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Letters

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Direct Binding of Bcl-2 Family Proteins by Quercetin Triggers Its Pro-Apoptotic Activity

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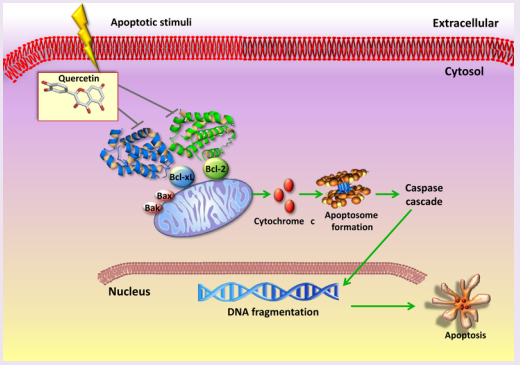
Supporting Information

ABSTRACT: Bcl-2 family proteins are important regulators of apoptosis and its antiapoptotic members, which are overexpressed in many types of cancer, are of high prognostic significance, establishing them as attractive therapeutic targets. Quercetin, a natural flavonoid, has drawn much attention because it exerts anticancer effects, while sparing normal cells. A multidisciplinary approach has been employed herein, in an effort to reveal its mode of action including dose−response antiproliferative activity and induced apoptosis effect, biochemical and physicochemical assays, and computational calculations. It may be concluded that, quercetin binds directly to the BH3 domain of Bcl-2 and Bcl-xL proteins, thereby inhibiting their activity and promoting cancer cell apoptosis.



Apoptosis is an essential physiological process and aberrant impairment or attenuation of apoptotic molecular machinery allows the expansion of tumor cell clones that eventually develop resistance to drug-induced apoptotic cell death.[1] The current knowledge on the process of apoptosis suggests that it is regulated by a dynamic interplay between pro- and antiapoptotic members of the Bcl-2 family proteins (Bcl-xL, Bcl-w, Mcl-1, A1) of regulatory proteins.[2] Binding of the antiapoptotic Bcl-2 family members by the BH3-only subfamily of proteins is a common denominator of all models ("direct activation", "derepression", and "embedded together") by which the Bcl-2 proteins are postulated to interconnect in regulating MOMP (Mitochondrial Outer Membrane Permeabilization).[3] Thus, apoptosis is triggered when the concentration of the pro-apoptotic BH3-only proteins exceeds the binding capacity of the hydrophobic pocket formed by the folding of the BH1, BH2, and BH3 domains of the antiapoptotic Bcl-2 proteins. *BCL-2* gene chromosomal translocations, reduced levels of Bax protein, elevated levels of Bcl-2, Mcl-1, or Bcl-xL expression are some of the key components, which commonly occur in hematopoietic malignancies.[4]

Designing small molecules that mimic the BH3 domain of the pro-apoptotic Bcl-2 family proteins is a currently challenging topic aiming at developing agents that inhibit antiapoptotic Bcl-2 proteins driving cancer cells to apoptosis.[5] For instance, ABT-263, a small molecule that inhibits Bcl-2 and Bcl-xL, has demonstrated clinical activity in Bcl-2-dependent hematological cancers, whereas its successor ABT-199, a next generation and more potent orally bioavailable Bcl-2 inhibitor, is currently in early phases of clinical development for the treatment of Bcl-2-dependent hematological cancers.[6] ABT-199 and ABT-737 both function as BH3 mimetics and demonstrated significant efficacy against ER-positive breast cancer by markedly enhancing tumor response to the antiestrogen tamoxifen, thus, providing rationale evidence for their clinical investigation need.[7]

Natural products are a rich source of bioactive compounds[8,9] with BH3 mimetic activity and thus consist a sound basis for such an approach. Indeed, (−)-gossypol, (AT-101), the active enantiomer of gossypol (Supporting Information (SI) Figure S1), a natural polyphenol derived from the cotton plant, is a BH3 mimetic that interacts directly with Bcl-xL and is currently under clinical investigation in chemotherapy combinations for the treatment of refractory metastatic cancers.[10,11] Moreover, (−)-gossypol down-regulates the Mcl-1 levels by acting directly or indirectly on its mRNA stability and protein degradation,

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mimicking in this respect the activity of quercetin.[12,13] Quercetin, a dietary consumed polyphenolic flavonoid (SI Figure S1), has drawn increased attention as a potential chemopreventing agent by significantly reducing cancer risk[14] and has been reported to intervene with the antiapoptotic proteins of the Bcl-2 family.[12,13] However, the exact mode of apoptotic-driven action of quercetin remains elusive. On the basis of the structural homology between gossypol and quercetin, we explored the possibility that quercetin directly interacts with Bcl-2 family of proteins, and the potential outcome of such interactions by employing vector- and Bcl-2-transduced human T-leukemic cells.

In the present study, we provide biochemical (pull down assays), physicochemical ($^{1}H-^{15}N$ HSQC NMR spectroscopy-chemical shift perturbation mapping and fluorimetric approaches) and computational (docking calculations) evidence that quercetin binds directly to the BH3 domain of the antiapoptotic Bcl-2 and Bcl-xL proteins exhibiting BH3-mimetic properties. We first investigated the effects of quercetin on the survival of human T-leukemic Jurkat cells stably transduced with an empty retroviral vector conferring puromycin resistance or with the same retrovector carrying a human Bcl-2 cDNA.[15] The forced expression of Bcl-2 protein protected cancer cells from quercetin induced toxicity. Specifically, after 24 h of exposure to quercetin Jurkat Puro cells demonstrated a decrease in cell number reaching 50% at 50 μmol/L, whereas Jurkat Bcl-2 cells displayed a markedly different growth profile (Figure 1A). Jurkat Bcl-2 cell numbers remained stable, following treatment with quercetin, even at the highest concentration of 100 μmol/L. This result suggested that quercetin probably interacts directly with Bcl-2, the entire experimental outcome being reminiscent of the interaction of gossypol with Bcl-xL.[16] Moreover, a dose–response analysis of Jurkat Puro cells revealed a moderate increase in apoptosis, 24 h after exposure to quercetin at 25 μmol/L and extensive apoptosis at 50 μmol/L or greater, using Annexin-V/PI staining (Figure 1B, SI Figure S2). Interestingly, apoptosis was inhibited at all quercetin concentrations used in Jurkat Bcl-2, suggesting that forced expression of Bcl-2 significantly protected cells from quercetin-induced apoptosis. Similar results were obtained by western blot analysis of poly(ADPribose) polymerase-1 (PARP-1), a substrate of caspase-3 and possibly other caspases, indicating that quercetin induced caspase-dependent apoptosis, an effect inhibited by Bcl-2 overexpression (SI Figure S3).

To validate whether quercetin interacted directly with Bcl-2 family proteins, we investigated the binding of quercetin to Bcl-2 and Bcl-xL proteins using pull down assays. Thus, the binding of Bcl-2 from Jurkat Bcl-2 and Jurkat Puro cell lysates to quercetin-Sepharose 4B beads,[17] but not to control Sepharose 4B beads, was clearly demonstrated by immunoblotting (Figure 2A). Moreover, a flavonoid of similar structure to quercetin was used as a negative control. Taxifolin, a natural polyphenol that differs in the presence of C2–C3 double bond from quercetin (SI Figure S1), has demonstrated an apoptotic activity independent of Bcl-2 mRNA expression and was used as a negative control for our study. Indeed, no direct binding was observed in the case of taxifolin (Figure 2B). In the case of Bcl-xL from Jurkat Puro cell lysates, the *in vitro* pull down assay demonstrated that it binds to quercetin-Sepharose 4B beads but not to taxifolin (Figure 2C). In addition, the recombinant Bcl-xL protein was bound specifically to quercetin–Sepharose 4B and not to control Sepharose 4B beads (SI Figure S4). Taken together these results strongly suggested that quercetin

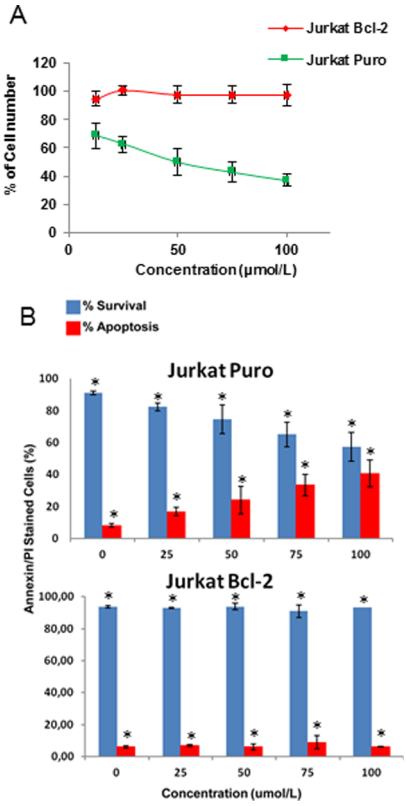

Figure 1. Forced expression of Bcl-2 protects cells from quercetin-induced apoptosis. Jurkat Puro and Bcl-2 cell lines were treated with vehicle (Ctrl) or increasing concentrations of quercetin for 24 h. Experiments were performed in duplicates. (A) Growth curve of Jurkat Puro (green) and Jurkat Bcl-2 (red) in the presence of quercetin after a 24 h treatment. Each point represents an average of duplicates. *t*-test $^{**}p < 0.0001$. (B) Evaluation of apoptosis by Annexin-V/PI staining in Jurkat Puro and Jurkat Bcl-2 cells was performed by treating with vehicle (Ctrl) or increasing concentrations of quercetin for 24 h. Blue bars represent viable cells and red bars represent apoptotic cells. *t*-test $^{*}p < 0.016$.

interacted directly with Bcl-2 and Bcl-xL members of the Bcl-2 protein family.

Having defined a direct binding of quercetin to Bcl-2 and Bcl-xL, 2D $^{1}H-^{15}N$ HSQC NMR experiments were performed to map the ligand–protein interfaces implicated in the interaction between these two molecules. The NMR chemical shift perturbation experiments (Figure 3A and B; SI Figures S5–S10) further proved a direct interaction between quercetin and a specific interface of Bcl-2 and Bcl-xL. Residues exhibiting ΔCS perturbation values over 0.015 and 0.03 ppm are projected on the protein structure (Figure 3A and B) in yellow and red colors, respectively. As it has been reported, residues illustrating chemical shift perturbation may or may not be directly related to the binding process.[18] From the residues exhibiting ΔCS perturbation values over 0.02 ppm for Bcl-2 protein, four amino acids were located on the BH1 domain, three located on the BH2 domain, and 13 found to span the BH3 binding groove. In the case of Bcl-xL, 14 residues were found to be located on the BH3 binding groove, whereas 8 and 10 were identified for domains BH1 and BH4, respectively. The NMR chemical shift perturbations on the amino acid residues of Bcl-2 and Bcl-xL upon quercetin binding were plotted against the amino acid sequences and the binding sites were

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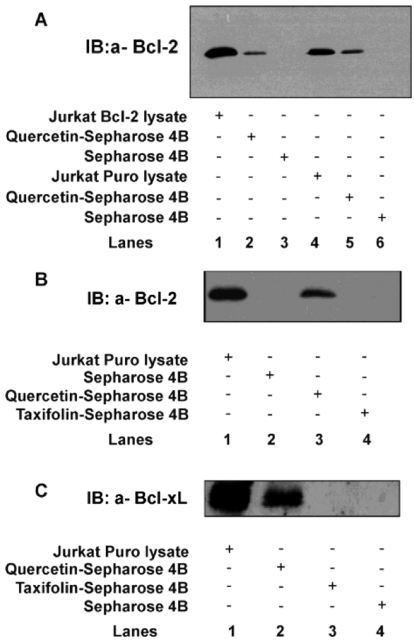

Figure 2. Pull down assays of quercetin with Bcl-2 family members. (A) Quercetin binds Bcl-2 from cell lysates, which was assessed by immunoblotting using a mouse antibody against Bcl-2. Lane 1, the input control, which contains the whole lysate from Jurkat Bcl-2 cells; lane 2, the Bcl-2 was pulled down using quercetin-Sepharose 4B beads; lane 3, the negative control, where Bcl-2 was not precipitated with Sepharose 4B. Lane 4, the input control for the whole cell lysate from Jurkat Puro cells; lane 5, the Bcl-2 was pulled down by quercetin-Sepharose 4B; lane 6, the negative control where Bcl-2 was not precipitated. (B) Quercetin binds Bcl-2 in Jurkat Puro lysates but not Taxifolin, which is used as a negative control. Lane 1, the input control for Bcl-2 from Jurkat Puro cells lysate; lane 2, Sepharose B as a negative control; lane 3 demonstrates the Bcl-2 protein, which is bound to Quercetin, whereas in lane 4, Taxifolin does not bind Bcl-2 (SI Figure S9). (C) Quercetin binds Bcl-xL in Jurkat Puro lysates but not Taxifolin, which is used as a negative control. Lane 1: the input control for Bcl-xL from Jurkat Puro cells lysate. Lane 2 demonstrates the Bcl-xL protein, which is bound to Quercetin, whereas in lane 3, Taxifolin does not bind Bcl-xL (SI Figure S9). Lane 4: Sepharose B as a negative control.

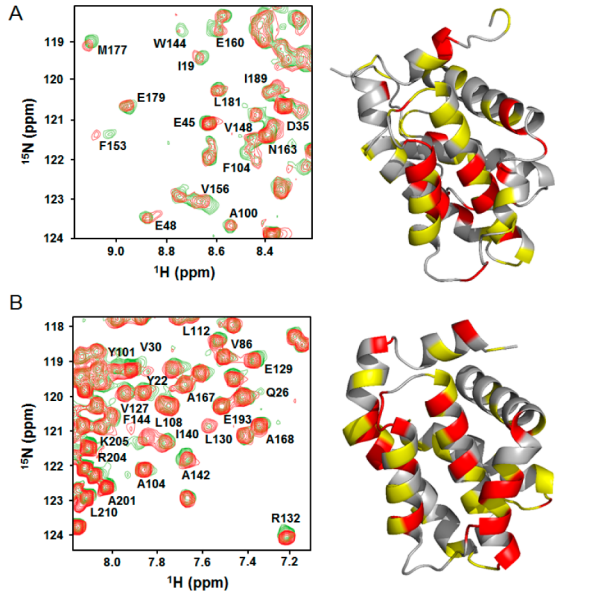

Figure 3. Mapping the Bcl-2-quercetin binding interface by NMR. $^{1}H-^{15}N$ HSQC NMR spectra of ^{15}N-labeled Bcl-2 (A) and Bcl-xL (B) proteins without (red crosspeaks) and with the addition of quercetin (green crosspeaks) (molar ratio 1:1). Binding site mapping of quercetin on the Bcl-2 (A) and Bcl-xL (B) structures. Weighted ΔCS values were calculated using the equation $\Delta CS = [(\Delta^{1}H)^2 + 0.2(\Delta^{15}N)^2]^{0.5}$. The residues showing ΔCS > 0.03 ppm are colored in red, whereas ΔCS > 0.015 ppm are colored in yellow.

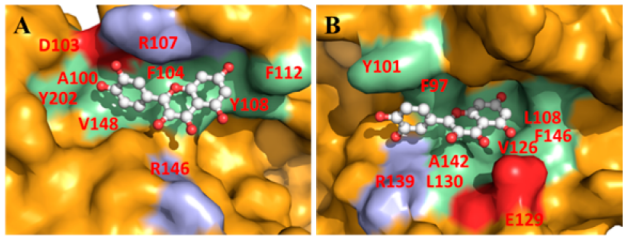

Figure 4. Modeling (3D) the complex of quercetin with Bcl-2 (A) and Bcl-xL (B). It is pinpointed that quercetin occupies the deep hydrophobic cleft in both proteins making an extended interaction network (SI Figure S10). The relevant residues are normally occupied by the proapoptotic Bak/Bad BH3 binding site in the complex.[19,20]

projected onto the three-dimensional structures (Figure 3A and B).

To further elucidate the structure of the quercetin-Bcl-xL and quercetin-Bcl-2 protein complexes, docking calculations were performed, which were guided on the basis of NMR chemical shift perturbation analysis. This combined strategy allowed us to deconvolute residues implicated in direct interaction from residues participating in structural changes that can be induced upon residues from the ligand far away from the binding site.[18]

Docking studies were performed with Autodock-vina[19] using the Bcl-2 conformation found in the complex with Bax BH3 peptide (PDB ID: 2XA0) and Bcl-xL complexed with ABT-737 (PDB ID: 2YXJ). Figure 4 illustrates the best solution of the relevant models of the two complexes. As it can be seen, quercetin occupies the deep hydrophobic cleft in both proteins making an extended network of interactions. In the case of Bcl-2, the A and C rings of quercetin form hydrophobic interactions with F104, R107, Y108, F112, whereas ring B develops favorable hydrophobic packing with F104 and V148, and it is in proximity to Y202. The −OH3′ group of the B ring is in hydrogen bonding distance with the side chain of R107. The relevant residues are normally occupied by the proapoptotic Bak/Bad BH3 binding site in the complex.[20,21]

In the model of the quercetin bound to Bcl-xL, the B and C rings of quercetin form hydrophobic interactions with F146, V126, L108, A142, whereas the CO(4) group is close to the carboxylic group of E129. The B ring forms stacking interaction with Y101 and its OH3′ group is in proximity to R139.

The influence of quercetin on the Bcl-xL fluoresence intensity was also exploited (SI Figure S11). Equilibrium binding of quercetin to Bcl-xL was quantitated using a change in the intrinsic fluorescence of Bcl-xL and the K_d was determined (K_d = 1.1 μM ± 0.1 μM). To further validate the NMR and *in silico* results the binding specificity to the BH3 site of Bcl-xL, competition experiments of epigallocatechin gallate, a selective BH3 inhibitor of Bcl-xL, with quercetin were performed.[22] Through this experiment, we observed a 10-fold increase of the measured K_d of quercetin (13.2 μM ± 1 μM) in the presence of epigallocatechin gallate, indicating the direct competition for the same site (BH3 site). An additional

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competitive experiment with HA14-1, a known BH3 binding site inhibitor of Bcl-2,[23] was also conducted. The presence of HA14-1 led to a 10-fold increase of the K_d of quercetin (16.5 μM $\pm$ 0.8 μM) confirming that quercetin binds to the BH3 site (SI Figure S12).

Although the two proteins are highly homologous in the BH3 binding groove, quercetin was found to bind in slightly discrete locations in the two proteins. This differentiation was rationalized due to alteration of L108 in Bcl-xL to M115 in Bcl-2. The validity of this observation was indirectly obtained through the NMR chemical shift perturbation binding experiments were it was found a ΔCS of 0.07 ppm and 0.02 for the case of L108 and M115, respectively.

A structural comparison between the Bcl-xL/quercetin and the Bcl-xL/ABT-737[24] complexes showed that quercetin binds to Bcl-xL in a similar manner to those of the BH3-mimetic antagonist. Six out of seven residues that appear in the binding site of quercetin with Bcl-xL are present also in the binding domain of the crystal structure of Bcl-xL with ABT-737. This suggested a potential molecular mechanism describing the ability of quercetin to inhibit the function of the antiapoptotic Bcl-2 family proteins to trigger intrinsic apoptotic pathway in cancer cells.

In conclusion, functional cytotoxicity assays of quercetin in Jurkat T-cell leukemic lines, Jurkat Bcl-2 and Jurkat Puro, indicated that quercetin binds to Bcl-2 protein. The direct binding of quercetin to Bcl-2 and Bcl-xL proteins was biochemically validated using pull down assays. NMR chemical shift perturbation experiments, fluorimetric spectroscopy, and docking calculations finally revealed that quercetin is bound to the pro-apoptotic BH3-binding site. This property classified quercetin as a natural flavonoid with BH3 mimetic activity capable of driving cancer cells to apoptosis. Of course, no other direct or indirect apoptotic effects of quercetin are excluded, but these results provide scientific evidence on the cytotoxic effects of quercetin on Jurkat cell lines and perhaps on other leukemic cell lines. Thus, the plant derived natural product quercetin through targeting key regulators of apoptotic cell death, the antiapoptotic Bcl-2 family proteins, could serve as a lead scaffold for sculpting more potent and selective analogues for the treatment of cancer.[25,26]

METHODS

Cell Culture. Human T-cell leukemic Jurkat Puro and Jurkat Bcl-2 cells were stably generated by retroviral infection of Jurkat cells, using either a control puromycin retrovector or the same retrovector carrying a human Bcl-2 cDNA, respectively.[15] Both cell lines were cultured as previously reported.

Cell Proliferation Assay. Cells were plated in 24-well plates and the next day were treated with vehicle (Ctrl) or five serial dilutions of quercetin and after 24 h the cells number for each well was counted (see Supporting Information).

Assessment of Apoptosis. Estimation of apoptosis was performed by flow cytometric analysis of Annexin-V binding and PI staining, according to Vermes.[27] Jurkat Puro and Bcl-2 cells were treated with vehicle (Ctrl) or increasing concentrations of quercetin for 24 h (see Supporting Information).

Protein Expression and Purification. Human Bcl-xL containing deletions in the C-terminus (Δ197−233) and the internal loop between helix R1 and helix R2 (Δ45−84) was expressed as a 6xHis-tagged protein in *E. coli* strain BL21. The protein was purified in one step by Ni^{2+}-affinity chromatography by Äkta Prime FPLC (GE Healthcare).

***In Vitro* Pull-down Assay.** Recombinant Bcl-xL or a Jurkat Bcl-2 and Jurkat Puro cellular supernatant fraction were incubated with quercetin (or taxifolin)-Sepharose 4B (or Sepharose 4B as control) beads in reaction buffer (see Supporting Information). After incubation with gentle rocking overnight at 4 °C, the beads were washed with the reaction buffer and bound proteins from the cell lysates were analyzed by immunoblotting with a mouse monoclonal Bcl-2 antibody whereas the recombinant protein was analyzed by coomassie staining.

NMR Binding Experiments. Truncated Bcl-2[28] and Bcl-xL[29] were expressed and purified for NMR experiments, as previously reported. All the NMR data were collected on Bruker Avance II 800 spectrometer at the Korea Basic Science Institute. The 2D $^{1}H-^{15}N$ HSQC spectra of Bcl-2 and Bcl-xL were obtained without and with the addition of 1:1 quercetin (see SI).

ASSOCIATED CONTENT

Supporting Information

Chemical structures, graphs for of flow cytometric and Western blot analysis of apoptosis evaluation, NMR chemical shift perturbations, the *in vitro* pull down assay protocol as also the different fluorimetry assays are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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