Biomedicine & Pharmacotherapy 83 (2016) 212–220

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Synergistic inhibition of sunitinib and ethaselen against human colorectal cancer cells proliferation

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ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form 14 June 2016
Accepted 16 June 2016

Keywords:
Sunitinib
Ethaselen
Colorectal cancer
Bcl-2/Bax
Cytochrome C
VEGFR

ABSTRACT

Sunitinib, a multi-targeted tyrosine kinase inhibitor, has been widely used in the therapy of advanced renal cell cancer and imatinib-resistant gastrointestinal stromal tumors. However, little benefits could be obtained from sunitinib for patients with other types of solid tumors including colorectal cancer (CRC). Ethaselen (BBSKE), a specific thioredoxin reductase 1 inhibitor, has shown convincing anticancer effects both *in vivo* and *in vitro*. In this study, we explored the combinatory effect of sunitinib and BBSKE in human CRC cell lines LoVo, HT-29 and RKO. Cotreatment of BBSKE and sunitinib with the ratio of 2:1 for 24 h displayed synergistic effect against CRC cells proliferation. Apoptosis analysis also revealed that combination treatment of BBSKE and sunitinib (2:1) for 24 h induced higher apoptosis rate than either single treatment. The synergistic effect against LoVo cells proliferation may be explained by sharp reduction of Bcl-2/Bax protein expression ratio, decrease of pro-Caspase-3 protein expression along with significantly augmented Caspase-3 enzymatic activity, and release of cytochrome C from mitochondria to cytoplasm in the combination treatment group. The significant inhibition of vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation might also account for the synergism in cotreatment group. In short, sunitinib plus BBSKE is perhaps a promising strategy for colorectal cancer therapy.

1. Introduction

Sunitinib is an orally multi-targeted tyrosine kinase inhibitor (TKI) agent, which mainly targets vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), as well as other tyrosine kinases such as FLT-3, c-KIT and RET [1]. Sunitinib has been approved by US Food and Drug Administration for the treatment of advanced renal cell cancer and imatinib-resistant gastrointestinal stromal tumors. However, in patients with other types of solid tumors, including breast cancer, colon cancer, non-small cell lung cancer (NSCLC), sunitinib showed low objective response rates in phase II clinical trials [2]. Besides, a series of side effects (eg. arterial thromboembolic events, hematologic toxicities, fatigue, etc) have been reported with sunitinib utilization [3–5]. Therefore, combination treatments with other targeted agents, cytotoxic chemo-therapy or radiation therapy are now under active investigation aiming to improve efficacy and reduce adverse events of sunitinib [2]. Other alternative treating approaches including new generations of TKI are also under research and development.

The thioredoxin (Trx) system, which consists of thioredoxin reductase (TrxR), Trx and NADPH, plays a pivotal role in regulating intracellular redox balance. Three isoforms of TrxRs have been identified in mammals: cytosolic TrxR1, mitochondrial TrxR2 and testis-specific TrxR3 [6]. It has been demonstrated that TrxR1 is over-expressed in many human malignancies compared with normal tissues, indicating that TrxR1 is a promising target for anticancer drug development [7]. Trx system overexpression was proved to promote growth, evade apoptosis and sustain angiogenesis in cancers [8]. Ethaselen (BBSKE) is a selenazole-based compound targeting TrxR1 and is on phase II clinical trial in China. By suppressing TrxR1 activity, elevating reactive oxygen

Abbreviations: TKI, tyrosine kinase inhibitor; VEGFRs, vascular endothelial growth factor receptors; PDGFRs, platelet-derived growth factor receptors; NSCLC, non-small cell lung cancer; Trx, thioredoxin; TrxR, thioredoxin reductase; BBSKE, ethaselen; ROS, reactive oxygen species; CRC, colorectal cancer; SRB, sulforhodamine B; CI, combination index; DRI, dose-reduction index; MI, maximum inhibition rate.

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http://dx.doi.org/10.1016/j.biopha.2016.06.040
0753-3322/

species (ROS) level and subsequently inducing apoptosis and cell cycle arrest *in vivo* and *in vitro*, BBSKE showed antitumor effects against prostate cancer, NSCLC and gastric cancer etc. [9,10]. Furthermore, in combination with BBSKE, cytostatic dosage of cisplatin against colon cancer cell line LoVo was reduced and G2/M phase arrest induced by cisplation was reversed [11].

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths with rising incidence in the world. In the past decades, multiple chemotheraputic strategies have been widely used, such as FOLFIRI (5-FU, leucovorin and irinotecan) and FOLFOX (5-FU, leucovorin and oxaliplatin) with or without molecular targeted agents. The median overall survival has notably increased from 12 months with 5-FU-based standard care, to around 30 months in the clinical trials with chemotherapy plus bevacizumab or cetuximab [12]. Nonetheless, the 5-year survival rates of patients with metastatic CRC still remains below 8% [13]. Hence, novel combined therapeutic regimen for CRC is urgently needed. It has been reported that in a double-blind phase III study, sunitinib plus FOLFIRI was not superior to FOLFIRI alone in previously untreated metastatic CRC, and has a poorer safety profile [14]. In this study, we aimed to investigate the effect of BBSKE in combination with sunitinib in CRC cell lines LoVo, HT-29 and RKO along with potential mechanisms involved.

2. Materials and methods

2.1. Reagents

BBSKE was synthesized in our laboratory (State Key Laboratory of Natural and Biomimetic Drugs, Beijing, China). Sunitinib was obtained from Ouhe (Beijing, China). BBSKE and Sunitinib stock solution was prepared at 5 mM in DMSO.

2.2. Cell culture

Human CRC cell lines LoVo, HT-29 and RKO were obtained from Cell Resource Center at Peking Union Medical College. CRC cells were cultured in Dulbecco's modified Eagle's medium (Macgene, Beijing, China) supplemented with 10% fetal bovine serum (BI, Israel) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Cell proliferation assay

The standard sulforhodamine B (SRB) colorimetric assay was used to measure inhibitory effect of BBSKE alone, Sunitinib alone or combination treatment against CRC cells as previously described [15]. Briefly, LoVo cells (4000/well), HT-29 cells (3000/well) and RKO cells (2000/well) were seeded in 96-well plates. After 24 h, cells were treated with BBSKE (1, 3, 5, 10, 20 μM), sunitinib (1, 3, 5, 10, 20 μM) or a combination of BBSKE and sunitinib (2:1) for indicated time periods. Then the OD values were measured at 492 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, MA, USA). The 50% inhibitory concentration (IC_{50}) values were calculated using the software Origin 7.5.

2.4. Chou-Talalay median-effect analyses

The median-effect analysis based on the median-effect principle was used to measure the combination treatment effect of BBSKE and Sunitinib [16]. Results from at least three independent experiments were pooled and mean values were used to calculate the combination index (CI) and dose-reduction index (DRI). CI values reflect the ways of interaction between two drugs. CI > 1.1, 0.9 < CI < 1.1, and CI < 0.9 indicate antagonism, additive effect and synergism respectively. DRI values reflect how many folds the dose of each drug could be reduced at a given effect level in a combination treatment, compared with the doses of each drug alone. DRI >1 implies a favorable dose reduction and a greater DRI value indicates a greater dose reduction effect. The software CompuSyn was used to analyze the Chou-Talalay median-effect.

2.5. Apoptosis assay

Cell apoptosis was detected by flow cytometry (BD FACSCalibur, NJ, USA). Briefly, exponentially growing LoVo cells were planted into 60 mm dishes (6×10^5/dish). After cultured for 24 h, cells were exposed to single drug or a combination for 24 h. Then cells were collected and fixed in 70% pre-cold ethanol at −20 °C overnight. Cells were digested with 0.1 mg/mL RNase (Tiangen Biotech, Beijing, China) in water bath at 37 °C for 30 min. After filtered through a 400-pore sieve, cells were stained with 50 μg/mL propidium iodide for 15 min protected from light. The sub-G1 analysis was employed to assess the percentage of apoptotic cells.

2.6. Western blotting

To obtain whole cell lysates, adherent cells were washed twice with pre-cold PBS and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer after drug treatment for 24 h. Mammalian protease inhibitor cocktail was added to the buffer before use. Cell suspension were collected by centrifugation at 1000*g* for 2 min and combined with adherent cells. Cells were lysed on ice for 30 min and lysates were then centrifuged at 13,000*g* for 15 min to collect supernatant.

To obtain cytosolic fraction and mitochondrial fraction, adherent cells were washed with pre-cold PBS, digested with trysin-EDTA then washed twice with ice-cold isotonic buffer (10 mM Tris–HCl (pH 7.4), 1 mM EDTA and 250 mM sucrose). Suspension cells were also collected. Cells were resuspended in 200 μL isotonic buffer with protease inhibitor, and were fully homogenized by 60 strokes in a Dounce homogenizer. After centrifugation at 1000*g* for 10 min at 4 °C, the supernatant was centrifuged at 17,000*g* for 15 min at 4 °C. The resulting supernatant was soluble cytosolic fraction, and the mitochondrial sediment was lysed in 30 μL RIPA buffer.

Protein concentration was measured by BCA method (Applygen Technology, Beijing, China). Cellular protein samples (30 μg) were resolved by SDS-PAGE and electrophoretically transferred onto PVDF membranes (Applygen Technology, Beijing, China). The membranes were blocked by 10% skimmed milk at room temperature for 60 min and incubated with primary antibodies, including β-actin, Bax, Bcl-2 (Santa Curz Biotech, CA, USA), caspase-3 (Cell Signaling Technology, MA, USA), cytochrome c, VDAC1/Porin, VEGFR2 (Proteintech, IL, USA) and Phospho-VEGFR2 (Tyr1214) (EnoGene, Nanjing, China) overnight at 4 °C. Then the primary antibody complex was incubated with HRP-conjugated secondary antibodies for 1 h at room temperature (Santa Curz Biotech, CA, USA). Proteins were visualized by ECL kit (Advansta, Menlo Park, CA, USA).

2.7. Caspase-3 activity measurement

Caspase-3 activity of LoVo cells lysates was measured by a Caspase-3 activity kit (Beyotime Institute of Biotechnology, Nantong, China). LoVo cells were seeded in 60 mm dishes (6×10^5/dish). After incubation with indicated drugs for 3 h, cells were harvested, 30 μL lysis buffer (provided by manufacturer) was added and cells were lysed for 15 min on ice. The lysates were centrifuged at 16 000*g* for 15 min at 4 °C and supernatant was collected. Bradford method was used to determined protein concentrations. Then 40 μg protein extracts (less than 40 μL)