RESEARCH ARTICLE

Luteolin Sensitizes Two Oxaliplatin-Resistant Colorectal Cancer Cell Lines to Chemotherapeutic Drugs Via Inhibition of the Nrf2 Pathway

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Abstract

Oxaliplatin is a first-line therapy for colorectal cancer, but cancer cell resistance to the drug compromises its efficacy. To explore mechanisms of drug resistance, we treated colorectal cancer cells (HCT116 and SW620) long-term with oxaliplatin and established stable oxaliplatin-resistant lines (HCT116-OX and SW620-OX). Compared with parental cell lines, IC_{50}s for various chemotherapeutic agents (oxaliplatin, cisplatin and doxorubicin) were increased in oxaliplatin-resistant cell lines and this was accompanied by activation of nuclear factor erythroid-2 p45-related factor 2 (Nrf2) and NADPH quinone oxidoreductase 1 (NQO1) . Furthermore, luteolin inhibited the Nrf2 pathway in oxaliplatin-resistant cell lines in a dose-dependent manner. Luteolin also inhibited Nrf2 target gene [NQO1, heme oxygenase-1 (HO-1) and GSTα1/2] expression and decreased reduced glutathione in wild type mouse small intestinal cells. There was no apparent effect in Nrf2-/- mice. Luteolin combined with other chemotherapeutics had greater anti-cancer activity in resistant cell lines (combined index values below 1), indicating a synergistic effect. Therefore, adaptive activation of Nrf2 may contribute to the development of acquired drug-resistance and luteolin could restore sensitivity of oxaliplatin-resistant cell lines to chemotherapeutic drugs. Inhibition of the Nrf2 pathway may be the mechanism for this restored therapeutic response.

Keywords: Luteolin - colorectal cancer - oxaliplatin-resistant cell lines - Nrf2 - sensitization

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Introduction

Oxaliplatin is a third generation platinum chemotherapeutic which binds to DNA to form platinum-DNA adducts that inhibit DNA synthesis and repair to confer cytotoxicity and anti-tumor activity. Compared to cisplatin, carboplatin, and other platinum compounds, oxaliplatin has broad anti-tumor activity and low toxicity; thus, it is a first-line treatment for colorectal cancer. Over time, colorectal cancer cells may become resistant to oxaliplatin, reducing therapeutic efficacy (Yoo et al., 2010; Wang et al., 2011). Therefore, investigating mechanisms of tumor cell oxaliplatin resistance may offer data to reverse this reduced efficacy and provide promising targets for new clinical trials.

Nuclear factor erythroid-2 p45-related factor 2 (Nrf2), a cap'n'collar basic leucine zipper transcription factor, was identified as a critical intracellular regulator in the adaptive response via regulation of a wide array of cytoprotective enzymes[e.g. NADPH quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), aldo-keto reductase family 1, member C1 (AKR1C) and glutathione S-transferase] (Wakabayashi et al., 2010). Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) whereby Keap1 maintains Nrf2 at low concentrations in the cytoplasm (Itoh et al., 2010; Magesh, et al., 2012). However, dysfunction of this pathway leads to constitutive activation of Nrf2 and chemoresistance in many cancer cells types (DeNicola et al., 2011; Konstantinopoulos et al., 2011; Niture and Jaiswal, 2012). In addition, activation of Nrf2 may contribute to the development of cancer cell resistance to chemotherapeutic drugs. Doxorubicin resistance in an ovarian carcinoma cell line (A2780DR) was accompanied by an elevation in Nrf2 activity, which restored sensitivity to doxorubicin after stable transfection of A2780DR cells with Nrf2-shRNA (Shim et al., 2009). Nrf2 also confers resistance to chemotherapeutic drug 5-FU in gastric cancer cells (Hu et al., 2013). Therefore, Nrf2 is a potential target for reversing tumor resistance (Jeong et al., 2006; Kwak and Kensler, 2010; Pandurangan and Esa, 2013).

Luteolin (3, 4, 5, 7-tetrahydroxy flavone), a flavonoid with antioxidant, anti-inflammatory, cardiovascular protection and anti-cancer effects has been identified as a potential Nrf2 inhibitor (Bagli et al., 2004; Lin et al., 2008; Attoub et al., 2011). Luteolin can promote the

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degradation of Nrf2 mRNA, leading to down-regulation of the antioxidant response element (ARE)-gene battery and enhancing the sensitivity of A549 cells to anti-cancer drugs (Tang et al., 2011).

Here, we established stable colorectal cancer oxaliplatin-resistant cell lines via long-term oxaliplatin treatment. We confirmed that oxaliplatin-resistant cell lines developed multidrug resistance and that this was accompanied by an activation of the Nrf2 pathway. Luteolin inhibited the Nrf2 pathway in oxaliplatin-resistant cell lines and reversed multidrug resistance. Thus, oxaliplatin-resistant cell lines are appropriate models for investigating multidrug resistance and the underlying mechanism behind that resistance in colorectal cancer. Using this model, we could sensitize colorectal cancer cells to chemotherapeutic drugs via luteolin-modulated Nrf2 pathway inhibition.

Materials and Methods

Chemicals and reagents

Luteolin, doxorubicin, and cisplatin were obtained from Sigma-Aldrich Co., Ltd. (Shanghai, China). Oxaliplatin was purchased from Sanofi Company (Laboratoires Thissen, Belgium). Unless otherwise stated, all antibodies for Western blot were purchased from Santa Cruz Biotechnology Company (Shanghai, China). The Gstα1/2 antibodies were provided by Professor John Hayes (University of Dundee, Scotland). The secondary antibody goat anti-rabbit polyclonal antibody was purchased from Gene Company (Hong Kong, China).

Cell culture and generation of colorectal cancer oxaliplatin-resistant cell lines

Both the human colorectal cancer cells HCT 116 and SW620 were obtained from Institute of Cell Biology of Chinese Academy of Science (Shanghai, China), They were cultured in medium (Mccoy'5A and RPMI1640) supplemented with 10% fetal bovine serum plus 1% antibiotics and incubated 5% CO_2 at 37°C, respectively. The resistant cell lines were generated in our laboratory. Briefly, HCT 116 and SW620 were exposed to oxaliplatin (1 μM) for two months, then cells were screened for stable oxaliplatin-resistant cells, which were named as HCT 116-OX and SW620-OX, respectively.

Cytometry

Cells (3×10^5) were seeded in 6-well plates and cultured for 24, 48 and 72h, respectively, and counted daily with cell counting chamber. The cell growth curve was drawn by the average value. The cell population doubling time (TD) was calculated according to linear regression.

Cell viability assay

Cell viability was determined using a MTS cell proliferating assay kit (Promega, China). Cells were seeded in 96-well plates at 104 cells/well. After overnight recovery, the cells were incubation in fresh culture medium containing oxaliplatin, cisplatin and doxorubicin alone or in combination with luteolin for 24 h. The half-maximal inhibitory concentration (IC_{50}) and the combination index (CI) were calculated as described (Chou et al., 1994).

Animals

C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Nrf2 (-/-) transgenic mice (C57BL/6) were provided by Dr. Masayuki Yamamoto (University of Tsukuba, Japan). Male C57BL/6 mice aged of 6 weeks were used and randomly allocated into two groups (n=3), control (0.5% carboxymethylcellulose, CMC) and luteolin (in 0.5% CMC), by intragastric gavage once a day for 14 consecutive days. Mice were housed in specific pathogen-free conditions in Animal Center of Zhejiang University School of Medicine. They were routinely fed and given free access to water. All experimental procedures strictly complied with the approval of the Laboratory Animals Ethics Committee of Zhejiang University. The health of the animals was monitored by measuring body weight. At the end of the experiments, the animals were sacrificed and the intestine were removed and cleaned with pro-cooled PBS, and then frozen in liquid nitrogen and stored at -80°C.

Western blot analysis

Preparation of cancer cells and intestinal cytosol were described as elsewhere (McMahon et al., 2001; Tang et al., 2011). Protein content was measured using bicinchoninic acid (BCA) method. The protein samples were subjected to SDS-PAGE and western blot was performed according to the standard protocol. The protein gray value was scanned on an Odyssey scanner (LI-COR Biosciences) and the band density were analyzed by its imaging system software. The relative levels of protein were calculated and normalized to actin.

Reduced glutathione assay

Reduced glutathione was measured as described previously, All operations should be conducted in the dark light and on ice (Wang et al., 2010).

Statistical analysis

The experimental data were presented as mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA by LSD test; $p<0.05$ was considered as statistically significant.

Results

Construction and identificationn of colorectal-oxaliplatin resistant cell line.

Our previously study indicated that pretreatment of colorectal cancer cells with oxaliplatin lead to multidrug resistance (MDR) and activation of Nrf2 signaling pathway might be one of important mechanism. In this study, we established the stable colorectal oxaliplatin resistant cell lines via long term exposed to oxaliplatin. Cytotoxicity was determined using MTS assay. Compare to the parent control, IC_{50}s of oxaliplatin, cisplatin and doxorubicin were increased by 2 folds approximately in oxaliplatin-resistant cell lines (Table 1). It is suggest that oxaliplatin-resistant cell lines were less sensitive to

Table 1. The IC_{50} of Chemotherapeutic Drugs in Human Colorectal Cell Lines

	Oxaliplatin (μM)	Cisplatin (μg/ml)	Doxorubicin (μM)
HCT 116	67.3±6.2	8.8±0.7	8.4±0.8
HCT 116-OX	148.0±7.6**	23.0±3.9**	17.2±1.9**
SW 620	108.6±8.9	14.6±0.9	6.8±0.4
SW620-OX	327.8±6.0**	33.1±5.3**	19.9±2.5*

*The human colorectal cells were treated with oxaliplatin (50-200 μM), cisplatin (3-30 μg/ml) and doxorubicin (1-10 μg) for 24 h. Cytotoxicity test were conducted by MTS assay. Compare to the parental cell lines (HCT116 or SW620), the IC 50 of oxaliplatin, cisplatin and doxorubicin were increased in the colorectal oxaliplatin resistant cell lines (HCT116-OX or SW620-OX), respectively. Results are from at least three separate experiments, Statistical evaluation was performed with one-way ANOVA test; **$p < 0.01$

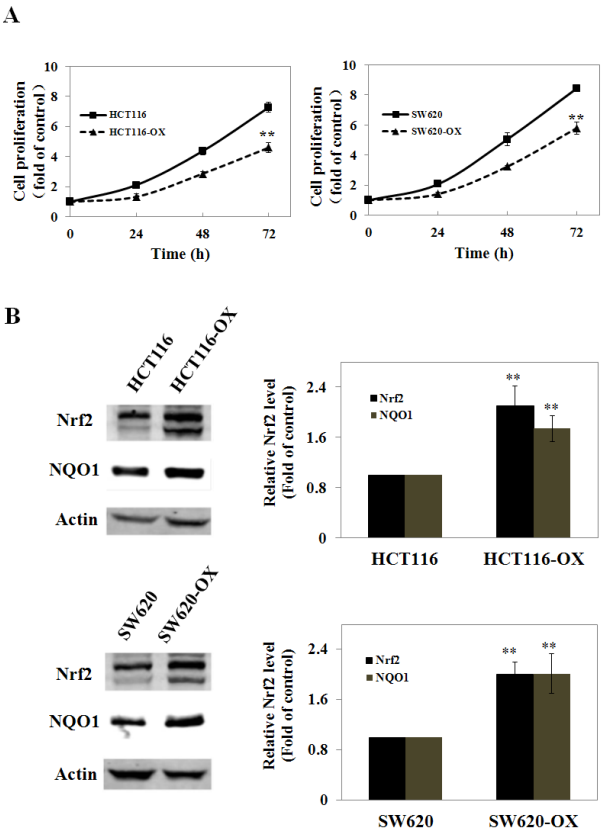

Figure 1. The IC_{50} of Chemotherapeutic Drugs in Human Colorectal Cell Lines. The human colorectal cells were treated with oxaliplatin (50-200 μM), cisplatin (3-30 μg) and doxorubicin (1-10 μg) for 24 h. Cytotoxicity test were conducted by MTS assay. Compare to the parental cell lines (HCT116 or SW620), the IC_{50} of oxaliplatin, cisplatin and doxorubicin were increased in the colorectal oxaliplatin resistant cell lines (HCT116-OX or SW620-OX), respectively. Results are from at least three separate experiments, Statistical evaluation was performed with one-way ANOVA test; **$p<0.01$

various chemotherapeutic agents and therefore developed a multidrug resistance.

Nrf2 activation in oxaliplatin-resistant colorectal cell lines

To investigate biological characteristics of oxaliplatin-resistant cell lines, we measured proliferation via cytometry and observed that cell proliferation of oxaliplatin-resistant cell lines were lower than the parent cell lines. The cell doubling time (TD) was 23.2 h in HCT116 cells and this was increased to 34.9 h in HCT116-

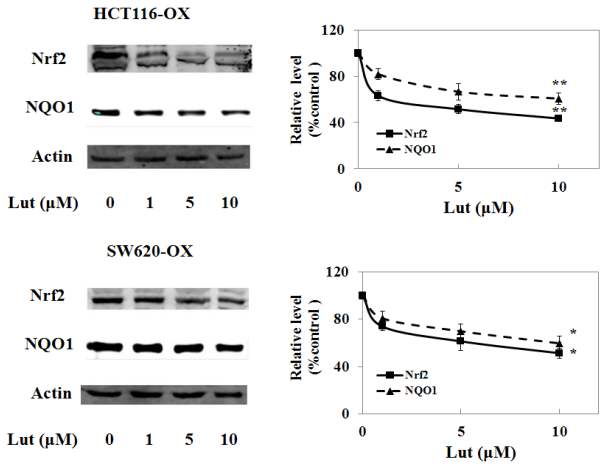

Figure 2. The Biological Characteristics of the Oxaliplatin Resistant Cell Lines. A). The proliferation rate of the oxaliplatin resistant cell lines were lower than their parent cell lines. Cells were seeded in 6-well plates at 3×10^4cells/well and cultured for 24, 48 and 72 h, respectively, The proliferation curve were drawed by cytometry method. The control (HCT116 or SW620) was set at 1. Values are mean±SD. Results are from at least three separate experiments. **B**). The Nrf2 pathway was activated in colorectal oxaliplatin resistant cell lines. Cell proteins were prepared as whole cell lysate protocol, Immunoblots of the expression of Nrf2 pathway. Nrf2 and NQO1 were normalized to actin. The immunoblots are typical of three replicates. Compare to the control, the level of Nrf2 and NQO1 were elevated in the colorectal oxaliplatin resistant cell lines (HCT116-OX or SW620-OX). Columns, mean (n = 3); bars, SD; Statistical evaluation was performed with one-way ANOVA test; **$p < 0.01$

OX cell lines. Likewise, TD was 21.9h in SW620 cells, and the TD was increased to 31.1 h in SW620-OX cells (Figure 1A). The phenomenon was also observed in other chemoresistant cell lines. We hypothesized that cell cycle modulation in oxaliplatin-resistant cell lines after long-term treatment with oxaliplatin may be affected and caused diminished cell proliferation as well as less chemotherapeutic sensitivity (O'Connell et al., 2000).

Then, we examined the expression of Nrf2 and its target gene NQO1 in the oxaliplatin resistant cell lines by immunoblotting. It was revealed that the expression of Nrf2 pathway in oxaliplatin resistant cell lines increased obviously. Compare to the control, the level of Nrf2 were elevated by 2.1 folds and NQO1 by1.7 folds in HCT116-OX. Similarly, the level of Nrf2 were elevated by 2.0 folds and NQO1 by 2.0 folds in SW620-OX (Figure 1B). It is demonstrated that the Nrf2 pathway has been activated in the the oxaliplatin resistant cell lines.

Luteolin attenuated activation of the Nrf2 pathway in oxaliplatin-resistant cell lines

Previous we reported that luteolin inhibits the Nrf2 pathway in nonsmall-cell lung cancer (NSCLC), we attempt to investigate whether inhibition occurs in oxaliplatin-resistant colorectal cells. The cell lines were treated with luteolin (1, 5 and 10μM) for 24 hours. Immunoblot analysis showd that luteolin treatment decreased the protein level of Nrf2 by 30-60% and NQO1 by 15-40% in HCT116-OX cell lines, respectively. With

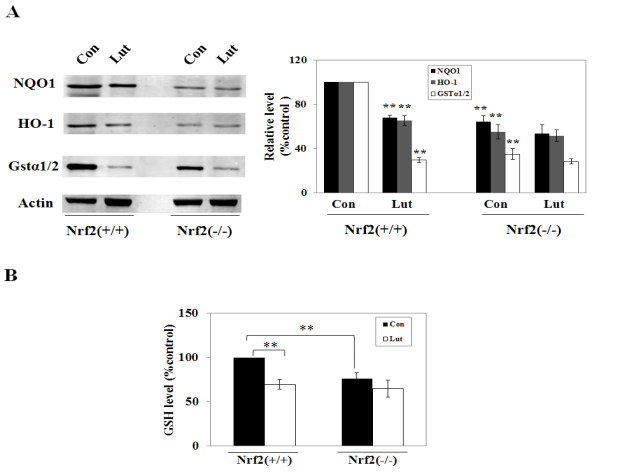

Figure 3. Luteolin Inhibited the Expression of Nrf2-Regulated Genes *in vivo*. A). Luteolin inhibited the expression of ARE-drivengenes *in vivo*. Male Nrf2+/+ and Nrf2-/- mice were intragastric gavage either CMC or luteolin (40 mg/kg) once a day for 14 days. Cytosol from the small intestine of wild-type and KO mice were analyzed by Western blotting, NQO1, HO-1, GSTα1/2 were normalized to actin. The immunoblots are typical of at least three replicates. **B)**. Luteolin reduced the level of glutathione *in vivo*. Cytosol from the small intestine of mice were analyzed for GSH levels. The control (CMC in wild-type mice) wasset at 100%. Values are mean±SD (n=3). Results are from at least three separate experiments. Statistical evaluation was performed with one-way ANOVA test. $^{**}p < 0.01$

the same concentration gradient, Luteolin treatment also decreased the protein level of Nrf2 by 20-50% and NQO1 by 15-40% in SW620-OX cell lines, respectively (Figure 2). Thus, luteolin inhibited expression of the Nrf2 pathway in oxaliplatin-resistant colorectal cells in a dose-dependent manner.

*Luteolin inhibits the expression of Nrf2-regulated genes **in vivo***

We confirmed that luteolin inhibits the Nrf2 pathway in oxaliplatin-resistant colorectal cell lines and we investigated whether this inhibitory effect on the Nrf2 pathway depended on Nrf2 in Nrf2+/+ and Nrf2-/- mice. we treated the mices with luteolin (40 mg/kg) for 14 days continuously. the small intestine were harvested and analyzed by Western blotting. luteolin could inhibits the expression of Nrf2 target genes in the small intestine of mice. Compare to the control group of Nrf2 +/+ mice, Luteolin treatment decreased the protein level of NQO1 by 32.1%, HO-1 by 34.7% and GSTα1/2 by 70.6% (Figure3 A). Next, we further determined the effect of luteolin on the level of GSH in the small intestine. compare to the control group. The GSH level was reduced by 29% in luteolin group. In contrast, there were no conspicuous responses in the Nrf2-/- mice (Figure 3B). that were consistent with the prior report (McMahon et al., 2001). Thus, luteolin mediated-inhibition is dependent of Nrf2.

Luteolin reduces resistance to anticancer drugs in oxaliplatin-resistant colorectal cancer cell lines

Adaptive activation of Nrf2 has been reported to contribute to chemoresistance in cancer cells. Thus, we

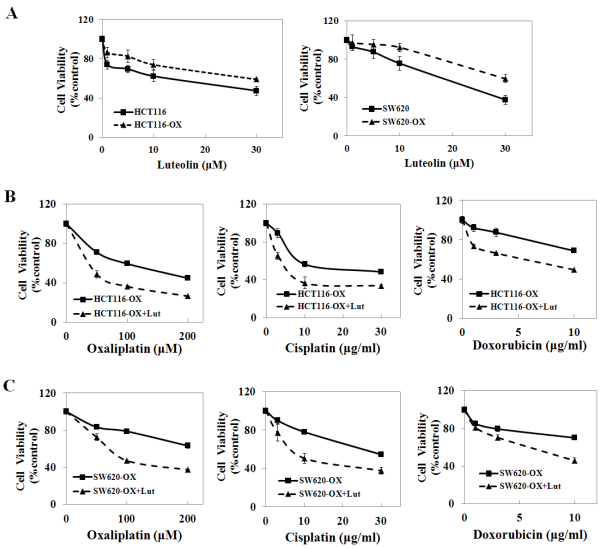

Figure 4. Luteolin Sensitizedthe Colorectal Oxaliplatin-Resistant Cell Lines to the Anticancer Drugs. A). The cytotoxicity of luteolin to the colorectal oxaliplatin resistant cell lines. The cell lines were treated with luteolin (1-30 μM) for 24 hours.The cell viability was measured by the MTS assay. Compared to the control (HCT116 or SW620). The IC_{50} of luteolin were increased in the colorectal oxaliplatin-resistant cell lines (HCT116-OX or SW620-OX). **B&C).** Luteolin sensitized the colorectal oxaliplatin-resistant cell lines to the anticancer drugs.The colorectal oxaliplatinresistant cell lines were treated with oxaliplatin (50-200 μM), cisplatin(3-30 μg/ml) and doxorubicin (1-10 μg/ml) alone or in combination with luteolin (5 μM) for 24h. The combination index (CI) values were below 1,which indicated that there were synergistic effect. Values are mean ±SD (n = 3). Results are from at least three separateexperiments. Statistical evaluation was performed with one-way ANOVA test; $^{*}p < 0.05$; $^{**}p < 0.01$

investigated whether luteolin influenced the susceptibility of oxaliplatin-resistant colorectal cell lines to therapeutic drugs via Nrf2 inhibition. We also measured luteolin cytotoxicity in the cell lines. The results showed that IC_{50}s of luteolin were largely raised in oxaliplatin-resistant cell lines, which demonstrated that the colorectal oxaliplatin resistant cell lines also developed resistance to luteolin (Figure 4A). Because 5 μM luteolin inhibited the Nrf2 pathway and had low cytotoxicity in oxaliplatin-resistant cell lines, we investigated luteolin-induced sensitization to chemotherapeutics using luteolin in combination with other anti-cancer drugs. In HCT116-OX cell lines, IC_{50}s in the oxaliplatin, cisplatin and doxorubicin were decreased by luteolin treatment from 153.3 μM, 25.6 μg/ml and 18.6 μg/ml to 78.1 μM, 6.9 μg/ml and 9.2 μg/ml, respectively. It also happened in SW620-OX cell lines, IC_{50}s of oxaliplatin, cisplatin and doxorubicin were decreased by luteolin treatment from 332.0 μM, 33.1 μg/ml and 21.1 μg/ml to 125.5 μM, 17.8 μg/m and 8.5 μg/ml, respectively (Figure 4B, C). In all combinations tested, combination index (CI) values were less than 1.0, suggesting synergy and that luteolin could sensitize oxaliplatin-resistant colorectal cancer cell lines to therapeutic drugs.

Discussion

Colorectal cancer is a common malignant

gastrointestinal tract cancer, and although oxaliplatin is a first-line chemotherapeutic for the disease, frequently colorectal cancer cells become resistant to oxaliplatin. Therefore, understanding the mechanisms of drug-resistance will enable us to better apply chemotherapy and reduce cell resistance. To investigate this, we induced drug resistance in a strain of colorectal cancer cells with oxaliplatin. The resistant cells were then treated with various chemotherapeutics and we observed multidrug resistance.

Oxaliplatin resistance may occur through several mechanisms. For example, mutations in tumor cells during proliferation may confer resistance. Oxaliplatin can induce mutations that confer resistance to future treatment. Also, multidrug resistance-related protein (MRP) activation may occur such that cancer drugs are transported out of tumor cells, decreasing therapeutic concentrations and efficacy (Conseil et al., 2005). Finally, Nrf2 activation can contribute to drug-resistance in digestive tract cancers. AKR1C was reported to be elevated in an oxaliplatin-resistant human gastric carcinoma cell line, and knockout of Nrf2 can decrease AKR1C expression and reverse drug-resistance to oxaliplatin in S3 cells (Chen et al., 2013). Moreover, we found that oxaliplatin activate the Nrf2 signaling pathway *in vitro* and *in vivo*. Therefore, tumor treatment via inhibition of the Nrf2 pathway using small molecules to sensitize drug-resistant cancer cells is a novel concept. Studies have reported that Apigenin (APG) can sensitize adriamycin-resistance cells (BEL-7402/ADM) by decreasing Nrf2, while the combination of APG and adriamycin can synergistically inhibit the growth of transplanted tumors (Gao et al., 2013). In addition, We also demonstrated that luteolin was a potential Nrf2 inhibitor and enhanced sensitivity of A549 cells to chemotherapeutics by promoting degradation of Nrf2 mRNA (Tang et al., 2011). Notably, luteolin had different effects on the Nrf2 signaling pathway in different cell types if activated Nrf2 expression in PC12 and C6 neural cells and protected them from oxidative stress mediated by ERK (Wruck et al., 2007; Lin et al., 2010). Luteolin can induce HO-1 expression in myocardial H9c2 cells and protect them by activating Akt and ERK to activate the Nrf2 signaling pathway (Sun et al., 2012).

Luteolin blocked Nrf2 signaling activation in drug-resistant cells and inhibited expression of Nrf2-targeted genes in small intestinal cells of wild type mice, but had no significant effects in knockout mice. Luteolin combined with other chemotherapeutics had greater anti-cancer activity and CI values were much less than 1.0, indicating synergistic action. Therefore, luteolin reversed drug-resistance by inhibiting Nrf2 expression and recovered its sensitivity to chemotherapeutics. Oxaliplatin-resistant cells used here are ideal for investigating mechanisms for drug-resistance in colorectal cancer, and future studies are warranted to investigate mechanisms of drug resistance in colorectal cancer and ideally its reversal *in vivo*.

Acknowledgements

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