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Original article

Rosiglitazone elevates sensitization of drug-resistant oral epidermoid carcinoma cells to vincristine by G2/M-phase arrest, independent of PPAR-γ pathway

Hong-Yuan Wang, Ying Zhang, Yue Zhou, Yu-Yin Lu, Wen-Fang Wang, Ming Xin, Xiu-Li Guo*

Department of Pharmacology, Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan, 250012, China

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ABSTRACT

Rosiglitazone (ROSI), an oral antidiabetic agent, has been reported the anti-cancer properties recent years. In this paper, the potency of ROSI as a synergistic drug for vincristine (VCR) on resistant oral cancer cells was investigated. We found that ROSI potently enhanced the susceptibility of KB cells or KB/V cells to VCR in a dose manner and the synergy in KB/V cells was much more prominent than that in KB cells. The synergistic anti-proliferative effect of ROSI and VCR was associated with inhibition on tubulin polymerization, cell cycle arrest in G2/M phase and cell apoptosis induction, but has no effect on drug efflux-protein P-gp and was independent with PPARγ. The combination treatment of ROSI and VCR could regulate the PTEN/PI3K/AKT survival pathway with an upregulation of PTEN and down-regulation of p-AKT. The effect of G2/M phase arrest was associated with the upregulation of cyclin B1 and downregulation of p-cdc2. The apoptosis induction of ROSI and VCR was partly due to an upregulation of cleaved PARP and downregulation of Bcl-2/Bax ratio. In addition, combination treatment of ROSI and VCR had also shown anti-angiogenic effect by suppressing the migration and blocking the capillary tube formation of HUVECs. More importantly, this combination treatment induced an acceptably weak cytotoxicity in human normal HL-7702 cells, GES-1 cells and HUVECs. Taken together, ROSI may be used as a potential compound for combinatorial therapy or as a complement to VCR for treatment on oral cancer, especially on that have acquired resistance to VCR therapy.

1. Introduction

Oral cancer is still a serious and growing problem worldwide. The annual estimated incidence is around 275,000 for oral cancer, ranked the sixth most common cancer in the world [1]. The areas characterized by high incidence rates for oral cancer are mainly some European and Asian countries with tobacco epidemics [2]. In China, oral cancer was reported 2.61 per 100,000 as incidence rate and 1.11 per 100,000 as mortality rate in 2010 [3].

Surgery and/or radiotherapy, as well as chemotherapy are the main treatments for oral cancer. Despite the advances in diagnosis and treatment, oral cancer still has lower 5-year survival rate [4–6]. Vincristine (VCR), a classical microtubule-destabilizing agent, is potent and widely used for hematologic malignancies and some solid tumors including oral cancer, due to its well-defined mechanism of the demonstrated anti-cancer activity and its ability to be combined with other agents [7]. However, the treatment of VCR is limited by the development of multidrug resistance (MDR), the same as other chemotherapeutics in oral cancer. Though great efforts have been made in developing new anti-cancer drugs for enhancing curative effect or reversing MDR, the results are not satisfactory due to either a lack of potency or unacceptable side-effects [6,8]. Recently, some drugs (such as quercetin and the COX-2 inhibitor celecoxib) with anti-tumor activity have been reported to elevate sensitization of drug-resistant oral epidermoid carcinoma cells to VCR, which provide an exciting method for overcoming VCR-drug resistance of tumors [9,10].

Rosiglitazone is a member of thiazolidinediones (TZDs) which act as high-affinity agonists of the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) and are highly effective oral medications for type 2 diabetes [11]. Other than regulating

* Corresponding author at: No. 44 Wen Hua Xi Road, Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, PR China.
E-mail address: guoxl@sdu.edu.cn (X.-L. Guo).

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glucose and adipose metabolism, PPARγ has also been shown to induce terminal differentiation, inhibit cell proliferation, promote apoptosis and inhibit innate inflammation in many cancer models [12]. Based on these properties of PPARγ, a number of preclinical and clinical studies with PPARγ agonists (especially TZDs) has been performed in human cancer. Though the anti-tumor action of TZDs in liver-, colon-, prostate- and some other carcinomas has been documented in pre-clinical studies [13–15], clinical trials involving some epithelial carcinomas showed no beneficial effect using TZDs as a monotherapy [16]. Emerging evidences indicate that TZDs in combination with conventional chemotherapeutic drugs (such as cisplatin and 5-FU) might increase the therapeutic efficacy with unclear mechanisms [17–19].

In the present study, we examined the anti-tumor efficacy and possible molecular mechanisms of combination treatment of vincristine and rosiglitazone in human oral epidermoid carcinoma cell line (KB cells) and the vincristine-resistant KB cell line (KB/V cells).

2. Materials and methods

2.1. Reagents

Rosiglitazone, GW9662 and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) with a concentration less than 0.1%. Vincristine Sulfate for Injection was purchased from Lingnan Pharmaceutical LTD. (Guangzhou China). Verapamil Hydrochloride Injection was purchased from Shanghai Harvest Pharmaceuticals CO., LTD. (Shanghai, China). RPMI-1640 medium, fetal bovine serum and L-glutamine were purchased from GINCO BRL (Grand Island, NY, USA). Penicillin/streptomycin, trypsin solution, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), RNase, Hoechst33342, rhodamine123 (Rh123), bovine serum albumin (BSA), radio immunoprecipitation assay (RIPA) lysis buffer and phenylmethane sulfonyl fluoride (PMSF, 100 mM) were purchased from Solarbio technology co., LTD. (Beijing, China). Cell counting kit-8 (CCK-8 kit) was purchased from Dojindo Molecular Technologies, INC. (DOJINDO, Kyushu, Japan). Annexin V-FITC apoptosis detection kit was purchased from 4A Biotech Co., Ltd. (Beijing, China). BCA protein assay kit was purchased from Thermo Fisher Scientific Pierce (Sunnyvale, CA, USA). Monoclonal antibodies against α-tubulin, Bax, Bcl-2, PARP, Phospho-cdc2 (p-cdc2, Tyr15), cyclin B1, Phospho-AKT (p-AKT, Ser473), PTEN, and P-glycoprotein (P-gp) were purchased from Cell Signaling Technology (CST, Boston, MA, USA). Monoclonal antibody against β-actin was purchased from ZS Bio. (Beijing, China).

2.2. Cell culture and treatments

Human oral epidermoid carcinoma cell line (KB cells) and human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (Rockville, USA). The vincristine selected MDR KB/V cells were obtained from Sun Yat-Sen University of Medical Sciences (Guangzhou, China). Human normal liver cell line HL-7702 cells were purchased from the China Cell Bank (Shanghai, China). Human normal gastric epithelial cell line GES-1 cells were purchased from Boshun joint experiment center (Shanghai, China). All cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin/streptomycin and 2 mM L-glutamine. KB/V cells were maintained in medium with 20 ng/mL vincristine for at least one week and then incubated in drug-free medium for one week prior to experimental use [6]. All cells were maintained at 37 °C in an incubator with humid atmosphere (5% CO_2-95% air) and were used from passage 3–8. Exponentially growing cells were seeded into cell culture plates and allowed to attach overnight, then treated with VCR (0.5, 2 nM) and/or ROSI (80 μM) for KB cells, or treated with VCR (50, 200 nM) and/or ROSI (100 μM) for KB/V cells according to the experimental design.

2.3. Cytotoxic assay

After appropriate treatments, cell proliferation was determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and cell counting kit-8 (CCK-8) assay. For MTT assay, KB cells or KB/V cells were seeded in 96-well pates with 1×10^3 cells/well and treated with or without various concentrations of VCR (0.5, 1, 2, 5 and 10 nM) and/or ROSI (20, 40 and 80 μM) for KB cells, VCR (10, 50, 250, 500 and 1250 nM) and/or ROSI (25, 50 and 100 μM) for KB/V cells for 48 h. HL-7702, GES-1 and HUVEC cells were treated with VCR (0.5, 2 nM) and/or ROSI (100 μM) for 48 h. 10% of MTT solution (5 mg/mL) was then added to each well for 4 h at 37 °C. The supernatant was removed and the cell pellet was re-suspended in 150 μL DMSO and the absorbance was measured at 570 nm on Thermo Multiskan GO microplate reader (Thermo-1510, CA, USA) [19]. For CCK-8 assay, KB or KB/V cells were seeded into 96-well pates (1×10^3 cells per well) and treated with or without VCR (0.5, 2 and 5 nM) and/or ROSI (80 μM) for KB cells, VCR (50, 200 and 500 nM) and/or ROSI (100 μM) for KB/V cells for 48 h. Then, 10% of CCK-8 solution was then added to the plates for 2 h at 37 °C. The absorbance was measured at 450 nm on Thermo Multiskan GO microplate reader [20]. The percentage of cell viability and the median inhibitory concentration (IC_{50}) value were calculated as described previously. The enhanced activity of ROSI was described as synergistic fold for KB cells and reversal fold for KB/V cells, both equated to the ratio of IC_{50} of VCR alone/IC_{50} of VCR combined with ROSI [19,21].

2.4. Colony formation assay

KB or KB/V cells were seeded in 6-well plates (300 cells per well). After appropriate treatments of VCR or ROSI for 24 h, cells cultured in drug-free medium for two weeks. Colonies were fixed with cold methanol-glacial acetic acid (3:1, v/v) and stained with 0.05% crystal violet. Then the colonies (greater than 50 cells) were imaged and counted under the microscope (CKX31SF, Olympus, Japan). The inhibition of colony formation was expressed by the percentage of vehicle control cells [6,22].

2.5. Rhodamine123 (Rh123) accumulation assay

KB/V cells were seeded in 24-well plates (6×10^4 cells per well) and treated with VCR (50, 200 nM) and/or ROSI (100 μM) or verapamil (VP, 10 μM) for 1 h at 37 °C, KB cells were used as a blank control. Then cells were exposed to Rh123 solution (5 μM) for additional 1.5 h in dark. After washing cells with phosphate buffered saline (PBS) repeatedly, the cell-associated mean fluorescence intensity (MFI) was determined by 1420 Vitor3 Multi label plate reader (Perkin Elmer, Waltham, MA, USA) with excitation/emission wave lengths of 485/535 nm and the images were taken by fluorescence microscope (100×, TE2000-S; Nikon, Tokyo, Japan) [21,23].

2.6. Hoechst 33342 staining

KB and KB/V cells seeded in 24-well plates (6×10^4 cells per well) were treated with VCR and/or ROSI for 24 h. Then cells were fixed with cold methanol-glacial acetic acid (3:1, v/v) and stained with Hoechst 33342 (10 μg/mL) in dark at room temperature for 15 min. After three washes with 1% PBS-TX (Triton X-100 in PBS, v/v), cells were visualized with fluorescence microscope