Supplementary methods

Targeting cancer cells via the reactive oxygen species-mediated unfolded protein response with a novel synthetic polyphenol conjugate

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Quantitative structure–activity relationship (QSAR)

Thirty-five known polyphenols, including sixteen flavones, eight isoflavones, ten flavanones, and one chalcone (listed in Supplementary Table S1), were all purchased from Indofine Chemical Co. Inc. (Hillsborough, NJ). Their purities were determined to be over 98% by HPLC analysis (data not shown). To calculate the QSAR, three-dimensional (3D) structures were determined based on molecular modeling. The flavone ligand contained in the protein data bank (PDB) file 4HKI was adapted as a template for the sixteen flavones. Similarly, 5,7-dihydroxy-4'-methoxysioflavone (2QYO) and naringenin (2BRT) were adapted as the templates for the isoflavones and flavanones, respectively. Finally, the solved X-ray crystal structure of 2'-hydroxy-3,5-dimethoxy-3',4'-benzochalcone (1) was used as the template for the chalcones. All structures were then built using the Sketch module provided by SYBYL 7.3 (Tripos, St. Louis, MO). To find the most stable structure, a conformational search was performed using the SYBYL Grid search method. During the grid search, selected bonds were rotated from 0° to 360° at increments of 15°. At each increment, the conformation was minimized using the Tripos force field and Gästeiger–Hückel charge. The maximum iteration for each minimization was set to 1000, and the minimization process was terminated at the

convergence criteria of the total energy (0.05 kcal/mol·Å). Based on the template structure, the 3D structures of all 35 polyphenols were modified and minimized (2).

To assess the relationship between the structures of plant-derived polyphenols and their inhibitory effects on the clonogenicity of tumor cells, 3D QSAR studies were performed by comparative molecular field analysis (CoMFA) using the database alignment module provided by the SYBYL program. The 35 polyphenols were randomly divided into a training set of twenty-seven compounds, and a test set of seven compounds (2, 6, 9, 10, 15, 22, and 34). To validate whether the test set belonged to different structural groups, hierarchical clustering analysis was performed using the SYBYL program. The seven compounds in the test group each belonged to different structural clusters, and so could validate the QSAR models created (data not shown). The aligned molecules were placed in a 3D cubic lattice with a grid spacing of 2.0 Å in the *x*, *y*, and *z* directions. An sp^3 hybridized carbon atom with a charge of +1 was used as the probe atom to calculate steric and electrostatic fields. The energy values were truncated to +30 kcal/mol. Five CoMFA models were then built using the region focusing method. Region focusing was used to enhance the cross-validation correlation coefficient (q^2) and non-cross-validation coefficient (r^2) values, and to refine the models. It was weighted by standard deviation coefficient values ranging from 0.3 to 1.2 and grid spacing ranging from 0.5 to 1.5. The CoMFA model showing the best cross-validation correlation coefficient or non-cross-validation coefficient was selected. To confirm the CoMFA models, the inhibition of clonogenicity predicted by CoMFA was compared with the experimental data (3).

The negative logarithmic values of the half maximal growth inhibitory concentrations ($pGIC_{50}$) are listed in Supplementary Table S1. The CoMFA model showing the best cross-validation correlation coefficient (q^2 = 0.518) was chosen for further analysis, whereas the

non-cross-validation correlation coefficient (r^2) was 0.919. To establish a linear relationship between the half maximal growth inhibitory concentrations and the resulting field matrix of polyphenols, partial least-squares (PLS) analysis was performed. The optimal number of components obtained from the LOO method was six, and the standard error of estimate and F values were 0.053 and 39.809, respectively. To evaluate the CoMFA model, $pGIC_{50}$ values of the polyphenols in the training set were predicted and compared with the experimental data (Supplementary Table S2). The differences between the experimental and predicted values for the training set ranged from 0.06−2.27%. To validate the QSAR model, seven polyphenols (compounds 2, 6, 9, 10, 15, 22, and 34) were selected as the test set. Their residuals ranged between 0.69−5.88%, which confirmed that the CoMFA model was reliable (Supplementary Fig. S2A).

To visualize relationships between the structures of the polyphenols and their inhibitory effects on clonogenicity, CoMFA contour maps were generated using SYBYL 7.3. The steric and electrostatic field descriptors contributed 56.2% and 43.8%, respectively. For the steric field, the steric bulk-favored region contributed 29%, whereas the disfavored region contributed 71%. In the electrostatic field, the electron-donating group-favored region contributed 69%, and the electron-withdrawing group-favored region contributed 31%. The steric field contour map showed that the bulky substituents at the C-1 and C-3 positions of the A-ring decreased the activity, and those at C-3' of the C-ring and C-4 of the A-ring increased activity (Supplementary Fig. S2B). The electropositive substituents at C-7 of the B-ring and C-5' of the C-ring increased activity, whereas electronegative substitutions at the C-3' and C-4' positions increased activity.

Synthesis of novel polyphenol conjugates that inhibit the clonogenicity of tumor cells

Based on the interpretation of the CoMFA, we synthesized additional eleven polyphenol conjugates (Supplementary Table S3), as described previously (4-5).

Antibodies

Rabbit polyclonal antibodies against Beclin-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and microtubule-associated protein 1 light chain 3 (LC3) was from Abcam (ab48394, Cambridge, UK). Antibodies against cleaved caspase-7 (Asp198), cleaved caspase-9, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), Ki-67 (8D5), and p53 were obtained from Cell Signaling Technology (Beverly, MA).

***In vivo* tumor xenografts**

Tumors were produced by subcutaneous inoculation with HCT116 cells (1×10^6 in 0.1 ml serum-free DMEM). Tumor growth was measured every 2−3 days using calipers. The tumor volume was calculated as $(L \times W^2)/2$, where L = length and W = width in mm. When the tumors reached a mean volume of ~100 mm^3, 100-μL PBS (control group) or DPP-23 (10 mg/kg) was injected intraperitoneally once daily. Mice were sacrificed by exposure to CO_2 on day 30, and tumor size was compared.

Supplementary References

1. Lee HJ, Lim Y, Koh D. (E)-3-(3,5-Dimeth-oxy-phen-yl)-1-(1-hy-droxy-naphthalen-2-yl)prop-2-en-1-one. Acta Crystallogr Sect E Struct Rep Online. 2012;68:o3403.
2. Shin SY, Yoon H, Ahn S, Kim DW, Kim SH, Koh D, et al. Chromenylchalcones showing cytotoxicity on human colon cancer cell lines and in silico docking with aurora

kinases. Bioorg Med Chem. 2013;21:4250-8.
3. Hyun J, Shin SY, So KM, Lee YH, Lim Y. Isoflavones inhibit the clonogenicity of human colon cancer cells. Bioorg Med Chem Lett. 2012;22:2664-9.
4. Hwang D, Jo G, Hyun J, Lee SD, Koh D, Lim Y. Synthesis of methoxybenzoflavones and assignments of their NMR data. Magn Reson Chem. 2012;50:62-7.
5. Goodarzi M, Duchowicz PR, Wu CH, Fernandez FM, Castro EA. New hybrid genetic based Support Vector Regression as QSAR approach for analyzing flavonoids-GABA(A) complexes. J Chem Inf Model. 2009;49:1475-85.