RESEARCH ARTICLE

Preparation and Antitumor Activity of a Tamibarotene-Furoxan Derivative

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Abstract

Multi-target drug design, in which drugs are designed as single molecules to simultaneously modulate multiple physiological targets, is an important strategy in the field of drug discovery. QT-011, a tamibarotene-furoxan derivative, was here prepared and proposed to exert synergistic effects on antileukemia by releasing nitric oxide and tamibarotene. Compared with tamibarotene itself, QT-011 displayed stronger antiproliferative effects on U937 and HL-60 cells and was more effective evaluated in a nude mice U937 xenograft model *in vivo*. In addition, QT-011 could release nitric oxide which might contribute to the antiproliferative activity. Autodocking assays showed that QT-011 fits well with the hydrophobic pocket of retinoic acid receptors. Taken together, these results suggest that QT-011 might be a highly effective derivative of tamibarotene and a potential candidate compound as antileukemia agent.

Keywords: Tamibarotene - NO donor - anti-tumor compound - retinoic acid receptors

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Introduction

Retinoids, which have been successfully used in the control and treatment of cancer, can serve as *in vivo* ligands of retinoic acid receptors (RARs) or retinoid X receptors to exert antitumor effects. The effective response rate of all-trans retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia (APL) is at least 80% (Huang et al., 1988). The 13-cis retinoic acid is used in therapies for squamous cell carcinoma of the cervix and skin (Lippman et al., 1992). Retinoids also exhibit antiproliferative activities against some tumor cell lines of breast cancer (Wu et al., 1997) and lung cancer (Wang et al., 2013). Tamibarotene, a derivative of retinoids, is an effective drug for relapsed or refractory APL. Compared with other retinoid derivatives, tamibarotene exhibits higher differentiation-inducing effects on APL cells and lower drug resistance because of its low affinity to cellular retinoic acid binding protein. However, the clinical application of tamibarotene is limited by its inevitable toxicity and side effects, such as hypertriglyceridemia, hypercholesterolemia, rashes, bone pain, retinoid acid syndrome, and strong teratogenic effect (Miwako et al., 2007). Therefore, the development of novel tamibarotene derivatives with low toxicities and good antitumor activities is crucial. Multi-target therapy, such as combination chemotherapy, is a conventional method with improved efficacy and safety. For example, the combined treatment with RAR agonists (e.g., ATRA) and histone deacetylase inhibitors (e.g., butyrate, valproic acid, SAHA, FK228, MS-275, and TSA) can remarkably improve efficacy, reduce side effects, and decrease retinoid resistance against acute leukemia (e.g., APL) (Warrell, Jr. et al., 1998; Kitamura et al., 2000; Savickiene et al., 2006; Bellos et al., 2008; Li et al., 2013). Thus, conventional multi-target therapy provides an important strategy (i.e., multi-target drug design) in drug design and discovery.

Multi-target drug design, in which drugs are designed as single molecules to simultaneously modulate multiple physiological targets directly or indirectly through metabolites, is an important strategy in the field of drug design and discovery. Through synergic multi-target actions, multi-target drugs might provide significantly enhanced efficacy, reduced side effects, or decreased drug resistance during the treatment of cancers, which involve many pathological pathways. Generally, multi-target drugs are obtained through the conjugated pharmacophore approach, in which two distinct pharmacophores (or drugs) are combined into one molecule by a cleavable or non-cleavable spacer.

In this work, tamibarotene was used as a basic model drug to be coupled with furoxans, which are NO donors that display remarkable antitumor activities (Wang et al., 2002; Cerecetto et al., 2005; Huerta et al., 2008; Hirst et al., 2010), to synthesize a new compound named QT-

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011. QT-011 was proposed to exert synergistic effects at multiple target sites and significantly enhance efficacy by releasing NO and tamibarotene through *in vivo* metabolism. In the present study, we synthesized QT-011 and determined its *in vitro* pharmacodynamic effects on human leukemic cell lines (U937 and HL-60) and human ovarian carcinoma cell lines (ES-2 and 3AO). We also determined the *in vivo* antitumor effect of QT-011 on a U937 xenograft model.

Materials and Methods

General

Tamibarotene was obtained from Beijing Bode United Chemical Co., Ltd. N-tert-butoxy-carbonyl-amino-acetic acid was from Aladdin Industrial Inc. Benzaldehyde, 1- (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and Nhydroxyenzotrizole were obtained from China National Medicines Corporation Ltd.

Commercially available tamibarotene and benzaldehyde were used without further purification. All solvents were dried on 3 Å molecular sieves prior to use.

^{1}H and ^{13}C spectra were recorded in $CDCl_3$ or DMSO-d_6 solution at 600 MHz on a Bruker AVANCE 600 instrument using TMS as internal reference. MS spectra were recorded on a Thermo Fisher LTQ Orbitrap XL instrument using electrospray ionization (ESI).

General procedure for synthesis of compound 1

Compound 1 was prepared according to the literature and the synthetic route was outlined in Scheme 1. In brief, to a stirred solution of benzaldehyde (212.2g, 2.0mol) in aq NaOH (1000mL, 0.5mol/L) containing PEG600 (42g, 0.07mol) 40% acetaldehyde (3.0mL, 330.4g) was added dropwise in 1.0 h. The reaction mixture was kept at 30°C for 4.0 h and then added with 500mL NaCl saturated solution. The organic layer was washed with H2O until pH7 and then collected 130~140°C fraction in 3 kPa to give the relevant compound 1-a (123.3g, yield 48.9%). Compound 1-a (66.1g, 0.50mol) was dissolved in methanol (200mL) and was drop-wised to a stirred solution of sodium borohydride (11.3g, 0.30mol) in tetrahydrofuran (100mL) in 1.0 h. The mixture was refluxed at room temperature for 1.0h, then acetone (20mL) was added and the mixture was stirred for another 0.5 h. The mixture was filtered and the filtrate evaporated in vacuo. The residue was distilled in vacuo and collected the 130~150°C fraction to give relevant compound 1-b (60.5g, yield 90.2%). Compound 1-b (53.7g, 0.40mol) and sodium nitrite (55.2g, 0.80mol) were dissolved in chloroform (500mL) under stirred at room temperature. Glacial acetic acid was added dropwise to the solution in 1.0 h and the mixture was stirred at room temperature for another 1.0h. Then the mixture was filtered and the filtrate was washed with H2O twice and dried over Na2SO4 to give a residue. The residue was purified on Si gel column (ethyl acetate / petroleum ether 1:5) to afford compound *1-c* (48.0g, yield 62.4%). Compound *1-c* (38.4, 0.20mol) and pyridine (32.2mL, 0.40mol) were dissolved in methylene chloride (600mL). After dissolved absolutely at room temperature, the mixture was cooled in ice bath to 0~5°C and sulfoxide chloride (28.4mL, 0.40mol) was added dropwise in 1.0 h. The mixture was stirred at room temperature for 12.0 h and then dumped slowly in 500mL ice water under vigorous stirring. The organic layer was washed in sequence with aq NaCl, aq $NaHCO_3$, aq NaCl and H_2O, dried over Na_2SO_4 and the solvent evaporated in vacuo. The residue was purified on Si gel column [ethyl acetate / petroleum ether (bp 40-60°C), 1:20] and placed in the refrigerator for 24 h to afford compound 1 (32.7g).

Compound 1 was obtained in 74% yield as a yellow solid; mp 54-56°C; ^{1}H NMR ($CDCl_3$) δ 7.78 (m, 2H), 7.58 (m, 3H), 4.58 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 155.96, 131.58, 128.56, 127.57, 125.79, 112.91, 110.01, 77.03, 32.66; ESI-MS (m/z): 211.1 $[M+H]^+$; which was in agreement with those reported in the literature (Boiani et al., 2001).

General procedure for synthesis of compound 2

The synthetic route of compound 2 was outlined in Scheme 2. A solution of compound 1 (2.1g, 10 mmol), N-tert-butoxy-carbonyl-amino-acetic acid (1.8g, 10 mmol) and K_2CO_3 (1.4g, 10 mmol) in N, N-dimethyl formamide was stirred at room temperature for 4.0 h and dumped in 300mL H_2O. The organic layer was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated in vacuo. The residue was dissolved in methylene chloride (60mL) containing trifluoroacetic acid (15mL). The mixture was stirred at room temperature for 1.0 h and then evaporated in vacuo. To the residue, aq NaHCO3 (200mL) and ethyl acetate (80mL) was added. The mixture was stirred and the organic layer washed with aq NaCl. The pooled extracts were dried over Na_2SO_4 and then taken to dryness. The residue was purified on Si gel column [ethyl acetate / petroleum ether (bp 40-60°C), 1:1] to give compound 2 (2.2g).

Compound 2 was obtained in 63% yield as a yellow oil; ^{1}H NMR (DMSO-d_6) δ 7.81 (m, 2H), 7.54 (m, 3H), 5.28 (s, 2H), 4.06 (s, 2H), 1.54 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 167.75, 157.59, 131.99, 129.90, 128.22, 126.05, 112.27, 55.42; ESI-MS (m/z): 250.2 [M+H]+; which was in agreement with those reported in the literature (Boiani et al., 2001).

General procedure for synthesis of QT-011

The synthetic route of QT-011 was outlined in Scheme 3. 1- (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.0g, 5.2mmol) and Nhydroxyenzotrizole (0.7g, 5.2mmol) were added to a solution of tamibarotene (1.0g, 2.8mmol) in tetrahydrofuran (30mL). The mixture was stirred at room temperature for 1.0 h and then the solution of compound 2 (0.7g, 2.8mmol) in tetrahydrofuran (60mL) was added dropwise to the mixture. After stirred for 4.0 h, the mixture was poured out into 400mL H2O. The organic layer was extracted with ethyl acetate, dried over Na_2SO_4 and evaporated in vaco. The residue was purified on Si gel column [ethyl acetate / petroleum ether (bp 40-60°C), 1:1] to afford QT-011 (1.2g).

QT-011 was obtained in 74% yield as a white powder; ^{1}H NMR (DMSO-d_6) δ 10.24 (s, ^{1}H), 9.21 (s, ^{1}H), 8.08~7.58 (m, 11H), 7.30-7.29 (m, 1H), 5.24 (s, 2H), 4.11 (s, 2H), 1.64 (s, 4H); 1.26~1.24 (m, 12H); ^{13}C NMR (DMSO-d_6) δ 169.76, 166.57, 164.93, 157.60, 145.04,

140.52, 138.14, 136.26, 131.92, 129.86, 128.17, 127.76, 126.90, 126.15, 118.86, 118.67, 112.45, 54.95, 41.79, 35.14, 35.09, 34.46, 34.02, 32.13, 32.10; ESI-MS (m/z): 599.2 $[M+Na]^+$.

Cell culture

human leukemic monocyte lymphoma cell line U937, Human promyelocytic leukemia cells HL-60, human ovarian carcinoma cells ES-2 and 3AO were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS). These cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assays

1×10^4 per well of U937, HL-60 cells or 5000 per well of ES-2, 3AO cells were seeded in 96-well plates and allowed to grow for 4h, and the compounds were added at various concentration. Two days after treatment, cells in each well were treated with 1% of 0.5mg/mL MTT reagent and incubated for an additional 4 h. After that, the culture was removed and 100 μL DMSO was added. Absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay reader (M5; MD), and absorbance at 630 nm was used as a reference. The inhibition rate of compounds was calculated by (ODcontrol-ODtested)/ ODcontrol×100%, where OD is the mean value of three replicate wells. The IC_{50} values were determined using ORIGIN 7.5 software.

The NO-releasing assay

The NO-releasing assay was determined by Griess test (Wang et al., 2002), and NO kit (Cat. A012) was purchased from Nanjing Jiancheng Bioengineering Institute. Briefly, compound was incubated in PBS solution with a large excess of L-cysteine for 8h at 37°C. Then the supernant was used to determine the NO-releasing according to the procedure of NO kit.

In vivo anti-tumor assay against U937 xenograft

In vivo anti-tumor efficacy study was performed using nude mice xenagraft model as described previously. Briefly, $2*10^7$ U937 cells were inoculated subcutaneously in the right shoulder of female athymic nude mice (4-5 weeks old, Slac Laboratory Animal, Shanghai). Six days after injection, tumors were palpable and mice were randomized into treatment and control groups (5 mice per group). The treatment groups were administrated with 100=mg/kg/d intragastricly, the control group was administrated with equal volume of PBS solution. During treatment, subcutaneous tumors were measured with a vernier caliper every three days, and body weight was monitored regularly. Tumor volumes (V) were estimated using the equation ($V=ab^2/2$, where a and b stand for the longest and shortest diameter, respectively). After 18 days of administration, the mice were executed and the tumor weight was measured by an electronic balance. This work was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

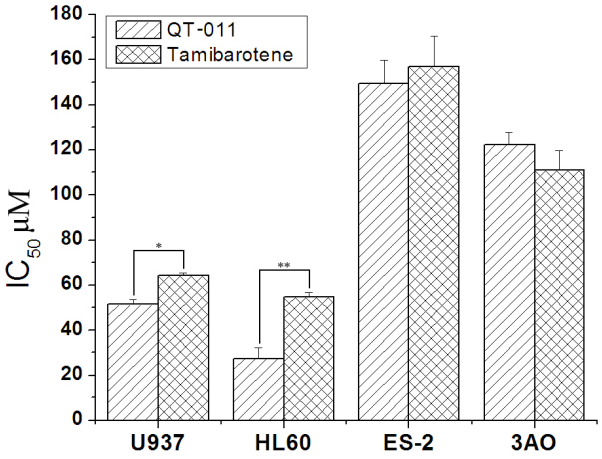

Figure 1. Antiproliferative Activities of QT-011 and Tamibarotene Against Human Tumor Cell Lines as Measured Using the MTT Assay. Values are presented as the mean of three independent experiments±SD. *p<0.05, **p<0.01

Autodock of Binding model between RARs and QT-011

Autodock, as one of the most widely used programs, has been applied with great success in the predicting of bound conformations of protein-ligand complexes. For the purpose of exploring the binding model between RARs and QT-011, docking approach was carried out using the Autodock 4.0 program. Automatic receptor preparation was performed, including merging of nonpolar hydrogens, and solvent molecules were removed. The corresponding residues which constituted the hydrophobic pocket (Ile112, Leu268, Phe230, Trp227, Phe304, Leu307, Leu416 and Ala397) were identified as flexible. The applied scoring grid had 0.375A grid spacing , which dimensions of 50×50×50 (x, y and z), centered on the hydrophobic pocket, to ensure the treatment of all possible binding poses. Default docking parameters were used. The docking conformations of QT-011 were clustered on the basis of RMSD and ranked on the basis of free energy of binding. The top-ranked conformations were visually analyzed.

Statistical Analysis

Data are expressed as mean ±SE. Mann-Whitney t test was used to determine whether two experimental values were significantly different.

Results

The synthesis of QT-011

To synthesize tamibarotene, tamibarotene-NO-donor derivatives were first prepared by multi-step reactions, with benzaldehyde as the starting material. The furoxan NO donor 3- (hydroxymethyl)-4-phenyl-1, 2, 5-oxadiazole-2-oxide was obtained through Claisen-Schmidt condensation reaction, NaBH4 reduction, and nitrosation reaction from benzaldehyde, followed by chlorination, etherification, hydrolysis, and esterification reactions to produce various key intermediates. The produced intermediates were reacted with tamibarotene to produce QT-011. The synthetic routes are outlined in Schemes 1 to 3. And supplementary materials are the NMR data of compound 1, 2 and QT-011.

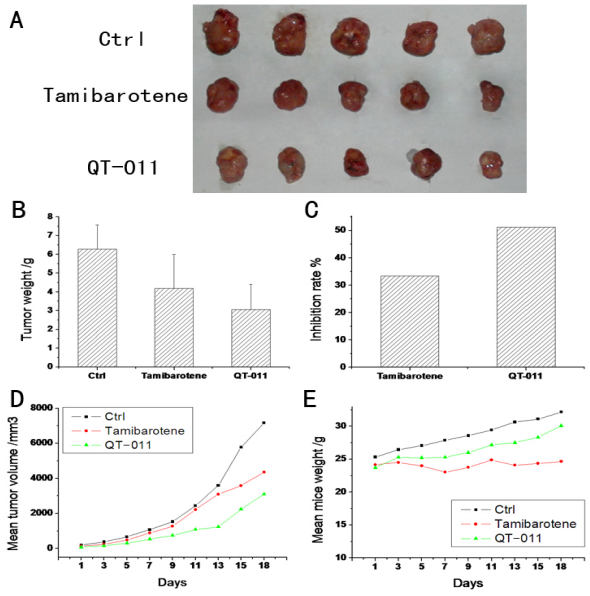

Figure 2. Antitumor Activity Against U937 Human Tumor Xenografts Implanted in Nude Mice. The mice were executed after 18 d of intragastric administration, and the tumors were weighed. The tumor volumes and body weights were measured every 3 d. **A)** Dissected tumor tissues. **B)** Tumor-weight plot. **C)** Inhibitory-ratio plot. D: Tumor-volume plot. **E)** Nude-mice-body-weight plot

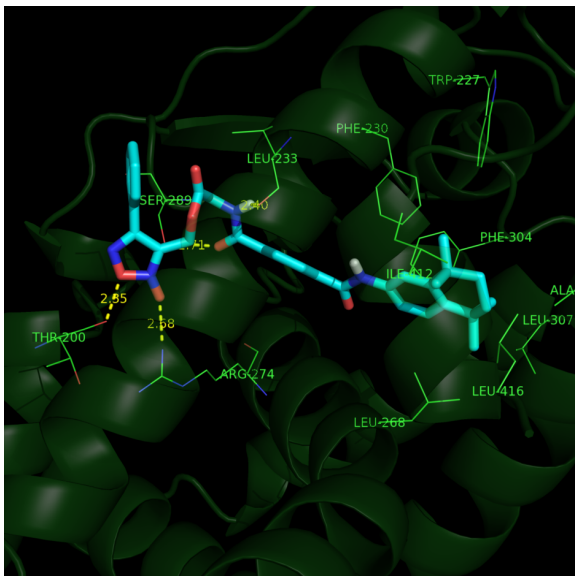

Figure 3. Binding Model Between RARs and QT-011 Obtained Using the Autodock Approach. The docked QT-011 is represented by sticks (carbon atoms in cyan, oxygen atoms in blue, and nitrogen atoms in blue), the protein is represented by ribbons, the residues are represented by lines (carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue), and the H-bonds are represented by yellow dashed lines

The activity evaluation of QT-011

The antiproliferative activity of QT-011 against U937, HL-60, ES-2, and 3AO cells were evaluated using the MTT assay (Figure 1). Results showed that QT-011 and tamibarotene exhibited stronger antiproliferative activity against the leukemic cell lines U937 and HL-60 than the ovarian carcinoma cell lines ES-2 and 3AO. Compared with the positive control tamibarotene, QT-011 displayed stronger antiproliferative effects on U937, HL-60, and ES-2 cells, particularly on HL-60 cells.

A nude-mice xenograft model was used to evaluate the antitumor activity of QT-011 *in vivo*. The mice were executed after 18 d of intragastric administration, and the tumors were weighed. The tumor weights and volume data indicated that QT-011 was more active than tamibarotene (Figure. 2A to 2D). The lack of body weight loss observed in the mice revealed that all of the compounds were safe (Figure 2E). No evident toxicity was detected in the livers and spleens (data not shown).

The release of NO from QT-011

The percentage of NO released *in vitro* from the compound was determined using Griess test. The reduced thiol groups from endogenous L-cysteine, glutathione, or proteins could mediate the release of NO from furoxan derivatives. QT-011 exhibited poor dissolubility. QT-011 was insoluble in an 8% (v/v) DMSO solution, with an NO release percentage of 0.187±0.0193%, but was soluble in a 66.67% (v/v) DMSO solution, with an NO release percentage of 4.16±0.21%. Tamibarotene can't release NO.

Docking of QT-011 with RAR

Docking results are shown in Figure 3. The tetrahydronaphthalene derivative group of QT-011 acted as the hydrophobic region of the molecule, which fit well with the hydrophobic pocket of the RARs (Ile112, Leu268, Phe230, Trp227, Phe304, Leu307, Leu416, and Ala397). These results are consistent with the study of Yasuyuki (Endo et al., 2001). In addition, three hydrogen bonds were observed between the remainder of the QT-011 and the Thr200, Arg274, and Ser289 of RARs.

Discussion

Recently, multi-target drugs, such as nitric oxide (NO) donor hybrids are designed as single molecules to modulate multiple physiological targets simultaneously. Konstantin et al. reported that conjugation of doxorubicin with NO donor inducd high cytotoxicity in doxorubicin-resistant human colon cancer cells (Konstantin et al., 2011). NO-releasing derivatives of tetrahydroisoquinoline had anticancer effects, as well as multidrug resistance reversal effects (Zou et al., 2011). As an important class of NO donor, furoxans have been found to possess a NO-related biological activity, antileukemic activity, and have been expected as promising lead compounds to develop novel antileukemic agents (Bian et al., 2011).

In this study, we synthesized QT-011, the tamibarotene-furoxan derivative. The antiproliferative activity in vitro and in vivo of QT-011 was evaluated. The compound exhibited stronger antiproliferative activity against the leukemic cell lines U937 and HL-60 than the ovarian carcinoma cell lines ES-2 and 3AO (Figure 1). In nude mice U937 xenograft model, QT-011 was more active than tamibarotene (Figure 2). And there was no evident toxicity. QT-011 was hydrophobic. Therefore it can enter the cells easily, and released significant levels of NO. This data also suggested that the antiproliferative activity of QT-

011 might partly come from NO released from NO donor.

In conclusion, we prepared a double-target antitumor drug (QT-011) and compared its in vitro and in vivo preliminary antitumor activity with tamibarotene. Compared with tamibarotene, QT-011 displayed stronger antiproliferative effects and was more active against tumor growth. Autodock showed that QT-011 fit well with the hydrophobic pocket of the RARs. QT-011 was proven to be a potential antitumor drug. Further studies should be conducted to elucidate the drug's mechanism of action.

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There are no conflicts of interest to disclose.

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