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Research Article

A Novel Structural Modification Strategy to Incorporate LAT-1 Transporter Inhibitory Activity

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Abstract

L-type Amino Acid Transporter 1 (LAT-1), over expressed on the membrane of various tumor cells, was considered as a potential target for solid tumor therapy. It was hypnotized that conjugating a LAT-1 affinity molecule upon cell toxic agent could enhance the tumor targeting. In this study, quercetin was modified by overhanging a LAT-1 affinity group, with the aim of developing an effective LAT-1-mediated chemotherapeutic agent. On the other hand, the poor water solubility of quercetin limited its clinical utility in fact; this obstacle was ameliorated by conjugating the LAT-1 affinity group.

The anti-tumor assays in this study as certained that the novel compound possessed LAT-1 inhibitory activity in high affinity which lead to progress of anti-tumor activity of quercetin, and increase on water-solubility of quercetin which enhanced the bioavailability of quercetin. All those data suggested that chem-conjugating a LAT-1 affinity group with traditional cellular toxic agent might be a powerful approach aiming to re-utilize and ameliorate its original anti-tumor characterizations.

Keywords: Quercetin; LAT-1 transporter; Solubility

Introduction

The amino acid transporters on the plasma membrane is crucial for mediating the amino acid transportation in and out of cells and organelles which is essential for cellular physiological function [1]. The L-type Amino Acid Transporter-1 (LAT-1), a Na^+-independent neutral amino acid transporter [2,3] which has been identified as potential as a target for tumor therapy since LAT-1 transporters is not only over expressed in various tumor cells but also in organ barrier system including blood brain barrier, testis barrier and placental barrier. The existence of these barriers extremely decreased the drug bioavailability following the lesion in drug efficacy [4-6].

Quercetin (3,3',4',5,7-pentahydroxyflavone), a natural flavonoid found in various vegetables and fruits, was reported to possess anti-carcinogenic properties against a wide range of human cancer [7,8]. It was ascertained that this compound plays its effects by inhibiting tyrosine kinases involved in cell signaling pathways, including HMGB1, JAK-STAT, and TSLP [9-11]. Following its comprehensive characterization, quercetin has been approved by FDA A as a therapeutic medicine for the treatment of prostatic cancer.

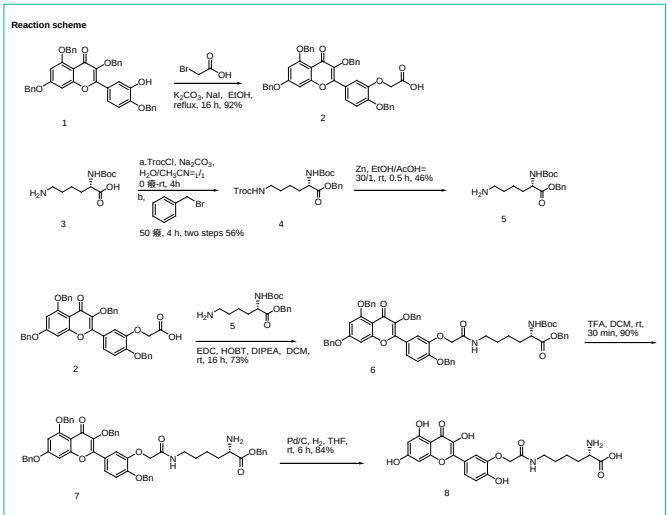
However, the poor water solubility of quercetin limited the efficacy in clinical utility of quercetin, the maximal solubility of quercetin of 0.1mg/ml even in 100% DMSO solution.

In this study, quercetin was conjugated with various amino acids possessing high affinity to the LAT-1 transporter containing a hydrolysis linker, with a view to enhancing affinity to LAT-1 over expressed on tumor cells and water solubility relative to parent quercetin. The increased water solubility induced by the introduced amino acid led to improved bioavailability. Moreover, as expected, the derivatives synthesized in this study possessed higher LAT-1 affinity, which facilitated increased quercetin transport into tumor cells.

Materials and Methods

Reaction Scheme for synthesis of compound 2

Step 1: A suspension of starting material (0.9g, 1.36mmol, 1eq), 2-bromoacetic acid (940mg, 6.79mmol, 5eq), K_2CO_3 (1.5g, 10.86mmol, 8eq), and NaI (40mg, 0.27, 0.2eq) in EtOH (50mL) was refluxed overnight. The solvent was removed under vacuum and the residue re-dissolved in a mixture of ethyl acetate/water. HCl (2N) was added to neutralize the mixture until pH reached 2-3. The desired product was extracted twice with EA. After drying and concentration, the residue was purified using silicagel column chromatography (Pure



Reaction Scheme for synthesis of compound 2

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PE/EA = 2/1 to DCM/MeOH = 20/1) to obtain target compound as a yellow solid (0.9g, 92%).

Step 2: The starting material (4.2g, 17mmol, 1eq) was dissolved in H_2O/CH_3CN (50/50mL).Na_2CO_3 (1.81g, 17.05mmol, 1eq) was added and stirred to dissolve into the mixture. TrocCl was added (3.97, 18.76, 1.1eq) within 15 min and further stirred for 4 h at room temperature. LC-MS analysis revealed the formation of an intermediate, which was reacted with BnBr (3.21g, 18.76, 1.1eq). The mixture was warmed to 50°C and stirred for an additional 4 h. EA was added to the reaction mixture, the organic phase separated, and the aqueous layer extracted once with EA. The combined organic phase was dried and concentrated. The residue obtained was purified directly *via* silicagel column chromatography (PE/EA = 8/1 to 5/1) to obtain the target compound as a colorless oil (4.88g, 56%).

Step 3: The starting material (4.88g, 9.53mmol, 1eq) was dissolved in a solution of EtOH/AcOH (60/2mL). Zn powder (6.23g, 95.3mmol, 10eq) was added and the mixture stirred for 0.5 h at rt. The reaction suspension was filtered and washed with MeOH. The filtrate was concentrated under vacuum and re-dissolved in a mixture of DCM/water. After separation of the DCM phase, the aqueous phase was extracted once with DCM. The combined organic layer was dried and concentrated, and the residue further purified using a silicagel column [DCM/MeOH = 20/1 to 10/1 to 5/1(3% NH_3))] to obtain target material as a yellow oil (1.4g, 46%).

Step 4: The starting acid material (502mg, 0.7mmol, 1eq), EDC (336mg, 2.09mmol, 3eq), and HOBt (292mg, 2.09mmol, 3eq) were dissolved in dry DCM (30ml) and stirred for 5 min, followed by the addition of DIPEA (280mg, 2.09mmol, 3eq) and amino compound (291mg, 0.84mmol, 1.2eq). The mixture was stirred for 16 h at rt and the reaction mixture concentrated. The residue was re-dissolved in EA and washed once with HCl, once with Na_2CO_3, and once with brine. After drying and removal of solvent, the residue was purified using column chromatography (PE/EA = 2/1 to 3/2) to obtain TM as a yellow oil (600mg, 73%).

Step 5: The starting material (600mg, 0.58mmol) was dissolved in DCM (10mL). After addition of TFA (3mL), the mixture was stirred for 30 min at rt. Next, the solvent and TFA were removed, and the residue re-dissolved in DCM (20mL). A Na_2CO_3 aqueous solution (20mL) was added and the mixture stirred for 15 min. The DCM layer was separated, dried, and concentrated. The residue was purified using silica-gel column chromatography (PE/acetone = 2/1 to DCM/MeOH = 15/1, 0.5% NH_3) and TM obtained as a yellow oil (488mg, 90%).

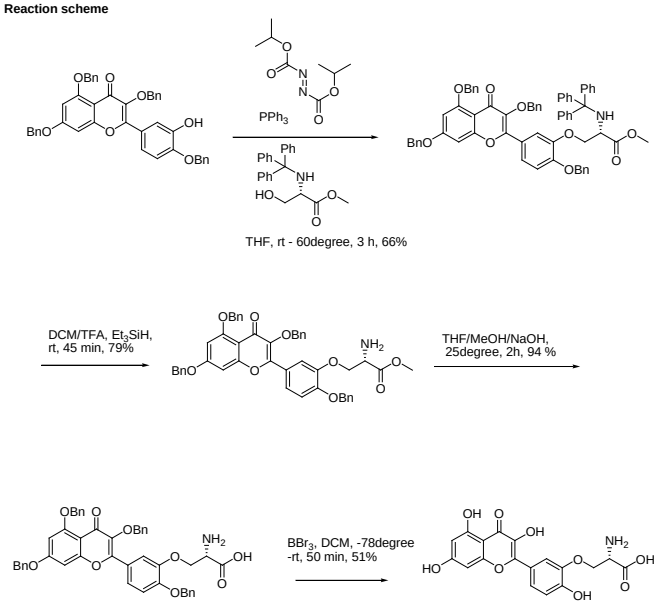
Step 6: SM (150mg) was dissolved in THF and Pd/C (10%, 200mg) added under a hydrogen atmosphere, and stirred for 6 h. Then the residue was dissolved into DMSO and filtered to obtain a DMSO solution of the desired compound. The solution was purified directly using reverse C18 column chromatography (CH_3CN/H_2O=90/1 to 80/20, 3% NH_3HCO_3) to obtain TM as a yellow solid (65mg, 83%).

The Mass spectrum and NMR spectrum of this compound was showed in supplementary Figure 1.

Reaction Scheme for synthesis of compound 3

Step 1: To a solution of SM (1g, 1.5 mmol, 1eq), alcohol (818mg, 2.26mmol, 1.5eq), and PPh_3 (790mg, 3.02mmol, 2eq) in dry THF (30mL), DIAD was added (0.61mL, 3.02mmol, 2eq) at rt. The mixture was stirred for 10 min and warmed to 60°C for 3 h. Following removal of solvent under vacuum, the residue was purified directly via silicagel column chromatography (pure DCM to DCM/MeOH = 200/1) to obtain the target compound as a yellow oil (1g, 66%).

Step 2: The starting material (1g, 1mmol) was dissolved in DCM (10mL), followed by sequential addition of TFA (3mL) and Et3SiH (0.3mL). The mixture was stirred for 45 min at rt and concentrated under vacuum. After re-dissolving the residue in DCM (50mL), aqueous saturated Na_2CO_3 (50mL) was added and stirred for 10 min. The DCM phase was separated, dried over anhydrous Na_2SO_4, and concentrated. The generated residue was purified using silicagel column chromatography (PE/EA = 2/1 to DCM/MeOH = 40/1) to



Reaction Scheme for synthesis of compound 3

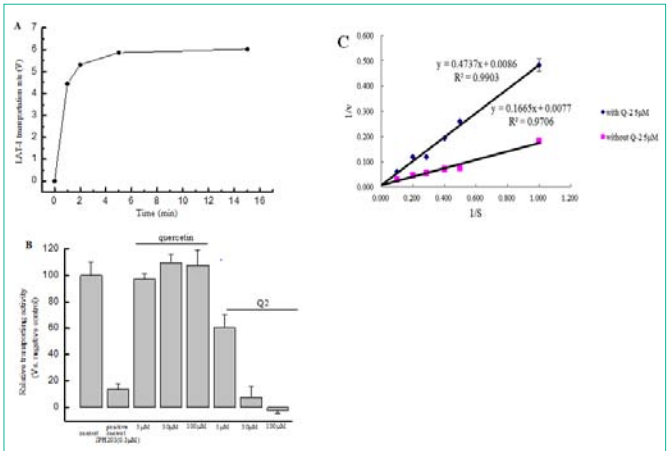


Figure 1: Cellular uptake assay.
1A: Measurement of the transporting rate of compound 2 by LAT1-1.
1B: Measurement of the relative transportation efficiency of compound 2.
1C: Q2 was competitive inhibitor against LAT-1 transporter.
The result indicated that the compound 2 (Q2) exhibited LAT-1 inhibitory characterization, and the transportation rate was saturated in 15 minutes.

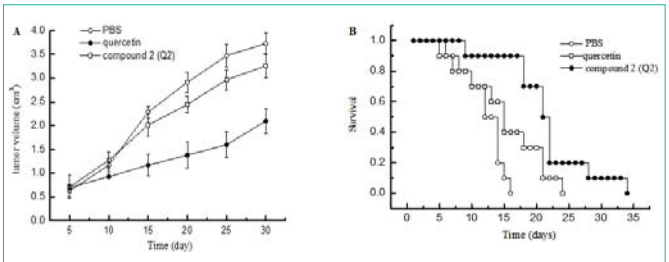


Figure 2: Anti-tumor efficacy of compound 2 on human tumor bearing mice.
Panel A: Effect of compound 2 on human xenograft tumor model mice bearing A549 cells.
Panel B: Anti-tumour effect of the compound 2in tumor-bearing mice.
Condition: Xenograft mice bearing A549 cells were treated with the quercetin and compound 2, respectively. The antitumor efficacy of compound 2 and quercetin was measured by monitoring the tumor volume after treatment from tumor bearing mice. Survival time was recorded in days after tumour injection. All data obtained for repeated experiments were pooled and utilized for statistical analysis.
Legend: Results indicated that tumor size possessed more reduction in group treated with compound 2 than those treated with quercetin in A549 bearing mice. In addition, the group treated with the compound 2 (●) exhibited efficient anti-tumor activity as shown. Experimental animals showed an improved survival time compared to quercetin (□), $p<0.05$ (n=10).

obtain the target compound as a yellow oil (0.6g, 79%).

Step 3: The starting material (0.6g, 0.78mmol) was dissolved in THF/MeOH (4/4mL) solution, followed by addition of NaOH aqueous solution (2mL, 2N). After stirring the mixture for 2 h at rt, TFA was added to neutralize the reaction solution. Next, the solution was concentrated under vacuum and a mixture of MeOH/H_2O (~1/1) added to partly re-dissolve the residue. The resultant suspension was purified using reverse C-18 column chromatography [CH_3CN/H_2O = 20/80 (0.3% TFA) to 75/25 (0.3% TFA)] to obtain TM (0.55g, 94%) as a yellow oil.

Step 4: The starting material (0.55g, 0.73mmol) was dissolved in dry DCM and cooled to -78°C. BBr_3 (2.4g, 10mmol, 13eq) was added to the mixture, and the suspension stirred for 20min at -78°C and warmed to rt for an additional 30min. The reaction was quenched with water, DCM removed, and the aqueous solution purified directly using the reverse C-18 column (pure H_2O to CH_3CN/H_2O of 10/90 to pure H_2O to 0.3% ammonia aqueous solution). Purified TM was obtained as a yellow solid (142mg, 51%).

The purity of original compounds 2 and 3 was assessed *via* high-performance liquid chromatography (HPLC) and stored as a powder at -20°C.

Water solubility measurement

Quercetin, compounds 2 and 3 were weighed and dissolved into 0.9% NaCl, PBS and Hanks solution (pH 6.5 and 7.4). Quantitative measurement of samples was performed *via* LC-MS/MS.

Cellular uptake analysis of quercetin and modified compounds *in vitro*

The lung cancer cell line, A549, expresses high levels of LAT-1, supporting the utility of these cells for our experiments. Initially, the time-dependent cellular uptake of chemicals in A549 cells was investigated.

A549 cells were cultured in the medium at a density of 2.0×10^5 cells/mL, followed by inoculation into 24-well cell culture plates (1mL/well). After 48 h cultured at 37°C and 5% CO_2, the medium was removed. Cells were washed with PBS and treated with test compound and control (quercetin) (25μM) in PBS for 1, 2, 5, and 15 min, respectively. The reaction was terminated by washing three times with cold PBS solution, followed by addition of 300μL methanol to each well. Liquid solution (50μL) was removed,mixed with ddH2O (50μL), methanol (50μL), and IS solution (50μL), and analyzed using LC-MS/MS.

Inhibition assay of modified quercetin against LAT-1

The uptake assay was performed on A549 cells cultured at 37°C and 5% CO_2 in 24-well plates for 48 h. Cell groups were incubated with test compounds (quercetin and compound 2 (Q2), negative control (JPH203; -3μM), and positive control (JPH203; -0.3μM). After removal of culture medium, cells were washed with PBS. Following incubation with warm PBS for 10 min, cells were treated with either radioactive-labeled or non-labeled leucine for 2 min. After termination of the reaction with cold PBS, cells were incubated with lysis solution (0.1M NaOH). Lysates were mixed with 3ml ULTIMA Gold scintillation solution and analyzed using theTri-Carb 2910TR liquid scintillation counter. Ki values were calculated using the Line weaver-Burk equation.

MTT assay

Cells were seeded in 96-well flat-bottom microtiter plates at a density of 5 × 104cells per well and allowed to adhere overnight. Next, cells were treated with quercetin or compound 2 (final concentration of 10 μg/mL) for 24, 48, and 72 h. Simultaneously, control and zero-adjustment wells were set. Subsequently, 20 μl MTT (5mg/mL; Sigma, St. Louis, MO, USA) solution in sterile deionized water was added at 24, 48, and 72 h, followed by incubation for a further 4 h. After termination of the reaction, the supernatant was removed and the formazan precipitate was dissolved in 150 μL DMSO (Sigma). Absorbance values at 490 nm were read on a microtiter plate reader (Thermo Scientific, West Palm Beach, FL, USA). Five replicates were examined for each MTT assay. The inhibitory rate of cell proliferation was calculated according to the formula: [1-(experimental absorbance value-zero absorbance value)/(control absorbance value-zero absorbance value)] × 100.

Treatment of human tumor xenograft mice

Sixweek-old female DBA/2 mice were obtained from Shanghai Laboratory Animal Co. (China Academy of Sciences, Shanghai, China). Animal studies were conducted in accordance with the ALAARK protocol guidelines in Tianjin GLP laboratory, China. Female DBA/2 mice (weighing 16-20 mg) were housed in barrier facilities under a 12 h light/dark cycle and supplied food and water ad libitum.

On day zero, one group was inoculated *via* i.p. injection with A549 tumor cells (2×10^5) in 0.5mL RPMI1640. Treatment was initiated after the tumor was allowed to grow to a volume of~100 mm^3 on the back of the mouse. This experimental protocol is intended to mimic the clinical situation whereby treatment is initiated after establishment of tumor. Animals were treated with the specified dose of quercetin or compound 2 (25mg/kg body weight) every day. During the 25-day experimental period, survival time was recorded as days after tumor

Table 1: Saturating concentrations of test compounds in various solvents.

	Chemical	Solvent	Theoretical Conc (mM)	Measured Conc (μM)
Q1	Quercetin		4	9.577
Q2	Compound 2	0.9% NaCl		24.823
Q3	Compound 3			11.354
Q1	Quercetin		4	7.569
Q2	Compound 2	PBS		22.158
Q3	Compound 3			9.184
Q1	Quercetin		4	13.874
Q2	Compound 2	Hanks- pH 6.5		13.321
Q3	Compound 3			13.785
Q1	Quercetin		4	10.096
Q2	Compound 2	Hanks- pH7.4		67.244
Q3	Compound 3			14.746

injection. Mean and median survival times and statistical significance of data were determined with a two-tailed Wilcoxon's rank test. All data obtained from replicate experiments were pooled and utilized for statistical analysis.

Statistical analysis

Statistical significance was evaluated using the two-tailed Student's t-test. Data were considered significant at $P < 0.05$.

Results

As proposed, structural modification of quercetin improved its water solubility. Notably, water solubility of compound 2 in various buffers was significantly higher than that of quercetin, as shown in Table 1.

Data from the cellular uptake assay indicated that compound 2 is efficiently transported into A549 cells owing to increased affinity for the LAT-1 transporter Figure 1A. The initial transport rate was 2 min and reached at plateau at 15 min. We observed considerably higher accumulation of compound 2 in A549 cells, compared to native quercetin Figure 1B. According to previous findings, the α–free carboxyl group is crucial for targeting the LAT-1 transporter. Compound 2 clearly retained LAT-1 inhibitory activity following structural modifications. More important, the compound 2 exerted a competitive inhibitory effect Figure 1C.

The cellular MTT assay was employed to measure the anti-tumor activity of compound 2. In A549 cells over expressing LAT-1, treatment with compound 2 resulted in significant loss of cell viability, signifying LAT-1 inhibitory activity Table 2. To establish the effects of modified quercetin *in vivo*, A549 cell xenograft-bearing mice were treated with compound 2 and quercetin, and antitumor activities determined by monitoring tumor volumes after treatment Figure 2. Compound 2 was administered at a dose of 8mg/kg body weight for 30 days and quercetin administered at an identical dose and frequency as control. Notably, tumor sizes of the groups treated with compound 2 were remarkably reduced, compared to mice administered quercetin alone Figure 2A. Moreover, administration of compound 2 increased the survival rates of experimental mice, as shown in Figure 2B.

Table 2: MTT assay to determine the effect of compound 2 relative to quercetin on A549 tumor cells.
Experimental conditions: Cells (1×10^5 cells/100 μL/well) were seeded in 96-well plates under 37°C and 5% CO_2. Aqueous solutions of quercetin and compound 2 were added into the culture medium following the assay protocol.
Legend: Measurement of anti-proliferative activity of compound 2 in A549 cells with high LAT-1 transporter expression.

	A549	
Time	Quercetin	Compound 2
24 h	23.01 ± 3.16	43.75 ± 3.32
48 h	35.42 ± 4.02	68.71 ± 7.97
72 h	46.63 ± 3.45	84.95 ± 5.64

Our collective *in vitro* and *in vivo* results demonstrate that modification of quercetin *via* additional amino acid conjugation enhances its anti-tumor activity. The improved therapeutic benefits of modified quercetin may be attributable to both increasedLAT-1 inhibitory activity and water solubility.

Conclusion

In this study, structural modification of quercetin was performed to improve its water solubility and bioavailability based on earlier literature showing that the conjugation of amino acids is capable of improving biological properties. The transporter protein, LAT-1, is over expressed in several tumor types. Among the two compounds synthesized in this study, compound 2 exhibited enhanced therapeutic properties. The solubility of compound 2 was three-fold higher than that of quercetin, indicative of higher bioavailability for clinical utility. Moreover, compound 2 possessed LAT-1 inhibitory activity, as expected, leading to higher accumulation in tumors. The collective results support the efficacy of compound 2 as a potential anti-tumor agent. Moreover, the structural modification strategy reported in this study may be employed to optimize other therapeutic agents.

Acknowledgement

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Ethics Approval and Consent to Participate

All animal experiments were approved by the General Hospital of Tianjin Medical University and performed following the Guide for the Care and Use of Laboratory Animals and Stroke Treatment.

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