Icotinib Enhances Bufalin-Induced Apoptosis via the Suppression of PI3K/Akt Signaling Pathway in Human Colon Cancer Cells

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ABSTRACT
This study sought to uncover whether icotinib can enhance bufalin-induced apoptosis of human colon cancer cells and the roles of PI3K/Akt signaling pathway in the apoptosis. The cell proliferation of human colon cancer cell lines RKO, HT29, CACO-2 and SW480 treated by bufalin was detected by MTT assay. Then, the apoptosis rate of RKO and SW480 cells treated by bufalin alone and combination of bufalin and icotinib was detected by flow cytometry. Afterwards, the levels of Cbl-b, p-AKT, p-ERK, PARP, Bax and Bcl-2 in RKO and SW480 cells were determined by Western blotting. Additionally, the effect of K-Ras silencing on the synergy of bufalin and icotinib was assessed. Bufalin decreased RKO, HT29, CACO-2 and SW480 (EX12 mutation) cell viability in a dose-dependent manner and induced apoptosis of RKO and SW480 cells in vitro. In the cells treated by combination of bufalin and icotinib, the levels of Cbl-b, cleaved PARP and Bax were increased, while the levels of p-Akt and Bcl-2 were reduced, comparing with that in the bufalin treated cells. Furthermore, K-Ras silencing (including EX12 mutated K-Ras and wild-type K-Ras) did not significantly affect the apoptosis rate of cells treated by the combination of bufalin and icotinib. Icotinib synergizes with bufalin to induce the apoptosis of colon cancer cells through PI3K/Akt signal pathway and regulating the apoptosis related proteins (Bcl-2, Cbl-b, cleaved PARP and Bax). K-Ras may not participate in the combination of bufalin and icotinib induced apoptosis in human colon cancer cells.

Key words: Colon cancer, PI3K/Akt, bufalin, icotinib, apoptosis

INTRODUCTION
Colon cancer is a common malignant tumor in digestive system (Allemani et al., 2015). Despite of improved therapy methods, the median overall survival of colon cancer patients is still low (Allemani et al., 2015; Tashiro et al., 2014). Therefore, it is urgent and needful to find new treatment strategies of this disease.

In recent years, the emerging targeted drugs contributes to the treatment of cancer to some extent. Bufalin, a Chinese medicine, is extracted from parotid venom glands and skin of toads and is able to induce apoptosis of colon cancer cells (Xie et al., 2011). A previous study has reported that bufalin induces autophagy-mediated cell death in CACO-2 and HT-29 human colon cancer cells through Reactive Oxygen Species (ROS) generation and e-Jun NH 2-terminal kinase (JNK)
activation (Xie et al., 2011). There is other evidence that bufalin induces apoptosis of colon cancer SW620 cells by transiently activating of p-stat3 that significantly inhibits the activation of stat3, activates caspase-3, up-regulates Bax (BCL2-associated X protein) expression, as well as down-regulates livin and Bcl-2 (B-cell CLL/lymphoma 2) expression (Zhu et al., 2012a). Icotinib is an epidermal growth factor receptor (EGFR) Tyrosine Kinase Inhibitor (TKI) that can specifically bind to the tyrosine kinase domain of EGFR, block the related signal conduction and thereby inhibit the growth of advanced Non-Small-Cell Lung Cancer (NSCLC) and other solid tumors (Zhao et al., 2011). However, it is unknown that whether icotinib can inhibit the growth of human colon cells in combination with bufalin.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is a downstream signaling pathway of EGFR and regulates many normal cellular processes including cell proliferation, apoptosis and motility that are critical for tumorigenesis (Vivanco and Sawyers, 2002; Sahlberg et al., 2012). There are evidences that bufalin induces cancer cell apoptosis by inhibiting PI3K/Akt pathway (Li et al., 2009; Zhu et al., 2012b; Kang et al., 2013). Besides, study has reported that PI3K/Akt pathway is involved in icotinib induced apoptosis of NSCLC cells (Mu et al., 2013). Furthermore, Fang and Liu (2001) reported that the ubiquitin ligase Cbl proto-oncogene B (Cbl-b) could regulate PI3K/Akt negatively through PI3K ubiquitination in T cells. Nevertheless, whether Cbl-b regulates the PI3K/Akt pathway during apoptosis of tumor cells, especially colon cancer cells is still unclear.

In the present study, the effect of bufalin and icotinib on the cell proliferation of human colon cancer cell lines RKO, HT29, CACO-2 and SW480 was assessed. Furthermore, the effect of bufalin and icotinib on the expression levels of several proteins associated with apoptosis (Cbl-b, phosphorylated Akt (p-Akt), phosphorylated ERK(p-ERK), poly (ADP-ribose) polymerase (PARP), Bel-2 and Bax) was detected. Besides, the effect of K-Ras (Kirsten rat sarcoma viral oncogene homolog) expression blocking on the synergy of bufalin and icotinib was detected. These findings might contribute to revealing the effect of icotinib on bufalin-induced apoptosis and provide new information for the clinical treatment of colon cancer.

**MATERIALS AND METHODS**

**Cell culture:** Human colon cancer cell lines RKO, HT29, CACO-2 and SW480 (EX12 mutation) purchased from Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 μg mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin in 5% CO₂ humidified atmosphere at 37°C. Once cells had reached confluence (passage 1), they were subcultured with 0.25% Trypsin-EDTA. Cells in the logarithmic phase were used to subsequent experiments.

**Methyl Thiazolyl Tetrazolium (MTT) assay:** Cultures were seperated into four groups: control (no medicine), bufalin group (treated with bufalin (Sigma, St Louis, Missouri, USA) at 5, 10, 20, 40, 80 and 160 nmol L⁻¹ for 24, 48 and 72 h, respectively), icotinib group (treated with icotinib (BETTA, Zhejiang, China) at 0.01, 0.1, 1, 10, 100 and 1000 μmol L⁻¹ for 72 h, respectively), bufalin+icotinib group (treated with icotinib (10 μmol L⁻¹) and bufalin (80 nmol L⁻¹) for 72 h). Cells were seeded at 1×10⁴ cells/well in 96-well plate and treated with bufalin (at 5, 10, 20, 40, 80 and 160 nmol L⁻¹, respectively) for 24, 48 and 72 h, respectively. Cells of the control were cultured in RPMI-1640 medium alone. Before termination of culture, MTT at 20 μL was added to each well for 4 h. Following 200 μL dimethyl sulfoxide (DMSO) being added in each well, the Optical Density (OD) was measured at 570 nmol L⁻¹ using a microplate reader (BioTek, Winooski, VT, USA). Each experiment repeated three times. The following formula was used:

\[
\text{Inhibition rate} = \frac{A_{570} \text{(experimental)}}{A_{570} \text{(control)}} \times 100
\]

Besides, the IC5₀ (half maximal inhibitory concentration) values for bufalin were determined.

**Flow cytometry analysis:** RKO and SW480 cells (1×10⁶ cells) in the control, bufalin and icotinib groups were fixed in 70% ice-cold ethanol at 4°C overnight. Then cells were washed two times with pre-cooled Phosphate Buffered Saline (PBS) and incubated with 20 μg mL⁻¹ RNase A for 30 min at 37°C, followed by staining with 10 μg mL⁻¹ Prodim Iodide (PI) for 30 min in the dark at 37°C. Finally, the samples were evaluated by a flow cytometry (BD Biosciences, San Jose, USA) and the data was analyzed by WinMDI software (provided by J. Trotter, The Scripps Research Institute, La Jolla, CA).

**Western blot analysis:** To assess the molecular mechanisms underlying the induction of apoptosis by bufalin (80 nmol L⁻¹) and icotinib (10 μM) in RKO and SW480 cells, the expression levels of Cbl-b, p-AKT, AKT, p-ERK, ERK, PARP, Bcl-2 and Bax were detected by Western blot. Approximately 1×10⁷ cells from bufalin group, icotinib group, bufalin+icotinib group and control group were harvested and lysed in RIPA lysis buffer (0.1% SDS, 1% Triton-100, 150 mmol L⁻¹ NaCl, 1 mmol L⁻¹ EDTA, 10 mmol L⁻¹ Tris-HCl (pH 7.5)) for 30 min in ice. Subsequently, the lysate was transferred to 1.5 mL eppendorf tubes, homogenized and then centrifuged at 12000 rpm for 30 min at 4°C. The supernatant was transferred to a fresh tube and mixed with equal volumes of Sodium Dodecyl Sulphate (SDS) sample
buffer and boiled for 10 min. Afterwards, an equal volume of sample (containing 50 μg of protein) was fractionated by 10% SDS-polyacrylamide gels (PAGE) and transferred onto nitrocellulose membrane for 2 h. After blocking non-specific binding sites with 5% skimmed milk in TBST (50 mmol L⁻¹ of Tris, 150 mmol L⁻¹ of NaCl, 0.1% Tween 20, pH 7.6) for 1 h, membranes were probed with mouse anti-human antibodies (p-ERK 1:1000, ERK 1:500, p-AKT 1:1000, AKT 1:1000, Cbl-b 1:250, PARP 1:1000, Bcl-2 1:300, Bax 1:300, β-actin 1:1000, all from Santa Cruz, California, USA) at 4°C overnight, washed four times with TTBS (10 mmol L⁻¹ of Tris, 150 mmol L⁻¹ of NaCl, 0.05% Tween 20, pH 7.4) and incubated with Horse Reddish Peroxidase (HRP)-conjugated secondary antibodies (1:800) for 30 min at room temperature. The membrane was visualized with ECL Western blotting analysis system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the protein expression levels were quantitated by image densitometry and ratios of (Cbl-b, p-AKT, AKT, β-actin) signals were statistically analyzed, respectively.

K-Ras gene silencing: To investigate whether K-Ras gene mutation play a role in the synergistic effect of bufalin and icotinib, RNA interference technique was used to knockdown the endogenous K-Ras protein expression in RKO and SW480 (EX12 mutation) cells. The shRNA-K-Ras/pRNA-U6.1, which is a expressing vector of eukaryotic plasmid containing targeted short hairpin loop of K-Ras gene, was constructed by Genscript (Nanjing, China) according to the protocol previously published (Qu et al., 2009). The expressing vectors were transfected into human colon cancer cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. After transfection for 48 h, the cells were switched using the medium containing G418 (600 μg mL⁻¹). The colonies, in which the expression of K-Ras was less than 10% of control group, was selected as the positive colonies and 3-5 positive colonies with different inhibition ratio were chosen for further study. The shRNA/pRNA-U6.1, as a control vector, was constructed at the same time. At 48 h after transfection, the cells were treated with 80 nmol L⁻¹ bufalin in the absence or the presence of 10 μmol L⁻¹ icotinib for 24 h.

Statistical analysis: Data was presented as Mean±Standard Deviation (SD) from three separate experiments performed in duplicate. Statistical analysis of inhibition rate of colon cancer cells was performed by t-test; statistical analysis of apoptotic rate of colon cancer cells was performed by chi-square test and statistical analysis of relative expression level of proteins was performed by one-way analysis of variance. All statistical analyses were conducted using SPSS version 16.0. Differences were considered significant if p<0.05.

RESULTS

Bufalin inhibits colon cancer cell proliferation: The MTT results demonstrated that bufalin significantly inhibited the proliferation of RKO (Fig. 1a), HT29 (Fig. 1b), CACO-2 (Fig. 1c) and SW480 cells (Fig. 1d) in time/dose dependent manners following incubation (p<0.05). The maximum inhibition of bufalin was 57.34±3.64, 57.92±3.76, 66.5±4.74 and 50.8±4.18% for RKO, HT29, CACO-2 and SW480 cells, respectively. Besides, the IC50 of bufalin on CACO-2 cells at 48 and 72 h was the lowest (80.71 and 60.54 nmol L⁻¹, respectively), compared with that on others (Table 1). However, icotinib alone at the concentrations tested for 72 h did not significantly inhibit proliferation of colon cancer cells (p>0.05, Fig. 1c). Icotinib (10 μmol L⁻¹) and bufalin (80 nmol L⁻¹) showed clearly synergistic effect on inhibition of proliferation of RKO, HT29, CACO-2 and SW480 cells at 72 h following incubation (p<0.01, bufalin+icotinib vs. icotinib; p<0.05, bufalin+icotinib vs. bufalin, Fig. 1f). Among them, the synergistic effect on CACO-2 cells was most significant.

Icotinib enhanced bufalin-induced apoptosis in RKO and SW480 cells: Exposure of RKO cells to bufalin (40 and 80 nmol L⁻¹) for 24 h resulted in cell cycle arrest at the G2/M phase and the apoptotic rate in Sub-G1 phase representing early apoptosis was 5.4 and 7.8%, respectively (Fig. 2a). When cells were exposed to icotinib (10 μmol L⁻¹) for 24 h, icotinib by itself had little effect on apoptosis of RKO and SW480 cells, while it significantly enhanced bufalin-induced apoptosis in RKO cells (5.4 vs. 11.1%; 7.8 vs. 19.7%, p<0.05, Fig. 2a) and SW480 cells (6.9 vs. 14.1%; 11.7 vs. 23.5%, p<0.05, Fig. 2b), respectively.

Bufalin regulates the expression of proteins related to apoptosis in RKO and SW480 cells: The relative expression level of p-ERK was relatively stable in either bufalin-treated RKO cells or bufalin-treated SW480 cells. Reversely, there was a significant transient increase of relative p-AKT inhibition ratio were chosen for further study. The shRNA/pRNA-U6.1, as a control vector, was constructed at the same time. At 48 h after transfection, the cells were treated with 80 nmol L⁻¹ bufalin in the absence or the presence of 10 μmol L⁻¹ icotinib for 24 h.

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Table 1: Cytotoxicity of bufalin and the mutation status of K-Ras gene in human colon cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>K-Ras gene</th>
<th>Bufalin IC₅₀ (mmol L⁻¹)</th>
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<tbody>
<tr>
<td>RKO</td>
<td>WT</td>
<td>133.50±8.28</td>
</tr>
<tr>
<td>HT29</td>
<td>WT</td>
<td>219.77±6.33</td>
</tr>
<tr>
<td>CACO-2</td>
<td>WT</td>
<td>80.71±3.28</td>
</tr>
<tr>
<td>SW480</td>
<td>EX12 mutation</td>
<td>207.40±5.27</td>
</tr>
</tbody>
</table>

Values shown are Mean±SD for 3 separate experiments performed in duplicate, WT: Wild type
expression level in both RKO and SW480 cells at 3 and 6 h (p<0.05), while over time, a distinct decrease was observed. Besides, the relative expression levels of Cbl-b were increased steadily and significantly (p<0.05) both in RKO and SW480 cells during the bufalin treatment (Fig. 3a and b).

**Concurrent treatment of bufalin and icotinib modulates the expression of proteins associated with apoptosis in RKO and SW480 cells:** Bufalin alone significantly increased the expression of Cbl-b (p<0.05) and also decreased Akt phosphorylation (p<0.05). Concurrent exposure to bufalin and icotinib resulted in a strong inhibition of Akt phosphorylation.
Fig. 2(a-b): Icotinib enhanced bufalin-induced apoptosis in colon cancer cells, (a) RKO and (b) SW480 cells were exposed to bufalin, icotinib and combination of bufalin and icotinib for 24 h. Sub-G1 phase: Early apoptosis cells, Bu40: 40 mmol L\textsuperscript{-1} bufalin, Bu80: 80 mmol L\textsuperscript{-1} bufalin, Bu160: 160 mmol L\textsuperscript{-1} bufalin, Ico10: 10 μmol L\textsuperscript{-1} icotinib. The percentage of apoptotic cells was analyzed by flow cytometry with PI staining. Data is a typical representative of three independent experiments and up-regulation of Cbl-b (p<0.05, Fig. 4a and b). Meanwhile, icotinib alone failed to induce the cleavage of PARP (p>0.05), which represented an active apoptotic process in these cells. Combining icotinib with bufalin enhanced the expression of cleaved PARP in both RKO and SW480 cells (p<0.05, Fig. 4a and b). Furthermore, the anti-apoptotic protein Bel-2, which plays a major role in evading apoptosis and prolonging survival of cancer cells, was substantially
Fig. 3(a-d): Bufalin induced apoptosis of RKO and SW480 cells in an Akt-dependent mechanism, (a) RKO and (b) SW480 cells were treated with bufalin (80 nmol L\(^{-1}\)) for 0-24 h, relative expression level of (c) RKO and (d) SW480 cells. The expression of Cbl-b, p-AKT, AKT, p-ERK and ERK proteins was analyzed by Western blotting. β-actin was used as the internal control. Band densitometry analysis of Cbl-b, p-AKT and p-ERK expression in RKO cells and SW480 cells normalized to β-actin, respectively. *p<0.05 vs. untreated control. Data is Mean±SD of three independent experiments.

**DISCUSSION**

Bufalin, which is obtained from parotid venom glands and skin of the toad, is one of the effective anticancer drugs, which primarily induce apoptosis by regulating apoptosis-associated signaling pathway (Khoo et al., 2010; Li et al., 2011). In the present study, bufalin decreased RKO, HT29, CACO-2 and SW480 (EX12 mutation) cell viability in a dose-dependent manner and induced apoptosis of RKO and SW480 cells *in vitro*, which was enhanced by icotinib. Furthermore, the expression levels of Cbl-b, p-AKT, PARP, Bax and Bcl-2 were significantly altered in RKO and SW480 cells treated by bufalin alone.

In our research, during the treatment of bufalin (80 nmol L\(^{-1}\)) on RKO and SW480 cells, there was a significant transient upregulation of p-AKT, followed by reduction of p-AKT and upregulation of Cbl-b. Increasing evidences have demonstrated that many cytotoxic drugs induce...
Fig. 4(a-d): Inhibit the PI3K/Akt signaling pathway was involved in the synergistic effect of bufalin and icotinib, (a) RKO and (b) SW480 cells were untreated, treated with 10 μmol L⁻¹ icotinib and/or 80 nmol L⁻¹ bufalin for 24 h, relative expression level of (c) RKO and (d) SW480 cells. The expression levels of Cbl-b, p-AKT, AKT, PARP, Bax and Bcl-2 were analyzed by Western blotting. β-actin was used as the internal control. Band densitometry analysis of Cbl-b, p-AKT, PARP, Bax and Bcl-2 expression in RKO cells and SW480 cells normalized to β-actin, respectively. *p<0.05 vs untreated control. Data is Mean±SD of three independent experiments.

Apoptosis in cancer cells via the inhibition of the PI3K/Akt pathway, which plays pivotal roles in mammalian cell survival and anti-apoptosis (Sos et al., 2009; Takezawa et al., 2011; De Luca et al., 2012; Harashima et al., 2012; Santarpia et al., 2012; Zhu et al., 2012a). Cbl-b, an upstream modulator of PI3K, can induce ubiquitination and degradation of PI3K (Qu et al., 2009). Furthermore, Cbl-b can inhibit the phosphorylation of Akt (Nakao et al., 2009). Cbl-b was significantly upregulated after cell treated with bufalin (80 nmol L⁻¹ L⁻¹) for 24 h. We speculated that bufalin might enhance the ubiquitination of PI3K by up-regulating Cbl-b and thereby inhibit the activation of Akt. Surprisingly, no alteration in the phosphorylation of ERK was observed in our study. A previous study found that the anomalous activation of ERK signal was essential for apoptosis induced by bufalin in the U937 cells (Watabe et al., 1996). For such discordant results, we speculated that different experimental cells might cause different effects on the ERK pathway; and it also might be associated with cell-specific effects of bufalin. Taken together, the PI3K/Akt rather than ERK signaling pathway might play a significant role in modulating bufalin-induced apoptosis in colon cancer cells.

Our data showed that icotinib slightly inhibited proliferation and induced apoptosis in colon cancer cells.
Fig. 5(a-b): Blocking of K-Ras expression does not affect the synergistic effect of bufalin and icotinib, (a) RKO and (b) SW480 (EX12 mutation) cells were transiently transfected with K-Ras specific shRNA for 48 h, followed by 80 nmol L\(^{-1}\) bufalin and combination of 80 nmol L\(^{-1}\) bufalin and 10 μmol L\(^{-1}\) icotinib for 24 h. The expression of K-Ras proteins was analyzed by Western blotting. Apoptosis was analyzed as a sub-G1 fraction by flow cytometric with PI staining. Data is Mean±SD of three independent experiments.

However, icotinib significantly enhanced bufalin-induced apoptosis in RKO and SW480 cells, suggesting that there was a synergistic effect between bufalin and icotinib. Furthermore, concurrent exposure to bufalin and icotinib resulted in the greatest inhibition of Akt phosphorylation and Bcl-2 expression, as well as up-regulation of Cbl-b, cleaved PARP and Bax in both cancer cells. The Bcl-2 (B-cell CLL/lymphoma 2) is an integral outer mitochondrial membrane protein that impedes the apoptosis of cells (Kroemer, 1997). There is evidence that both bufalin and icotinib hydrochloride can reduce the expression of the Bcl-2 protein and increase the expression of the Bax protein (Bcl-2-associated X protein) (Zhu et al., 2012b; Yang et al., 2013). Besides, Cbl-b was up-regulated and Akt phosphorylation was inhibited in icotinib-induced apoptosis of NSCLC (Mu et al., 2013). No study has reported that altered expression of cleaved PARP (ADP-ribose) polymerase in the icotinib-induced apoptosis so far. Collectively, icotinib might enhance the effect of bufalin-induced apoptosis by affecting the expression of apoptosis related proteins.

K-Ras is a Kirsten ras oncogene homolog that is a downstream mediator of EGFR-induced cell signaling. Previous studies have demonstrated that Colon tumors with K-ras codon 12 mutations have lower levels of apoptosis (Guerrero et al., 2000) and K-Ras mutations are associated with a lack of sensitivity to anti-EGFR drugs such as cetuximab and gefitinib (Park et al., 2010; Garcia-Alfonso et al., 2014). Our data demonstrated that combination of bufalin and icotinib significantly increased drug sensitivity of the cells comparing with bufalin alone and blocking of wild-type K-Ras and EX12 mutated K-Ras expression did not affect the synergistic effect of bufalin and icotinib. The results indicated that the K-Ras may not participate in the combination of bufalin and icotinib induced apoptosis in human colon cancer cells.

In conclusion, study provides supportive evidences that icotinib synergizes with bufalin to inhibit the activation of PI3K/Akt signal pathway by regulating Cbl-b and p-Akt, which is correlated with down regulated Bcl-2 expression, up-regulated cleaved PARP and Bax expression, resulting in
the apoptosis of colon cancer cells. This synergistic effect is not affected by K-Ras gene silencing.

**CONCLUSION**

Icotinib synergizes with bufalin to induce the apoptosis of colon cancer cells through PI3K/Akt signal pathway and regulating the apoptosis related proteins (Bcl-2, Cbl-b, cleaved PARP and Bax). The K-Ras may not participate in the combination of bufalin and icotinib induced apoptosis in human colon cancer cells.

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