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Inhibition of the mitochondrial NADH-oxidase (NADH-Coenzyme Q oxidoreductase) enzyme system by flavonoids: a structure–activity study

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Flavonoids have widespread biological activities [1–7] including inhibition of mitochondrial enzyme systems [6, 7]. Some of these activities, for example cytotoxicity, may be the result of alteration of cellular bioenergetics. Rotenone, a potent inhibitor of NADH-oxidase, is a flavonoid derivative [2, 8], and thus it could be expected that other flavonoids could affect mitochondrial function. In addition, rotenone inhibits the growth of Ehrlich ascites tumors (EAT) *in vitro* [9] and *in vivo* [10].

The antineoplastic agent 4′-demethyl-epipodophyllotoxin thenylidene glucoside (VM-26) is a lignan and as such is a biosynthetic relative to flavonoids [11]. VM-26 has been found to inhibit NADH-linked respiration in isolated mitochondria, submitochondrial particles, and suspensions of EAT cells [12]. Another inhibitor of mitochondrial respiration, antimycin A, inhibits the growth of EAT cells *in vitro* [13]. These findings suggest that the inhibition of mitochondrial enzyme systems may be an underlying mechanism for cytotoxicity and certain other biological effects of flavonoids. These findings further support the notion that inhibition of mitochondrial respiration may be a target for anticancer chemotherapy [10].

In a previous paper, we reported the results of a systematic structure–activity study of the inhibition of mitochondrial succinoxidase by flavonoids [7]. We now report the structure–activity relationships for the inhibition of mitochondrial NADH-oxidase by the same series of flavonoids and compare them to those previously found for succinoxidase inhibition.

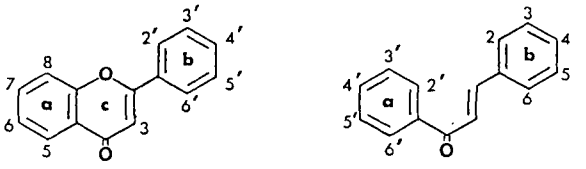
Methods

Heavy beef heart mitochondria (HBHM) were isolated by differential centrifugation and stored as described previously [14]. Aging of the mitochondria to uncouple respiration from phosphorylation was achieved by repeated freezing and thawing. Mitochondrial protein was assayed by the method of Lowry *et al.* [15]. The activity of HBHM NADH-oxidase was determined manometrically in the absence and presence of the various test compounds [16, 17]. The test compounds were added in ethanol except for cyanidin, delphinidin and quercetagetin which were dissolved in dimethyl sulfoxide (DMSO). The solvent was maintained at a constant concentration in all assays (0.1 ml solvent/3 ml of reaction mixture). In each experiment, a 5-min preincubation was employed to ensure thermal equilibrium and depletion of endogenous substrates. The I_{50} values (dose required to inhibit enzyme activity by 50%) were estimated by extrapolation from titration curves.

Results and discussion

The potencies of a series of fifteen structurally related flavonoids for inhibiting NADH-oxidase are shown in Table

Table 1. I_{50} Values of various flavonoids towards mitochondrial NADH-oxidase and succinoxidase enzyme systems



Compound	Class	Hydroxylation patterns	I_{50} value: NADH-oxidase* nmole/mg protein	I_{50} value: Succinoxidase† nmole/mg protein
Fisetin	flavonol	3,7,3′,4′	15	45
Quercetin	flavonol	3,5,7,3′,4′	145	715
Morin	flavonol	3,5,7,2′,4′	430	730
Quercetagetin	flavonol	3,5,6,7,3′,4′	177	104
Cyanidin	anthocyanidin	3,5,7,3′,4′	500	290
Delphinidin	anthocyanidin	3,5,7,3′,4′,5′	1000	740
Butein	chalcone	3,4,2′,4′	18	21
Myricetin	flavonol	3,5,7,3′,4′,5′	35	45
Luteolin	flavone	5,7,3′,4′	48	32
Fustin	dihydroflavonol	3,7,3′,4′	127	148
Taxifolin	dihydroflavonol	3,5,7,3′,4′	173	220
Kaempferol	flavonol	3,5,7,4′	>1800	>1800
Galangin	flavonol	3,5,7	>1800	>1800
Catechin	catechin	3,5,7,3′,4′	1800	>1800

* Number of replicates was from 6–26, and the protein concentration varied from 0.9–1.4 mg/flask.

† Previously published values [7].

1. Compounds that did not inhibit NADH-oxidase activity by 50% or more at the initial test dose of 1800 nmol/mg protein were considered inactive and were not tested further. This dose was chosen because of solubility limitations of the test flavonoids in solvent.

Comparing the I_{50} values of flavonoids from different classes but with identical hydroxyl configurations suggested that two structural features of the flavonoids are important for inhibition of NADH-oxidase. First, the importance of the $C_{2,3}$ double bond is indicated by comparing the relative potencies of the flavonols fisetin and quercetin (I_{50} values of 15 and 145) with their corresponding dihydroflavonols fustin and taxifolin (I_{50} values of 127 and 173) respectively. This suggests that planarity of the chromone (pyrone) structure enhances inhibitory activity. Second, the importance of the C_4 keto group for inhibition of NADH-oxidase is shown by the low activity of the anthocyanidins, cyanidin chloride and delphinidin chloride, and especially the catechin stereoisomers.

The importance of the $C_{2,3}$ double bond is further exemplified by the finding that the chalcone butein and its corresponding flavone luteolin were both more potent inhibitors of NADH-oxidase than the two dihydroflavonols fustin and taxifolin. Since flavonols were the most potent NADH-oxidase inhibitors, a detailed structure–activity comparison was conducted on a series of 3,5,7-trihydroxy flavones differing in the number and position of the b ring hydroxyl groups. Myricetin, containing a pyrogallol configuration, was the most potent inhibitor of NADH-oxidase, followed by quercetin, containing a catechol configuration, then morin with a meta-hydroxyl group, then kaempferol which contains a mono-hydroxyl group and galangin containing no b ring hydroxyls. The effects of these hydroxyl configurations on potency are consistent with the general order of potencies of a series of model phenolic compounds toward the mitochondrial respiratory chain [16, 18].

NADH-oxidase and succinoxidase exhibited different sensitivities to various flavonoids (Table 1). Fisetin, quercetin and morin were more potent inhibitors of the NADH-oxidase system than succinoxidase, demonstrating that these three flavonoids act primarily in complex I, the NADH-CoQ reductase of the inner mitochondrial membrane. Conversely, quercetagetin, cyanidin chloride and delphinidin appear to inhibit succinoxidase activity more with I_{50} values lower than those observed for the NADH-oxidase system. This greater inhibition of succinoxidase suggests that these flavonoids tend to preferentially inhibit the succinate-CoQ reductase (complex II) portion of the mitochondrial respiratory chain. However, the variability, particularly for quercetagetin in succinoxidase, was such that statistically the titration curves and, hence, the I_{50} values are not significantly different, although the trend suggests that succinoxidase is more sensitive to these compounds.

Butein, myricetin, luteolin, fustin and taxifolin inhibited NADH-oxidase and succinoxidase activities almost equally; thus, they either inhibit complexes I and II to about the same extent, or they act further down the respiratory chain between Coenzyme Q and cytochrome oxidase, a portion common to both the succinoxidase and the NADH-oxidase enzyme systems.

Virtually all of the flavonoids that are inhibitory towards the succinoxidase system ($I_{50} < 500$) contain a catechol group on the b ring. The exceptions are the unexpectedly inactive quercetin ($I_{50} = 715$), which contains a catechol, and the potent myricetin ($I_{50} = 45$), which contains a pyrogallol structure on the b ring. In addition, catechin, which contains a catechol on the b ring is inactive [7]. This succinoxidase inhibition pattern is similar to that reported for a series of model phenolic compounds [16, 18], is consistent with the findings of Bartlett [19] on chalcones, and supports the conclusion that phenolic compounds capable of a redox reaction inhibit succinoxidase.

Carpenedo *et al.* [20] previously reported that quercetin inhibits mitochondrial electron transport and that this inhibition is reversed by cysteine. They found quercetin to be a better inhibitor of succinoxidase than NADH-oxidase, the opposite to our results. This discrepancy remains unexplained, but it may be the result of using different beef heart mitochondria preparations or assay conditions. The exact site in the complexes and the mechanism(s) of inhibition by quercetin are unknown. The finding that cysteine totally reversed the inhibition suggests that interaction with sulfhydryl groups is involved [20]. This is consistent with our previous report demonstrating redox cycling and the production of active oxygen species by redox-active flavonoids [7]. Redox active quinones have been shown to generate toxic oxygen species, deplete mitochondrial thiol groups, and inhibit mitochondrial electron transport [21, 22]. The results of this study suggest that flavonoids may act in an analogous fashion.

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