SHORT COMMUNICATION

Apoptosis induced by DICO, a novel non-aromatic B-ring flavonoid via a ROS-dependent mechanism in human colon cancer cells

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ABSTRACT
5,7-Dihydroxy-2-(1,2-isopropylidioxy-4-oxo-cyclohex-5-enyl)-chromen-4-one (DICO) is a novel non-aromatic B-ring flavonoid, isolated mainly from \textit{Macrothelypteris viridifrons} and has anti-tumour properties. In this study, we investigated the cytotoxicity and underlying biochemical pathways leading to cell death, in response to DICO treatment of a human colon cancer cell line HT-29. Our results indicated that DICO induced apoptosis by elevating the generation of reactive oxygen species, which could be quenched by the antioxidants N-acetyl cysteine. In addition, activation of signal transducer and activator of transcription 3 and suppression of nuclear factor kappa B played a crucial role in DICO-induced apoptosis. Overall, our results provide mechanistic insights into the apoptotic action of a potential anti-tumour drug, DICO.

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1. Introduction

Macrothelypteris viridifrons (Tagawa) Ching is widely found in southern China and has been used as a folk medicine for the treatment of diseases, such as cancer, hydropsy, and traumatic bleeding. (Ding 1982). In our previous report, the anti-tumour and anti-angiogenic effects of M. viridifrons were confirmed, and the phytochemical and pharmacological potential of its total protoflavones were preliminarily studied (Wei et al. 2012, 2019). These results supported that protoflavones, as the characteristic constituents of M. viridifrons, are worthy of further development and systematic research.

Protoflavones, a less widespread, unique class of natural flavonoids with a non-aromatic B-ring and a hydroxyl group at C-1, exert significant anti-tumour activities, which have attracted increasing interest from scientists because of their novel structure and potent activity (Liu et al. 2011; 2012; Hunyadi et al. 2014; Csekes et al. 2020). It is a typical example of protoapigenone, that inhibits the growth of various of cancer cells in vitro and in vivo (Chang et al. 2008a, 2008b; Chen et al. 2011). To date, almost all chemical compounds of protoflavones have been isolated from the genus Macrothelypteris (Fu et al. 2009; Tang et al. 2009; Fang et al. 2011; Wei et al. 2011a, 2011b). In our phytochemical study, 5,7-Dihydroxy-2-(1,2-isopropylidenoxy-4-oxo-cyclohex-5-enyl)-chromen-4-one (DICO) (shown in Figure S1), a protoflavone isolated from M. viridifrons, induced significant cytotoxicity, potential G2/M cell cycle arrest, and apoptosis in hepatoma cells (Wei et al. 2011a, Zhou et al. 2013). In this study, we examined whether DICO could manipulate the signalling transduction via reactive oxygen species (ROS), and evaluated the underlying molecular mechanisms of DICO-induced cytotoxicity in a human colon cancer cell line (HT-29).
2. Result and discussion

2.1. DICO induced apoptosis and cell cycle arrest in HT-29 cells

We evaluated the cytotoxicity of DICO using the MTT assay in six human cancer cell lines - HepG2 (liver), PC-3 (prostate), A549 (lung), MCF-7 (breast), MOLT-4 (leukaemia), and HT-29 (colon). The results indicated that DICO significantly inhibited the growth of all cancer cells in a concentration-dependent manner. Of all cell lines tested, HT-29 cells showed the highest sensitivity to DICO (IC50 values of 6.28 and 4.13 µg/mL at 24 h and 48 h respectively) and were therefore chosen for further follow-up. As well as DICO exhibited less cytotoxic to normal human colon mucosal epithelial cell NCM460. When treatment with 12.5 µg/mL DICO for 24 h and 48 h, the cell viability of NCM460 were 68% and 56%, respectively. Flow cytometry was performed to investigate the effects of pyrrolidine dithiocarbamate (PDTC, nuclear factor kappa B [NF-κB] inhibitor) and N-acetyl cysteine (NAC, ROS scavenger) in combination with DICO (5.0 µg/mL) or DICO alone for 24 h on the cell cycle and apoptosis in HT-29 cells. As shown in Figure S2(A) and S2(B), treatment with DICO significantly increased apoptosis and cell cycle arrest at the G2/M phase. When the cells were pre-treated with PDTC, the fraction of apoptotic cells and cells arrested in the G2/M phase increased by approximately 1.5- and 2.1-fold respectively (p < 0.05). However, pre-treatment with NAC significantly reversed the DICO-induced G2/M cell arrest and apoptosis.

2.2. Effect of DICO on intracellular reactive oxygen species (ROS) in HT-29 cells

To confirm the relationship between DICO-induced HT-29 cell death and oxidative stress, we measured ROS levels by flow cytometric analysis of an oxidant-sensitive fluorescent probe dichlorofluorescein-diacetate (DCFH-DA) (Feng et al. 2021). Figure S2(C) shows that the intracellular levels of DCFH-DA increased by 1.8- and 3.0-fold upon treatment with 5.0 µg/mL DICO alone or with DICO and PDTC respectively (p < 0.05), relative to untreated controls. The elevation in ROS levels with DICO treatment was significantly diminished by the presence of NAC with 32 percent.

2.3. Expression of apoptosis-associated proteins in DICO-treated HT-29 cells

We investigated the effect of DICO treatment on the expression of several apoptosis- and cell cycle-related proteins by western blotting, including phosphor-IkB, phosphor-signal transducer and activator of transcription 3 (STAT3), p53, p21, cyclin B1, phosphor-CDK1, Bcl-2, and Bax (Figure S2(D) and S2(E)). Exposure of HT-29 cells to DICO for 24 h resulted in an increase in the Bax/Bcl-2 ratio in a concentration-dependent manner, which was in agreement with earlier studies in HepG2 cells (Zhou et al. 2013). Since the complex CDK1/cyclin B1 promotes G2/M transition, the phosphorylation of CDK1 and downregulation of cyclin B1 expression following DICO treatment may be responsible for cell cycle arrest in G2/M phase coupled with a concurrent increase in the levels of p53 and p21 proteins. DICO treatment induced a dose-dependent decrease in the levels of phosphor-STAT3, revealing the key role of activation of STAT3.
signalling in DICO-induced HT-29 apoptosis. DICO also activated NF-κB likely via enhanced phosphorylation and subsequent degradation of IκB.

2.4. Effects of NF-κB inhibitors and ROS scavengers on the expression of apoptosis-associated molecules

In order to better understand the roles of ROS, STAT3, and NF-κB in DICO-induced apoptosis of HT-29 cell, we analysed the expression of key proteins in the presence or absence of PDTC or NAC. Compared to cells treated with DICO alone, there were no significant differences in the protein levels of phosphor-STAT3, NF-KB, and p53 in the NAC pre-treatment group. However, pre-treatment with PDTC in HT-29 cells dramatically downregulated the protein levels of NF-KB and phosphor-STAT3, which was accompanied by an increase in the level of p53 protein. These results indicated that NAC and PDTC mitigated the DICO-induced apoptosis via different mechanisms.

3. Conclusions

The present study demonstrated that DICO induced apoptosis and G2/M cell cycle arrest via the negative regulation of STAT3 signal transduction in an ROS-dependent manner. Additionally, activation of NF-κB may play a key role in DICO-induced apoptosis. We summarize the molecular mechanisms of DICO-induced apoptosis in HT-29 cells in the model as follows in Figure 1.

Figure 1. The molecular mechanisms of DICO-induced apoptosis in HT-29 cells.

There are several limitations in our study. Firstly, our experiments were mainly performed in vitro, we need to further confirm the anti-tumour effect of DICO on animal model. Meanwhile, we focus on the effects of NF-κB inhibitors and ROS scavenger inhibitors on the expression of apoptosis-associated molecules, the underlying signal pathway need to research with cell transfection, gene knockout mice, or rescue
experiment. In conclusion, this is the first report on the mechanism of DICO-induced cytotoxicity in human colon cancer cells via ROS, STAT3, and NF-κB signalling. Our work lays out a framework for the discovery and mechanistic dissection of promising natural compounds for cancer treatment.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Supplementary material**

The experimental section, Figure S1 and S2 and the uncropped raw data are available in the supplementary materials.

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