Design and Synthesis of Biotinylated Troglitazone as an Active Affinity Probe

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A biotin-tagged analogue of troglitazone was designed, synthesized and applied to affinity chromatography to pulldown EGFR from 293T cell lysates overexpressing EGFR, a membrane protein assumed to be troglitazone's direct binding target by previous work. The results indicate the feasibility of the biotinylated probe as a vigorous tool to clarify the molecular mechanisms for troglitazone's antitumor activities.

Keywords antitumor agent, molecular mechanism, structure-activity relationship, biotinylated probe, affinity chromatography

Introduction

Troglitazone (TGZ) is a synthetic high-affinity ligand of peroxisome proliferator-activated receptor γ (PPARγ),[1] a nuclear transcription factor that controls genetic programs involved in glucose and lipid homeostasis, energy metabolism, adipocyte differentiation, and maturation.[2] Recent studies have well established that TGZ, like other members of the thiazolidinedione class of drugs, causes growth inhibition, induces differentiation, and triggers apoptosis of various human malignant cells in addition to its well-known activity to increase insulin sensitivity.[3-6] However, the underlying mechanism remains inconclusive.[7] As TGZ has already shown anticancer efficacy in humans and represents a promising new anticancer agent, molecular mechanism for its anti-tumor activity has become the focus of investigation.[5,8] To date, numerous studies have shown that TGZ exerts growth inhibitory effects independently of PPARγ and may result from multiple mechanisms.[9-14] In the long term, validating TGZ's target proteins may help to define its molecular mechanisms and facilitate the design of improved therapeutic agents. The modification of TGZ with biotin is among the most direct strategies to address this question.[15] By streptavidin-biotin affinity chromatography, the biotinylated probe should be suitable for purification of TGZ's target proteins. In this paper, we reported the design, synthesis and preliminary evaluation of a biotin-tagged analogue of troglitazone (TGZ-biotin) as an activity-based probe to study the molecular mechanism of thiazolidinedione class of drugs related to cross-activation of EGFR-Erk1/2 pathway.[16-18]

Results and discussion

Design and synthesis of TGZ-biotin

To ensure that the biotinylated probe would maintain sufficient potency, design of the probe was based on the structure-activity relationship of TGZ and its analogues (Figure 1). Since all TGZ analogues with antiproliferative activities have the thiazolidinedione motif in their structures, it seems that this moiety is essential to keep the desired probe's activity. On the other hand, it has been reported that protection of the hydroxyl group in the chroman ring of TGZ with benzyl retains its euglycemic and hypolipidemic activities *in vivo*.[19] This fact, together with the observation that tocopherol moiety alone does not induce physiological response similar to TGZ in HCT-116 cells,[10] suggests that the modification of the hydroxyl group in the chroman moiety would be well tolerated. To ensure a large conformational freedom of the bioactive TGZ motif, a poly(ethylene glycol) (PEG) linker with rational length was introduced to link it with the biotin tag.[20]

Preparation of the probe was commenced with (*R/S*)-Trolox.[19] Reduction of the carboxyl acid group with $LiAlH_4$ yielded **1**, which was then attached with the PEG linker to give **2**. Reaction of **2** with 4-fluorobenzaldehyde afforded **3** with low yield, owing to partially denatured KOBu-*t*. Aldol reaction of **3** with thiazolidine-2,4-dione gave **4**, which was reduced with MeOH-Mg to furnish **5** as a mixture of two diastereomer pairs (2*R*-5*R*/2*S*-5*S* and 2*R*-5*S*/2*S*-5*R*). Re-

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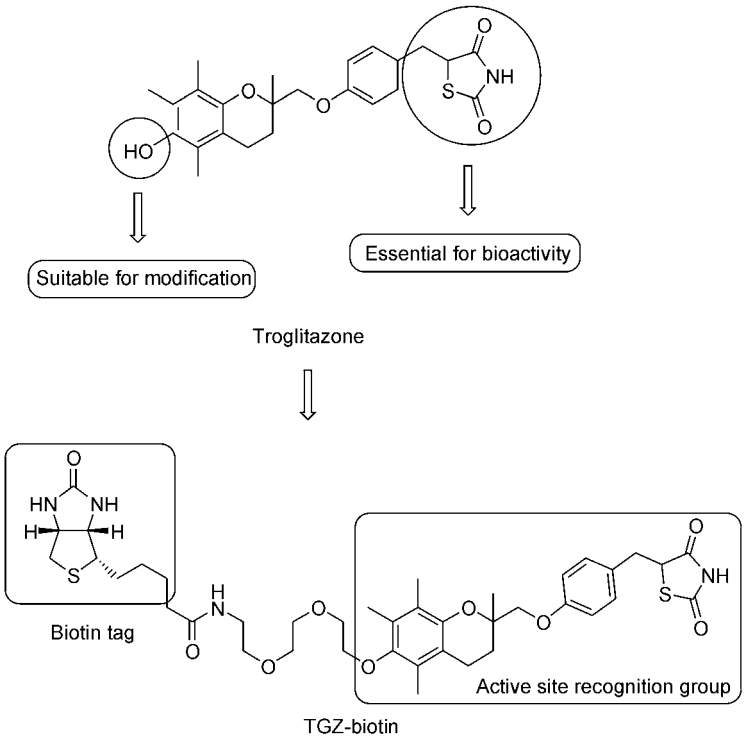
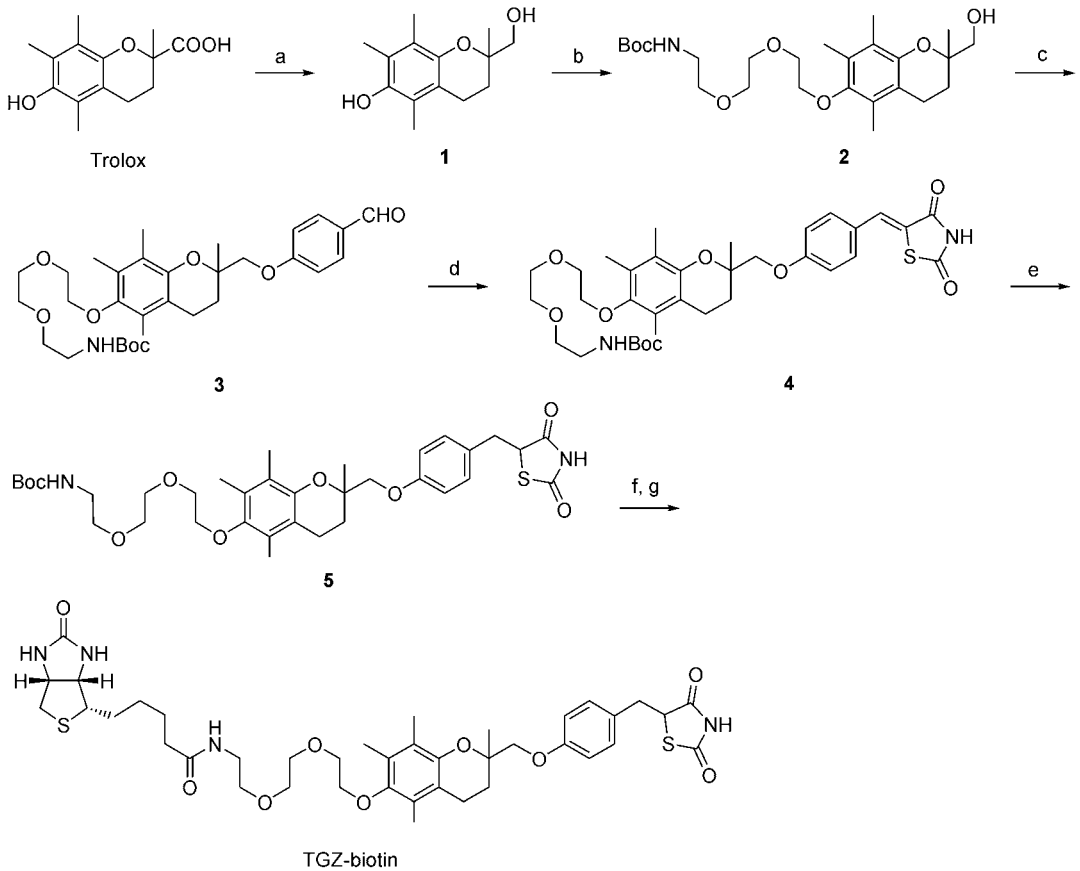


Figure 1 Structures of TGZ and its biotinylated analogue designed.

Scheme 1 Synthesis of TGZ-biotin



Reagents and conditions: (a) $LiAlH_4$, THF, 70 ℃, 3 h, 93.6%; (b) *tert*-butyl 2-[2-(2-bromoethoxy)ethoxy]ethylcarbamate, K_2CO_3, *n*-Bu_4NI, 80 ℃, butanone, 24 h, 59.0%; (c) (i) KOBu-*t*, DMF, room temperature, 1 h; (ii) 4-fluorobenzaldehyde, room temperature, 15 h, 29.2%; (d) thiazolidine-2,4-dione, piperidine, benzoic acid, reflux to separate water, 4 h, 59.6%; (e) Mg, CH_3OH, 45 ℃, 8 h, 70.8%; (f) (i) 2 mol•L^{-1} HCl in EtOAc, room temperature, 1 h; (ii) (*D*)-biotin-*N*-succinimidyl ester, *i*-Pr_2NEt, CH_2Cl_2, room temperature, 12 h, 58.4%.

moval of the *tert*-butyloxycarbonyl protecting group of **5** and subsequent incorporation of the biotin tag furnished TGZ-biotin at last.

Affinity chromatography employing TGZ-biotin

Previous work has shown that TGZ triggered activation of Erk1/2 dependent on epidermal growth factor receptor (EGFR) instead of PPARγ.[16] Besides, pre-incubation of porcine aorta endothelial (PAE) cells stably expressing human EGFR with TGZ caused a dose and time-dependent inhibition of rhodamine tagged EGF binding to cell surface at 4 ℃.[16] These results implied that TGZ might direct bind EGFR for signaling. To validate this assumption, 293T cells overexpressing EGFR were simulated with TGZ-biotin (50 μmol•L^{-1}) for 1 h at room temperature. The cells were then rendered to trypsin digestion and cell lysates were then incubated with streptavidin magnetic beads at room temperature for 1h. The bound proteins were resolved in SDS-PAGE and immunoblotted with anti-EGFR antibody. As shown in Figure 2, the affinity chromatography could extract EGFR from the cells. While in contrast, EGFR could not be extracted if the cells were not treated with the probe first. These results further validate the notion that TGZ functionally binds EGFR and shed a new light on its anti-tumor mechanisms.

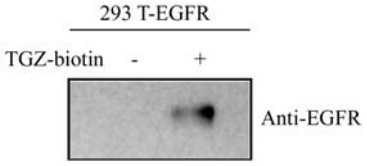


Figure 2 TGZ-biotin direct binds EGFR. Streptavidin-biotin affinity chromatography could extract EGFR from lysates of 293T cells which overexpressed EGFR and were first stimulated with the probe (50 μmol•L^{-1}). The bound protein was detected to be EGFR by western blotting with anti-EGFR.

Conclusion

We have developed a biotinylated TGZ analogue for its target validation via affinity chromatography. The biotin-tagged probe turned out to be useful for the pull-down of TGZ's direct binding target by affinity chromatography, which helps to gain a new insight concerning the mechanisms for TGZ's antiproliferative and pro-apoptotic effects. Considering that TGZ has shown anticancer efficacy in humans and might exert this activity through several different pathways, this probe represents an appropriate tool for further biological studies to clarify the molecular mechanisms of the promising antitumor reagent.

Experimental

General methods

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Dichloromethane (DCM) was distilled from CaH_2. Toluene and tetrahydrofuran (THF) were distilled from sodium. DMF was distilled *in vacuo*. Reactions were monitored by thin-layer chromatography (TLC). ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian Mercury VX300 Fourier transform spectrometer. The chemical shifts δ were reported using the δ=7.26 signal of $CDCl_3$ as internal standard. Low-resolution mass data were obtained on an Agilent 6100 Series Quadrupole LC/MS systems. High-resolution mass data were obtained on a Micromass Q-Tof Ultima^{TM} spectrometer.

Synthesis

(2*R*/*S*)-(6-Hydroxy-2,5,7,8-tetramethylchroman-2-yl)-carbinol (**1**) To a stirred solution of trolox (215 mg, 0.86 mmol) in dry THF (15 mL) was added $LiAlH_4$ (33 mg, 0.86 mmol) and the mixture was heated to reflux and stirred for 3 h while maintaining refluxing. Na_2SO_4•nH_2O (200 mg) was then added at 0 ℃ to quench the reaction and the mixture was stirred at 0 ℃ till becoming clear. The solid was removed by filtration and the filtrate concentrated under reduced pressure to give a brown gum (190 mg, 93.6%), which was used in the next step without further purification.[19] ^{1}H NMR ($CDCl_3$) δ: 1.22 (s, 3H), 1.64—1.75 (m, 1H), 1.95—2.06 (m, 1H), 2.12 (s, 6H), 2.21 (s, 3H), 2.67 (brs, 2H), 3.58—3.68 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 11.50, 12.00, 12.40, 20.37, 20.47, 27.89, 69.41, 75.14, 117.38, 119.15, 121.73, 126.61, 144.95, 145.20; ESIMS *m/z*: 259.1 $(M+Na)^+$.

(2*R*/*S*)-(6-(2-(2-(2-(*tert*-Butoxycarbonylamino)ethoxy)ethoxy)ethoxy)-2,5,7,8-tetramethylchroman-2-yl)-carbinol (2) Anhydrous K_2CO_3 (439 mg, 3.17 mmol) and *n*-Bu_4NI (78 mg, 0.22 mmol) were added to a solution of **1** (500 mg, 2.12 mmol) and *tert*-butyl 2-(2-(2-bromoethoxy)ethoxy)ethylcarbamate (990 mg, 3.17 mmol) in butanone (35 mL). After being stirred at 80 ℃ for 24 h, H_2O was added at room temperature to quench the reaction and the product was extracted into EtOAc and purified by chromatography on a silica gel eluted with PE∶EtOAc (*V*∶*V*=1.5∶1) to get a yellow oil (584 mg, 59.0%). ^{1}H NMR ($CDCl_3$) δ: 1.22 (s, 3H), 1.42 (s, 9H), 1.70—1.78 (m, 1H), 1.92—2.02 (m, 1H), 2.07 (s, 3H), 2.15 (s, 3H), 2.18 (s, 3H), 2.63 (brs, 2H), 3.25—3.38 (m, 2H), 3.50—3.60 (m, 2H), 3.60—3.65 (m, 2H), 3.65—3.75 (m, 2H), 3.75—3.80 (m, 2H), 3.82 (s, 4H), 5.00—5.10 (m, 1H); ESIMS *m/z*: 490.3 $(M+Na)^+$.

4-(6-(2-(2-(2-(*tert*-Butoxycarbonylamino)ethoxy)-ethoxy)ethoxy)-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzaldehyde (3) To a stirred solution of **2** (111 mg, 0.24 mmol) in dry DMF (2.5 mL) at room temperature was added *t*-BuOK (81 mg, 0.72 mmol). The mixture was stirred for 1 h first and then 4-fluorobenzaldehyde (51 μL, 0.48 mmol) in dry DMF (20 mL) was added. The mixture was continued to be stirred for 15 h at room temperature and was then

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quenched with water (5 mL) and extracted with EtOAc thrice. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was chromatographed over silica gel using PE∶EtOAc ($V∶V$=2∶1) as eluent to give 40 mg (29.2%) of **3** as a viscous liquid. ^{1}H NMR ($CDCl_3$) δ: 1.41 (s, 12H), 1.85—2.00 (m, 1H), 2.05 (s, 3H), 2.10—2.20 (m, 1H), 2.14 (s, 3H), 2.18 (s, 3H), 2.61 (brs, 2H), 3.25—3.40 (m, 2H), 3.50—3.62 (m, 2H), 3.62—3.70 (m, 2H), 3.70—3.80 (m, 2H), 3.82 (s, 4H), 3.97—4.08 (m, 2H), 5.05—5.15 (m, 1H), 7.04 (d, J=8.4 Hz, 2H), 7.82 (d, J=8.4 Hz, 2H), 9.89 (s, 1H).

5-(4-(6-(2-(2-(2-(*tert*-Butoxycarbonylamino)ethoxy)ethoxy)ethoxy)-2,5,7,8-tetramethylchroman-2-ylmethoxy)phenylmethylene)thiazolidine-2,4-dione (4) A mixture of **3** (40 mg, 0.07 mmol), thiazolidine-2,4-dione (10 mg, 0.084 mmol), benzoic acid (2 mg, 0.016 mmol) and piperidine (1 μL, 0.01 mmol) in toluene (4 mL) was refluxed for 4 h with continuous removal of water using a Dean-Stark water separator. The reaction mixture was cooled to room temperature, and the solvent evaporated. The resulting residue was chromatographed on a silica gel to give the product as a yellow gum (28 mg, 59.6%). ^{1}H NMR ($CDCl_3$) δ: 1.25 (s, 3H), 1.41 (s, 9H), 1.80—1.95 (m, 1H), 2.04 (s, 3H), 2.05—2.10 (m, 1H), 2.14 (s, 3H), 2.17 (s, 3H), 2.60 (brs, 2H), 3.30—3.38 (m, 2H), 3.55—3.65 (m, 2H), 3.65—3.70 (m, 2H), 3.70—3.80 (m, 2H), 3.82 (s, 4H), 3.93—4.08 (m, 2H), 5.10—5.25 (m, 1H), 7.01 (d, J=8.4 Hz, 2H), 7.44 (d, J=8.4 Hz, 2H), 7.78 (s, 1H), 9.69 (s, 1H).

5-(4-(6-(2-(2-(2-(*tert*-Butoxycarbonylamino)ethoxy)-ethoxy)ethoxy)-2,5,7,8-tetramethylchroman-2-ylmethoxy)phenylmethyl)thiazolidine-2,4-dione (5) A suspension of **4** (28 mg, 0.042 mmol) and magnesium turnings (18 mg, 0.75 mmol) in dry MeOH (5 mL) was stirred at 45 ℃ for 8 h. The reaction mixture was cooled to room temperature and Boc_2O (15 mg, 0.086 mmol) was added. The mixture was stirred at room temperature for 2 h and neutralized with 1 mol•L^{-1} HCl and extracted with EtOAc four times. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The crude product was chromatographed over silica gel using $CHCl_3$∶MeOH ($V∶V$=50∶1) as eluent to yield **5** (20 mg, 70.8%). ^{1}H NMR ($CDCl_3$) δ: 1.42 (s, 12H), 1.80—2.00 (m, 2H), 2.06 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.60 (brs, 2H), 3.03—3.11 (m, 0.5H), 3.35—3.40 (m, 2H), 3.40—3.50 (m, 0.5H), 3.55—3.65 (m, 2H), 3.65—3.72 (m, 2.5H), 3.72—3.80 (m, 2H), 3.80 (s, 4H), 3.80—4.00 (m, 3H), 4.40—4.50 (m, 0.5H), 5.05—5.15 (m, 1H), 6.87 (d, J=8.4 Hz, 2H), 7.11 (d, J=8.4 Hz, 2H).

Biotin-TGZ To a stirred solution of **5** (10 mg, 0.015 mmol) in EtOAc (1 mL) was added 2 mol•L^{-1} HCl in EtOAc (2 mL) dropwise at 0 ℃. After being stirred at room temperature for 1 h, the solvent was evaporated under reduced pressure and the resulting residue dissolved in 2 mL of dry DCM, to which *i*-Pr_2NEt (5.2 μL, 0.03 mmol) was added at 0 ℃, followed by the *N*-hydroxylsuccinimide ester of Biotin (6 mg, 0.018 mmol). The mixture was stirred at room temperature for 12 h and concentrated under reduced pressure. Purification by chromatography on a silica gel column eluted with $CHCl_3$∶MeOH ($V∶V$=20∶1) yielded the product Biotin-TGZ as a white solid (7 mg, 58.4%). ^{1}H NMR ($CDCl_3$) δ: 1.20—1.40 (m, 2H), 1.42 (s, 3H), 1.50—1.70 (m, 4H), 1.80—1.95 (m, 2H), 2.00—2.20 (m, 11H), 2.55—2.65 (m, 2H), 2.67—2.73 (m, 1H), 2.85—2.95 (m, 1H), 3.10—3.25 (m, 2H), 3.30—3.40 (m, 1H), 3.40—3.55 (m, 2H), 3.60—3.65 (m, 2H), 3.65—3.80 (m, 4H), 3.80—4.00 (m, 6H), 4.25—4.35 (m, 1H), 4.45—4.55 (m, 2H), 5.80 (brs, 1H), 6.28 (brs, 1H), 6.69 (brs, 1H), 6.85 (d, J=7.8 Hz, 2H), 7.11 (d, J=7.8 Hz, 2H); ESIMS *m/z*: 821.4 $(M+Na)^+$ and 797.4 $(M-H)^-$; HRMS calcd for $C_{40}H_{54}N_4O_9S_2$ 821.3230, found 821.3208.

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