Plant phenolics regulate neoplastic cell growth and survival: a quantitative structure–activity and biochemical analysis[1]

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Abstract: The anti-tumour activities of many plant phenolics at high concentrations (>100 μmol/L) suggest their potential use as dietary supplements in cancer chemoprevention and cancer chemotherapy. However, it is not clear what impact phenolic compounds have at the physiological concentrations obtained through consumption of high phenolic diets on neoplastic cells. In the present study, 54 naturally occurring phenolics were evaluated at physiologically relevant concentrations for their capacity to alter PC12 cell viability in response to serum deprivation, the chemotherepeutic agent etoposide, and the apoptogen C2-ceramide. Surprisingly, novel mitogenic, cytoprotective, and antiapoptotic activities were detected. Quantitative structure–activity relationship modelling indicated that many of these activities could be predicted by compound lipophilicity, steric bulk, and (or) antioxidant capacity, with the exception of inhibition of ceramide-induced apoptosis. Where quantitative structure–activity relationship analysis was insufficient, biochemical assessment demonstrated that the benzoate orsellinic acid blocked downstream caspase-12 activation following ceramide challenge. These findings demonstrate substantive mitogenic, cytoprotective, and antiapoptotic biological activities of plant phenolics on neoplastic cells at physiologically relevant dietary concentrations that should be considered in chemopreventive and chemotherapeutic strategies.

Key words: chemotherapy, cytoprotection, mitogenic, phenolic acids, flavonoids, tannins, antiapoptotic, dietary antioxidants/phenolics.

Résumé : Les activités anti-tumorales de nombreux composés phénoliques végétaux à concentrations élevées (>100 μM) laissent entrevoir une utilisation possible comme suppléments alimentaires dans la chimioprévention et la chimiothérapie des cancers. Toutefois, ne sait pas exactement quel impact les composés phénoliques aux concentrations physiologiques obtenues par une alimentation riche en phénols ont sur les cellules néoplasiques. Dans la présente étude, on a évalué 54 composés phénoliques naturels à des concentrations physiologiques capables de modifier la viabilité des cellules PC12 en réponse à la privation de sérum, à l'agent chimiothérapeutique étoposide et à l'apoptogène C2-céramide. Fait étonnant, on a détecté de nouvelles activités mitogéniques, cytoprotectrices et antiapopotiques. La modélisation de la relation structure–activité quantitative (RSAQ) a indiqué que beaucoup de ces activités pouvaient être prédites par la lipophilicité, l'encombrement stérique et l'activité antioxydante des composés, ou les trois, à l'exception de l'inhibition de l'apoptose induite par le céramide. En complément à l'analyse RSAQ, l'évaluation biochimique a démontré que l'acide orsellinique (benzoate) a bloqué l'activation en aval de la caspase 12 après l'épreuve avec céramide. Ces résultats démontrent que les composés phénoliques végétaux ont des activités biologiques mitogéniques, cytoprotectrices et antiapoptotiques significatives sur les cellules néoplasiques aux concentrations alimentaires physiologiquement pertinentes, et qu'elles devraient être considérées dans les stratégies chimiopréventives et chimiothérapeutiques.

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Mots-clés : chimiothérapie, cytoprotection, mitogénique, acides phénoliques, flavonoïdes, tannins, antiapoptotiques, composés phénoliques/antioxydants alimentaires.

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Introduction

Plant-derived phenolic compounds have received considerable attention as dietary antioxidants, particularly with regard to aging and its associated health problems (Middleton et al. 2000). Yet, individual compounds trigger a plethora of other biologically relevant effects in mammalian cells, including proliferation, cell-cycle inhibitor, antiapoptotic, and cytotoxic actions that are not necessarily attributable to their antioxidant capacities (Tsuda et al. 2004). As multiple (and sometimes conflicting) therapeutic potentials are elucidated, it becomes increasingly important to identify structure–activity relationships at the circulating concentrations that would be obtained through consumption of phenolic-rich diets (Balunas and Kinghorn 2005; Ji and Peterson 2004; Karakaya 2004; Spencer 2003).

In the current study, 54 phenolic compounds, many with proven cytostatic or cytotoxic effects at concentrations >100 μmol/L, were screened for mitogenic, cytoprotective, antiapoptotic, cytotoxic, and proapoptotic actions at concentrations between 0.6 and 7.3 μmol/L (Table 1). This range was chosen to fall within the physiologically relevant concentration of plasma phenolic concentrations detected in individuals on high phenolic diets (Lotito and Frei 2006). Bioactivities of plant-derived phenolics were assessed in response to the following: (*i*) growth factor deprivation, (*ii*) DNA damage induced by the topoisomerase II inhibitor etoposide, and (*iii*) endoplasmic reticulum stress triggered by the lipid second messenger C2-ceramide (Beck et al. 2001; Darios et al. 2003; Ogretmen and Hannun 2004). These assays tested effects of dietary phenolics on neoplastic cells cultured under conditions designed to promote either growth arrest or apoptosis characteristic of cancer chemopreventive or chemotherepeutic strategies. PC12 cells were used as they respond to growth factor deprivation by growth arrest and, in the presence of nerve growth factor, differentiation to a neuronal phenotype (Eaton and Duplan 2004). Unlike untransformed adrenal cells, phenotypic differentiation is not terminal and is reversed by exposure to exogenous mitogens (Eaton and Duplan 2004). As such, this cell line offers an ideal model system to screen rapidly the impact of dietary phenolics on neoplastic cell growth and survival under physiological conditions as compared with untransformed or tumour cell types.

To identify underlying structural determinants within classes of phenolics, bioactivities were modelled by quantitative structure–activity relationship (QSAR) analyses. Bond dissociation enthalpy (BDE) was calculated as a measure of oxidant reactivity (Cheng et al. 2003; Wright et al. 2001). Physiochemical parameters of steric bulk and lipophilicity were used to predict compound bioavailability at the cellular level. Molecular refractivity (MR) was used to estimate steric bulk (Hansch et al. 2003). Lipophilicity was determined as the log value of the compound's partition coefficient (log P). A second term, $(\log P)^2$, was also introduced to account for the parabolic relationship between biological activity and lipophilicity relative to partitioning within the cellular environs (Marles et al. 1991; Rosenkranz and Thampatty 2003). Where QSAR analysis was insufficient to account for the variance in observed results, the ability of antiapoptotic phenolics to inhibit executioner caspase activity was assessed. Surprisingly, we detected significant proliferative and prosurvival activities in 78% of the 54 compounds tested that could impact upon their use as cancer chemopreventive or cancer therapeutic adjuvants.

Materials and methods

Cell culture

PC12–AC cells, a clonal derivate of the PC12 rat adrenal pheochromocytoma cell line (American Tissue Culture Collection), were maintained in complete media (RPMI 1640 containing 10% horse serum and 5% newborn calf serum *v*/*v*) and treated in serum-free RPMI 1640 supplemented with 0.025% *w*/*v* BSA (treatment media) at 37 °C in a 5% CO_2 atmosphere. Cell culture reagents were obtained from Invitrogen. The PC12–AC cell line is a homogenous subclone of the parental PC12 line generated in Bennett's laboratory to ensure genetic homogeneity within a neoplastic background (Bonin et al. 2004; Brewer et al. 2002; Chichirau et al. 2005; Flueraru et al. 2005; Martineau et al. 2006; Ryan et al. 2007; Spoor et al. 2006). This line undergoes growth factor arrest in serum-deprived media and is responsive to nerve growth factor, but is more adherent than the original population, and thus more amenable to 96-well bioactivity assays.

Primary bioactivity screen

Phenolic compounds were from the phytochemical collections of C. Nozzilillo (University of Ottawa, Canada), T. Yoshida (Okayama University, Japan), and J.T. Arnason (University of Ottawa, Canada). Compounds were solubilized in dimethylsulfoxide (DMSO) and used at a final concentration of 1 μg/mL. Ceramide (C2-ceramide, Biomol) and etoposide (Sigma) were prepared in EtOH and used at a final concentration of 20 μmol/L or 25 μmol/L, respectively, based on dose–response analysis establishing approximately 50% cell loss in PC12–AC cells after a 24 h treatment. Cells were seeded in 96-well plates (1.25×10^4 cells/well) and allowed to adhere at 37 °C in 5% CO_2 overnight. Cultures were deprived of growth factors in the presence of plant phenolics (1 μg/mL) or vehicle (0.1% DMSO – 0.1% EtOH) or challenged with apoptogen and plant phenolic (1 μg/mL) or vehicle. Negative controls included cultures incubated in treatment media only. Following 24 h of treatment, the cell proliferation reagent (formazan dye) WST (Roche Diagnostics) was added to each well and incubated for 60 min before spectrophotometric analysis at 420 nm (formazan) and at 620 nm (reference). Cultures were

Table 1. Structures, molecular masses, physicochemical properties, and test concentrations of phenolics screened for biological activity.

No.	Compound	Substitutions	Mol. mass	Log *P*	MR, m^3/mol	BDE, kcal/mol	[C][a], μmol/L
Benzoic acids							
1	Salicylic acid	C_2 = OH	138.1	2.27	33.90	95.1	7.2
2	3-Hydroxybenzoic acid	C_3 = OH	138.1	1.49	33.90	89.5	7.2
3	Resorcyclic acid	C_2,C_4 = OH	154.1	1.50	35.72	89.2	6.5
4	Gentisic acid	C_2,C_5 = OH	154.1	1.37	35.72	83.6	6.5
5	Protocatechuic acid	C_3,C_4 = OH	154.1	0.81	35.72	80.3	6.5
6	Vanillic acid	C_3 = OMe, C_4 = OH	168.2	1.35	41.15	88.2	6.0
7	Orsellinic acid	C_2 = Me, C_4,C_6 = OH	168.2	1.30	41.62	88.8	6.0
8	Gallic acid	C_3,C_4,C_5 = OH	170.1	0.05	37.53	75.5	5.9
9	Veratric acid	C_3,C_4 = OMe	182.2	1.34	46.59	N/A	5.5
10	3,5-Dihydroxy-4-methoxybenzoic acid	C_3,C_5 = OH, C_4 = OMe	184.2	0.58	42.67	87.7	5.4
11	Syringic acid	C_3,C_5 = OMe, C_4 = OH	198.2	0.95	48.40	86.8	5.0
Benzoids							
12	*m*-Digallic acid	C_3 = 0-galloyl, C_4,C_5 = OH	322.2	1.13	72.49	75.5	3.1
13	Bergenin	$C_{14}H_{16}O_9$ (see Fig. 1)	328.3	–1.40	72.29	85.2	3.0
Cinnamic acids							
14	Cinnamic acid		148.1	1.00	42.85	N/A	6.8
15	*trans*-Cinnamic acid		148.1	1.93	42.85	N/A	6.8
16	*o*-Coumaric acid	C_2 = OH	164.2	1.57	44.75	91.1	6.1
17	*m*-Coumaric acid	C_3 = OH	164.2	1.54	44.67	89.3	6.1
18	*p*-Coumaric acid	C_4 = OH	164.2	1.54	44.76	84.9	6.1
19	Caffeic acid	C_3,C_4 = OH	180.2	1.15	46.48	75.7	5.6
20	Ferulic acid	C_3 = OMe, $C_{4'}$ = OH	194.2	1.42	51.92	83.5	5.1
21	Sinapic acid	C_3,C_5 = OMe, C_4 = OH	224.2	1.29	59.17	78.1	4.5
Phenylpropanoic acids (PPA)							
22	3-(*m*-Hydroxy)-PPA	C_3 = OH	166.2	1.57	44.75	86.6	6.0
23	3-(*p*-Hydroxy)-PPA	C_4 = OH	166.2	1.56	44.75	84.5	6.0
24	Hydrocaffeic acid	C_3,C_4 = OH	182.2	1.15	46.45	75.3	5.5
Phenylacetic acids							
25	2-(*m*-Hydroxy)-phenylacetic acid	C_3 = OH	152.2	1.15	38.22	86.6	6.5
Coumarins							
26	Coumarin		146.1	1.82	42.09	N/A	6.8
27	Umbelliferone	C_7 = OH	162.1	1.44	43.91	83.9	6.2
28	Esculetin	C_6,C_7 = OH	178.1	1.05	45.72	73.9	5.6
Flavonoids							
29	7-Hydroxyflavone	C_7 = OH	240.3	2.41	68.04	88.8	4.2
Flavones							
30	Apigenin	C_5,C_7,$C_{4'}$ = OH	270.2	1.00	73.51	82.3	3.7
31	Cosmetin[b]	C_5,$C_{4'}$ = OH, C_7 = O-glucose	432.4	0.06	107.46	82.3	2.3
32	Lucenin[b]	C_5,C_7,$C_{3'}$,$C_{4'}$ = OH, C_6,C_8 = C-glucose	610.5	–2.94	141.98	73.1	1.6
Flavonols (C_3 = OH)							
33	Quercetin	C_3,C_5,C_7,$C_{3'}$,$C_{4'}$ = OH	302.2	0.35	76.51	73.1	3.3
34	5-Methoxy-quercetin	C_5 = OMe, C_3,C_7,$C_{3'}$,$C_{4'}$ = OH	316.2	0.61	80.26	73.1	3.2
35	Quercitrin[b]	C_5,C_7,$C_{3'}$,$C_{4'}$ = OH, C_3 = O-rhamnose	448.4	–0.54	106.36	73.1	2.2
36	Myricitrin[b]	C_5,C_7,$C_{3'}$,$C_{4'}$,$C_{5'}$ = OH, C_3 = O-rhamnose	464.4	–0.92	110.23	68.2	2.2
37	Kaempferitrin[b]	C_5,$C_{4'}$ = OH, C_3,C_7 = O-rhamnose	578.5	–1.13	135.56	82.3	1.7
38	Rutin[b]	C_5,C_7,$C_{3'}$,$C_{4'}$ = OH, C_3 = O-rutinose	610.5	–2.28	141.69	73.1	1.6
Flavanones (saturated C_2- C_3 bond)							
39	Flavanone	—	224.3	2.80	66.23	N/A	4.5

Table 1 (*concluded*).

No.	Compound	Substitutions	Mol. mass	Log *P*	MR, m^3/mol	BDE, kcal/mol	[C][a], μmol/L
40	Naringenin	C_5,C_7,$C_{4'}$ = OH	272.3	1.90	71.67	84.5	3.7
41	Hesperetin	C_5,C_7,$C_{3'}$ = OH, $C_{4'}$ = Ome	302.3	1.5	78.92	85.2	3.3
42	Naringin[b]	C_5,$C_{4'}$ = OH, C_7 = O-rutinose	580.5	–1.10	137.52	84.5	1.7
Flavanonols (C_3 = OH)							
43	Taxifolin	C_3,C_5,C_7,$C_{3'}$,$C_{4'}$ = OH	304.3	0.58	75.43	75.3	3.3
Flavan-3-ols (saturated C_2- C_3 no O at C_4)							
44	Epicatechin	C_3,C_5,C_7,$C_{3'}$,$C_{4'}$ = OH	290.3	1.50	74.05	75.3	3.4
45	Epigallocatechin gallate	C_3 = O-galloyl, C_5,C_7,$C_{3'}$,$C_{4'}$,$C_{5'}$ = OH	458.4	2.07	110.79	70.4	2.2
Isoflavones (B-ring at C_3)							
46	Daidzein	C_7,$C_{4'}$ = OH	254.2	2.13	69.13	82.3	3.9
Anthocyanins (no O at C_4)							
47	Malvidin-3,5-diglucoside[b]	C_7,$C_{4'}$ = OH, $C_{3'}$,$C_{5'}$ = OMe, C_3,C_5 = O-glucose	655.6	N/A	N/A	81.2	1.5
Dihydro-chalcones (open C-ring)							
48	Phloridzin[b]	C_5 = O-glucose, C_7,C_9,$C_{4'}$ = OH	436.4	–0.48	109.73	83.5	2.3
Tannins							
49	Geraniin	(See Fig. 1)	952	0.51	205.53	75.3	1.1
50	Strictinin	(See Fig. 1)	618	0.14	138.22	75.3	1.6
51	Pedunculagin	(See Fig. 1)	918	0.74	171.17	75.3	1.1
52	Oenothein B	(See Fig. 1)	1569	N/A	N/A	74.9	0.6
53	Casuarinin	(See Fig. 1)	936	0.82	206.82	74.9	1.1
54	Tannic acid	(See Fig. 1)	1701	N/A	N/A	75.5	0.6

Note: Log partition coefficient (log *P*), molecular refractivity (MR), and bond dissociation enthalpy (BDE) values were calculated as described in Materials and methods.

[a]Screening concentration of 1 ppm phenolic in micromoles per litre.

[b]Flavonoid glycoside.

blanked against cell-free treatment media incubated for the same period. Cell number per well was calculated from standard curves derived from wells containing known cell densities. Standardization allowed data to be compared across replicates. Each compound was tested in quintuplicate over 2 or 3 independent experiments (n = 10–15). Data from control cultures were combined across plates (n = 25–80). Percentage viability was calculated as follows: % viability = cell number$_{(\text{treatment well})}$/mean cell number$_{(\text{negative control})}$.

Validation of cell survival, cell death, and mitogenicity

For the Live/Dead Viability/Cytotoxicity assay (Molecular Probes), cells were grown in 96-well black plates with clear bottoms. Viability was determined by the ability of intracellular esterases to cleave calcein-AM, producing a fluorescent wavelength of 515 nm. Cytotoxicity was measured by the ability of ethidium homodimer-1 to enter cells with damaged membranes and intercalate between DNA strands, emitting a fluorescent wavelength of 635 nm. Cells were incubated for 5 min in calcein-AM (1.25 μmol/L) and ethidium homodimer-1 (0.75 μmol/L). Counts were performed on a Leica DMIL inverted epifluorescent microscope equipped with a Qimaging QICAM fast 1394 digital camera (Quorum Technologies). Phase contrast and fluorescent photos were taken of 5 fields per well and counts were averaged to yield a single data point per well in duplicate experiments (n = 4–6).

In bromodeoxyuridine (BrdU) incorporation experiments, PC12–AC cells were seeded overnight on 0.1% gelatin-coated glass cover slips in complete media. Cells were subsequently synchronized for 36 h in serum-free treatment media. BrdU (20 μg/mL, Boehringer, Germany) was coadministered with 0.1% DMSO or compounds 4, 38, 39, or 43 to cells over a 30 min pulse followed by a 2.5 h chase with DMSO or phenolics only. Incorporated BrdU was labelled with anti-BrdU fluorescein isothiocyanate (Roche). Hoechst 33258 was used as a nuclear counterstain at 2 μg/mL. Cells were photographed (9 shots per cover slip) using a Leica DMXRA2 epifluorescent microscope. BrdU-positive ($BrdU^+$) cells were counted from fluorescence images and expressed as a percentage of total cells per field. Each compound was tested in triplicate in replicate experiments (n = 6).

Western analysis of caspase activation

Proteins were collected in RIPA buffer (10 mmol/L PBS, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 30 μL/mL aprotonin, 10 mmol/L Na-orthovanadate, 1 μL/mL phenylmethanesulfonyl fluoride, 1 μL/mL NaF). Protein samples (30 μg) were separated under reducing conditions by using SDS–PAGE. Western analyses were performed with monoclonal anti-poly(ADP-ribose) polymerase (PARP, 1:10000, Clontech), detecting both the uncleaved 116 kDa fragment and the cleaved 85 kDa PARP fragment, or polyclonal anti-caspase-12 (1:1000, Stressgen), detecting both uncleaved (55 kDa) and cleaved (17 kDa) caspase fragments. Monoclonal actin (1:1000, Sigma) was used as a loading control. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:2000, Jackson Immunolabs).

Calculation of physicochemical properties of test compounds

Using ChemDraw Pro Volume 7.0 (CambridgeSoft Laboratory Solutions), the molecular weight, log *P*, andMR of test compounds were determined. Due to computational limitations, no log *P* or MR values were determined for malvidin 3,5-diglucoside and the more structurally complex tannins. BDEs were predicted by analysis of phenolic substituent effects on proton-donating ability as previously described (Wright et al. 2001). BDE values for all hydroxyl groups located on phenolic rings were calculated and the lowest, corresponding to the most stable radical conformation and strongest antioxidant capacity, was recorded for use in QSAR analyses. In compounds with hydroxyl-free benzoic rings, BDE could not be estimated. Because the effects of some substituent patterns present in our collection have not been described previously, estimated values were used for calculation as follows: in the case of cinnamic acids, the combined effects of the vinyl and carboxyl groups were considered additive, whereas, because of reduced communication with the phenolic ring by saturation of the vinyl group, the 3-carbon chain in phenyl propanoic acids was considered as a methyl substituent. For hydroxyls located on flavonoids, glycoside groups were regarded as methoxy groups, whereas the C-ring substituent of the B-ring was considered a methyl group for flavanone-type molecules or as a vinyl group for flavone types. These assumptions were validated according to the lowest level method using Gaussian software as we have previously described (Wright et al. 2001). No additive effects were calculated through conjugated C-rings because the A-ring generally provides additional stability that can be generalized to the group. Similarly, the oxygen atom in the C-ring in most flavonoids provides increased stabilization (not in dihydrochalcones or positively charged anthocyanins) and was not included in our calculations. Thus, our predicted BDEs are conservative estimates.

Statistics

All statistics were performed with SYSTAT software (version 10). Biological data were analysed by analysis of variance (ANOVA) followed by Fisher's least squared differences (LSD) post hoc tests in screens or Dunnett's *t* tests for validation studies. QSAR modelling was conducted by simple regression between individual physicochemical parameters and measured biological activity. Multiple linear regressions were employed to identify more complex relationships between multiple parameters confirmed by backward stepwise regression analysis. Models with p values below 0.05 were considered significant, whereas models with $p < 0.08$ were subjected to further manipulations in search of stronger relationships.

Results

Fifty-four compounds, representing 5 classes of plant phenolics, were screened at a standard concentration of 1 ppm for their ability to alter viable cell number under conditions of growth factor deprivation or in response to etoposide- or ceramide-induced apoptosis. This protocol allowed us to test multiple phenolics within a physiological range falling between 0.6 and 7.3 μmol/L (Table 1) consistent with the circulating levels of multiple phenolic compounds obtained in plasma through consumption of a high phenolic diet (Lotito and Frei 2006). Viable cell number was initially established by mitochondrial dehydrogenase cleavage of the formazan dye WST relative to standardized controls of known cell densities and then validated by direct assessment of mitogenicity and cell death. The 5 phenolic classes studied were the following: (*i*) benzoic acid derivatives ($n = 13$); (*ii*) cinnamic acid derivatives, including phenylpropanoic acid and phenylacetic acid derivatives ($n = 12$); (*iii*) coumarins ($n = 3$); (*iv*) flavonoids ($n = 20$); and (*v*) hydrolysable tannins ($n = 6$) (Table 1, Fig. 1). The members of each class differed in terms of the type and position of benzyl side groups and the saturation of the vinyl group in the cinnamic acids or the C-ring in the flavonoids (Table 1, Fig. 1). In tannins (all gallotannins, Fig. 1), the number, position, and binding of gallic and ellagic acid residues varied among members. Purity of compounds was >95% based on HPLC analysis verified by HPLC/MS as described in Harris et al. (2007). Two different phenolic collections from the Yoshida and the Arnason/Nozzolillo laboratories were assessed separately, revealing no differences in biological activity of compounds common to both collections.

Effects on cell viability

Viability of cells treated for 24 h in serum-deprived media without vehicle was comparable with cells treated with 0.1% DMSO (phenolic vehicle) and 0.1% EtOH (apoptogen vehicle) and was standardized to 100% (Table 2). Twenty-four compounds (44%) increased viable cell number ($p < 0.01$) compared with vehicle-treated cultures in serum-free media, 26 had no effect, and 4 decreased viable cell number (dagger, $p < 0.01$) (Table 2). In etoposide-treated cells, 28 phenolics (52%) reduced etoposide-induced cell loss, 23 had no effect, and 3 potentiated toxicity. Similarly, 24 compounds increased cell number in ceramide-treated cultures, whereas 28 had no effect and only 2 increased cell loss (Table 2).

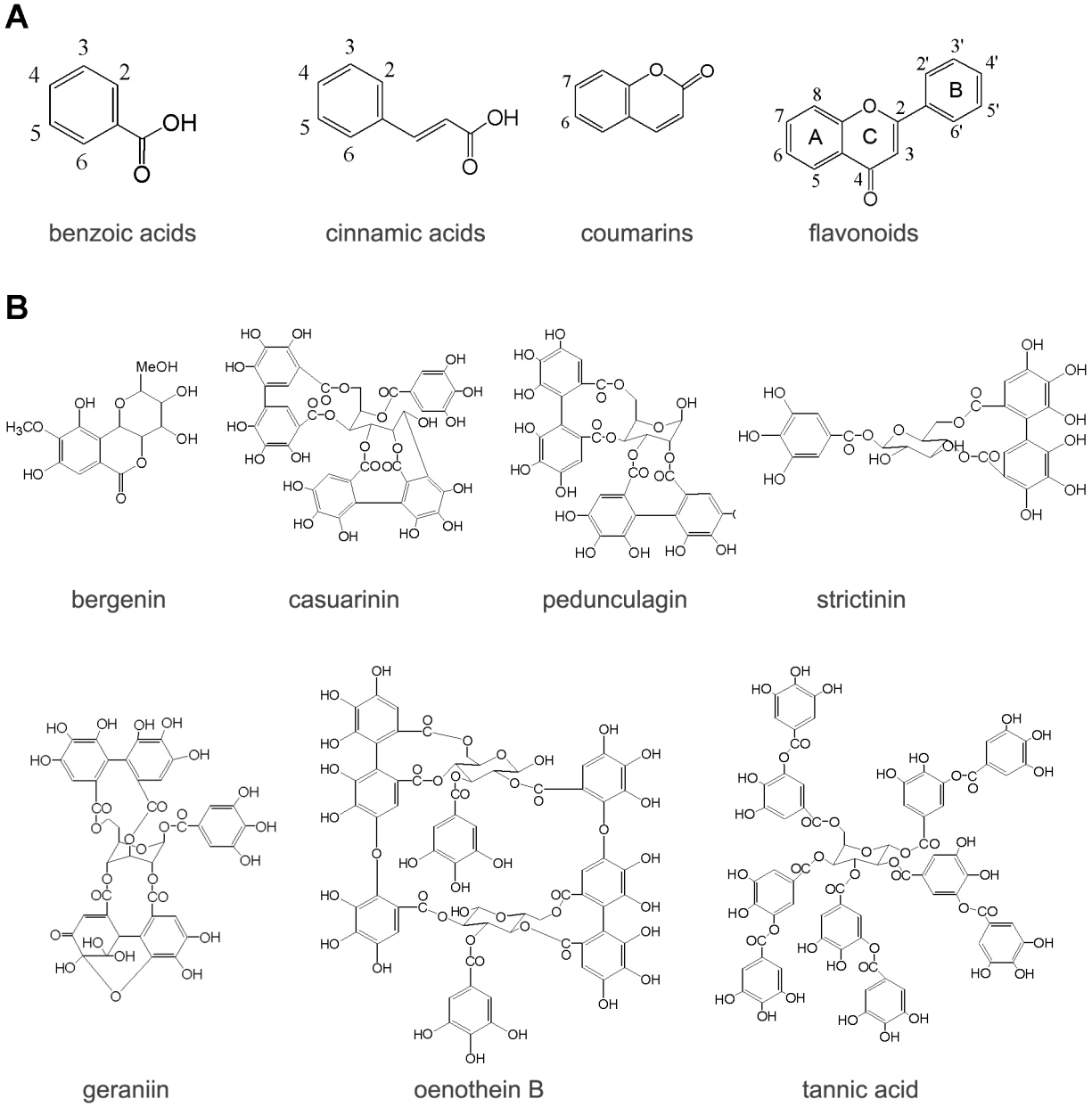
Compounds with no effect on cell viability under all growth conditions tested

Seven percent of tested compounds (3 benzoic acids and 1 flavonoid) had no effect on cell viability under any of the test conditions. These were compounds 1, 2, 5, and 39 (Table 2).

Cytoprotective and mitogenic activities

Cytoprotective compounds were defined as phenolics that promoted cell viablity in the absence of exogenous growth factors without protecting PC12–AC cells from etoposide or ceramide challenge. Compounds 8, 48, 42, and 54 met these criteria (7% of test compounds) (Table 2). To distinguish between cytoprotective and mitogenic activities, we compared effects on viable cell number in the 3 test paradigms. Mitogenic progression through the G1/S or G2/M checkpoints can protect tumour cell lines, including PC12 cells, from etoposide and ceramide-induced apoptosis (Kurosu et al. 2005; Lee et al. 2005*b*; Misasi et al. 2001). Nineteen phenolics (35% of test compounds) both increased cell number following serum deprivation and reduced etoposide- and

Fig. 1 Chemical structures of the plant phenolic compounds. (A) The structure and substitution locations of parental molecules for benzoic acids, cinnamic acids, coumarins, and flavonoids. (B) The structures of bergenin and the 6 hydrolysable tannins are indicated. MeOH, methyl alcohol. Structures were made using ChemDraw.



(or) ceramide-induced cell death. Compounds 4, 9, 11, 17, 21, 25, 26, 28, 31, 32, 34–36, 38, 43, 44, 47, 49, and 50 were categorized as mitogens (Table 2).

Since an increase in mitochondrial dehydrogenase activity and hence WST absorbance can occur in the absence of cell proliferation (Vogt et al. 2004), assigned bioactivities of selected compounds identified in 96-well screens were validated by direct assessment. Growth factor-deprived cultures were treated for 24 h with DMSO/EtOH (control) or compounds 4, 20, 28, and 39, and cell survival was quantified directly by Live/Dead assay. Viable cells were capable of cleaving calcein-AM to its fluorogenic product. Dead cells were identified by uptake of the membrane-impermeant ethidium bromide homodimer. Consistent with the WST screen (Table 2), compound 39 had no effect on cell survival, whereas compounds 4 and 38 increased the number of viable cells present in serum-deprived cultures without affecting the basal rate of cell death (Fig. 2A). By contrast, compound 20 increased WST absorbance (Table 2), but decreased the number of viable calcein$^+$/ethidium bromide$^-$ cells (Fig. 2A). These data pointed to an increase in mitochondrial enzymatic activity in dying cells. We further confirmed this cytotoxicity by quantifying terminally compromised ethidium bromide$^+$ cells (Fig. 2A, inset).

To confirm mitogenicity, PC12–AC cells were cell cycle synchronized by serum starvation for 36 h before stimulation with vehicle or test phenolics. After a 30 min pulse in the presence of the thymidine analog BrdU, cells were chased for 2.5 h with DMSO or phenolics. As predicted by WST results, compound 39 did not affect the number of

Table 2. Bioactivity of 54 plant phenolics in PC12–AC cells exposed to serum deprivation, etoposide, or ceramide.

No.	Compound	Viable cells, %[a]			Identified biological activity
		Serum withdrawal	Etoposide, 25 μmol/L	Ceramide, 20 μmol/L	
Vehicle controls					
	DMSO–ethanol (vehicles)[b]	100.0±1.1	42.8±1.6	44.4±2.4	Not applicable
Benzoic acids					
1	Salicylic acid	110.8±5.0	50.7±2.7	33.6±6.0	No effect
2	3-Hydroxybenzoic acid	112.7±5.3	40.9±4.8	64.3±9.6	No effect
3	Resorcyclic acid	91.4±5.5	57.6±4.1*	38.7±3.5	Antiapoptotic
4	Gentisic acid	170.9±13.6*	68.6±7.0*	104.9±13.4*	Mitogenic[c,d]
5	Protocatechuic acid	85.7±7.0	46.4±6.3	29.2±6.6	No effect
6	Vanillic acid	98.9±4.8	68.2±3.0*	39.±7.8	Antiapoptotic
7	Orsellinic acid	104.6±4.5	58.3±5.1*	67.6±5.4*	Antiapoptotic[c,e]
8	Gallic acid	137.9±2.5*	49.5±2.2	32.6±11.0	Cytoprotective
9	Veratric acid	154.5±6.7*	63.1±2.2*	63.7±12.2*	Mitogenic
10	3,5-Dihydroxy-4-methoxy-benzoic acid	96.0±2.4	65.0±2.6*	65.7±13.2*	Antiapoptotic
11	Syringic acid	137.7±10.3*	74.3±7.7*	40.6±8.9	Mitogenic
12	*m*-Digallic acid	65.4±7.1**	49.2±6.4	75.3±5.4*	Cytotoxic/anti-apoptotic
13	Bergenin	99.8±5.2	78.2±3.7*	26.2±6.3	Antiapoptotic
Cinnamic acids					
14	Cinnamic acid	108.1±9.3	53.4±1.8	84.8±12.3*	Antiapoptotic
15	*trans*-Cinnamic acid	105.0±6.4	37.4±4.7	69.4±9.6*	Antiapoptotic
16	*o*-Coumaric acid	83.7±2.6**	51.2±5.1	98.5±7.1*	Cytotoxic/anti-apoptotic
17	*m*-Coumaric acid	128.6±10.6*	66.8±8.6*	77.2±16.0*	Mitogenic
18	*p*-Coumaric acid	89.3±5.7	17.1±1.1**	56.7±5.7	Cytotoxic
19	Caffeic acid	103.7±8.0	30.3±1.1	96.2±6.8*	Antiapoptotic
20	Ferulic acid	120.3±6.5*	21.4±1.0**	39.1±9.0	Cytotoxic[c]
21	Sinapic acid	117.7±4.1*	41.7±1.1	71.9±10.2*	Mitogenic
22	3-(*m*-Hydroxy)-phenyl-propanoic acid	109.7±2.7	85.1±6.7*	42.1±3.4	Antiapoptotic
23	3-(*p*-Hydroxy)-phenyl-propanoic acid	57.7±4.2**	38.3±3.6	14.9±3.8**	Cytotoxic
24	Hydrocaffeic acid	95.0±2.9	64.1±4.4*	54.4±4.3	Antiapoptotic
25	2-(*m*-Hydroxy)-phenylacetic acid	120.4±6.7*	53.9±6.4	95.3±8.9*	Mitogenic
Coumarins					
26	Coumarin	158.4±13.3*	62.8±4.2*	127.7±14.2*	Mitogenic
27	Umbelliferone	120.5±7.5	58.8±6.8*	35.6±4.7	Antiapoptotic
28	Esculetin	142.2±10.7*	59.2±8.1*	91.8±10.4*	Mitogenic
Flavonoids					
29	7-Hydroxyflavone	83.9±5.8**	52.9±5.9	41.1±6.1	Cytotoxic
30	Apigenin	105.1±5.8	23.3±0.7**	58.7±5.7*	Pro-apoptotic/anti-apoptotic
31	Cosmetin[f]	169.7±5.6*	61.5±4.1*	40.6±3.3	Mitogenic
32	Lucenin[f]	139.7±9.1*	61.0±5.0*	27.0±4.1	Mitogenic
33	Quercetin	94.9±4.6	52.4±5.8	54.0±8.5*	Antiapoptotic[e]
34	5-Methoxy-quercetin	150.9±9.5*	41.2±2.5	57.0±9.5*	Mitogenic
35	Quercitrin[f]	125.5±3.9*	84.2±5.7*	36.1±3.5	Mitogenic
36	Myricitrin[f]	125.2±4.7*	62.7±4.1*	50.4±6.8	Mitogenic
37	Kaempferitrin[f]	92.2±6.7	75.1±4.1*	26.1±1.7	Antiapoptotic
38	Rutin[f]	130.4±9.0*	74.3±5.1*	29.4±4.3	Mitogenic[c,d]
39	Flavanone	105.3±11.0	32.1±4.4	26.9±5.9	No effect[c,d]
40	Naringenin	102.1±9.7	42.3±3.5	10.2±1.8**	Pro-apoptotic
41	Hesperetin	93.7±4.2	60.6±3.4	86.3±7.9*	Antiapoptotic

Table 2 (*concluded*).

No.	Compound	Viable cells, %[a] Serum withdrawal	Etoposide, 25 μmol/L	Ceramide, 20 μmol/L	Identified biological activity
42	Naringin[f]	117.6±5.1*	55.7±3.6	50.6±6.8	Cytoprotective
43	Taxifolin	149.1±7.2*	35.4±1.9	103.0±9.0*	Mitogenic[d]
44	Epicatechin	138.5±7.7*	55.9±4.1	56.8±8.2*	Mitogenic
45	Epigallocatechin gallate	116.8±2.2	60.8±3.7*	20.9±2.9	Antiapoptotic
46	Daidzein	85.4±3.1	74.4±4.0*	64.3±6.7*	Antiapoptotic
47	Malvidin-3,5-diglucoside[f]	134.0±5.8*	65.5±6.8*	69.0±11.4*	Mitogenic
48	Phloridzin[f]	120.3±4.3*	29.6±3.3	43.8±4.4	Cytoprotective
Tannins					
49	Geraniin	143.2±9.6*	74.0±6.0*	45.7±7.2	Mitogenic
50	Strictinin	137.1±10.9*	98.3±6.6*	84.6±10.8*	Mitogenic
51	Pedunculagin	104.7±5.4	100.7±4.6*	29.73±5.3	Antiapoptotic[c]
52	Oenothein B	103.4±7.5	115.1±9.0*	66.3±2.1*	Antiapoptotic
53	Casuarinin	73.4±2.1	117.6±6.9*	17.6±5.2	Antiapoptotic
54	Tannic acid	135.7±15.4*	66.8±7.2	51.7±3.5	Cytoprotective

Note: *, significantly greater than vehicle control (ANOVA, post hoc Fisher LSD, $p < 0.01$); **, significantly less than vehicle control (ANOVA, post hoc Fisher LSD, $p < 0.01$)

[a]Viable cell number was established by WST assay compared with standard curves of known cell number following treatment in one of the following media: (*i*) growth factor-deprived media (serum withdrawal column), (*ii*) with etoposide in growth factor-deprived media (etoposide), and (*iii*) with ceramide in growth factor-deprived media (ceramide) as defined in Materials and methods.

[b]Mean viable cell number for vehicle controls includes all experimental trials ($n = 110$). Note that statistics were performed by phenolic class using only the yoked controls run with each phenolic ($n = 10$–15) per group ($n = 25$–80).

[c]Biological activity was confirmed by Live/Dead assay as described in Materials and methods.

[d]Biological activity was confirmed by quantification of BrdU-positive ($BrdU^+$) nuclei following serum starvation as described in Materials and methods.

[e]Ability to inhibit caspase-3 and -12 cleavage in response to etoposide and ceramide was assessed by Western blot analysis as described in Materials and methods.

[f]Flavonoid glycoside.

$BrdU^+$ cells, whereas compounds 4, 48, and 43 promoted entry into S phase (Fig. 2B). Increased BrdU labelling strongly correlated with WST estimation of increased cell number (Fig. 2B, inset).

Cytotoxic effects of plant-derived phenolics

Of the 54 test compounds, only 8 (15%) reduced PC12–AC viability under 1 or more of the 3 test conditions. Six phenolics, compounds 12, 16, 18, 20, 23, and 29, decreased metabolic activity and (or) cell survival in growth factor-deprived media and were classified as cytotoxic (Table 2). Of these, 18 and 20 also potentiated etoposide-induced cell loss, whereas 23 increased ceramide-induced cell death. Four compounds exhibited more complex interactions. Compounds 12 and 16 reduced PC12–AC viability in the absence of exogenous growth factors but partially protected cells from ceramide-induced cell death. These phenolics were thus classified as both cytotoxic and antiapoptotic (Table 2). Compound 40 potentiated ceramide-induced cell death without affecting cell survival in serum-free conditions and was classified as pro-apoptotic. Compound 30 did not alter viability of growth factor-deprived cultures, augmented etoposide-induced toxicity, yet inhibited ceramide-induced apoptosis, and therefore was classified as both pro- and antiapoptotic, depending upon the apoptotic pathway initiated (Table 2).

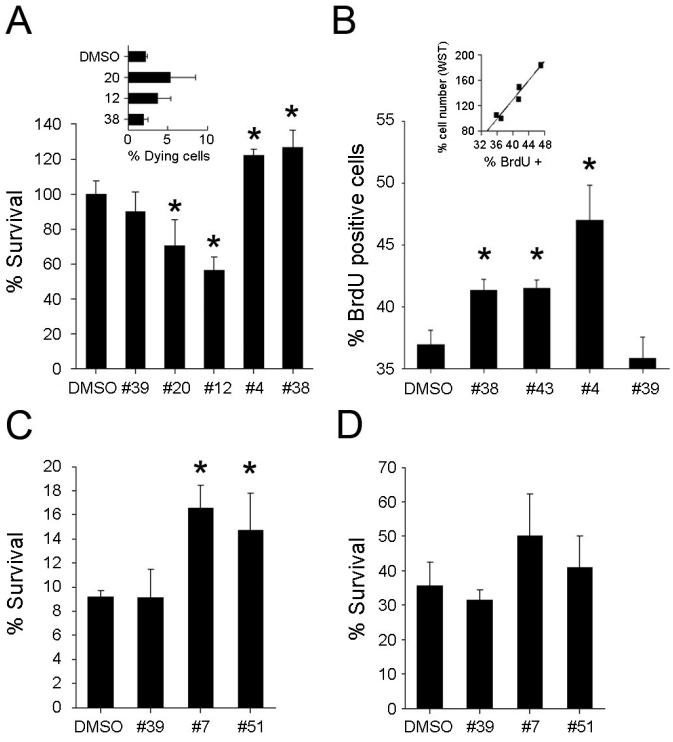
Antiapoptotic activities of plant-derived phenolics

Nineteen compounds inhibited ceramide- or etoposide-induced cell death without altering viability of vehicle-treated cultures. These antiapoptotic phenolics were: 3, 6, 7, 10, 13–15, 19, 22, 24, 27, 33, 37, 41, 45, 46, and 51–53 (Table 2). Including the 3 phenolics exhibiting more complex interactions (compounds 12, 16, 30), 22 phenolics (41%) exhibited significant antiapoptotic actions in 1 or both of the cell death models. The antiapoptotic activities of compound 7 and 51 were confirmed by Live/Dead assay following etoposide challenge (Figs. 2C and 2D).

Calculation of physicochemical parameters within phenolic families

To identify structural determinants underlying these phenolic bioactivities, lipophilicity (log *P*) and steric bulk (MR) values were calculated for each compound (Table 1). Only phenolics containing sugar moieties, including bergenin (compound 13) and flavonoid glycosides, produced negative log *P* values, indicating preferential partitioning to the hydrophilic phase. The tannins, which also contain sugars, were more lipophilic, producing scores between 0 and 1. All cinnamic acids, coumarins, and most of the benzoic acids and flavonoid aglycones produced values between 1 and 2. Compounds 1, 29, 39, and 46 were the only compounds with log *P* values greater than 2, signifying a 100-fold preference for lipophilic conditions. As expected, calculated MR values were lowest for the simple phenolics (benzoic acids, 34–72 m^3/mol; cinnamic acids, 38–59 m^3/mol; coumarins, 42–46 m^3/mol) and higher for flavonoids (68–141 m^3/mol) and tannins (>138 m^3/mol), particularly those containing glycosidic, galloyl (trihydroxyphenolic), or hexahydroxydiphenolic residues.

Fig. 2 Validation of assigned bioactivities. (A) PC12–AC cells were cultured in growth factor-deprived media for 24 h in the presence of the control vehicle (0.1% DMSO – 0.1% ethanol) or test phenolics. Cell survival was assessed by Live/Dead assay. Data represent % survival standardized to vehicle-treated cells ± SE. Inset depicts the number of ethidium bromide$^+$ dead cells detected in treated cultures (n = 4–6 performed over duplicate experiments). 39, flavanone; 20, ferulic acid; 12, *m*-digallic acid; 4, gentisic acid; 38, rutin. (B) PC12–AC cells were synchronized by serum starvation for 36 h and pulse chased with BrdU. Data represent the percentage of BrdU-labelled cells after a 30 min pulse and 2.5 h chase (n = 6–9 performed over duplicate experiments). 38, rutin; 43, taxifolin; 4, gentisic acid; 39, flavanone. Inset depicts linear regression comparing WST assay and BrdU labelling, validating the WST assay as a measure of cell proliferation (r^2 = 0.947, $p < 0.01$). (C) Cell survival following etoposide or (D) ceramide treatment was assessed by Live/Dead assay. 39, flavanone; 7, orsellinic acid; 51, pedunculagin. Statistics were ANOVA and post hoc Dunnett's t test (*, $p < 0.05$).



We determined theoretical antioxidant capacity (Wright et al. 2001). To validate these calculations, we compared our predicted values with empirical measures reported in the literature for the same phenolics (Baderschneider and Winterhalter 2001; Cai et al. 2006; Meyer and Frankel 2001; Pannala and Rice-Evans 2001). Linear regression detected a significant correlation between predicted BDE values and those obtained using the radical 2,2′-azobis-3-ethyl-benzthiazoline-6-sulphonic acid and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assays, demonstrating that our antioxidant calculations are comparable with previously published experimental values (data not shown). BDE is an indication of chain-breaking antioxidant ability with lower energies providing superior activity. A BDE value below, near, or above 80 kcal/mol designates strong, weak, and poor antioxidant potential, respectively, with regards to lipid peroxidation. In general, cinnamic acids were predicted to be better antioxidants than benzoic acids, but weaker than flavonoids and other polyphenolics (Table 1). Catechol (3,4-dihydroxy) and galloyl (3,4,5-trihydroxy) substitution patterns decreased BDE values. Of the benzoic acids, only compound 8, its condensation product compound 12, and, to a lesser extent, compound 5 possessed BDEs indicative of good antioxidant potential. Similarly, compounds 19, 21, and 24 were the only cinnamic acids with BDEs below 80 kcal/mol. Compound 28, a catechol-containing coumarin, presented the lowest BDE among phenolic acid derivatives (73.9 kcal/mol). Flavonoids are recognized antioxidants and, correspondingly, many members presented BDE values between 70–79 kcal/mol, resulting in family-wide mean of 78.5 kcal/mol. Unlike log P and MR values, glycosylation of flavonoids did not strongly influence BDE values. Though the calculated BDEs across tannins remained fairly constant (74.9–75.5 kcal/mol), the number of trihydroxyphenolic substituents varied from compound to compound, resulting in more or fewer sites of potential radical scavenging. For instance, compounds 50 and 52 contain 9 and 22 benzene-associated hydroxy groups, respectively, but shared nearly identical BDEs (Table 1).

Analysis of covariance between the physicochemical parameters revealed several significant relationships within families. For benzoic acids, steric volume (MR) increased with decreasing log P value (p = 0.023, r^2 = 0.386), as substitution of the phenolic ring with polar side groups resulted in greater bulk and hydrophilicity. Within the cinnamic acids, log P values were all greater than 1, thus $(\log P)^2$ values showed near perfect covariance. Additionally, the calculated BDEs of cinnamic acids decreased with decreasing log P values (p = 0.017, r^2 = 0.531), again due to substitution of the phenolic ring. The flavonoids displayed a similar but much stronger relationship between log P and MR ($p < 0.001$, r^2 = 0.866) as larger glycosidic compounds were markedly more hydrophilic than aglycones.

QSAR modelling of phenolic bioactivities

Compounds exhibiting similar biological activities within given families were analyzed as subgroups. Subgroups with fewer than 6 compounds were not considered. Coumarins are derived from the phenylacrylic skeleton of cinnamic acids. As such, the cinnamic acids were assessed with and without the coumarins as analytical subgroups. Owing to limited physicochemical data, no QSAR analyses were conducted on the tannins.

Structure–activity relationships were identified for the flavonoids and benzoic acids but not for the cinnamic acids/coumarins. The physicochemical properties of flavonoids were strongly correlated with their mitogenic and cytoprotective activities. As expected, phenolic capacity to increase PC12–AC viability in growth factor-deprived cultures depended, in part, on relative antioxidant potential, but, surprisingly, when all 20 flavonoids were analyzed, only a weak correlation was identified (p = 0.062, r^2 = 0.190, Fig. 3A). However, when we restricted our analysis to the aglycones, the antioxidant model was significantly strengthened (p = 0.037, r^2 = 0.437, Fig. 3B). This separation also revealed a second structure–activity relationship within the glycosides wherein effects on cell viability under conditions

of serum deprivation was a function of lipophilicity (Fig. 3C). According to this model, viability was optimized at log *P* approaching –0.8, suggesting that the glycosides are acting in the aqueous phase.

Antiapoptotic activities in the etoposide paradigm were strongly correlated with physicochemical properties. Among flavonoids, protection increased with MR (Fig. 4). Although this model was not improved by separate analysis of flavonoid subgroups, the relationship largely reflects the finding that the bulky glycosides displayed significant activity, whrereas the smaller aglycones were generally inactive (Table 2). This model was not improved by separate analysis of flavonoid subgroups and appeared to be an underlying property of flavonoid activity. Within the benzoic acids, antiapoptotic activity following etoposide treatment was associated with lipophilicity and steric bulk.

Interestingly, the antiapoptotic effects in the ceramide death paradigm could not be modelled with the considered physicochemical parameters for any phenolic class beyond the presence of a catechol group associated with reduced ceramide toxicity among flavonoid aglycones (compounds 33, 34 , 43 and 44) (Table 2).

Compound 7 (orsellinic acid) inhibits caspase-12 cleavage

In the absence of a strong QSAR model, the ability of select phenolics to protect PC12–AC cells from ceramide-induced apoptotic signalling was assessed biochemically. We chose a catechol-containing antiapoptotic flavonoid (compound 33) and an antiapoptotic benzoic acid (compound 7) for mechanistic evaluation. Both etoposide and ceramide elicit caspase-dependent apoptosis with caspase-3 activation (Scorrano et al. 2003) but only ceramide triggers an endoplasmic reticulum stress pathway (Darios et al. 2003; Mandic et al. 2003), activating caspase-12 in PC12 cells (Smith et al. 2005). Figure 5 confirms that etoposide and ceramide treatment activated caspase-3, indicated by cleavage of the caspase-3 substrate PARP, but only ceramide activated caspase-12, determined by cleavage of procaspase-12 to its active form, in PC12–AC cells. Exposure to compounds 7 and 33 had no effect on PARP cleavage following etoposide (data not shown) and ceramide treatment (Fig. 5), whereas compound 7, but not 33, inhibited caspase-12 cleavage in ceramide-treated cultures (Fig. 5).

Discussion

Dietary phenolics have been promoted as an adjuvant means of reducing cancer incidence and sensitizing tumour cells to existing chemotherapeutic agents (d'Ischia et al. 2006; Landis-Piwowar et al. 2006; Lee and Lee 2006). Underlying molecular mechanisms have only begun to be elucidated. In this study, we present the results of an in vitro screen designed to compare the bioactivities of a panel of natural phenolic compounds at concentrations ranging between 0.6 to 7.3 μmol/L (Table 1). Whereas similar studies have focused on the pharmacological activities of phenolics at higher concentrations (>50 μmol/L), few have examined effects on cell viability at the physiologically relevant dietary concentrations. We compared 54 phenolics (Table 1) for their capacity to enhance or reduce PC12–AC cell viability under conditions of growth-factor deprivation or following challenge with C2-ceramide or etoposide. Our test compounds included phenolics previously shown to potentiate tumour cell death in response to chemotherapeutic agents (i.e., epigallocatechin gallate and protocatechuic acid) as well as phenolics with purported chemopreventive potential (d'Ischia et al. 2006; Kulkarni et al. 2006; Lee and Lee 2006; Narayanan 2006; Stuart et al. 2006; Tseng and Lee 2006). Antiapoptotic and, surprisingly, mitogenic activities were most commonly identified for phenolics in the benzoic acid, cinnamic acid, coumarin, flavonoid, and tannin classes.

Fig. 3 Quantitative structure–activity relationship (QSAR) models of flavonoid activity in growth factor-deprived and etoposide-treated cells. (A) QSAR analysis of flavonoid activity in growth factor-deprived cells and compound antioxidant capacity (BDE). (B) QSAR analysis as in (A) but of the aglycone subgroup. (C) QSAR analysis as in (A) but of the glycoside subgroup and lipophilicity, log *P*. Trend lines represent the expected values predicted by the given QSAR model. Refer to Table 1 for individual physicochemical values.

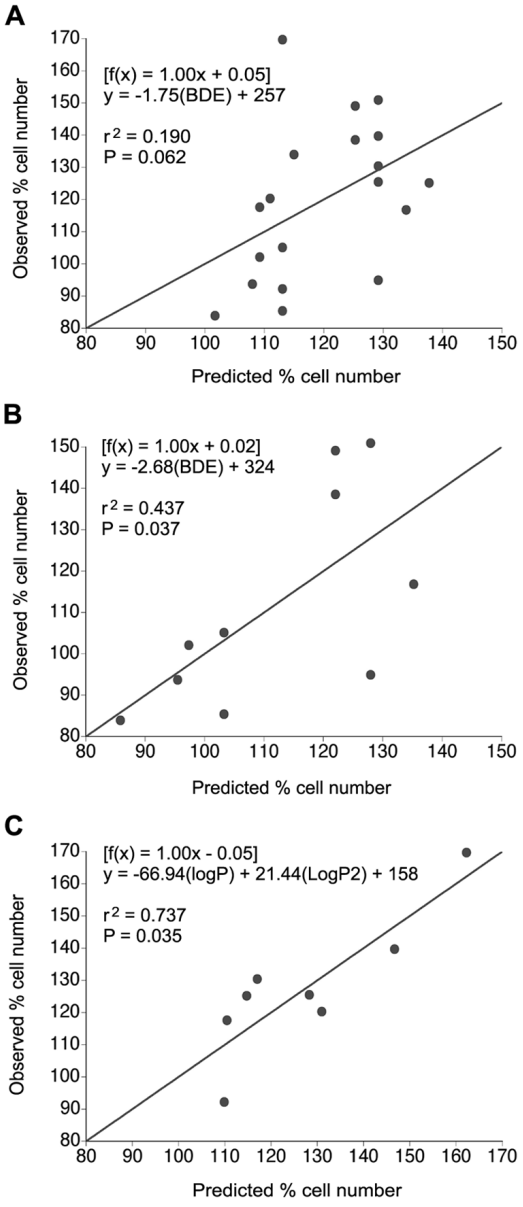
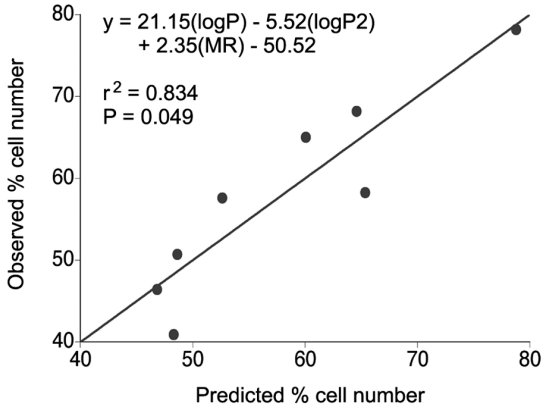


Fig. 4 Quantitative structure–activity relationship (QSAR) models using lipophilicity (log *P*) and steric bulk (MR) of benzoic acid activity in etoposide-treated cells. Trend lines represent the expected values predicted by the given QSAR model. Refer to Table 1 for individual physicochemical values.



These activities could be modelled in some, but not all, cases by QSAR analysis. As summarized in Table 2, 39% of compounds were identified as antiapoptotic (<6 μmol/L), reducing cell death triggered by etoposide and (or) ceramide, 35% were identified as mitogenic (1.5–7 μmol/L), increasing PC12–AC cell proliferation in the absence of exogenous growth factors, and another 7% were cytoprotective (2–5.5 μmol/L), promoting cell survival during serum withdrawal without altering cell cycle kinetics. Only 8 (15%) of the tested compounds were cytotoxic or pro-apoptotic. Three of these exhibited multiple activities dependent upon the stressor present in the microenvironment.

The high percentage of mitogenic phenolics detected in this study was unexpected. Although consistent with reports that coumarins and flavonoids regulate G1/S and G2/M cell cycle check-point transitions, our data conflict with findings that many of the same phenolics inhibit cell cycle progression and augment the toxicity of chemotherapeutic DNA damaging agents (Finn et al. 2004; Kuo et al. 2006; Lacy and O'Kennedy 2004; Singh and Agarwal 2006; Wang et al. 2002; Yeh et al. 2005). We argue that phenolic bioactivity is dramatically affected by concentration. Previous studies have documented similar dose-dependent activation of prosurvival and cytotoxic pathways in other cell systems. For example, esculetin (compound 28) at concentrations <30 μmol/L inhibits cell death triggered by disruptions in calcium homeostasis or by excitotoxicity (Kim et al. 2000; Wie et al. 2001), yet initiates a dose-dependent mitochondrial apoptotic response when concentrations exceed 100 μmol/L (Yang et al. 2006). Interestingly, salicylic acid (compound 1) and protocatechuic acid (compound 5) did not exhibit any detectable bioactivity in this study at concentrations <10 μmol/L, despite reports of cytotoxicity on carcinoma tumour cells at >100 μmol/L (Nunez et al. 2006; Yip et al. 2006).

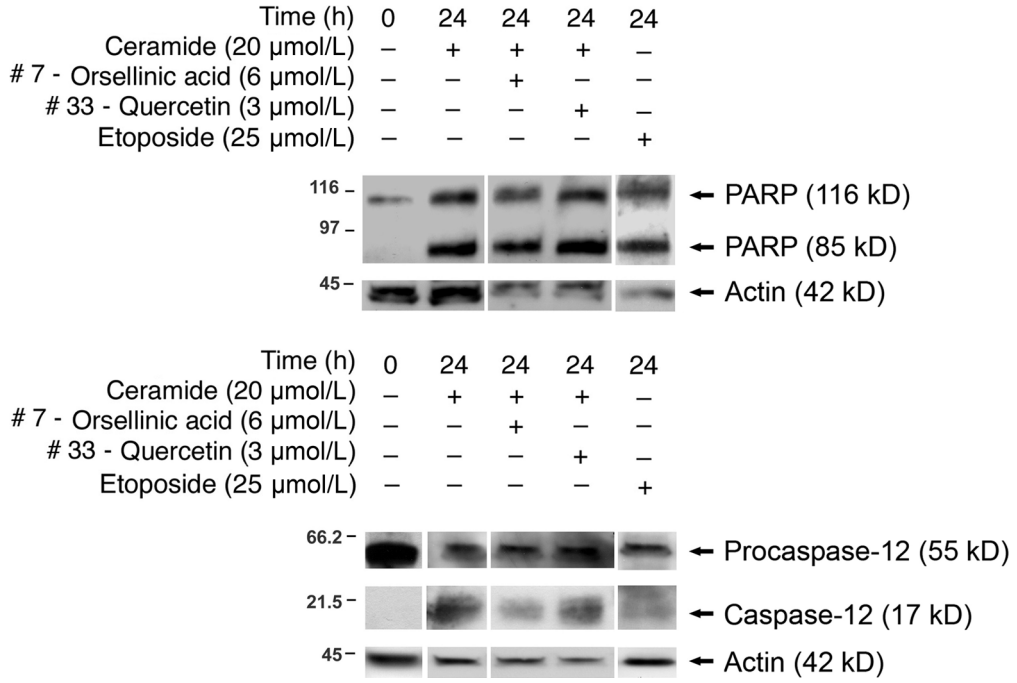
The antiapoptotic interactions detected here also raise important considerations for use of natural health products as adjuvants with chemotherapeutic agents. Here, two thirds of tannins (<1.6 μmol/L) completely inhibited etoposide-induced death, despite reported pro-apoptotic activity at higher concentrations (Wang et al. 2001). The ability of strictinin (compound 50) and oenothein B (compound 52) to reduce etoposide toxicity is nonetheless consistent with previous reports (Choi et al. 2002; Wei et al. 2002). Interestingly, within the benzoic acids, the aldehyde vanillin, but not its major metabolite vanillic acid (compound 6), has been shown to have growth-inhibitory effects on tumour cells at concentrations >250 μmol/L (Lirdprapamongkol et al. 2005). The antiapoptotic activity of vanillic acid (6 μmol/L) observed was consistent with reports that vanillic acid derivatives alter NF-κB and Akt kinase signalling in tumour cells (Kumar et al. 2003). Together, these data suggest that the metabolism of potential anti-tumour phenolics may inhibit their efficacy as chemosensitizers.

We found that the protective effects of low-dose phenolics could be accurately modelled by their physicochemical properties. The 20 flavonoids included in our collection are composed of several structurally distinct groups with a variety of phenolic ring substituents (Table 1). Subdividing these compounds into glycosides and aglycones (no sugar moiety) revealed 2 distinct structure–activity profiles associated with both cytoprotection and mitogenicity. Moreover, the QSAR for flavonoid mitogenicity and cytoprotection differed from the QSAR for antiapoptotic activity. Within the aglycone group, lower BDE values (greater antioxidant capacity) correlated with increasing viability under conditions of serum deprivation as previously reported (Baderschneider and Winterhalter 2001; Lee et al. 2005*a*; Meyer and Frankel 2001; Ratty and Das 1988). Conversely, glycoside activity in the same conditions did not depend on antioxidant capacity but on lipophilicity. Nearly all the glycosides tested in this study enhanced viability; those with log *P* values close to –1 had the greatest effect. Such compounds are unlikely to passively diffuse across the cell membrane and therefore are predicted to interact with extracellular receptors or transporters where they may activate signalling cascades or be transported into the cell. In support of this hypothesis, the flavonoid glycosides quercitrin and kaempferitrin (compounds 35 and 37, respectively) have been shown to interact directly with glucose transporters or extracellular receptors (Jorge et al. 2004). Thus, glycosidic flavonoids are predicted to interact with critical effectors (in addition to their antioxidant capacities), whereas the ability of aglycones to promote cell viability appears owing, in large part, to their ability to act as effective antioxidants at low concentration.

The QSAR for antiapoptotic flavonoid activity in the etoposide paradigm was positively correlated with increasing MR values. With 7 of 9 glycosides, but only 2 of 11 aglycones, significantly reducing etoposide-induced death (Table 2), this relationship is likely a consequence of added bulk provided by glycosyl groups rather than an accurate model of activity within the entire class. Curiously, no significant model was identified within glycosides or aglycones. The strong association between glycosylation and protection suggests a distinct mode of action with glycoside-specific access to cellular targets or pathways. This activity may depend upon a closed C-ring as suggested by the observation that compound 48, a dihydrochalcone glycoside, failed to protect the cells from death (Table 2).

Structural correlates underlying the antiapoptotic activity of benzoic acids revealed an unexpected determinant with

Fig. 5 Western blot analysis of caspase cleavage following etoposide or ceramide treatment in the presence of phenolics. Biochemical assessment was performed for compounds for which quantitative structure–activity relationship (QSAR) analysis could not predict bioactivity. Etoposide and ceramide activated caspase-3, resulting in the cleavage of poly(ADP-ribose) polymerase (PARP), but only ceramide treatment induced cleavage of caspase-12 to its activated form. Neither phenolic prevented PARP cleavage. We identified a mechanism of action for orsellinic acid (compound 7) attenuating caspase-12 cleavage but not quercetin (compound 33).



in vivo impact upon the efficacy of the chemotherapeutic agent etoposide. QSAR analysis of antiapoptotic benzoic acids in the etoposide paradigm was highly correlated with MR and log *P*. This model became even stronger with inclusion of syringic acid (compound 11) that, together with bergenin (compound 13), represents the highest and most protective benzoic acid derivative. Moreover, syringic acid and bergenin were the only 2 phenolic acids tested in this study that possess a 3,5-dimethoxy 4-hydroxybenzyl structure. Etoposide, a semisynthetic derivative of the plant lignan podophyllotoxin, contains an exposed syringic acid residue. If this residue is directly involved in topoisomerase II interaction, free syringic acid-like compounds may provide protection by occupying the site of etoposide–topoisomerase association and preventing DNA damage. If such is the case, the lesser (yet significant) protection provided by structurally similar benzoic acids (compounds 6, 9, and 10, Table 1) may reflect lower affinities for the site of association and the involvement of lipophilicity in the QSAR model may reflect the required passage through plasma and nuclear membranes.

Surprisingly, the antiapoptotic activity of benzoic acids in the ceramide model did not correspond with a distinct structural profile, but to an activity profile. With the exception of *m*-digallic acid (compound 12), all of the benzoic acids that reduced ceramide toxicity also inhibited etoposide-induced cell death, perhaps indicative of additional direct effects on cell survival and (or) death pathways. In comparing the ability of the antiapoptotic flavonoid quercetin (compound 33) with the antiapoptotic benzoic acid orsellinic acid (compound 7), we show, for the first time, that orsellinic acid inhibits cleavage of procaspase-12 to its active form during endoplasmic reticulum stress triggered by ceramide, providing direct evidence of a direct signalling effect.

Conclusion

In summary, a comparison of 54 phenolics revealed an unexpected degree of cytoprotective, mitogenic, and antiapoptotic potential at dietary concentrations. QSAR analyses indicate that many of these activities can be predicted a priori by compound lipophilicity, steric bulk, and (or) antioxidant capacity. Notably, antioxidant capacity appears to be an important determinant of aglycone cytoprotection, but, surprisingly, less predictive of the protective effects of the bulky glycosides in which steric bulk and lipophilicity were more strongly correlated with bioactivity. These data are suggestive of bioactivity directed at discrete cellular targets predominantly in the aqueous phase. To begin to characterize these phenolic targets, we identified a novel caspase-12 inhibitor in orsellinic acid (compound 7). These findings highlight the need to continue to mechanistically evaluate the bioactivities of phenolics at physiologically relevant concentrations on normal, neoplastic, and tumour cells to be able to predict potential biological activity under different environmental conditions.

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