targeted chemotherapeutic that can produce potent activity against established sc tumors. Hence, this report has a great significance in pharmacology and clinical medicine as well as methodology.

5. Experimental

5.1. Materials and apparatus

Rhaponticin (RHA) was isolated by authors from Rheum hotaoense, and its purity was evaluated to be above 99%. Folic acid (FA), peptide synthesis reagents, human serum albumin (HSA) and L-glutathione (GSH) were purchased from Sigma Chemical Company. Stock solution of protein was prepared by dissolving HSA in PBS (0.05 M, pH 7.4) and stored at 0–4 °C. The SpectraPor dialysis membranes (molecular weight cutoff 13–14 kDa) were purchased from Spectrum Laboratories Inc. (Los Angeles, CA, USA). All amino acid were purchased from Xi'an zhou dingguo biotech.co.itd. A 0.05 M PBS was prepared by maxing of 19 mL 0.05 M NaH_2PO_4 solution (by dissolving of 0.78 g $NaH_2PO_4{\cdot}2H_2O$ in 100 mL volumetric flask with water) and 81 mL 0.05 M Na_2HPO_4 solution (by

dissolving of 1.79 g $Na_2HPO_4{\cdot}12H_2O$ in 100 mL volumetric flask with water). Methyl alcohol and acetonitrile were chromatographic grade, all other materials were of analytical reagent grade and double distilled water was used throughout. KB cells (FR-positive cells, xenograft), M109 cells (FR-positive cells, syngeneic), 4T1 cells (FR-negative cells) and six week old female Balb/c mice, were purchased from the Fourth Military Medical University, China.

EQUINOX55 Fourier transformed infrared spectrometry (Bruker Company, Germany); AVANCE300 MHz digital superconducting NMR spectrometer (Bruker Company, Germany); MicroTOF-Q II mass spectrometer (Bruker Company, Germany); Agilent 1200 series high performance liquid chromatograph (Agilent Technolgies, America); F-7000 fluorescence spectrometer (Hitachi, Japan); All pH measurements were made with a PHS-3CF acidity meter (Shanghai, Leici Instrument Co., Ltd, China).

5.2. Synthesis, purification, and analytical characterization of FRHA

The thiol-containing FA-Spacer **1** is a folate-containing peptide consisting in sequence of pteroic acid (Pte), L-glutamic acid (Glu), L-aspartic acid (Asp), L-arginine (Arg), L-serine (Ser) and L-cysteine (Cys) to tailor the water-solubility of the final drug conjugate. It was synthesized using standard fluorenylmethyloxycarbonyl-based solid-phase peptide synthesis (Fmoc SPPS) on a Wang-resin polymeric support, as described by Leamon et al.[18] Pteroic acid served as the N-terminus, whereas the thiol group of Cys was selected as the attachment site for the cleavable linker. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphoniumhexafluorophosphate (PyBOP) was applied as the activating reagent to ensure efficient coupling using low equivalents of amino acids. Fmoc protecting groups were removed after every coupling step under standard conditions (20% piperidine in DMF). After the last assembly step, the peptide was cleaved from the polymeric support by treatment with 92.5% trifluoroacetic acid containing 2.5% ethanedithiol, 2.5% triisopropylsilane, and 2.5% deionized water. This reaction also resulted in simultaneous removal of the *t*-Bu, Boc and trityl protecting groups. Finally, the trifluoroacetyl moiety was removed in aqueous ammonium hydroxide to give compound **1**. The crude compound **1** was purified by preparative HPLC (Eclipse XDB-C_{18} 4.6 × 250 mm) with 10 mM NH_4OAc, pH 5 (solvent A) and acetonitrile (solvent B) using a gradient consisting of 1–20% B over 40 min at a flow rate of 1.5 mL/min.[13] The structure of compound **1** was confirmed by ^{1}H NMR (D_2O, 300 MHz) analysis: δ 8.68 (s, 1H), 7.57 (d, 2H, J = 8.4 Hz), 6.67 (d, 2H, J = 9.0 Hz), 4.40–4.75 (m, 5H), 4.30 (s, 2H), 3.98 (d, 2H, J = 6.3 Hz), 3.02 (m, 2H), 2.85 (d, 2H, J = 7.0 Hz), 2.67 (m, 2H), 2.00–2.30 (m, 4H), 1.69 (m, 4H).

Activated carbonate **3** served as an important hetero-bifunctional cross-linker for conjugate synthesis since it has been found to react under mild conditions with many *N*- and *O*-nucleophiles.[24] In this experiment, it has been shown to be a convenient and universal tool for the incorporation of reductively labile disulfide linkages into a wide variety of drug conjugates. Generally, activated carbonate **3** could be readily prepared from 2-mercaptoethanol in two steps adopting the method by Vlahov et al.[10,19] The structure of **3** was confirmed by ^{1}H NMR analysis and the same as Vlahov's.[10]

Thiol-reactive derivative of RHA **2** was prepared in three steps from RHA which was isolated by our laboratory. Briefly, as shown in Scheme 1, RHA (100 mg, 0.238 mmol) was treated with $(PhO)_3P{-}CH_3I$ (0.1 mL) in DMF for 18 h at room temperature, followed by ammonolysis in an autoclave for 12 h to yield amino-reactive derivatives of RHA **4**. Compound **4** was treated with the activated carbonate **3** and diisopropylethylamine (DIPEA, 0.2 mL) in DCM for 1 h to yield **2**, followed by chromatographic purification (silica gel, 10% MeOH/$CHCl_3$ follow by 35% MeOH/$CHCl_3$), **2** was isolated with 72.3 mg. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.23 (d, 1H, J = 7.9 Hz), 7.63 (d, 1H, J = 7.2 Hz), 7.22 (m, 1H), 6.96 (d, 2H, J = 6.8 Hz), 6.86 (d, 1H, J = 7.6 Hz), 6.75 (s, 1H), 6.64 (d, 1H, J = 7.6 Hz), 6.45 (s, 2H), 6.13 (s, 1H), 5.88 (d, 1H, J = 5.7 Hz), 4.75 (m, 1H), 4.35 (t, 2H, J = 7.2 Hz), 4.25 (m, 1H), 3.85 (t, 1H, J = 5.8 Hz), 3.73 (s, 3H), 3.43 (d, 2H, J = 5.9 Hz), 3.17 (d, 2H, J = 7.9 Hz), 2.93 (t, 2H, J = 7.2 Hz); ^{13}C NMR (DMSO-d_6) δ 160.2, 158.8, 156.9, 149.8, 148.4, 147.1, 142.8, 136.7, 131.0, 127.9, 124.8, 119.8, 115.4, 114.3, 105.3, 104.1, 100.6, 98.9, 71.4, 69.5, 68.7, 67.3, 65.0, 56.5, 44.1, 33.8; HRMS (ESI): $(M+H)^+$ calcd for $C_{29}H_{32}N_2O_{10}S_2$ 633.1577, found 633.1572.

Treatment of a suspension of FA-Spacer **1** (79 mg) in PBS under argon with 0.1 N $NaHCO_3$ resulted in a clear yellow solution at pH > 6.5. This mixture was added at once under vigorous stirring to a solution of **2** (50 mg) in THF. The formation of the conjugate, FRHA, was monitored by analytical HPLC. Then, this product was purified by preparative HPLC (Eclipse XDB-C_{18} 4.6 × 250 mm) with PBS, pH 7.4 (solvent A) and acetonitrile (solvent B) using a gradient consisting of 10–50% B over 30 min at a flow rate of 1.5 mL/min. Acetonitrile was removed from the collected fractions in vacuo and the residue subjected to freeze-drying to give 87.6 mg (70% yield) of FRHA. ^{1}H NMR, ^{13}C NMR and HRMS signals were in agreement with the expected structure: ^{1}H NMR (D_2O, 300 MHz): δ 8.67 (s, 1H), 7.35 (d, 2H, J = 7.8 Hz), 6.96 (d, 2H, J = 6.8 Hz), 6.88 (d, 1H, J = 8.3 Hz), 6.75 (s, 1H), 6.64 (d, 1H, J = 7.6 Hz), 6.51 (d, 2H, J = 8.7 Hz), 6.45 (s, 2H), 6.13 (s, 1H), 5.88 (d, 1H, J = 5.7 Hz), 4.53–4.84 (m, 5H), 4.35 (t, 2H, J = 7.2 Hz), 4.30 (s, 2H), 4.25 (m, 1H), 3.94 (d, 2H, J = 6.3 Hz), 3.85 (t, 1H, J = 5.8 Hz), 3.73 (s, 3H), 3.43 (d, 2H, J = 5.9 Hz), 3.17 (d,2H, J = 7.9 Hz), 3.02 (m, 2H), 2.93 (t, 2H, J = 7.2 Hz) 2.88 (d, 2H, J = 7.0 Hz), 2.63 (m, 2H), 2.00–2.30 (m, 4H), 1.69 (m, 4H); ^{13}C NMR (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.

5.3. Treatment of FRHA with a reducing agent

A 2 mL solution of FRHA in PBS (pH 7.4) with the concentration of 50 mM was treated with 20 mL L-glutathione (GSH) at 37 °C.[25] Before this, GSH was dissolved in PBS to achieve the concentration of 2 mM. At different length of time (t = 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 h), a sample of solution was taken to be monitored at λ = 324 nm by HPLC-MS as a function of time.

5.4. Cytotoxicity assay

5.4.1. Cell culture

KB cells (FR-positive cells, xenograft), M109 cells (FR-positive cells, syngeneic) and 4T1 cells (FR-negative cells) were employed as in vitro and in vivo models. The cells were cultured in the folate-free RPMI medium (FFRPMI) containing 10% heat-inactivated fetal calf serum (HIFCS) and 100 IU/mL penicillin and 100 μg/mL streptomycin. They were incubated in a 37 °C water-jacketed incubator equilibrated with 5% CO_2/95% relative air-humidified atmosphere with no antibiotics. The HIFCS contained its normal complement of endogenous FA which enabled the cells to sustain growth in this more physiologically relevant medium.[13] The medium was replenished every other day until confluence was achieved. The cells were then washed with PBS and harvested with 0.125% Trypsin–EDTA solution.[26] All cell experiments were performed using FFRPMI containing 10% HIFCS and 5% penicillin–stretomycin as the growth medium.

5.4.2. In vitro cytotoxicity

The cytotoxicity of FRHA were measured using the MTT proliferation assay as described before.[26,27] Briefly, 150 μL of KB/4T1 cells were seeded at a density of 1.0×10^4 cells/well in 96-well transparent plates and allowed to adhere overnight. The growth medium was replaced with fresh medium containing FRHA or parental RHA or the RHA derivative (compound **2** or compound **4**) at various drug concentrations (0.1, 0.5, 1, 5, 10, 50, 100, 500 nM) in the presence or absence of 0.01 mM FA (as a competitor to determine the targeting specificity) in the medium. They were then incubated for another 48 h before replacing the medium within each well with 0.1 mL of fresh growth medium and 20 μL of MTT solution (5.0 mg/mL in PBS). After incubation for another 3 h, the growth medium was removed and 150 μL of DMSO was added to each well to dissolve any formazan crystals formed. The plates were vigorously shaken before measuring the relative color intensity using a microplate reader at 570 nm. To eliminate any effect from drugs incorporated into the cells on absorbance measurement, an identical experiment without MTT (with all other conditions fixed) was carried out and the corresponding wells (after addition of DMSO) were used to prepare the baseline for subsequent absorbance measurement, respectively. Cell viability for that particular concentration of sample was expressed as a percentage of the intensity of the controls ± standard deviation. Each experiment was repeated 5 times at each polymer concentration.

5.5. The plasma protein-binding properties of FRHA

Equilibrium dialysis was exploited to determine the HSA binding ability of FRHA. The dialysis membranes were activated by immersing in phosphate buffer for 30 min, and rinsed twice with water. And then, they were placed into the equilibrium dialysis unit between the two compartments of dialysis chamber to separate free and total FRHA. FRHA in various concentrations (1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0 μM) were mixed with HSA (10.0 μM) and 1 mL of the sample was placed into one side of the cell, while 1 mL of phosphate buffer was added to the other side of the cell. The cell was then placed on a dialysis cell shaker at 37 °C for 1 h. At the end of the experiment, 0.1 mL samples were taken from each side of the cell for analyzing. The free FRHA concentration in the PBS compartment (F) and the total FRHA concentration in the HSA compartment (T) were measured by fluorescence spectroscopy. The excitation wavelength was set at 377 nm, and the emission spectra were recorded in the range of 385–550 nm. The protein binding rate was calculated using the following equation:

$$\text{protein binding} \quad (\%) = [(T - F)/T] \times 100 \qquad (1)$$

5.6. Serum stability

The stability of FRHA and parental RHA in human serum were determined by an in vitro assay including a solid-phase extraction procedure for sample cleanup followed by HPLC analysis.[9] Briefly, FRHA or RHA was added to human serum at a final concentration of 200 μM, and the sample was incubated in a 37 °C water bath for 24 h. 200 μL of serum sample ($n = 3$) was removed at the designated time interval (1–3, 5, 8, 12, 18 and 24 h) after initiation of incubation, and FRHA/RHA was recovered from serum by solid-phase extraction with Oasis HLB cartridges (1 cm^3/30 mg; Waters Corporation). First, 800 μL of guanidine (6 M, pH adjusted to 7.4 with 0.1 M NaOH) was added to each serum sample to reduce non-specific protein binding of the drug. Next, the cartridges were conditioned with 1 mL of methanol and equilibrated with 1 mL of guanidine (6 M, pH 7.4). Samples were then loaded onto the cartridges followed by a wash with 5% methanol in water (1 mL). Finally, samples were eluted with 1 mL of methanol, evaporated to dryness, and reconstituted in 200 μL of PBS, pH 7.4. Ten microliters of the reconstituted sample was analysed with HPLC (equipped with a Eclipse XDB-C_{18} 4.6 mm × 150 mm × 5 μm column, a UV detector at 375 nm, the mobile phase was a 28:72 (v/v) mixed solution of acetonitrile and 1% aqueous formic acid at the flow rate of 1 mL/min). The %RSD for each individual time point was <2%.

5.7. Blood Clearance

Six week old female Balb/c mice which treated with FR-positive M109 cells or FR-negative 4T1 cells along with normal mice were used to investigate blood clearance. In order to confirm the possible results, six week old female normal mice were also used in this experiment. After three weeks on a folate-free diet, M109 cells or 4T1 cells (0.75×10^6 per mouse) were inoculated in the peritoneal cavity and allowed to proliferate for 4 days. The mice were maintained on a folate-free diet prior to and beyond the onset of dosing. Then, all mice were received an 2 μmol/kg intravenous dose of FRHA or parental RHA, and blood samples were taken with time (10, 25, 50, 100, 160, 240, 320, 400 min) and transferred into heparinized tubes. The samples were treated and analysed by the method by Zhao Y. Y. et al.[28]

5.8. In vivo antitumor experiments

Six- to seven-week-old female Balb/c mice were maintained on a standard 24 h light–dark cycle and fed *ad libitum* with folate-free diet for the duration of the experiment. KB, M109, or 4T1 cells (1×10^6 per mouse) in 100 μL were injected in the subcutis of the dorsal medial area. Tumors were measured in two perpendicular directions using vernier calipers every 2-3 days and their volumes calculated as $0.5 \times L \times W^2$, where L is the measurement of longest axis in millimeters and W is the measurement of axis perpendicular to L in millimeters.[23] Then, all mice were received a 2 μmol/kg intravenous dose of FRHA or parental RHA or only PBS (for control groups) when tumors were approximately 50–110 mm^3. All test articles were freshly prepared and injected through the lateral tail vein under sterile conditions in a volume of 200 μL. Growth of each sc tumor was followed by measuring the tumor three times per week (TIW) two or three week schedule. As a general measure of toxicity, changes in body weights were determined on the same schedule as tumor volume measurements. Survival of animals was monitored daily while animals that were moribund (or unable to reach food or water) were euthanized by CO_2 asphyxiation. For individual tumors, a complete response (CR) was defined as a disappearance of measurable tumor mass (<2 mm^3) at some point after tumor implantation. Cures were defined as CRs without tumor regrowth within the 90 day study time frame.[17]

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