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# Quercetin sensitizes pancreatic cancer cells to TRAIL-induced apoptosis through JNK-mediated cFLIP turnover

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent that can selectively kill cancer cells. Nonetheless, many cancers are resistant to TRAIL, and the molecular mechanisms of TRAIL resistance in cancer, particularly pancreatic cancer, are still unclear. In this study, we tested the hypothesis that quercetin, a flavonoid, induces apoptosis in TRAIL-resistant pancreatic cancer cells. Although quercetin alone had no significant cytotoxic effect, when combined with TRAIL, it promoted TRAIL-induced apoptosis that required mitochondrial outer membrane permeabilization. A BH3-only protein BID knockdown dramatically attenuated TRAIL/quercetin-induced apoptosis. The expression levels of cellular FLICE-like inhibitory protein (cFLIP) decreased in a dose-dependent manner in the presence of quercetin, and overexpression of cFLIP was able to robustly rescue pancreatic cancer cells from TRAIL/quercetin-induced apoptosis. Additionally, quercetin activated c-Jun N-terminal kinase (JNK) in a dose-dependent manner, which in turn induced the proteasomal degradation of cFLIP, and JNK activation also sensitized pancreatic cancer cells to TRAIL-induced apoptosis. Thus, our results suggest that quercetin induces TRAIL-induced apoptosis via JNK activation-mediated cFLIP turnover.


## 1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant tumors. It is the fourth leading cause of cancer-related deaths in the western world, and the 5-year survival rate has barely improved in the past decades (Jemal et al., 2010). One reason for the dismal prognosis of PDAC is the resistance to cytotoxic chemotherapy, radiotherapy, and immunotherapy (Hamacher et al., 2008). One of the hallmarks of cancer is the inherent or acquired resistance to apoptosis. Deregulation of apoptosis is an important pathological factor of various human diseases (Sayers, 2011). Therefore, there is a growing need for a better understanding of the molecular mechanisms underlying tumor resistance to apoptotic cell death in order to design more effective therapies for pancreatic cancer.

Apoptosis is a regulated distinct form of cell suicide that enables homeostasis in a multicellular organism by eliminating no longer needed and damaged cells. There are two well-characterized signaling pathways that activate caspase cascades, leading to activation of apoptosis: the death receptor (extrinsic) pathway and mitochondrial (intrinsic) pathway. The extrinsic pathway is mediated by various death receptors, which are activated by binding of death ligands belonging to the TNF family including tumor necrosis factor (TNF) α, FasL, and TRAIL (Walczak and Krammer, 2000). The binding of death ligands to their receptors initiates recruitment of intracellular FADD and procaspase-8, which cluster into the death-inducing signaling complex (DISC) (Ashkenazi and Dixit, 1998). The formation of DISC promotes activation of procaspase-8, and after that, the latter activates the effector caspase-3, leading to apoptosis (Ashkenazi and Herbst, 2008). The intrinsic pathway is activated by intracellular stress and involves an imbalance of

*Abbreviations:* AIF, apoptosis-inducing factor; cFLIP, FLICE-like inhibitory protein; AMPK, AMP-activated protein kinase; DISC, death-inducing signaling complex; FITC, fluorescein isothiocyanate; JNK, c-Jun N-terminal kinase; PDAC, Pancreatic ductal adenocarcinoma; MMP, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeabilization; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; Smac/DIABLO, second mitochondrial activator of caspases/direct IAP-binding protein with low pI; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone.

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pro- and anti-apoptotic members of the BCL-2 protein family (Adams and Cory, 2007). This imbalance then activates pro-apoptotic BCL-2 family members including BAX and BAK, which induce mitochondrial outer membrane permeabilization (MOMP) (Son et al., 2010). After MOMP, apoptotic proteins are released into the cytoplasm, including cytochrome *c*, apoptosis-inducing factor (AIF), and second mitochondrial activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO) (Coultas and Strasser, 2003). The released cytochrome *c* binds to Apaf-1, and this event leads to formation of the apoptosome, which activates procaspase-9. Active caspase-9 activates the effector caspase-3, leading to apoptosis.

TRAIL was discovered as a pro-apoptotic ligand that belongs to the TNF gene family (Pitti et al., 1996). TRAIL has emerged as a cancer therapeutic agent because cancer cells are susceptible to TRAIL while normal cells are relatively insensitive to it (Walczak et al., 1999). Despite its promise as a cancer therapeutic agent, the intrinsic or acquired resistance to TRAIL is a serious problem for TRAIL-based therapy. Many studies have revealed the mechanism that renders cancer cells resistant to TRAIL-induced apoptosis. TRAIL decoy receptors, TRAIL-R3 and TRAIL-R4, have been shown to inhibit TRAIL-induced apoptosis by competing with TRAIL-R1 (death receptor 4) and TRAIL-R2 (death receptor 5) for TRAIL (LeBlanc and Ashkenazi, 2003). TRAIL-induced apoptosis is also inhibited by overexpression of cFLIP, which competes with procaspase-8 for binding to FADD, thereby blocking cell death (Longley et al., 2006). In addition, overexpression of antiapoptotic BCL-2 family members including BCL-2, BCL-xL, and MCL-1 is known to contribute to TRAIL resistance (Chawla-Sarkar et al., 2004). Thus, there is a strong impetus to understand the mechanisms underlying TRAIL resistance in cancer cells.

Flavonoids, a group of phytochemicals, have been reported to have antiviral, anti allergic, anti-inflammatory, anticancer, and antioxidant activities. Quercetin, a member of the flavonoid family and a natural free-radical scavenger, has been shown to play a critical role in a variety of cellular processes, including apoptosis, autophagy, and cell migration (Senthilkumar et al., 2011). Particularly, quercetin induces the dissociation of BAX from BCL-xL, leading to activation of caspases; it also enhances TRAIL-induced apoptosis in prostate cancer cells via upregulation of death receptor 5 (DR5) (Jung et al., 2010). Quercetin treatment results in the activation of apoptosis via AMP-activated protein kinase (AMPK) activation and p53-dependent apoptotic cell death in HT-29 colon cancer cells (Kim et al., 2010). Furthermore, quercetin induces TRAIL-induced apoptosis by inhibiting survivin expression through ERK-MSK1-mediated deacetylation of histone H3 (Kim et al., 2008b). However, the precise molecular mechanisms underlying the regulation of cFLIP by quercetin during TRAIL-induced apoptosis remain unclear.

In this study, our aim was to explore the mechanisms responsible for TRAIL resistance in pancreatic cancer cells. We found that quercetin induces the proteasomal degradation of cFLIP through JNK activation, leading to induction of TRAIL-induced apoptosis. Thus, our findings suggest that cFLIP may be a promising target for TRAIL-based therapy.

## 2. Materials and methods

### 2.1. Cell culture and reagents

All cell lines were acquired from the American Type Culture Collection or the German Collection of Microorganisms and Cell cultures and were tested regularly for mycoplasma contamination. All the cells were maintained in a humidified atmosphere containing 5% of $CO_2$ at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% of FBS (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA, USA). Quercetin (A15807) was purchased from Alfa Aesar, and zVAD-fmk (FMK001) was obtained from R&D Systems (Minneapolis, MN, USA). Tetramethylrhodamine ethyl ester (TMRE) (T669) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anisomycin (1290) was purchased from Tocris Bioscience (Avonmouth, Bristol, UK). Bortezomib (1846) was obtained from Biovision Incorporated (Milpitas, CA, USA).

### 2.2. Lentiviral-mediated short hairpin RNA (shRNA) transfection

The RNAi Consortium clone IDs for the shRNAs used in this study are as follow: BID: TRCN0000312746.

### 2.3. Western blotting

Cells were lysed in RIPA lysis buffer containing a protease inhibitor cocktail Sigma-Aldrich (St Louis, MO, USA), and the lysates were assayed for protein concentration by the BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of lysates (by protein) were mixed with Laemmli loading dye and boiled for 10 min. The lysates were subjected to SDS-PAGE, and the proteins were transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20 (TBS-T), prior to incubation with the primary antibody overnight at 4 °C and then washed with TBS-T followed by exposure to the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. The immune complexes were visualized on Kodak X-ray film using the Enhanced Chemiluminescence (ECL) Detection System (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies to BCL2 (2870), BCL-xL (2764), BAD (9232), BAK (6947), BAX (2772), BID (2002), caspase-3 (9662), cleaved- caspase-3 (9664), caspase-8 (9746), caspase-9 (9502), PARP (9542), MCL-l (5453), P-JNK (4668), and P-cJUN (9261) were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-FLIP antibody (ALX-804-961-0100) was acquired from Enzo Life Science. Anti-cJUN (SC1694) and anti-JNK (SC7345) antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA), and antibody to DR4 (1139) and DR5 (2019) was obtained from ProSci Inc (Poway, CA, USA).

### 2.4. The annexin V/PI assay

Apoptotic cell death was detected by the Annexin-V/fluorescein isothiocyanate (FITC) assay. Cells were harvested by trypsinization and washed with PBS, and pelleted cells were resuspended in annexin-V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and labeled with annexin-V, fluorescein isothiocyanate (FITC), and propidium iodide (PI). FITC- and/or PI-labeled cell populations were counted on a flow cytometer (Beckman-Coulter).

### 2.5. Digitonin-based subcellular fractionation

Cells were washed with PBS and lysed for 10 min on ice in 100 μL of digitonin lysis buffer (75 mM KCl, 1 mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$, 250 mM sucrose, 60 μg/mL digitonin) containing the protease inhibitor cocktail. The supernatants (cytosol) were obtained by centrifugation at 15,000 × *g* for 10 min. The remaining pellet (mitochondria) was then resuspended in lysis buffer containing the protease inhibitor cocktail and incubated for 20 min on ice.