Targeting thioredoxin reductase by ibrutinib promotes apoptosis of SMMC-7721 cells

Xiao Han, Junmin Zhang, Danfeng Shi, Yueting Wu, Ruijuan Liu, Tianyu Liu, Jianqiang Xu, Xiaojun Yao and Jianguo Fang

State Key Laboratory of Applied Organic Chemistry and College of Chemistry and Chemical Engineering (X. H., D. S., Y. W., T. L., X. Y., J. F.), School of Pharmacy (J. Z., R. L.), Lanzhou University, Lanzhou 730000, China.

School of Life Science and Medicine & Panjin Industrial Technology Institute (J. X.), Dalian University of Technology, Panjin Campus, Panjin 124221, China.
Running title: TrxR as a new target of ibrutinib

Corresponding author:

Prof. Jianguo Fang
Address: State Key Laboratory of Applied Organic Chemistry and College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China
Tel: +86 931 8912500;
Fax: +86 931 8915557.
E-mail: fangjg@lzu.edu.cn

Dr. Junmin Zhang
Address: School of Pharmacy, Lanzhou University, Lanzhou 730000, China
Tel: +86 13669305351;
Fax: +86 931 8915685.
E-mail: zhangjunmin@lzu.edu.cn

Number of Text Pages: 55
Number of Figures: 9
Number of References: 66
Number of Words in Abstract: 106
Number of Words in Introduction: 538
Number of Words in Discussion: 1274
**Abbreviation:** ABCB1, ATP-Binding Cassette Subfamily B member 1; Ac-DEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide; Akt, v-akt murine thymoma viral oncogene homolog; ARE, antioxidant response element; BSO, L-buthionine-(S, R)-sulfoximine; BTK, Bruton tyrosine kinase; CLL, chronic lymphocytic leukemia; DCFH-DA, 2',7'-dichlorfluoresceindiacetate; DTNB, 5,5'-Dithiobis-(2-nitrobenzoic acid); ErbB, human epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IBT, ibrutinib; Keap1, Kelch-like ECH-associated protein 1; NAC, N-acetyl-L-cysteine; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PMSF, phenylmethylsulfonyl fluoride; SecTRAPs, selenium-compromised thioredoxin reductase-derived apoptotic proteins; Trx, thioredoxin; TrxR, thioredoxin reductase.

**Recommended Section Assignment:**

Cellular and Molecular, Chemotherapy
Abstract

Ibrutinib (IBT), the first-in-class inhibitor of Bruton’s tyrosine kinase (BTK), has demonstrated clinical activity against various B-cell malignancies. Aside from its therapeutic mechanism through BTK inhibition, IBT has other target sites reported for cancer therapy, leading us to investigate whether IBT has unreported targets. Our study revealed that IBT can inhibit SMMC-7721 cells through irreversible inhibition of mammalian thioredoxin reductase enzymes. Further study demonstrated that IBT can cause cellular reactive oxygen species elevation and induce cancer cells apoptosis. The discovery of a new target of IBT sheds light on better understanding its anticancer mechanisms and provides a theoretical foundation for its further use in clinical therapy.
Introduction

Liver cancer has become a serious threat to the health of human. It was called “The king of cancer” because of the properties such as diagnostic difficulties, rapid development, high malignancy, easy metastasis and high mortality etc. Liver cancer was ranked by WHO the third highest mortality cancer in 2018 (Bray et al., 2018). The treatment of liver cancer has a poor prognosis with of only 6-12 months median survival, and the recurrence rate was as high as 19%-25% in the first year (Torzilli et al., 2013). Due to the properties mentioned above, clinical treatments for liver cancer haven’t made remarkable progress. However chemotherapy was still the main clinical method for liver cancer therapy; systemic chemotherapy can obviously extend patients’ lives, especially the patients in advanced stage and has metastasized. Hence, finding a low-toxicity and high-efficiency drug has become the top priority.

Ibrutinib (IBT) (Fig. 1), also named PCI-32765, is a first-in-class inhibitor of BTK. It was initially modified from the Src family kinase selected inhibitor PP1 by Arnold and Pan to treat rheumatoid arthritis (Arnold et al., 2000). IBT was the second approval through the U.S. Food and Drug Administration Breakthrough Therapy Designation Pathway; it was also approved by European Medicines Agency to treat mantle cell lymphomas and chronic lymphocytic leukemia. Currently, numerous phase I and phase II clinical studies have demonstrated that IBT can significantly reduce the tumor burden and slow the progression of various lymphomas (Harrison, 2012; Byrd et al., 2013). It has an overall response rate of 70% in both relapsed/refractory and newly-diagnosed chronic lymphocytic leukemia (CLL) patients (Herman et al., 2011). It has been reported that the therapeutic effect for B-cell
malignancies was through its irreversible inhibition for BTK (Byrd et al., 2013), further leading to blocking the expression of downstream of BCR including NF-κB, v-akt murine thymoma viral oncogene homolog (Akt) and extracellular signal-regulated kinase (ERK) etc. (Herman et al., 2011; Chong et al., 2018). It has been reported that IBT could also inhibit ErbB receptor phosphorylation and stimulate the ATPase activity of ATP- Binding Cassette Subfamily B member 1 (ABCB1) (Grabinski and Ewald, 2014; Zhang et al., 2017a), and have therapeutic effects on glioblastoma, breast cancer, and gastric cancer etc. These reports make us consider whether IBT possess other molecular targets for cancer therapy.

TrxR is a component of the thioredoxin (Trx) system, also contains Trx and NADPH, which is one of the most important systems to balance the redox state. Not only can Trx scavenge ROS, it also has an abroad substrate such as ASK1 caspase3 P53 and NF-κB in vivo (Zhang et al., 2017b). As the main electron receptor of TrxR, the Trx activity is directly depended on the activity of TrxR. It has been reported that TrxR was highly expressed in most of cancer cells (Arner and Holmgren, 2006). High expression of TrxR can help to scavenge ROS and resist cell death. And various recent literatures have reported that irreversible inhibition of TrxR could induce cancer cells apoptosis (Stafford et al., 2018; Zhuge et al., 2018; Zhang et al., 2019). Our studies have provided preliminary evidence that IBT could target mammalian TrxR for cancer therapy, which will give us more viewpoints to understand the cancer therapy mechanism.
Materials and methods

Materials

IBT (440.50 MW Da, 99% purity) was purchased from Easybio (Shanghai, China), a 100 mM IBT in DMSO stored at -20 °C as a stock solution. Dulbecco's modified Eagle’s medium (DMEM), dithiothreitol (DTT), bovine insulin, yeast glutathione reductase (GR), reduced glutathione (GSH), oxidized glutathione (GSSG), hydrogen peroxide (H₂O₂), N-acetyl-L-cysteine (NAC), 2’, 7’-dichlorofluoresceindiacetate (DCFH-DA), L-buthionine-(S, R)-sulfoximine (BSO), 2-vinylpyridine, 3-(3-Cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS), Hoechst 33342 and N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-actin antibody (AA128-1), Trypan blue, bovine phenylmethylsulfonyl fluoride (PMSF) and sodium orthovanadate (V) (Na₃VO₄) and serum albumin (BSA) were purchased from Beyotime (Nantong, China). 5’, 5’-dithiobis-2-nitrobenzoic acid (DTNB) was obtained from J&K Scientific (Beijing, China). Poly-vinylidene difluoride (PVDF) membrane and NADPH were obtained from Millipore (Billerica, MA) and Roche (Mannheim, Germany). The Annexin V (Annexin V-FITC)/propidium iodide (PI) double stain apoptosis kit were obtained from Zoman Biotech (Beijing, China). The Western Blotting chemiluminescence kit purchased from GE Healthcare Life Science. The cell cycle kit (BB-4104) purchased from shanghai BestBio Science. Streptomycin, Penicillin and 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Amresco (Solon, OH). Anti-rabbit IgG-HRP (sc-2004), anti-mouse IgG-HRP (sc-2031) and TrxR1 primary antibody (sc-28321) were products of Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS) was
product of Sijiqing (Hangzhou, China). All others reagents were analytical reagents.

Cell cultures

SMMC-7721, HepG2, HeLa, A549, and HEK 293T cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. DMEM was supplemented with 100 units/ml penicillin/streptomycin, 2 mM glutamine, and 10% FBS with 5% CO$_2$ to culture the cells at 37 °C. HeLa-shNT and HeLa-shTrxR1 cells stably transfected with shNT plasmid and shTrxR1 plasmid which were generated in our lab (Duan et al., 2014) were incubated with the same conditions of the above cells with an additional supplement of 1 μg/ml puromycin in DMEM.

Trypan blue exclusion assay

The indicated concentrations of IBT was used to culture 4×10$^4$ SMMC-7721 cells at 37 °C in 24-well plates at for 48 h, and cells in control group were treated with 0.1% DMSO alone for 48 h. After incubated with 48 h, 0.4% trypan blue was used to trypsinize and incubate the cells. Dead cells were stained while live cells were non-stained, cell viability was determined as: (Cell viability) % = living cells / (control living cells) × 100.

MTT assay

Cells (8×10$^3$) were cultured with above concentrations of IBT in a 96-well plate in triplicate at 37 °C for indicated times until the final volume was 100 μl. The same cells incubated with 0.1% DMSO alone was used as control, the blank group was incubated with
0.1% DMSO alone without cells. Then adding 10 μl 5 mg/ml MTT to the DMEM for 4 h incubation. After adding 100 μl extraction buffer (0.1% HCl, 5% isobutanol and 10% SDS), the cells were further incubated at 37 °C for 8 h. Microplate reader (Thermo Scientific Multiskan GO, Finland) was used to assay the absorbance at 570 nm. The viability of cells was calculated as: (Cell viability) % = (A_{IBT} − A_{Blank}) / (A_{Control} − A_{Blank}) × 100.

**Cell cycle assay**

After seeded in 6-well plate, 5×10^5 SMMC-7721 cells were incubated overnight at 37 °C, and then cultured with indicated concentrations of IBT for 24 h. The cells washed with PBS were trypsinized. They were suspended with iced PBS and fixed with iced ethanol with a final concentration of 70%. Then cells were harvested in -20 °C for more than 4 h. PBS was used to wash the cells twice, which were incubated with 400 μl of PI solution. The samples were finally analyzed using FACSCanto™ flow cytometer (BD Biosciences, USA).

**Wound healing assay**

SMMC-7721 cells (1×10^6) seeded in 6-well plate were incubated at 37 °C for 24 h. The even scratches were scratched by the 10 μl pipette tips. After aseptic PBS was used to wash the plate twice, the cells were cultured with the indicated concentrations of IBT in 1% FBS medium. Before and after 24 h incubation, the bright field images were obtained by Floid Cell Imaging Station (Life Technologies, USA).

**Molecular docking simulation**
A covalent docking by Schrödinger Software Suite (Schrödinger, LLC: New York, NY, 2015) was carried out to reveal the probable interaction between compound IBT and TrxR1. A crystal structure of rat TrxR1 with Sec\textsubscript{498} not mutant (PDB accession number: 3EAN, Chain A and B) (Cheng et al., 2009) were downloaded from the Protein Data Bank. To investigate the interaction, the structure was prepared by the Protein Preparation Wizard module. The active site to reacting with IBT was the residue Sec\textsubscript{498}, which was set to be centroid of the binding site. The docking simulation was implemented by using the default parameters.

**Cell lysis**

Cells (2\times 10^6) were seeded in 100 mm diameter plastic Petri dish to grow overnight at 37°C. Afterwards, they were cultured with indicated concentrations of IBT for 24 h and washed with fresh PBS. Store them at -20°C for further study if necessary. RIPA buffer (50 mM Tris-HCl pH 7.5, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM PMSF, 1% Triton X-100, 150 mM NaCl, 0.1% SDS, 2 mM EDTA and 0.5% deoxycholate) was employed to lyse the samples for 30 minutes on ice, the homogenate was centrifuged (Thermo Scientific Sorvall Legend Micro21R Centrifuge) at 13,000 rpm for 10 min to remove cellular debris. The cell suspension was collected, and the Bradford procedure was used to measure the protein quantification. Samples were stored at -20°C for further study if necessary.

**Endpoint insulin reduction assay**

The TrxR activity of cells was determined using the endpoint insulin reduction assay which has been reported in our published methods (Duan et al., 2014). The cells were
extracted and measured as mentioned above. 100 mM Tris-HCl buffer (pH 7.6) was added with the samples which contained 20 μg total proteins to a final volume of 50 μl (containing 3 mM EDTA, 660 μM NADPH, 15 μM *E. coli* Trx and 0.3 mM insulin,) for 30 min at 37 °C. Then adding 200 μl 6 M guanidine hydrochloride (pH 8.0 including 1 mM DTNB) to the systems terminate the reaction. The blank group, containing everything but Trx, was incubated in the same way with experienced groups. The TrxR activity was calculated by the absorbance at 412 nm as: (TrxR activity) % = (A_{IBT} - A_{Blank}) / (A_{Control} - A_{Blank}) × 100.

**Fluorescence microscopy imaging by TRFS-green**

The fluorogenic probe TRFS-green was reported by our lab to selectively determined intracellular TrxR activity (Zhang et al., 2014). SMMC-7721 cells (5×10^5) were cultured in 12-well plate overnight at 37 °C, and then they were cultured with indicated concentrations of IBT for 20 h. The cell culture medium was added with 1 μl 10 mM TRFS-green for 4 h. Floid Cell Imaging Station obtained the images.

**Gpx activity assay**

The samples were extracted and measured as mentioned above. Samples contained 20 μg protein was added to 65 μl TE buffer (0.2 mg/ml BSA). Then 2 μl 50 mM NADPH (or TE buffer for control group), 20 μl 10 mM GSH and 10 μl 40 U/ml GR was added to the samples. After 100 μl 1 mM H_2O_2 was added to the mixture, the absorbance at 340 nm was immediately read every 10 s for 5 min. The Gpx activity was reflected by the rate of increase in absorbance of 340 nm. The Gpx activity was calculated as: (Gpx activity) % = (R_{IBT} -
R_{Blank} / (R_{Control} - R_{Blank}) \times 100 \text{ (Zheng et al., 2017).}

**GR activity assay**

The samples were extracted and measured as mentioned above. Samples contained 30 μg protein was added to 95 μl TE buffer (0.2 mg/ml BSA) for 30 minutes at 37 ℃. Then 2 μl 50 mM NADPH or TE buffer was added to the mixture. As soon as 100 μl 10 mM GSSG was added to the groups, the absorbance at 340 nm was immediately read every 30 s for 10 min. The GR activity was reflected by the rate of increase in absorbance of 340 nm (Zhang et al., 2018).

**Western blot analysis**

The expressions of TrxR1 in control and experienced group were detected through western blot analysis. The cells were extracted and measured as mentioned above. Each sample contains equal quantity of protein and was heated at 100 ℃ for 10 minutes to denature. 12% SDS-PAGE was used to separate 50 mg protein in the presence of DTT (100 mM). Next the protein was transferred to PVDF membranes. 5% non-fat milk was resolved in TBST (10 mM Tris-HCl pH 7.5 containing 0.1% Tween-20 and 150 mM NaCl) to block the PVDF membranes at 25 ℃ for 2 h. 5% non-fat milk containing 0.1% (v/v) specific primary antibodies was used to incubate the PVDF membranes at 4 ℃ for 8 h. The PVDF membranes was washed with TBST for three times and incubated with the 0.025% secondary antibodies for 1 h at 25 ℃. Finally, TBST was used to wash the PVDF membranes for 10 min three times. The immunoreactive bands were obtained by an enhanced chemiluminescence kit.
**Measurement of intracellular ROS levels**

The cellular ROS levels caused by IBT were determined by fluorescence microscopy imaging. SMMC-7721 cells (5×10^5) were cultured in 12-well plate overnight at 37 °C and cultured with indicated concentrations of IBT for 1 h. After the medium was removed, the plate was washed with PBS three times and replace with FBS-free medium. Each well was added with 1 μl 10 mM ROS probe DCFH-DA, which was incubated for another 30 min in dark. The images were obtained by the Leica inverted fluorescence microscope.

**Assessment of intracellular thiols**

Total cellular thiol content was measured via DTNB titration assay. The cells were extracted and measured as mentioned above. Cell lysate (10 μl) was mixed with 90 μl 6 M guanidine hydrochloride (pH 8.0 containing 1 mM DTNB) to 96-well plate. After incubating at 25 °C for 5 min, the absorbance at 412 nm was assayed by microplate reader. The total thiol levels were obtained by the calibration curve generated from several standards of GSH.

**Measurement of cellular glutathione**

Enzymatic recycling assay (Rahman et al., 2006; Zhang et al., 2018) was used to measure the cellular GSH and GSSG. The cells were cultured and harvested as mentioned above, and were extracted by precooling extraction buffer (5 mM EDTA, 0.6% sulfosalicylic acid Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.5). After the iced buffer was sonicated for 30 s twice, the homogenate was centrifuged at 3000 g for 5 minutes at 4 °C, so
that the cellular debris could be removed. The supernatant was collected as soon as possible, and the Bradford procedure was carried out to measure the protein quantification. To determine the total GSH level, 20 μl of sample was added to a 120 μl KPE solution which contained 1.66 units/ml GR and 0.83 mM DTNB. The buffer was added with 60 μl 0.66 mg/ml NADPH, and the absorbance at 412 nm was read at the interval of 15 s for 3 minutes. The total levels of GSH were linearly dependent the rates of increase of the absorbance. The total levels of GSH were calculated by the calibration curve which uses several concentrations of GSH as standard. To determine the GSSG level, adding 2 μl of 2-vinylpyridine to 100 μl cell lysis and vortexing as soon as the supernatant was collected, and then the mixture was incubated at 25 °C for 1 h. The reaction system was added with 6 μl of triethanolamine, which was incubated with the samples for 10 min at 25 °C. Levels of GSSG were calculated as mentioned above for GSH.

**Determination of caspase 3 activity**

Cells were extracted and measured as mentioned above. Lysis samples containing 50 μg total proteins were added to 50 mM HEPES (containing 0.2 mM Ac-DEVD-pNA, 10 mM DTT, 5% glycerol, 2 mM EDTA and 0.1% CHAPS, pH 7.5) to make the final volume to be 100 μl. The samples were incubated for 2 h at 37 °C. The blank group contained everything except the sample. The absorbance at 400 nm was read by microplate reader. The activity was calculated as: (Caspase 3 activity) % = (A_{IBT} − A_{Blank}) / (A_{Control} − A_{Blank}) × 100.

**Hoechst 33342 staining**
After seeded in 12-well plate overnight at 37 °C, SMMC-7721 cells (5x10^5) were cultured with indicated concentrations of IBT for 48 h. After adding 1 μl 5 mg/ml Hoechst 33342 to the DMEM, the mixture was incubated for 15 minutes at 37 °C. The images were obtained by the Leica inverted fluorescence microscope.

**Flow cytometry analysis**

Cell apoptosis was assayed using Annexin V-FITC/PI double staining kit. After seeded in 12-well plate overnight at 37 °C, SMMC-7721 cells (5x10^5) were cultured with indicated concentrations of IBT. Then the cells were trypsinized and washed by fresh PBS, next the original DMEM was added to the 12-well to inhibit the trypsinization. PBS was used to wash the cells again, which was resuspended by 500 μl binding buffer. Finally the samples were incubated with 5 μl Annexin V-FITC and 10 μl PI at 25 °C for 30 min avoiding light. Finally the samples were analyzed by FACSCanto™ flow cytometer.
Results

Inhibiting the growth of SMMC-7721 cells by IBT

To evaluate the therapeutic efficacy, we determined the cytotoxicity of IBT against several cancer cell lines (SMMC-7721, HepG2, HeLa and A549) by MTT assay. Amongst those cell lines, IBT exhibited the highest cytotoxicity towards the human hepatoma SMMC-7721 cells with the IC\textsubscript{50} value lower than 10 μM after 48 h treatment, while the IC\textsubscript{50} from other cancer cell lines were all above 20 μM (Fig. 2A). Our study also showed the time- and dose-dependency of IBT cytotoxicity to SMMC-7721 cells (Fig. 2B). In our work, trypan blue exclusion assay results (Fig. 2C) were confirmed with fairly consistent results using MTT assay. While IBT displayed a low cytotoxicity towards the normal HEK 293T cell line (Fig. 2D), which indicated its feasibility for cancer therapy.

Further studies demonstrated that IBT can arrest the cell proliferation and migration. The cell cycle result was analyzed by flow cytometry. It can be seen from Fig. 2E, SMMC-7721 cells were arrested at the G\textsubscript{0}/G\textsubscript{1} phase by IBT. The effects of IBT on cell migration were examined \textit{via} the wound healing assay. As illustrated in Fig. 2F, the scratch area was obviously wider in the IBT-treated group after 24 h incubation, which revealed inhibition of SMMC-7721 cell migration by IBT. Our results conclusively demonstrated that IBT caused significant inhibition against SMMC-7721 cells.

Molecular docking simulation

To investigate the potential mechanism of IBT cytotoxicity, we analyzed its chemical structure first to glean some preliminary insights. We found that IBT has an α, β-unsaturated
ketone reactive group which has been demonstrated to be an acceptable recognition site of the mammalian TrxR inhibitor. The approved structure, also including naphthoquinone, α-methylene-γ-lactone moiety and α, β-γ, δ-unsaturated lactone moiety, can inhibit TrxR activity due to its electrophilic propensity towards the nucleophilic groups of TrxR (Cai et al., 2012; Zhang et al., 2019).

To verify whether IBT could be an irreversible inhibitor of TrxR, we adopted a molecular docking approach to simulate the possible binding mode of IBT interactions with mammalian TrxR1. As shown in Fig. 3A, IBT mainly bound to the pocket of TrxR1 with good complementarity, and the α, β-unsaturated carbonyl group of IBT formed a covalent bond with the selenol of Sec498 by Michael addition. The surface presentation indicated good complementarity between the pocket and the structural scaffold of IBT (Fig. 3B). As illustrated in Fig. 3C, the binding pocket of IBT was formed by Phe406, Cys475, Ile478, Thr481, Ser483, Val484, Gln494, Gly496, Cys497 and Sec498, and a hydrogen bond is formed between the amino group of IBT and the carboxyl group of Thr481. The docking score of the nonbonding interaction is -5.38 kcal/mol. All these results present a clear view that IBT can irreversibly interact with mammalian TrxR1 and connect a covalent bond with Sec498 of TrxR1. Moreover the nonbonding interactions between the residues around Sec498 tend to form a suitable binding site for IBT.

Inhibition of cellular TrxR activity

The above results demonstrated that IBT could be an irreversible inhibitor of mammalian TrxR. To extend this discovery, we adopted classic Trx-mediated insulin reduction assay to
investigate it. SMMC-7721 cells were selected to research the physiological function between IBT and TrxR. The control group was treated with the maximum DMSO content of the experimental groups without IBT. It can be seen from Fig. 4A, IBT up-regulated the expression of cellular TrxR1, which was suspected to be caused by activation of the Keap1-Nrf2-ARE pathway (Kumar et al., 2011; Wilson et al., 2013), while the decrease of TrxR1 expression of the 40 μM group was suspected to be caused by over-cytotoxicity-induced inhibition of gene expression (Lopez-Alonso et al., 2013). It can be seen from Fig. 4B, incubation of SMMC-7721 cells with IBT caused a significant decrease of cellular TrxR activity in a dose-dependent manner. Only approximately 25% TrxR activity remained when treated with 40 μM IBT. To further confirm the insult of cellular TrxR by IBT, we employed a specific TrxR indicator, TRFS-green (Zhang et al., 2014), to measure the intracellular TrxR activity in SMMC-7721 cells. The results showed the fluorescence of SMMC-7721 cells, which was correlated with cellular TrxR activity, decreased dose-dependently (Fig. 4C). Despite increased expression of cellular TrxR1, total cellular TrxR activity significantly decreased, which indicated that IBT inhibited enzymatic activity through its direct inhibition to TrxR. As shown in Fig. 4D & E, IBT had a faint inhibition to GR and Gpx compared to the significant inhibition of TrxR, which indicated that IBT might selectively inhibit the activity of TrxR in our experimental condition.

Inhibition of TrxR contributes to the cytotoxicity of IBT

To confirm whether cytotoxicity to SMMC-7721 cells by IBT was associated with the inhibition of cellular TrxR activity, we investigated the cytotoxic effects in the TrxR inhibition
situation. *ShTrxR1* vectors, which can specifically target the TrxR1 gene and knock down the TrxR1 expression, were transfected into HeLa cells to generate HeLa-*shTrxR1* cells, while HeLa-*shNT* cells were transfected with *shNT* vectors. *ShTrxR1* and *shNT* vectors were obtained from Professor Constantinos Koumenis from the University of Pennsylvania School of Medicine (Javvadi et al., 2010). As shown in Fig. 5A & B, *shTrxR1* vectors significantly decreased the expression of TrxR1 and the activity of TrxR. Treatment of HeLa-*shNT* cells and HeLa-*shTrxR1* cells with the same concentration of IBT caused different cytotoxicity; IBT exhibited higher potency towards HeLa-*shTrxR1* cells than HeLa-*shNT* cells (Fig. 5C). Pharmacological inhibition of TrxR also obtained the constant results. Pretreatment with auranofin (AF) for 4 h significantly reduced the TrxR activity (Fig. 5D), and the pretreatment group was more sensitive to IBT-induced cell death (Fig. 5E). All the above results indicated that the cytotoxicity of IBT towards SMMC-7721 cells was related to TrxR inhibition.

**Induction of ROS by IBT**

The Trx system is one of the most important *in vivo* systems to scavenge ROS generated during cell metabolism and balance intracellular redox homeostasis. TrxR inhibitors, such as securinine (Zhang et al., 2017c) and alantolactone (Zhang et al., 2016), have been reported to interfere with TrxR to induce the accumulation of oxidized Trx, which subsequently reduced the burst of ROS. These compounds can also react with TrxR to generate modified TrxR, which can transfer electrons from NADPH to molecular oxygen and produce superoxide anions (Fang et al., 2005). Thus, we examined whether IBT has an ROS-generating effect in SMMC-7721 cells.
We first employed the commercial probe DCFH-DA to detect the overall ROS levels. The membrane-permeable dye DCFH-DA can be deacetylated to DCFH, which is membrane impermeable and nonfluorescent, by intracellular esterase. DCFH can be subsequently converted to fluorescent 2’, 7’-dichlorofluorescein (DCF) by intracellular ROS, and the cellular ROS content can therefore be reflected by the intensity of fluorescence. As shown in Fig. 6A, the fluorescence intensity increased with the rising of IBT concentration. The fluorescence of cells under basal conditions was undetectable, while the intense fluorescence in the experimental groups indicated a burst of ROS, especially the 40 μM group. Taken together, our findings indicated that IBT could promote ROS generation in SMMC-7721 cells.

Change of cellular thiols and glutathione

As the results of DCFH-DA staining assays are prone to artifacts as adequately discussed (Kalyanaraman et al., 2012), we assayed overall thiol contents to confirm the redox state in SMMC-7721 cells. The balance of thiols and disulfides is a key indicator of cellular redox state, and cellular thiols are also important antioxidants to resist ROS accumulation (Bindoli et al., 2008). Overall intracellular thiol levels were determined via the DTNB titration assay. It can be seen from Fig. 6B that total thiol levels in treated cells significantly reduced. To further confirm the results, we measured the change of cellular GSH homeostasis, another sensitive indicator of the cellular redox state. As illustrated in Fig. 6C, the ratio of GSSG/GSH in SMMC-7721 cells also showed a dose-dependent increase, and the ratio of 40 μM group increased to more than 2.5 times that of the control group. Taken together, all the above results confirmed that IBT could increase the intracellular oxidation level in SMMC-7721
Protection of cell death by NAC and promotion of cell death by BSO

The above results have indicated that IBT could induce ROS generation and disrupt the balance of cellular redox state. We further determined the effects of NAC supplementation and GSH depletion on IBT-induced cell death to explore whether the cytotoxicity of IBT was related to the oxidative stress. After SMMC-7721 cells were treated with indicated concentrations of IBT and NAC for 48 h, the results were detected via MTT assay. As shown in Fig. 7A, the cytotoxicity of IBT was gradually lost as the concentration of NAC increased; 5 mM NAC enhanced the survival rate to twice that of the control, while 10 mM NAC tripled it. Then, we measured the effect of GSH depletion on the cytotoxicity of IBT. The GSH system is an important backup for the Trx system and plays a significant role when the Trx system is dysfunctional (Du et al., 2012). SMMC-7721 cells were pretreated with 50 μM BSO for 24 h, and the cells were then washed and replaced with new medium before incubation with IBT. In our conditions, cellular GSH content decreased to less than 20% of the control group (Fig. 7B). As shown in Fig. 7C, pretreatment with BSO obviously enhanced the cytotoxicity of IBT, while incubation with BSO alone resulted in faint cytotoxicity. Both the NAC-induced decreased cytotoxicity and the GSH depletion-induced promoted to cytotoxicity further support the notion that oxidative stress is involved in the process of IBT-induced cell death.

Induction of apoptosis by IBT
Apoptosis is a component of normal physiological processes and is controlled by a series of complicated events. Inhibition of apoptosis has been thought to be the fundamental pathway that cancer cells prolong cell viability (Hanahan and Weinberg, 2011). ROS are a double-edged sword to cells; at a low level, ROS can function as signaling molecules (D'Autreaux and Toledano, 2007), but excess ROS are cytotoxic and can induce cell apoptosis (Davis et al., 2001). As described above, we demonstrated that IBT can induce oxidative stress contributing to the cytotoxicity. We further wondered whether IBT killed SMMC-7721 cells through apoptosis. Chemotherapeutic drugs and oxidative stress can induce intrinsic apoptosis pathway. Mitochondrion-dependent apoptosis is initiated by the release of cytochrome C and recruitment of caspase 9, which subsequently activates the effector caspases (caspase 3, 6 and 7) (Boatright and Salvesen, 2003; Fink and Cookson, 2005). Therefore, we first assessed the activation of caspase 3, the most common hallmark of early apoptosis. It can be seen from Fig. 8A, IBT obviously activated cellular caspase 3 in a dose-dependent manner. Then, we employed Hoechst 33342, a membrane-penetrating double-stranded DNA-binding stain, to characterize the nuclear morphological changes to confirm the results. The control group showed homogeneous round nuclei with diffuse weak fluorescence, while the experimental group showed condensed nuclei with more intense fluorescence (Fig. 8B), which was considered to be a characteristic morphology of apoptosis. Finally, an Annexin V-FITC/PI apoptosis detection assay was performed to determine the specific amounts of apoptosis. Living and apoptotic cells were quantified via flow cytometry. It can be seen from Fig. 8D, the control group consisted almost entirely of all living cells, while the experimental groups exhibited an increasing rate of apoptotic cells as the IBT
concentration increased. All the above results suggest that IBT predominantly triggered SMMC-7721 cell apoptosis.
Discussion

IBT has been approved for the treatment of several B-cell malignancies (Dreyling et al., 2016), and numerous phase I and phase II trials have demonstrated its clinical effects (Harrison, 2012; Byrd et al., 2013). It has been reported that IBT could block downstream B-cell receptor activation (Herman et al., 2011), abolish cell proliferation and induce cell apoptosis both in vitro and in vivo (Herman et al., 2013). As a clinical BTK inhibitor for various B cell malignancies therapy, IBT has been proved by extensive in vivo and in vitro studies. Although the IC$_{50}$ to BTK was in nanomole level, the IC$_{50}$ of IBT to CLL cells was in micromole level (Herman et al., 2011; Ponader et al., 2012), which made us consider if IBT has other target for its clinical therapy. For example, ErbB receptor and ABCB1 were also reported to be targeted by IBT (Grabinski and Ewald, 2014; Zhang et al., 2017a). Our results demonstrated that IBT can target mammalian TrxR to inhibit SMMC-7721 cells growth. TrxR is an antioxidant enzyme widely existing in all organisms, and it has recently got more and more attention in cancer therapy (Urig and Becker, 2006; Zhang et al., 2017b). Because BTK was mainly expressed in bone marrow, spleen and hematopoietic cells (Smith et al., 2001; Mohamed et al., 2009), the anticancer effect we researched maybe not relate with the BTK inhibition. Only in this condition, can we investigate other target of IBT. Novel targets of IBT could contribute positively to understanding its anticancer mechanism, and it’s easier to apply IBT in clinical therapy without a large number of clinical trials.

Among several cancer cell lines, the human hepatoma SMMC-7721 cell line was found to be the most sensitive cell line to IBT with an IC$_{50}$ value of 7.3 μM (Fig. 2A). The results also showed that IBT could arrest the cell cycle and migration (Fig. 2E & F), which further
indicated that IBT can significantly inhibit SMMC-7721 cell growth. As shown in Fig. 2D, the low cytotoxicity of IBT towards the HEK 293T cell line demonstrated its potential for cancer therapy.

The mechanism IBT inhibit BTK was that the skeleton of IBT was similar with ATP and could bind the ATP-binding site of BTK. Since there was a Cys residue in the binding site, the α, β-unsaturated ketone group was added to enhance the binding effect (Arnold et al., 2000; Pan et al., 2007). Our molecular docking results also suggested that IBT could be an irreversible inhibitor of mammalian TrxR (Fig. 3). Intracellular TrxR activity in SMMC-7721 cells was obviously inhibited by IBT (Fig. 4B & C). The IC_{50} to CLL cells was also in micromole level (Herman et al., 2011; Ponader et al., 2012), which indicated that TrxR could also be a potential target of IBT for cancer therapy. Under our experiential conditions, IBT caused obvious inhibition of cellular TrxR activity (Fig. 4B) but mild inhibition to cellular GR and Gpx activity (Fig. 4D & E), which indicated that IBT showed a high preference for mammalian TrxR compared to other sulfhydryl protein. There was a pocket around the active site GCUG of TrxR (Cheng et al., 2009), IBT binding with the pocket can inhibit the activity of TrxR. Although Gpx was also a selenoprotein which can directly scavenge the H_{2}O_{2} produced from superoxide anion (Dey et al., 2016), only in the presence of extensive reduced GSH, can Gpx exert the function. There was amount of ROS in living cells, especially cancer cells, IBT couldn’t react with the oxidized Gpx. While low inhibition of GR was due to the deficiency of Sec residues (Berkholz et al., 2008), which reduced the reactivity to IBT. The above results supposed that IBT maybe target mammal TrxR for cancer therapy. Due to the high homology between mammal TrxR1 and mammal TrxR2 (Mustacich and Powis, 2000),
IBT should also equally inhibit both TrxRs, which has been proved in our previous investigation (Duan et al., 2016). Inhibition of both TrxRs contributed to the cancer therapy (Urig and Becker, 2006; Topkas et al., 2016). It has also been reported that AF and IBT have a synergistic effect on EGFR-mutant non-small cell lung cancer (Hu et al., 2018). AF was also a reported irreversible inhibitor of TrxR (Becker et al., 2000). According to our results, IBT could inhibit the TrxR activity. Therefore, the synergistic effect could also relate with oxidative stress. Further cellular level evidence was obtained to support our hypothesis. Knockdown of TrxR1 and pharmacological inhibition of TrxR both sensitized cancer cells to IBT (Fig. 5C & F). It has been reported that inhibition of the Trx system can lead to arrest of cell cycle and migration (Boronat et al., 2017; Xie et al., 2017), which was also obtained in our experimental results (Fig. 2E & F). In conclusion, our results demonstrate that TrxR is a crucial target in the cellular effects of IBT.

Recently, the oxidative stress strategy has received increasing interest in relation to cancer therapy. Tumor cells are more sensitive to ROS promotion because they are more subject to oxidative stress than normal cells. Thereby oxidative stress can kill cancer cells at lower levels of cytotoxicity that are required to kill normal cells (Gorrini et al., 2013; Glasauer and Chandel, 2014). It has been reported that IBT can induce oxidative stress in several B-cell malignancies (Rotin et al., 2014). In our study, we have demonstrated that treatment with IBT can obviously insult the TrxR activity. Since Trx system is a vital antioxidant for scavenging ROS in vivo, the significant ROS elevation by IBT was also determined through various methods (Fig. 6A, B & C). NAC can act as a source of Cys for GSH biosynthesis in situation of severe GSH depletion, while it acts as an antioxidant when...
in the situation of no severe GSH depletion (Gleixner et al., 2017). Some literatures also reported that additional NAC usually act as an antioxidant but not a Cys provider for GSH biosynthesis (Patriarca et al., 2005; Gleixner et al., 2017). Treatment with IBT of SMMC-7721 cells didn’t lead to significant GSH decrease (Fig. 6D) and GR activity inhibition (Fig. 4D), so pretreatment with BSO sensitizing cells to IBT could be caused by synergistic effects for oxidative stress (Fig. 7C).

Upregulation of TrxR is one of the characteristics of cancer cells (Cha et al., 2009; Park et al., 2014). Not only can Trx, the substrate of TrxR, scavenge excess ROS produced by a vigorous metabolism, but TrxR can also help cancer cells resist apoptosis (Sasada et al., 1996; Kim et al., 2005). Resistance to apoptosis is the main way cancer cells evade cell death (Hanahan and Weinberg, 2011), thus inducing the apoptosis of cancer cells has been a feasible scheme for cancer therapy. Our study has determined IBT can induce the apoptosis of SMMC-7721 cells (Fig. 8), which has been previously reported (Schwamb et al., 2012; Amin et al., 2017). Inhibition of TrxR obviously weakened resistance to apoptosis and induced an increase in ROS (Fig. 6A), and excess ROS can further induce cellular apoptosis (Simon et al., 2000; Valko et al., 2007). It has been reported that compounds with electrophilic groups can connect with the Sec residue of mammalian TrxR to form selenium-compromised thioredoxin reductase-derived apoptotic proteins (SecTRAPs), and these SecTRAPs can also induce cell apoptosis (Anestal et al., 2008).

In conclusion, our findings proved that IBT could interfere with cellular TrxR and induce oxidative stress to promote the apoptosis of SMMC-7721 cells (Fig. 9). The discovery of a new target of IBT sheds light on better understanding its anticancer mechanisms and provides
a theoretical foundation for its further use in clinical therapy.
Acknowledgments

We thank Professor Constantinos Koumenis for providing shTrxR and shNT vector.
Authorship contributions

Participated in research design: Han, Zhang, Xu, Yao and Fang.

Conducted experiments: Han.

Performed molecular docking simulation: Shi.

Contributed results analysis: Han, Zhang, Ruijuan Liu, Tianyu Liu, Shi, Wu and Fang.

Contributed to the writing of the manuscript: Han, Zhang, Wu and Fang.
References


Association for Cancer Research 11:8425-8430.


Patriarca S, Furfaro AL, Domenicotti C, Odetti P, Cottalasso D, Marinari UM, Pronzato MA


Footnotes

This work was supported by grants from the National Natural Science Foundation of China (21572093 & 21778028), Natural Science Foundation of Gansu Province (18JR3RA296), Lanzhou University (the Fundamental Research Funds for the Central Universities, lzujbky-2018-39), Dalian University of Technology (the Fundamental Research Funds for the Central Universities and DUT17RC (4) 27) and the 111 project are greatly acknowledged.
Figure Legends

Fig. 1. The structure of ibrutinib (IBT).

Fig. 2. Inhibition of cell growth by IBT. (A) Cytotoxicity of IBT towards HeLa, A549, HepG2 and SMMC-7721 cells. IBT with the indicate concentration was used to treat the cells for 48 h, and MTT assay was used to determine the cell viability (B) Time-dependent cytotoxicity of IBT towards SMMC-7721 cells. IBT with the indicated concentrations was used to incubate the SMMC-7721 cells for 24, 48 or 72 h, and MTT assay was carried out to determine the cell viability. (C) After the cytotoxicity of IBT towards SMMC-7721 cells for 48 h, the trypan blue exclusion assay was used to determine the cell viability. (D) Cytotoxicity of IBT towards HEK 293T cells compared with SMMC-7721 cells. The viability of the cells treated by IBT with the indicated concentrations for 48 h was determined using the MTT assay. (E) IBT inhibits proliferation of SMMC-7721 cells. 10 or 20 μM IBT was used to incubate the SMMC-7721 cells for 24 h, and the cell percentage in G0/G1, S and G2/M phase was analyzed by flow cytometry. (F) IBT suppresses migration of SMMC-7721 cells. Before (upper) and after (lower) SMMC-7721 cells were incubated with 2.5 or 5 μM IBT for 24 h, the images were acquired by fluorescence microscope. Scale bars: 100 μm. Data were expressed as the average ± S.E. in the three experiments. **P < 0.01 vs. the control groups in (A) (B) and (C), **P < 0.01 vs. the normal cells groups in (D).

Fig. 3. The binding mode of IBT with mammal TrxR1. (A) The binding site of TrxR1 dimer with IBT is labeled out by red circle. The chain A and chain B of TrxR1 dimer are shown in
green and cyan cartoon respectively. (B) The surface representation of the binding site of IBT on TrxR1. The Sec$_{498}$ was shown in yellow. (C) The surrounding residues of IBT in the pocket. The structural scaffold of IBT is shown in orange sticks.

**Fig. 4.** Change of intracellular TrxR1 expression and TrxR, GR and Gpx activity by IBT. (A) TrxR1 protein levels in SMMC-7721 cells changed by IBT. IBT with different concentrations was used to incubate SMMC-7721 cells for 24 h, and cell lysis was conducted with western blot assay. (B) End point insulin reduction assay and (C) cell imaging were employed to determine the intracellular TrxR activity. After SMMC-7721 was incubated with IBT with different concentrations of for 24 h, the intracellular TrxR activity were assayed through the above mentioned method. The fluorescence images (bottom panel) and the bright filed images (top panel) were obtained by fluorescence microscope. Scale bars: 50 μm. After SMMC-7721 cells were treated with IBT for 24 h, the GR activity (D) and Gpx activity (E) was determined by the enzyme dynamic method. Data were expressed as average ± S.E. in three experiments. *P < 0.05 and **P < 0.01 vs. the control groups in (B), (D) and (E).

**Fig. 5.** Pharmacological intervention of TrxR and TrxR1-knockdown increase the cytotoxicity by IBT. (A) TrxR1 expression and (B) TrxR activity in HeLa-shNT/HeLa-shTrxR1 cells. The endpoint insulin reduction assay was implemented to determine the TrxR activity, and the expression of TrxR1 in both cells was analyzed by western blot. (C) IBT had cytotoxic effects on HeLa-shTrxR1 and HeLa-shNT cells, both of which were incubated through IBT with indicated concentrations for 48 h. The MTT assay was implemented to measure the cell
viability. (D) SMMC-7721 cells were incubated with indicated concentrations of AF for 4 h. The TrxR activity was determined by end point insulin reduction assay. (E) After cultured with indicated AF for 4 h, the SMMC-7721 cells were incubated with above concentrations of IBT for 44 h. The cell viability was determined by MTT assay. The data were expressed as average ± SE in three experiments. *P < 0.05 and ** P < 0.01 vs. the HeLa-shNT cells in (B), ** P < 0.01 vs. the 0 μM AF group in (D) and (E).

**Fig. 6.** Oxidative stress induced by IBT in SMMC-7721 cells. (A) The alteration of total thiols level by IBT in SMMC-7721 cells. IBT with the above mentioned concentrations was used to treat SMMC-7721 cells for 24 h, and DTNB titration quantified the intracellular total thiols. (B) Increased GSSG/GSH ratio in SMMC-7721 cells. The SMMC-7721 cells were treated by IBT with the above-mentioned concentrations for 24 h, and enzymatic recycling method was adopted to assay the intracellular GSSG and GSH level; the GSSG/GSH ratio was shown. (C) Imaging of intracellular ROS level in SMMC-7721 cells by DCFH-DA. IBT with the above mentioned concentrations was used to treat SMMC-7721 cells for 1 h. The fluorescence images (bottom panel) and the bright filed images (top panel) were obtained by fluorescence microscope. Scale bars: 50 μm. (D) The GSH and (E) GSSG content in SMMC-7721 cells treated with IBT for 24 h. Data were expressed as average ± SE of three experiments. **P < 0.01 vs. the control groups in (A), (B), (D) and (E).

**Fig. 7.** (A) Alleviation of IBT cytotoxicity against SMMC-7721 cells by NAC. IBT and NAC with the indicated concentrations were used to incubate the SMMC-7721 cells for 48 h, and
the MTT assay was carried out to measure the cell viability. (B) Total GSH content in SMMC-7721 cells treated with 50 mM BSO for 24 h. (C) Enhancement of IBT cytotoxicity against SMMC-7721 cells by GSH depletion. The SMMC-7721 cells were incubated with 50 μM BSO for 24 h, and incubated by IBT with the indicated concentrations for 48 h. The viability of cells was measured through the MTT assay. Data were expressed as average ± SE in three experiments. *P < 0.05 and **P < 0.01 vs. the groups without NAC in (A); **P < 0.01 vs. the groups without BSO in (C).

**Fig. 8.** Apoptotic cell death induced by IBT. (A) Activation of caspase 3 by IBT in SMMC-7721 cells. The SMMC-7721 cells were incubated with IBT with the indicated concentrations for 24 h. Based on the fluorescence intensity of free pNA, the activity of caspase 3 activity was assayed. (B) The nuclear morphological changes in SMMC-7721 cells by IBT. SMMC-7721 cells were incubated with IBT with the indicated concentrations for 48 h, the culture was added with the Hoechst 33342 staining. The fluorescence images (bottom panel) and the bright filed images (top panel) and were obtained by fluorescence microscope. Scale bars: 50 μm. (C) Quantification of the population of normal cells, apoptotic cells and necrotic cells from (D). (D) Apoptotic cell death analyzed by Annexin V/PI double staining assay. The SMMC-7721 cells were incubated by IBT with indicated concentrations for 24 h. Flow cytometry was used to analyze the percentage of apoptotic cells, necrotic cells and live cells. Cells were divided into four different cell populations in the scatter grams: PI-positively and FITC-negatively stained cells were necrotic cells (upper left, Q1), FITC and PI double-positive (stained) cells were late apoptosis (upper right, Q2). FITC and PI
double-negative (unstained) cells were live cells (lower left, Q3). FITC-positively and PI negative (stained) cells were early apoptosis (lower right, Q4). Data were expressed as average ± SE in three experiments. **P < 0.01 vs. the control groups in (A) and (C).

**Fig. 9.** Proposed mechanism for the apoptosis-inducing ability of IBT.
Figure files

Fig. 1. The structure of IBT.
Fig. 2. Inhibition of cell growth by IBT.
Fig. 3. The binding mode of IBT with mammal TrxR1.
Fig. 4. Change of intracellular TrxR1 expression and TrxR, GR and Gpx activity by IBT.
**Fig. 5.** Pharmacological intervention of TrxR and TrxR1-knockdown increase the cytotoxicity by IBT.
Fig. 6. Oxidative stress induced by IBT in SMMC-7721 cells.
Fig. 7. Alleviation of IBT cytotoxicity by NAC and enhancement of IBT cytotoxicity by BSO.
**Fig. 8.** Apoptotic cell death induced by IBT.
**Fig. 9.** Proposed mechanism for the apoptosis-inducing ability of IBT.