Flavonoids As Inhibitors or Enhancers of the Cytotoxicity of Tumor Necrosis Factor-α in L-929 Tumor Cells

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The effects of some selected flavonoids on tumor necrosis factor-α (TNF)-induced cytotoxicity in murine fibroblast L-929 cells were studied. All of the flavonols tested as well as a flavan, epicatechin, protected L-929 cells from TNF-induced cell death. Of the flavanones tested, hesperetin, isosakuranetin, and pinocembrin failed to modify TNF cytotoxicity, but the 3′,4′-dihydroxyflavanones, eriodictyol and taxifolin, showed a protective effect. Eriodictyol was the most potent protective agent of all the flavonoids tested, while a 4′-hydroxyflavanone, naringenin, rather showed enhancement of TNF cytotoxicity. Of the flavones tested, chrysin and apigenin markedly augmented the cytotoxicity of TNF, while luteolin showed a weak protective effect. The magnitude of protection and potentiation by these flavonoids were concentration-dependent and these effects were not altered when the flavonoids were added as much as 2 h after TNF treatment.

Tumor necrosis factor-α (TNF), also known as cachectin, is a protein product of activated macrophages that plays a central role in integrating and amplifying host response to infection and malignancy.[1,2] Through its interaction with cells like macrophages, fibroblasts, and endothelial cells, TNF promotes the immune response, local inflammatory processes, and wound repair.[1−5] TNF is also known to elicit cytotoxicity to tumor cells and mediate the wasting (cachexia) that accompanies disease states such as AIDS.[6,7] Modulators of TNF-mediated biological processes therefore have the potential for a wide range of therapeutic activities, for example, as anticancer, antiinflammatory, or anti-AIDS agents and for treatment of various vascular disorders.

Flavonoids are phenolic pigments of plants that possess various biological activities including, antiinflammatory,[8] cystostatic,[9] cytotoxic,[10] in vivo anticancer,[11] and antiviral[12] properties. The biological effects of flavonoids seem to occur mainly through their interaction with biomolecules like DNA[13] and regulatory enzymes.[14−19] Flavonoids are also known to scavenge free radicals directly and reduce the formation of free radicals indirectly through their interaction with redox metals such as iron ions.[20−25] Thus, flavonoids have been shown to prevent mammalian cytotoxicity caused by reactive oxygen species.[26,27] Although some free radical scavengers and metal chelators have been shown to inhibit TNF-mediated cell death,[28−31] the effects of flavonoids on TNF cytotoxicity have not yet been established. In the present report, the potentiation and inhibition of TNF-mediated cell death in L-929 cells by some selected flavonoids (Figure 1) and the structure−activity relationship established from the results are discussed.

Results and Discussion

TNF activity is usually studied using prototypical transformed murine L-929 tumor cells in which TNF induces cell death within 24 h.[28,31] A similar scenario was established in the present assay system. Exposure of L-929 cells to 200 pg/mL of TNF resulted in 40 ± 3 and 53 ± 6% (mean ± S.E., $n = 3$) reduction in the viability of cells when assessed by the crystal violet staining and thymidine incorporation assays, respectively. The addition of 1 μg/mL of MAb anti-hTNF-α prior to TNF totally abolished the cytotoxicity of TNF (data not shown), while nordihydroguaiaretic acid, a known inhibitor of TNF-mediated cell death, showed activity (Table 1) in close agreement with previous reports.[28,31] Using these quantitative cell viability assays, the effects of some selected flavones, flavonols, flavanones, and a flavan, epicatechin, against TNF were studied. All of the flavonoids studied possess the common 5- and 7-hydroxyl groups of the A ring but differ in their oxygenation pattern on B and C rings (Figure 1).

Because most of the flavonols tested did show some degree of direct cytotoxicity at 1 mM and above concentrations (data not shown), only lower, non-toxic concentrations were tested against TNF activity. All of the flavonols tested showed a concentration-dependent protection of L-929 cells from TNF-mediated cell death (Figure 2). EC_{50} values obtained from three separate experiments using the crystal violet staining and thymidine incorporation assays are also shown in Table 1. The order of potency in both assay systems was similar; that is, quercetin ≥ kaempferol > galangin > myricetin > morin > kaempferide > rutin. Structural comparison of these flavonols (Figure 1) with their inhibitory activity (Figure 2 and Table 1) clearly shows that the C-3 hydroxyl group plays a pivotal role for the observed protective effect. Evidence for this came from two facts: (a) rutin, in which the C-3 hydroxyl group is glycosylated, was 12.5 times less potent than quercetin, and (b) while galangin and kaempferol were protective, similar structures that lack the C-3 hydroxyl group (i.e., chrysin and apigenin) did not show a protective effect. The C-4′ hydroxyl group of flavonols also appears to be increasing the protective effect as a lack of this functional group (galangin) or replacement with a methoxyl group (kaempferide) reduced the protective effect of kaempferol. The higher potency of galangin than kaempferide may suggest that C-4′ methoxylation

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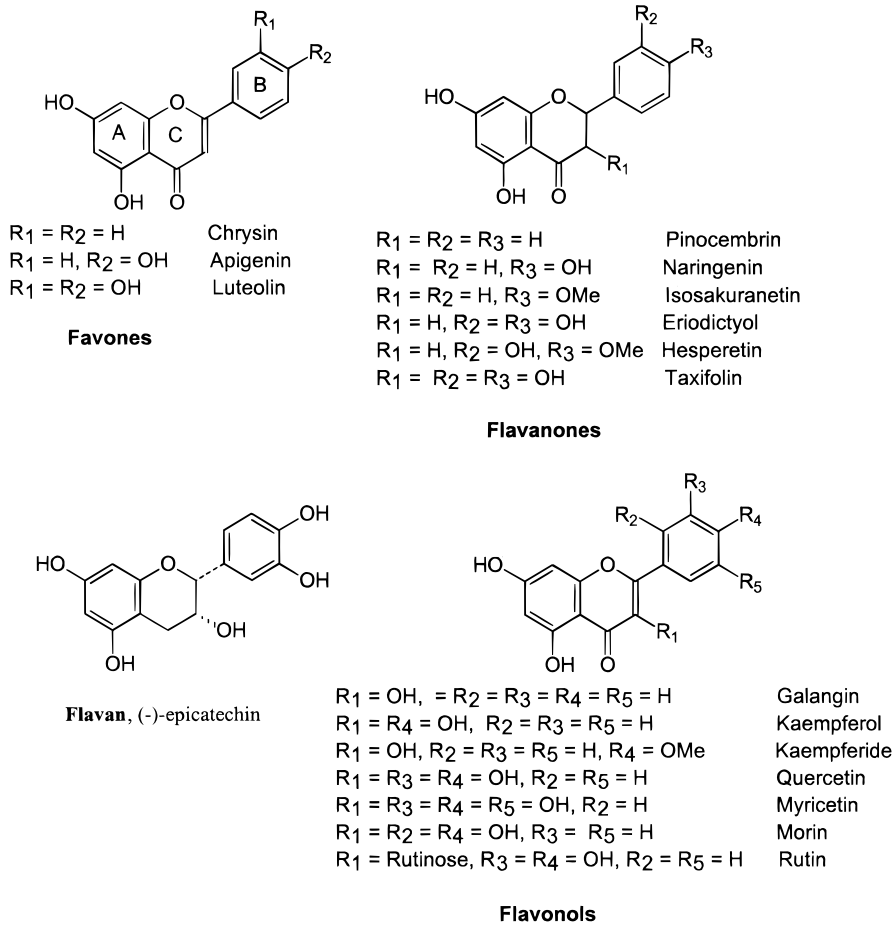

Figure 1. Structures of flavonoids studied.

Table 1. EC_{50} Values for the Protective Effects of Flavonoids and Nordihydroguaiaretic Acid[a]

compounds	EC_{50} values (μM) crystal violet staining assay	thymidine incorporation assay
apigenin	P[b]	P[b]
chrysin	P[b]	P[b]
epicatechin	300 ± 7	350 ± 10
eriodictyol	4 ± 0.3	6 ± 1
galangin	40 ± 3	50 ± 6
hesperetin	N[c]	N[c]
kaempferol	20 ± 6	25 ± 7
kaempferide	129 ± 10	140 ± 6
luteolin	>250	>250
morin	120 ± 8	115 ± 10
myricetin	55 ± 8	59 ± 6
naringenin	P[b]	P[b]
pinocembrin	N[c]	N[c]
quercetin	20 ± 4	26 ± 6
rutin	250 ± 9	>250
taxifolin	200 ± 10	240 ± 10
nordihidroguaiaretic acid	28 ± 6	19 ± 7

[a] Percent protection was calculated as described in the experimental section. EC_{50} values ± S.E. obtained from three separate experiments are shown. [b] P = potentiate the TNF activity. [c] N = no activity up to the concentration of 250 μM.

negatively contributed to the protective effect of flavonols. Further addition of a hydroxyl group *ortho* to the C-4′ hydroxyl group of kaempferol (i.e., quercetin) did not alter the potency, whereas addition of a *meta* hydroxyl group (i.e., morin) resulted in a six-times greater reduction of the protective effect. Addition of two hydroxyl groups *ortho* to the C-4′ hydroxyl group (kaempferol vs. myricetin) also reduced the protective effect.

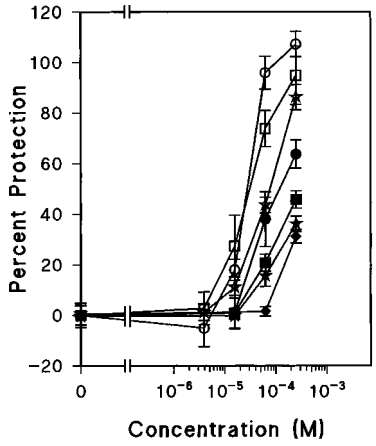

Figure 2. Protection of L-929 cells against TNF-mediated cell death by some flavonols. TNF activity and percent protection were measured using the crystal violet staining assay as described in the experimental section. Each point represents the mean ± S.E. of six determinations. The symbols indicate: ○, quercetin; □, kaempferol; ☆, galangin; ●, myrecitin; ■, morin; ★, kaempferide; ◆, rutin.

Unlike the flavonols, some of the flavones tested showed enhancement of the TNF cytotoxicity (Figure 3). Apigenin and chrysin which possess one (C-4′) hydroxyl group or none on the B ring, respectively, enhanced the cytotoxicity of TNF. All concentrations of flavones that potentiated the TNF-mediated cytolysis were not cytotoxic when tested under the same experimental condition (Figure 3). In contrast to their role in the protective effects of flavonols, C-4′ hydroxylation appears to be not important for the potentiation activity

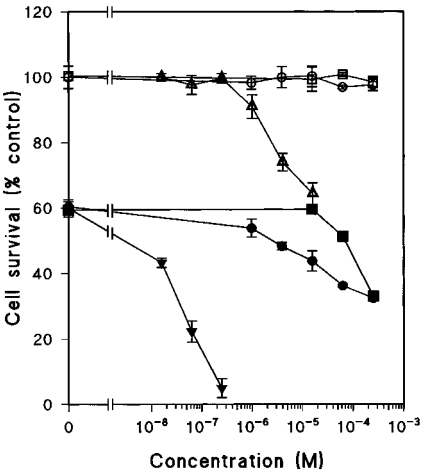

Figure 3. Enhancement of TNF-mediated cell death in L-929 cells by flavones. The concentration-dependent effects of two flavones and staurosporine on L-929 cell survival are shown. Symbols indicate: □, apigenin; ○, chrysin, and △, staurosporine in the absence of TNF and ■, apigenin; ●, chrysin, and ▼, staurosporine in the presence of 200 pg/mL TNF. Each point represents the mean ± S.E. ($n = 6$) obtained from the crystal violet staining assay. Similar results were obtained when cell viability was measured using thymidine incorporation assay.

of flavones, as chrysin was more potent than the C-4′ hydroxyl flavone, apigenin (Figure 3). Further addition of a hydroxyl group at C-3′ on the apigenin skeleton abolished the potentiation effect of flavones, luteolin actually seeming to show a protective effect (Table 1). The effect of a broad-spectrum kinase inhibitor, staurosporine, on TNF cytotoxicity was similar with apigenin and chrysin (Figure 3). Staurosporine showed direct cytotoxicity at concentrations higher than 1 μM, but its non-toxic concentrations enhanced the cytotoxicity of TNF in a concentration-dependent manner (Figure 3).

The biological effects observed for the flavanones were similar to those of flavones. At the highest non-toxic concentration tested (250 μM), the C-4′ hydroxyl flavanone, naringenin, enhanced the TNF cytotoxicity by 25 ± 4 and 31 ± 3% (mean ± S.E., $n = 3$) when assessed by crystal violet staining and thymidine incorporation assays, respectively. Pinocembrin and isosakuranetin failed to modify the TNF activity, while a related flavanone, eriodictyol, which bears *o*-dihydroxyl functional groups at C-3′ and C-4′, was the most protective compound of all the flavonoids tested (Table 1). Replacement of the C-3′ hydroxyl group of eriodictyol by a methoxyl group (i.e., hesperetin) abolished the protective effect, while addition of a further hydroxyl group at C-3 (i.e., taxifolin) reduced the protective effect, of eriodictyol by 50-fold. One flavan, epicatechin, also showed a protective effect (Table 1).

Comparison of EC_{50} values for the inhibitory effects of different classes of flavonoids (Table 1) against TNF cytotoxicity did reveal useful structural requirments for the observed biological activity. All of the flavonoids tested that possess the *o*-dihydroxy functional group on the B ring appear to have protective effects. Because eriodictyol is about > 62.5 and 5 times more potent than luteolin and quercetin, respectively, the C2−C3 double bond does not appear to be important for the inhibition of TNF cytotoxicity by flavonoids that possess the catecholic functional group. On the other hand, taxifolin was more potent than epicatechin, and thus the 4-oxo functional group of these flavonoids must have a role for the inhibition of TNF cytotoxicity. Within the flavonol group of compounds, the C-3 and C-4′ hydroxylation appears to be optimal for the protective effect, and the presence of catecholic function on the B ring does not increase potency. In contrast to the flavonols, C-3 hydroxylation appears to reduce the protective effect of 2,3-dihydroflavones (flavanones).

The mechanism whereby flavonoids inhibit or potentiate the cytotoxicity of TNF is yet to be established. The protective as well as potentiation effects of the flavonoids studied were not altered when they were added even at 2 h after TNF treatment (data not shown). Thus, the modulatory effect of flavonoids are unlikely to be through the possible interaction of flavonoids with TNF or its cell surface receptors. Several regulatory enzymes, including protein kinases, reverse transcriptase, DNA topoisomerase, cyclooxygenase, and lipoxygenases, are shown to play some role in the cytotoxicity of TNF following receptor activation.[18,28,31] Because flavonoids are potent inhibitors of most of these enzymes,[14−19] their modulatory role toward TNF-mediated cell death may be associated with these activities. Recently, several flavonoids have been shown to inhibit the TNF and other cytokines-induced expression of adhesion molecules at the transcription level.[32] This or similar mode of action, however, is unlikely to account for the protective effect of flavonoids as it would be shared by nonprotective flavonoids, in particular, apigenin. On the other hand, flavonoids are known to scavenge reactive oxygen species, interact with redox metals like iron ions, and protect cells from free radical-mediated cytotoxicity.[20−27,33] Because some metal chelators and free radical scavengers were shown to protect cells from TNF cytotoxicity,[28−30] the protective effects of flavonoids in the present study may be associated with their interaction with iron and/or active oxygen species. There appears to be a good agreement between the structure−activity relationship established for the flavonoids in the present study and their reported antioxidant activities.[20−27] The *o*-dihydroxy structure in the B ring of flavonoids is shown to be the active center for metal chelation, scavenging, and antioxidant activity.[24] In agreement with this finding, all of the flavonoids tested that possess this catecholic moiety (epicatechin, eriodictyol, luteolin, quercetin, and taxifolin) effectively inhibited the TNF cytotoxicity in the present study. A number of non-flavonoid aromatic compounds that possess catecholic structures have also been shown to inhibit the cytotoxicity of TNF (unpublished results). The importance of the catecholic functional group for the protective effects of flavonoids in the present study was also supported by the lack of protective activity of the flavones chrysin and apigenin and the flavanones, pinocembrin, naringenin, isosakuranetin, and hesperetin. Flavonoids that possess a 4-oxo function together with a 2,3-double bond in the C ring and/or the 3- and 5-hydroxyl groups in the A and C rings are also known to interact with iron and scavenge free radicals.[26] The good protective activity and the structure−activity relationship established for the flavonols in the present study closely follows these findings.

The potentiation effects of some flavonoids (apigenin, chrysin, and naringenin) in the present study was, however, not expected and may not be explained by the iron chelation and/or free radical scavenging mode of action. In agreement with previous reports,[38] a known

broad-spectum kinase inhibitor, staurosporine, potentiated the TNF cytotoxicity in L-929 cells. The potent inhibitory effects of flavonoids toward a variety of kinase enzymes[35−37] is, thus, likely to be involved for the enhancement of TNF cytotoxicity by flavonoids. Whether one or multiple modes of action are involved in the modulatory action of flavonoids against TNF cytotoxicity remains to be proved, but the present study clearly establishes the structure−activity relationship for potentiation and protective effects for the classes of flavonoids studied. Although protective flavonoids could be useful for combating diseases where TNF-mediated cell death have a significant role, flavonoids that enhance the cytotoxicity of TNF may have a therapeutic potential for cancer and related diseases. In both cases, the use of flavonoids for their modulatory effect toward TNF-mediated cell death needs careful analysis of substructures and/or nature of substituents on the flavonoids skeleton

Experimental Section

Chemicals and Reagents. Apigenin, chrysin, crystal violet, (−)-epicatechin, hesperetin, morin, myricetin, naringenin, nordihydroguaiaretic acid, quercetin, rutin, and staurosporine were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Eriodictyol, kaempferide, isosakuranetin, luteolin, pinocembrin, and taxifolin were from Apin Chemicals Ltd. (Milton Park, Oxon, U.K.). Galangin was obtained from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.), while rhTNF-α and MAb anti-hTNF-α were products of R & D System (Abingdon, Oxon, U.K.). [Methyl-^{3}H]thymidine (5 Ci/mmol) was purchased from Amersham International plc (Slough, U.K.).

Cell Line and Cytotoxicity Assay. The TNF-sensitive murine fibroblast cell line, L-929, was kindly supplied by Dr. B. Wilson (Department of Medical Microbiology, St. Mary's Hospital Medical School, London). Cells were maintained with Dulbeccos Minimum Extract medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 100 U/mL of penicillin and 100 μg/mL of streptomycin.

L-929 cells (2×10^4 cells/well in 100 μL) were plated in 96-well plates and incubated at 37 °C, 5% CO_2 for 24 h to establish a dense monolayer. After adding 50 μL of actinomycin D (1 μg/mL) containing media to each well, different concentrations of flavonoids or staurosporine were added and plates incubated at 37 °C for 15 min. TNF (200 pg/mL) was then added and plates incubated for an additional 24 h. Cell survival was determined using crystal violet staining[39] and thymidine incorporation assays. In the latter case, [methyl-^{3}H]-thymidine (0.5 μCi/well) was added during the last 6 h of incubation. After removing unincorporated thymidine by washing, cells were harvested using z Dynatech Automash 2000 cell harvester, and radioactivity was counted using liquid scintillation spectrometery. Percent protection was calculated as $(D - T) \times 100/(C - T)$, where D is the absorbance intensity [or radioactive count (dpm)] of TNF-treated cells in the presence of drugs, T is the mean absorbance (or dpm) of TNF-treated cells in the absence of drugs, and C is the mean absorbance (or dpm) obtained from control cells (no TNF).

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