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Title: Flazin as a promising Nrf2 pathway activator

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Abstract

Flazin is a β -carboline-derived alkaloid found in Japanese fermented foods. Here, the potential of flazin as an antioxidant food was studied with particular regard to its effect on the Keap1-Nrf2 system in human hepatocytes (C3A). Flazin, flazin analogues, and the decarboxylated derivative perlolyrine were chemically synthesized and compared with each other and with chlorogenic acid and curcumin. Among these compounds, flazin showed the lowest cytotoxicity ($IC_{50} < 500 \mu M$) and the highest capacity to activate Keap1-Nrf2 and provided the most cytoprotection against a pro-oxidant, although its radical absorbance capacity was relatively low. Flazin increased Nrf2-dependent protein expression and Nrf2-dependent phase II enzyme gene expression. The characteristics of flazin, including its combination of strong cytoprotective ability and a low log P , are shared by sulforaphane and 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA), suggesting the potential value of flazin and flazin-rich foods for the prevention and/or alleviation of health disorders involving oxidation processes.

1. Introduction

The balance of reactive oxygen species (ROS) is maintained by a cytoprotection system whose components can be divided into enzymatic components (superoxide dismutase [SOD], catalase [CAT], etc.) and non-enzymatic components (glutathione [GSH], vitamins, etc.)¹. Non-compensated overproduction of ROS damages cells, causing ageing² and various pathological conditions, such as neoplasia³, inflammation⁴, atherosclerosis⁵, and neurodegenerative diseases⁶. Ingestion of phenolic antioxidants in foods is crucially important for health and for the maintenance of physiological conditions resistant to ROS⁷.

Dinkova-Kostova and Talalay proposed classifying antioxidants into two groups: direct and indirect antioxidants⁸. Direct antioxidants scavenge ROS through redox reactions. During this process, the direct antioxidants are chemically oxidized and in some cases regenerated^{9,10}. The antioxidant potential of direct antioxidants can be evaluated through *in vitro* chemical analyses, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays or oxygen radical absorbance capacity (ORAC) assays. On the other hand, indirect antioxidants induce a series of antioxidant enzymes or low-molecular-weight substances by activating the Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and thus ultimately

biologically catalyse ROS breakdown in cells. Indirect antioxidants may be redox active, but this characteristic is not necessary for their indirect antioxidant activity ⁸.

Flazin is a β -carboline-derived alkaloid found in Japanese fermented foods such as Japanese rice wine lees, rice vinegar, soy sauce, and soy paste ¹¹; it has also been found in a marine bacterium ¹² and in a fungus from China ¹³. Flazin has been reported to induce NAD(P)H: quinone oxidoreductase (NQO1) in a murine hepatoma cell line ¹⁴. Since NQO1 belongs to the family of Nrf2-dependent phase II antioxidant enzymes, we aimed to uncover the possible relationship of flazin and its analogues with the Keap1-Nrf2 pathway in this study.

To this end, we chemically synthesized flazin, four analogues ¹³ and decarboxylated derivative perlolyrine in the present study. Furthermore, we compared these compounds with chlorogenic acid and curcumin, which are representative direct and indirect antioxidants, respectively ¹⁵, in assessments of cytotoxicity, ORAC, Keap1-Nrf2 pathway activation, and cytoprotection against oxidative stress.

In addition, we here propose a model for predicting the usefulness of antioxidants in which log *P* and cytoprotective effects against oxidative stress serve as determinant factors. Log *P*, or the partition coefficient ¹⁶, is common in drug design in the pharmaceutical and biotechnology industries as a measure of a solute's hydrophobicity

and as a proxy for its membrane permeability; however, this parameter has been uncommon in the food industry. Our proposed model reveals a unique position of flazin among antioxidants, indicating its remarkable potential with regard to the prevention and/or alleviation of health disorders involving oxidation processes.

2. Materials and methods

2.1. Chemicals

The chemicals used in this study were described in the Supporting Materials and Methods.

2.2. n-Octanol/water partition coefficients (log *P* values)

The n-octanol/water partition coefficients (log P_{ow} or log *P* values) of flazin and perlolyrine were obtained from the PubChem Open Chemistry Database (<https://pubchem.ncbi.nlm.nih.gov/search/>) maintained by the National Institutes of Health (NIH). The log *P* values for the flazin analogues, chlorogenic acid (**8**), and curcumin (**7**) were obtained from the Virtual Computational Chemistry Laboratory (<http://www.vcclab.org/lab/alogps/>) and the literature^{17,18}.

2.3. Chemical synthesis

2.3.1. General

The chemicals and procedures in chemical synthesis were described in the Supporting Materials and Methods.

2.3.2. Synthesis of flazin and its analogues

The structures of the compounds synthesized in this study as well as those of chlorogenic acid (**8**) and curcumin (**7**) are shown in Fig. 1. Flazin (**1**) and its analogues (**3-6**) were synthesized as reported by Tang *et al.*¹³, and each synthesized compound was confirmed to be identical to the previous report on the basis of NMR chemical shifts and the molecular weights determined by mass spectrometry.

2.3.3. Synthesis of perlolyrine

2.3.3.1. Synthesis of compound 10

To a solution of tryptamine (**9**) (3.33 g, 20.8 mmol) and activated 3Å molecular sieves (10 g) in anhydrous dichloromethane (210 mL), trifluoroacetic acid (0.41 mL: 5.41 mmol) and 5-acetoxy-2-furaldehyde (3.50 g, 20.8 mmol) were added, and the mixture was stirred for 2 days under a nitrogen atmosphere. After TLC indicated that the reaction was complete, the reaction mixture was passed through a pad of Celite. The solvent was removed under reduced pressure, and the crude product was purified with flash column chromatography (hexane/EtOAc = 2/1–0/1) to give cyclized **10** (3.60 g, 56%) as a brown solid. The data of proton and carbon NMR were described in the

Supporting Materials and Methods.

2.3.3.2. Synthesis of compound **11**

To a solution of **10** (3.50 g, 11.3 mmol) and trimethylamine (5.67 mL, 40.7 mmol) in DMF (20 mL), trichloroisocyanuric acid (2.63 g, 11.3 mmol) in DMF (10 mL) was added, and the mixture was stirred for 2 h. The reaction mixture was diluted with chloroform, washed with water, dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified with flash column chromatography (hexane/EtOAc = 5/1~3/1) to give oxidized **11** (545 mg, 16%) as a light-brown film. The data of proton and carbon NMR were described in the Supporting Materials and Methods.

2.3.3.3. Synthesis of perlolyrine (**2**)

Perlolyrine (**2**) was synthesized as shown in Fig. 2. To a solution of compound **11** (545 mg, 1.78 mmol) in dichloromethane/MeOH (1/1, 71 mL), NaOMe (19 mg, 0.36 mmol) was added, and the mixture was stirred for 3 h. When TLC showed the complete consumption of the starting material, the reaction was quenched by the addition of Dowex 50X8 resin until the pH was neutral (~7). The resulting mixture was filtered and concentrated. The crude product was purified with flash column chromatography (hexane/EtOAc = 2/1~0/1) to give perlolyrine (**2**) (250 mg, 53%) as a yellow solid. The

data of proton and carbon NMR were described in the Supporting Materials and Methods.

2.4. Cell culture

Human hepatoma-derived cells (C3A) were purchased from the ATCC. The cells were maintained in MEM supplemented with 10% (v/v) FBS and the PSN antibiotic mixture under an atmosphere of 5% CO₂ and 95% air in an incubator with controlled humidity at 37°C.

2.5. Cytotoxicity assay

All procedures were performed according to a previously described protocol, with minor modifications ¹⁹. Cytotoxicity assay in details was performed as indicated in the Supporting Materials and Methods.

2.6. Reporter gene assay

The ability of each compound at its non-cytotoxic concentrations to activate the Keap1-Nrf2 pathway was examined with a reporter gene assay using a Dual-Glo Luciferase Reporter Assay System (Promega) in which the antioxidant response element (ARE) drove transcription of the luciferase reporter gene according to a previously described protocol, with minor modifications ¹⁵. Reporter gene assay in details was performed as indicated in the Supporting Materials and Methods.

Chlorogenic acid (**8**) and curcumin (**7**) were used as negative and positive controls, respectively, in the reporter gene assay since we previously reported that curcumin significantly increases relative luciferase activity, while chlorogenic acid does not significantly affect it ¹⁵.

2.7. Cytoprotection assay

The cytoprotective effects of the antioxidants at its non-cytotoxic concentrations against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress in C3A cells was examined by cytoprotection assay. All procedures were performed according to a previously described protocol, with minor modifications ¹⁹. Cytoprotection assay in details was performed as indicated in the Supporting Materials and Methods.

2.8. First-strand cDNA synthesis and real-time PCR

Total RNA was extracted from C3A cells, and the mRNA was converted into first-strand cDNA for gene expression analysis. All procedures were performed according to a previously described protocol, with minor modifications ¹⁵. Gene expression induced by flazin in C3A cells was examined using real-time PCR. All procedures were performed according to a previously described protocol ¹⁵.

2.9. Western blot analysis

Protein expression induced by flazin (**1**) in C3A cells was examined using Western blotting. All procedures were performed according to a previously described protocol, with minor modifications ¹⁵. Western blot analysis in details was performed as indicated in the Supporting Materials and Methods.

2.10. Nuclear protein extraction and Western blot analysis

The effect of flazin (**1**) on the translocation of the Nrf2 protein from the cytoplasm to the nucleus was examined using nuclear protein extraction and Western blotting. Nuclear protein extraction and Western blot analysis in details was performed as indicated in the Supporting Materials and Methods.

2.11. Measurement of glutathione (GSH)

The effect of flazin (**1**) on GSH levels in C3A cells was examined. All procedures were performed according to a previously described protocol, with minor modifications ¹⁵. Measurement of glutathione in details was performed as indicated in the Supporting Materials and Methods.

2.12. Oxygen radical absorbance capacity (ORAC) assay

The free radical-scavenging activity of the antioxidants was examined using ORAC assays. All procedures were performed according to a previously described protocol ^{20,21}. The relative ORAC value (TE) was calculated as follows: relative ORAC value =

molarity of Trolox/molarity of the samples.

2.13. Statistical analysis

The data from the reporter gene assay, cell viability assay, and ORAC assay and the data from the nuclear protein, Nrf2 target gene and protein expression analyses are expressed as the mean $\pm$ SD and were analysed by one-way ANOVA using Prism 6.03.

The significance level was set at $p < 0.05$.

3. Results

3.1. Cytotoxicity

The half-maximal inhibitory concentration (IC_{50}) values for synthetic flazin (**1**), perlolyrine (**2**) and the flazin analogues are shown in Fig. 3A and Table 1. The IC_{50} values of flazin (**1**) and chlorogenic acid (**8**) were more than 500 μ M. In contrast, those of perlolyrine (**2**), curcumin (**7**), flazin analogue I (**3**), flazin analogue II (**4**), flazin analogue III (**5**), and flazin analogue IV (**6**) were low at 11.0, 109, 51.5, 88.2, 12.0, and 22.4 μ M, respectively.

3.2. ORAC assay

The relative ORAC values of the antioxidants are shown in Table 1. The relative ORAC values of flazin (**1**), perlolyrine (**2**), flazin analogue II (**4**), and flazin analogue III

(5) were 1.43 ± 0.112 , 1.60 ± 0.230 , 0.587 ± 0.124 , and 0.886 ± 0.0735 μmol of Trolox equivalents (TE)/ μmol , respectively. In contrast, those of flazin analogue I (3) and flazin analogue IV (6) were relatively high (3.65 ± 0.392 and 2.50 ± 0.287 μmol of TE/ μmol , respectively). Curcumin (7) and secondarily chlorogenic acid (8) showed the highest ORAC values: 8.12 ± 0.831 and 4.57 ± 0.303 μmol of TE/ μmol , respectively.

3.3. Reporter gene assay

The results of the reporter gene assay for the studied compounds at non-cytotoxic concentrations are shown in Fig. 3B and Table 1. Compared to the control treatment (0 μM), flazin (1) treatment at concentrations of 0-500 μM dose-dependently increased relative luciferase activity by 23.4 ± 0.861 -fold; these effects were comparable to those of curcumin (7) (23.8 ± 7.30 -fold increase at concentrations of 20-40 μM). Perlolryrine (2) increased relative luciferase activity by 4.64 ± 0.812 -fold at concentrations of 4-8 μM . Flazin analogue II (4) at concentrations of 10-20 μM increased the luciferase activity by 10.50 ± 4.5 -fold. However, chlorogenic acid (8), flazin analogue I (3), flazin analogue III (5), and flazin analogue IV (6) did not significantly affect the relative luciferase activity at any concentration.

3.4. Cytoprotection against pro-oxidants

The effects of the studied compounds at non-cytotoxic concentrations on the viability

of C3A cells exposed to the pro-oxidant (AAPH) are shown in Fig. 3C and Table 1. Flazin (**1**) significantly and dose-dependently increased cell viability by 5.21 ± 0.24 -fold at concentrations of 0-500 μ M. Curcumin (**7**) also significantly and dose-dependently increased viability by 2.66 ± 0.253 -fold at concentrations of 15-60 μ M, and flazin analogue II (**4**) increased viability by 2.00 ± 0.176 -fold at concentrations of 10-20 μ M. Perlolyrine (**2**), chlorogenic acid (**8**), flazin analogue I (**3**), flazin analogue III (**5**), and flazin analogue IV (**6**) did not significantly affect cell viability at any concentration.

3.5. Effect of flazin on Nrf2 nuclear translocation

The effect of flazin (**1**) on Nrf2 nuclear translocation in C3A cells is shown in Fig. 4A. Compared to the control treatment (0 μ M), flazin (**1**) treatment at concentrations of 0-500 μ M significantly and dose-dependently increased the levels of Nrf2 in the nucleus by approximately 1.5-fold.

3.6. Effect of flazin on the expression of Nrf2 target genes

The Nrf2 target genes induced by flazin in C3A cells are shown in Fig. 4B. Flazin (**1**) significantly induced the expression of the Nrf2 target genes *NQO1*, glutamate-cysteine ligase modulatory subunit (*GCLM*), glutamate-cysteine ligase catalytic subunit (*GCLC*) and glutathione S-transferase pi (*GSTP*) by more than 10-fold in a dose-dependent manner. More moderate but statistically significant induction was observed for *HO-1*,

SOD1, *GR*, *GPx*, *CAT*, and *GSTT*.

3.7. Effect of flazin on the expression of Nrf2 target gene products

The effects of flazin (**1**) on the expression of three Nrf2 target gene products, NQO1, GSTP and GSH (glutathione), are shown in Fig. 4C. Flazin (**1**) increased the protein expression of NQO1 and GSH in a dose-dependent manner. In addition, flazin (**1**) significantly increased GSTP expression levels at 500 μ M.

3.8. Cytoprotection and log *P*

The log *P* values of the studied compounds are shown in Table 1. The highest log *P* was observed for curcumin (3.29), while the lowest was observed for chlorogenic acid (0.69). The log *P* values of flazin (2.1), perlolyrine (2.2), and the flazin analogues (1.72-2.80) were in the middle range. For eight of the natural antioxidants (a - h), there was a strong correlation between cytoprotection and the log *P* value ($y = 0.400x + 0.880$, $r^2 = 0.766$, $p = 0.004$) (Fig. 6). For another group of four natural antioxidants (f - i), there was also a strong correlation between cytoprotection and the log *P* value ($y = 0.059x + 1.974$, $r^2 = 0.750$, $p = 0.333$) (Fig. 6). The antioxidants flazin, 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) and sulforaphane did not fit the correlation lines and were distributed in division I.

4. Discussion

Flazin has been reported to be present in traditional Japanese fermented foods, such as sake (rice wine) lees, rice vinegar, soy sauce (fermented soybean liquid), and miso (fermented soybean paste)¹¹, as well as in the fruiting bodies of *Suillus granulatus* (a fungus)¹³ and in the marine-derived bacteria *Streptomyces* spp.¹². With regard to biological function, flazin exhibits anti-HIV-1 activity¹³, NQO1-inducing activity¹⁴, leukaemic cell (HL-60) proliferation-inhibiting activity²², and xanthine oxidase-inhibiting activity²³. In our present study, flazin was found to activate the Nrf2 pathway (Fig. 3B and 4A) and to promote the expression of a battery of Nrf2 target genes and products, including NQO1 (Fig. 4B and 4C). Flazin exhibit a strong cytoprotective effect against AAPH despite its relatively low ORAC, suggesting that flazin essentially serves as an indirect antioxidant. The action mechanisms of flazin as an indirect antioxidant are summarized in Fig. 5.

The relationships between the chemical structures and anti-HIV activity of flazin and its 45 synthetic analogues have been reported previously¹³. In this study, the highest anti-HIV activity was observed for flazin analogue III (**5**), in which the carboxylic acid at carbon 3 of flazin has been converted to an amide¹³ (Fig. 1). Flazin itself was found not to exhibit anti-HIV-1 activity; thus, the activity was dramatically enhanced by the

introduction of an appropriate substituent at carbon 3. In contrast to anti-HIV-1 activity, indirect antioxidant activity requires the carboxylic at position 3. Naturally occurring flazin showed higher cytotoxicity, stronger Nrf2-activating ability, and greater cytoprotective effects than the flazin analogues or perlolyrine (Fig. 3, Table 1). Conversely, flazin analogue III (5) showed the lowest performance in cytoprotection.

The ORAC assay is one of the most common assays for measuring peroxyl radical-scavenging capacity *in vitro*, although its reaction mechanism remains unknown²⁴. In this study, the ORAC values of flazin analogues I (3) and IV (6) (3.65 ± 0.392 and 2.50 ± 0.287 μmol of TE/ μmol , respectively) were higher than that of flazin (1) (1.43 ± 0.112 μmol of TE/ μmol) (Table 1). In flazin analogues I (3) and IV (6), the carboxylic acid at carbon 3 of flazin has been replaced with a hydroxamic acid and aliphatic alcohol, respectively (Fig. 1). We hypothesize that the hydroxamic acid and aliphatic alcohol of the flazin analogues might easily form $\text{R-C(O)-NH-O}\bullet$ and $\text{R-C-O}\bullet$, respectively, to enable these analogues to scavenge peroxyl radicals in the ORAC assay more easily than flazin.

The potent cytoprotective effect of flazin can be attributed at least partly to the induction of a battery of antioxidant enzymes by flazin through activation of the Nrf2 pathway. Flazin induced the expression of Nrf2 protein and of the Nrf2 target genes

286 *HO-1*, *GCLM*, *GCLC*, *NQO1*, *SOD1*, *GR*, *GPx*, and *GSTP* (Fig. 4B and 4C). Of these,
287 *NQO1*, *GCLM*, *GCLC*, and *GSTP* and their products showed substantially increased
288 expression (Fig. 4B and 4C). The catalytic activity of *NQO1* is directed towards the
289 reduction and detoxication of highly reactive quinones and their derivatives. *NQO1* is
290 expressed in many tissues, and its expression is co-regulated by the Keap1-Nrf2
291 pathway ²⁵. *GSTP* is a type of cytosolic GST and plays an important role in the classic
292 GSH-dependent enzymatic detoxification pathway of electrophilic metabolites and
293 xenobiotics ²⁶. Nrf2 is one of the most important transcription factors recognized to
294 stimulate *GSTP* ²⁷. GSH is present at micromolar levels in all eukaryotic cells and
295 provides protection against ROS ²⁸. GSH is synthesized by glutamate-cysteine ligase
296 (GCL), which is composed of *GCLC* and *GCLM*, and GSH synthetase. GCL catalyses
297 the rate-limiting step in GSH synthesis and is the site of feedback inhibition by GSH
298 itself ²⁹. Both human *GCLC* and *GCLM* are regulated by the Keap1-Nrf2 pathway ²⁸. In
299 this study, the increased expression of *GCLC* and *GCLM* due to flazin-induced Nrf2
300 activation was associated with an increase in GSH (Fig. 4C). The increase in GSH,
301 however, might be too small (2.6-fold) to fully account for the observed increases in
302 *GCLM* and *GCLC* expression of 12.7- and 42.5-fold, respectively. We propose that
303 synthesized GSH inhibits the catalytic activity of GCL.

In our previous study, we examined $\log P$ in ten natural antioxidants¹⁵. In a scatter plot showing the cytoprotective effect plotted against the $\log P$ for each antioxidant, most antioxidants were distributed along a single curve. However, sulforaphane and DHMBA showed unique distributions. A new scatter plot including the present data is shown in Fig. 6, in which 4 divisions (I-IV) have been made on the basis of cytoprotection (dividing line at 3-fold greater than control values) and $\log P$ (dividing line at 3). Flazin is distributed in division I (upper left) together with sulforaphane and DHMBA; moreover, flazin occupies the highest position among them in terms of its cytoprotection. In contrast to flazin, the flazin analogues, perlolyrine and other antioxidants are distributed along a curve in division III. With regard to sulforaphane and DHMBA, studies have reported their usefulness in preventing and/or alleviating health disorders; for instance, anticancer, anti-diabetes, and anti-inflammatory effects have been reported for sulforaphane^{30,31}, and anti-non-alcoholic steatohepatitis (NASH) effects have been reported for DHMBA³². On the basis of the above evidence, flazin and flazin-rich foods may also have a promising future as functional foods.

As shown in the scatter plot, $\log P$ appears to increase the cytoprotective ability of the antioxidants in division III (Fig. 6). However, the effect of $\log P$ reaches a plateau in division IV. This can be explained by the association of higher $\log P$ values with higher

cytotoxicity (Table 1). It is a general rule that hydrophobic compounds (with higher log *P* values) are more toxic in the body than hydrophilic compounds (with lower log *P* values)¹⁶. Flazin and perlolyrine (e in Fig. 6) exhibited large differences in cytoprotection despite their similar log *P* values, indicating that log *P* is not the only determinant of cytoprotective ability. This difference must be due to the Nrf2-activating ability of flazin, since divisions I and III are clearly separated at the cytoprotection level of approximately 3-fold (Fig. 6). Since only a few antioxidants (sulforaphane, j; DHMBA, k; and flazin) have been specified as belonging to division I so far, further surveys of novel indirect antioxidants belonging to division I are warranted.

This study indicates that flazin is a potentially useful functional food for antioxidants *via* activating Keap1-Nrf2 pathway. However, possible adverse effect of flazin remains to be investigated. Flazin belongs to β -carboline derivatives, which is supposed to generate through the Maillard reaction as previously reported^{33,34}. The Maillard reaction products have both positive and negative effects on health such as antioxidants, bactericidals, antiallergenic, prooxidant, and carcinogens³⁵⁻³⁸. The utility of flazin as a functional food needs to be examined by animal model experiments and human trials in future.

5. Conclusion

Flazin activates the Nrf2 pathway and protects human hepatocytes from oxidative

stress without showing any significant cytotoxicity. The potential for flazin to be used as a functional food must be investigated in future animal model experiments and subsequent human trials. Since log *P* values are accessible in chemical databases and are useful for predicting the utility of individual antioxidants, a log *P*-based search for indirect antioxidant foods is recommended.

Competing interests

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Abbreviations used: **AAPH**, 2,2'-azobis(2-amidinopropane) dihydrochloride; **DHMB**, 3,5-dihydroxy-4-methoxybenzyl alcohol; **ORAC**, oxygen radical absorbance capacity.

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(Figure captions)

Figure 1. Structures of flazin (**1**) perlolyrine (**2**), the flazin analogues (**3-6**) and the antioxidants (**7 & 8**) used in this study.

Figure 2. Synthetic route of perlolyrine (**2**) used in this study.

Figure 3. The structure-activity relationships of flazin, perlolyrine and flazin analogues were studied with reporter gene assays (A) and cytoprotection assays against AAPH (B). (A) Reporter gene assay for flazin (**1**), perlolyrine (**2**), flazin analogues (**3-6**), curcumin (**7**), and chlorogenic acid (**8**). The data are expressed as the fold increase in luciferase activity compared to that under control conditions (0 μ M). (B) Cell viability in the presence of flazin (**1**), perlolyrine (**2**), flazin analogues (**3-6**), curcumin (**7**), and chlorogenic acid (**8**). The cells were incubated with 0 mM (-) or 15 mM (+) compound for 24 h. The data are expressed as the viability percentage relative to that under control conditions (0 μ M). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant (one-way ANOVA) (n = 6).

Figure 4. Nuclear translocation of Nrf2, and expression of Nrf2 and Keap1 by flazin (**1**) in C3A cells. (A) Nuclear Nrf2 levels. Also shown is a Western blot of the relative Nrf2 levels in which lamin B1 was used as the internal control. (B) Cytoplasmic Nrf2 levels. Also shown is a Western blot of the relative Nrf2 levels in which GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was as internal

control. (C) Nrf2 levels in total lysates. Also shown is a Western blot of the relative Nrf2 levels in which GAPDH was as internal control. (D) Gene expression levels of Nrf2 as determined by real-time PCR analysis. The level of GAPDH expression was used as an internal control. (E) Keap1 levels in total lysates. Also shown is a Western blot of the relative Keap1 levels in which GAPDH was as internal control. The data are expressed as the fold increase in expression relative to the expression under control conditions (0 μ M). * p < 0.05, ** p < 0.01, *** p < 0.001, ns: not significant versus the control (one-way ANOVA) (n = 6).

Figure 5. Expression of Nrf2 target genes and proteins induced by flazin in C3A.

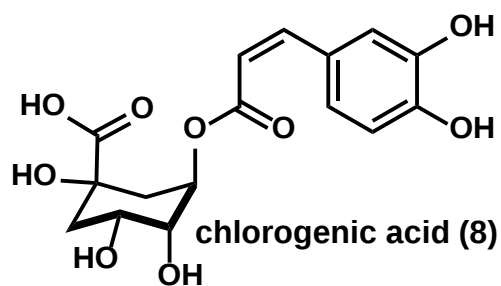
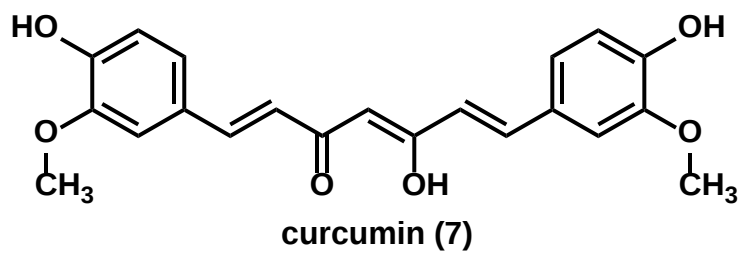
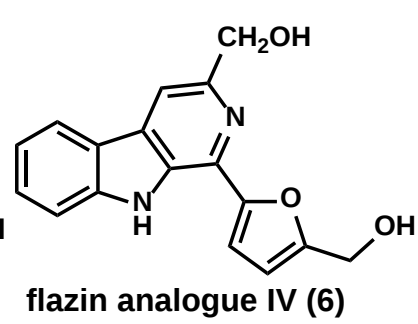
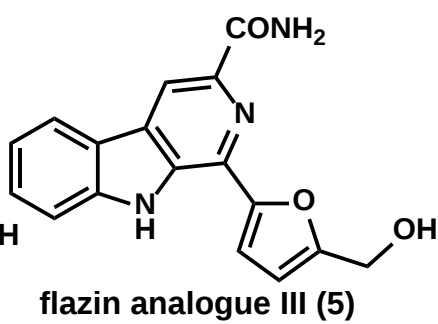
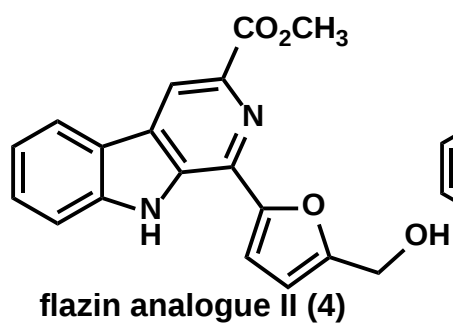
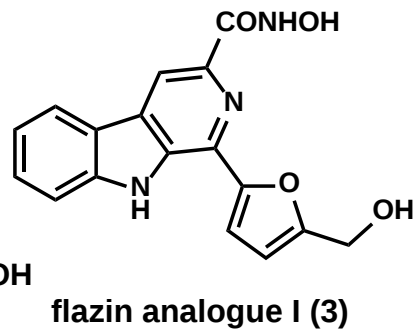
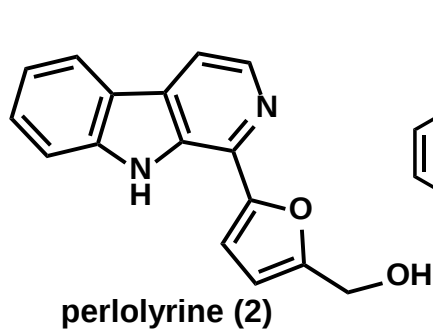
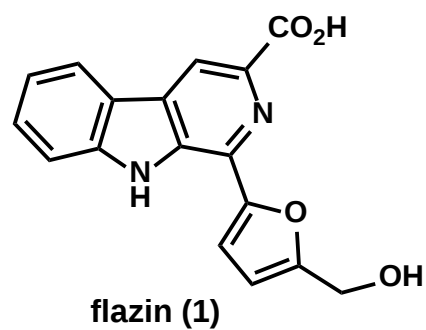
(A) Relative expression of Nrf2 target genes as determined by real-time PCR analysis. The level of GAPDH expression was used as an internal control. The data are expressed as the fold increase in expression relative to the expression under control conditions (0 μ M). (B) Protein expression levels and a Western blot of the relative expression levels of NAD(P)H: quinine oxidoreductase 1 (NQO1) (n=6); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control. The protein expression and a Western blot showing the relative expression levels of glutathione S-transferase pi (GSTP) (n=6) are also shown; GAPDH was used as the internal control. The concentrations of glutathione (GSH) (n=6), which were normalized to the levels of total protein (n=6), are shown. * p < 0.05, ** p < 0.01, *** p < 0.001, ns: not significant versus the control (one-way ANOVA) (n = 6). CAT, catalase; GCL, glutamate-cysteine ligase; GCLC, glutamate-cysteine ligase

catalytic subunit; GCLM, glutamate-cysteine ligase modulatory subunit; GSH, glutathione; GSS, glutathione synthetase; GSSG, oxidized glutathione; GSTM, glutathione S-transferase mu; GSTP, glutathione S-transferase pi; GSTT, glutathione S-transferase theta; GPx, glutathione peroxidase; GR, glutathione reductase; HO-1, haem oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1; SOD1, superoxide dismutase 1.

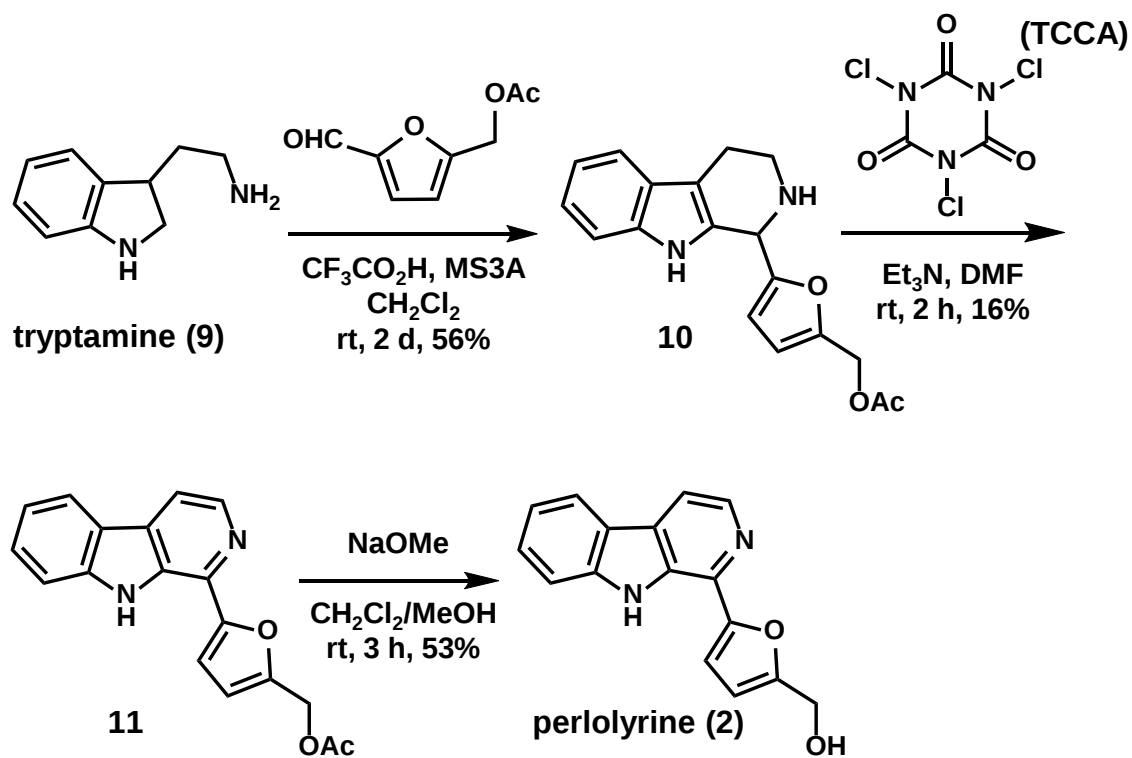
Figure 6. Schematic model of cytoprotection by flazin (**1**) *via* the Keap1-Nrf2 pathway. ARE, antioxidant response element; BVR, biliverdin reductase; CAT, catalase; Cys, cysteine; CO, carbon monoxide; CYP, cytochrome p450; GCL, glutamate-cysteine ligase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modulatory subunit; GSH, glutathione; GSS, glutathione synthetase; GSSG, oxidized glutathione; GST, glutathione S-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; HO-1, haem oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; sMaf, small Maf; SOD1, superoxide dismutase 1; γ GS, γ -glutamylcysteine.

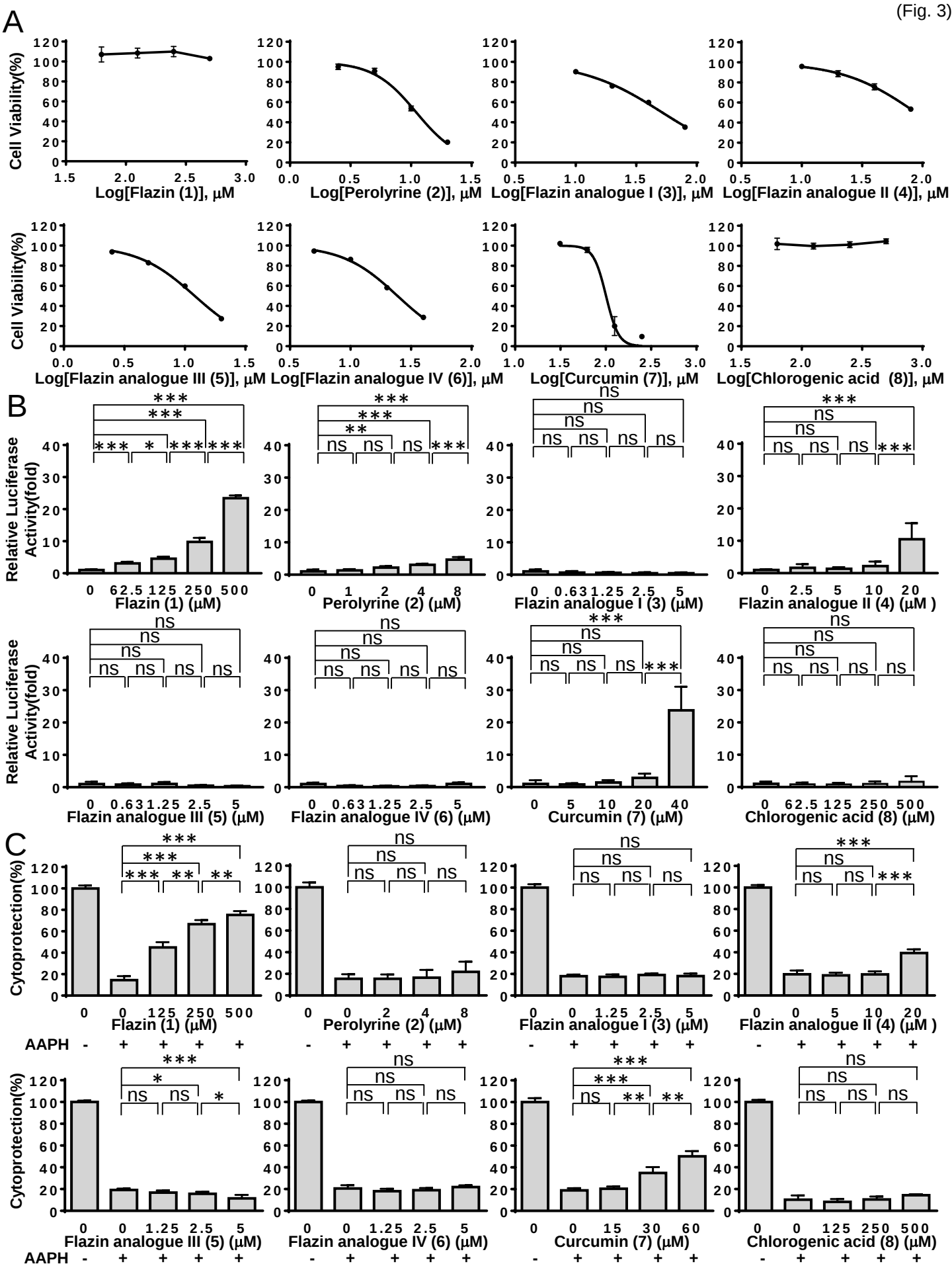
Figure 7. Correlation between the cytoprotective effects and log *P* values of natural antioxidants. The cytoprotection was expressed in the relative cell viability to that for the control (0 μ M antioxidants, 15 mM AAPH). The open triangles and the solid triangles indicate the direct antioxidants and the indirect antioxidants,

respectively, which fit the correlation line. The closed circles (flazin, j [sulforaphane] and k [DHMBA]) indicate the indirect antioxidants that do not fit the correlation line. In the scatter plot shown, 4 divisions (I-IV) have been made on the basis of cytoprotection (dividing line at 3-fold greater than control values) and $\log P$ (dividing line at 3). The letters represent the antioxidants, as follows: (a) cyanidin-3-O-glucoside, (b) chlorogenic acid, (c) gallic acid, (d) rosmarinic acid, (e) perlolirine, (f) quercetin, (g) isoliquiritigenin, (h) curcumin, (i) lycopene, (j) sulforaphane, and (k) DHMBA.



(Fig. 2)





Flazin (μM)	Relative Nrf2 Level
0	1.0
250	1.3
500	1.55

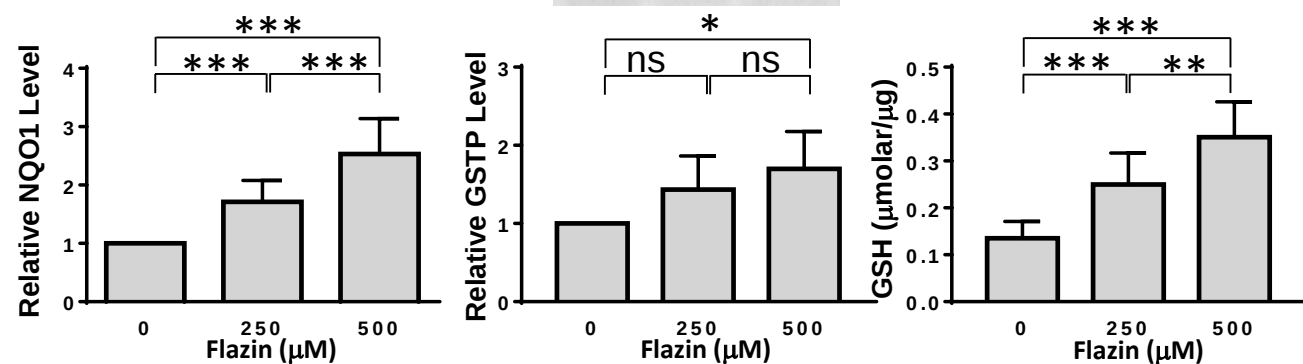
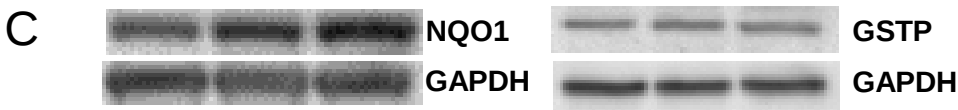
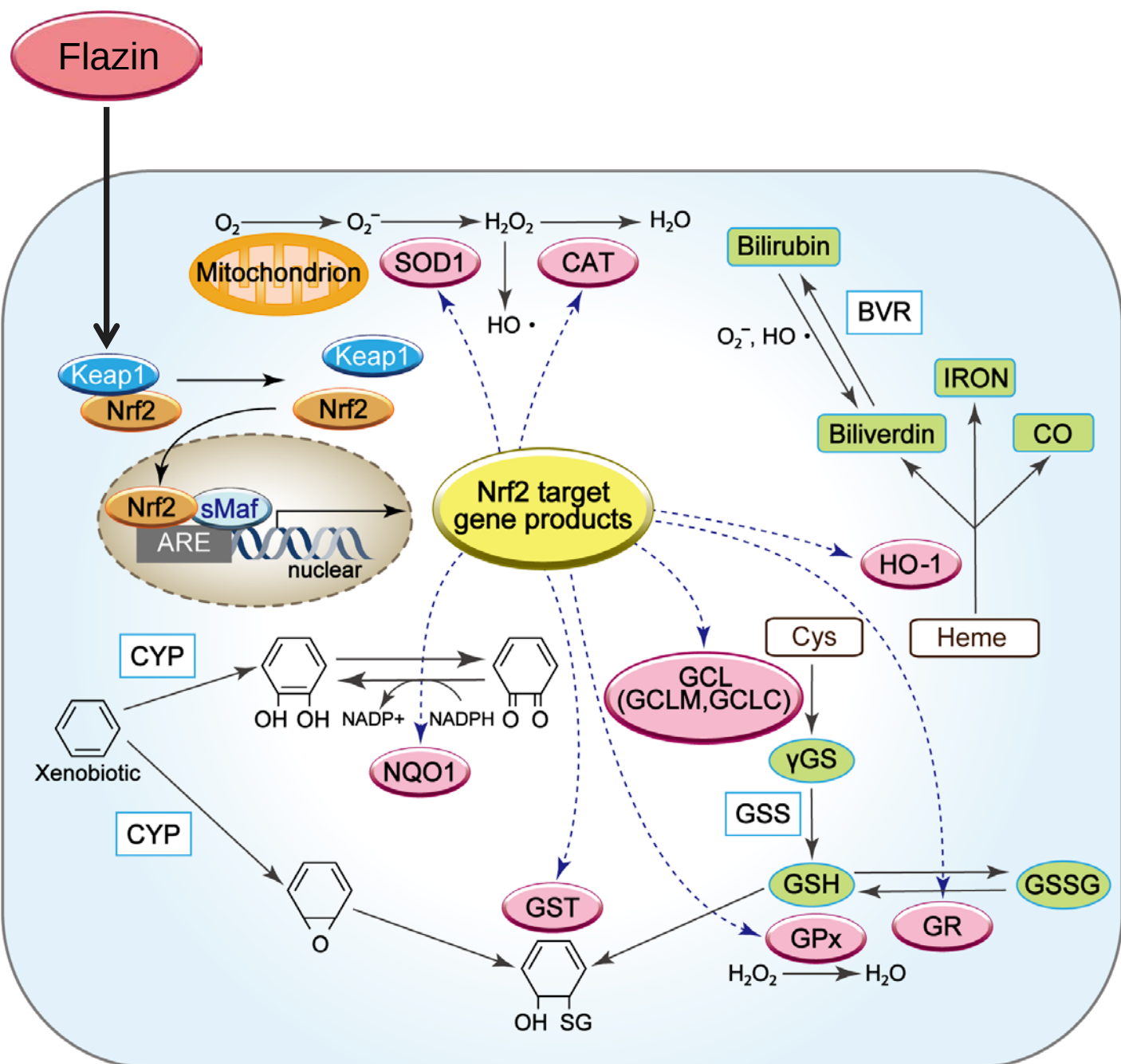


Table 1. Summarized characteristics of studied antioxidants.

Compounds	IC ₅₀ (μ M)	Relative ORAC value (μ mol of TE/ μ mol)	Keap1-Nrf2 activity (fold)	Cytoprotection (fold)	<i>n</i> -Octanol/water partition coefficient (log <i>P</i>)	References
Flazin (1)	500<	1.43 $\pm$ 0.11 2	23.4 $\pm$ 0.861	5.21 $\pm$ 0.245	2.1 ^a	this study
Perlolyrin (2)	11.0	1.60 $\pm$ 0.230	4.64 $\pm$ 0.812	1.41 $\pm$ 0.607	2.2 ^a	this study
Flazin analogue I (3)	51.5	3.65 $\pm$ 0.392	0.45 3 $\pm$ 0.263	1.01 $\pm$ 0.123	1.72 ^b	this study
Flazin analogue II (4)	88.2	0.587 $\pm$ 0.124	10.5 $\pm$ 4.95	2.00 $\pm$ 0.176	2.80 ^b	this study
Flazin analogue III (5)	12.0	0.886 $\pm$ 0.0735	0.332 $\pm$ 0.216	0.873 $\pm$ 0.103	2.01 ^b	this study
Flazin analogue IV (6)	24.4	2.50 $\pm$ 0.287	1.00 $\pm$ 0.480	1.06 $\pm$ 0.0809	2.45 ^b	this study
Cyanidin-3-O-glucoside	500 <	1.06 $\pm$ 0.0412	0.810 $\pm$ 0.124	1.29 $\pm$ 0.110	0.39 ^c	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Chlorogenic acid (8)	500 <	4.57 $\pm$ 0.303	0.912 $\pm$ 0.203	0.774 $\pm$ 0.320	0.69 ^d	Watanabe et al. (2012). Journal of Agricultural Food Chemistry, 60, 830-835; Fuda et al. (2015). Food Chemistry, 176, 226-233; Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Gallic acid	400 <	1.47 $\pm$ 0.121	0.901 $\pm$ 0.330	1.42 $\pm$ 0.219	0.91 ^d	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Rosmarinic acid	400 <	11.5 $\pm$ 0.562	1.13 $\pm$ 0.340	1.61 $\pm$ 0.268	1.6 ^e	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
DHMBA	500 <	1.47 $\pm$ 0.401	5.58 $\pm$ 0.634	3.50 $\pm$ 0.31 2	0.561 $\pm$ 0.190 ^f	Watanabe et al. (2012). Journal of Agricultural Food Chemistry, 60, 830-835; Fuda et al. (2015). Food Chemistry, 176, 226-233; Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Sulforaphane	296	n.d.	15.4 $\pm$ 4.75	2.97 $\pm$ 0.166	1.68 ^g	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Quercetin	199	1.57 $\pm$ 0.303	12.8 $\pm$ 1.11	2.15 $\pm$ 0.0312	2.74 ^h	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Isoliquiritigenin	153	6.50 $\pm$ 0.332	40.1 $\pm$ 9.41	2.00 $\pm$ 0.161	3.04 ⁱ	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Curcumin (7)	109	8.12 $\pm$ 0.831	23.8 $\pm$ 7.30	2.66 $\pm$ 0.253	3.29 ^j	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.
Lycopene	110	n.d.	18.7 $\pm$ 1.03	2.63 $\pm$ 0.248	11.11 ^k	Joko et al. (2017). Journal of Functional Foods, 35, 245-255.

Keap1-Nrf2 activity was expressed in the relative activity to that for the control (0 μ M). The cytoprotection was expressed in the relative cell viability to that for the control (0 μ M antioxidants, 15 mM AAPH). The values of cell cytotoxicity (IC₅₀) were determined by non-linear transform. Each value was expressed in average $\pm$ SD. n.d.: not detected. ^a PubChem. <https://pubchem.ncbi.nlm.nih.gov/> ^b Virtual Computational Chemistry Laboratory. <http://www.vcclab.org/> ^c Olivas-Aguirre et al. (2016). Molecules, 21, 1264. ^d Sergediene et al. (1999). FEBS Letter. 462, 392-396. ^e Zhu et al. Bioscience, Biotechnology, and Biochemistry. 79, 1178-1182 (2015). ^f Joko et al. (2017). Journal of Functional Foods, 35, 245-255. ^g Avdeef & Tam. (2010). Journal of Medicinal Chemistry, 53, 3566-3584. ^h Sergediene et al. (1999). FEBS Letter, 462, 392-396. ⁱ Tanemoto et al. (2015). Biochemistry and Biophysics Reports, 2, 153-159. ^j Ramalingam, Yoo, and Ko. (2016). Food Research International, 84, 113-119. ^k Goupy, Reynaud, Dangles, and Caris-Veyrat. (2012). New Journal of Chemistry, 36, 575-587.



(Fig. 6)

