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Inhibition of Rat Liver NAD(P)H:Quinone Acceptor Oxidoreductase (DT-Diaphorase) by Flavonoids Isolated from the Chinese Herb Scutellariae Radix (Huang Qin)

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SUMMARY

The glucuronide conjugates of oroxylin A and two other flavones, baicalein, and wogonin, were isolated from the methanol extract of the herb scutellariae radix (Huang Qin) and were found to be inhibitors of rat liver NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2). Baicalin (baicalein 7-*O*-glucuronide) and oroxylin-A 7-*O*-glucuronide are approximately 50-fold more potent than wogonin 7-*O*-glucuronide. The enzyme kinetic analysis revealed that oroxylin-A 7-*O*-glucuronide is a competitive inhibitor with respect to NADH (the electron donor), with a K_i value of 63 nM. Considering the similarities of their structures and inhibition kinetics to those of dicoumarol, it is thought that oroxylin-A 7-*O*-glucuronide and the other two flavonoids bind to an identical site and inhibit this quinone reductase in the same fashion as dicoumarol. The results also suggest that the inhibition of NAD(P)H:quinone acceptor oxidoreductase or another vitamin K reductase by oroxylin-A 7-*O*-glucuronide and the related flavonoids may be one of the steps associated with the anticoagulation action of the herb. These compounds are potentially useful anticoagulant drugs.

Scutellariae radix (Huang Qin), the root of *Scutellaria baicalensis* GEORGI (Labiatae), has been used in Chinese medicine as a remedy for inflammation, suppurative dermatitis, allergic diseases, hyperlipemia, and arteriosclerosis. Kubo *et al* (1) have shown that a 70% methanolic extract of this herb inhibited endotoxin-induced disseminated intravascular coagulation in hyperlipemic rats, and its fractions inhibited blood platelet aggregation and the conversion of fibrinogen to fibrin induced by thrombin. The above description makes it clear that scutellariae radix contains component(s) with broad influences on the many steps associated with the blood coagulation mechanism.

Recently, we have found that the methanol extract of this herb contains potent inhibitors of rat liver NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2; DT-diaphorase). Although the relevance of this enzyme as a major participant in the vitamin K cycle in humans remains controversial, rat liver quinone reductase can function physiologically as one of several vitamin K reductases in the vitamin K cycling involved in the hepatic biosynthesis of certain blood coagulation factors (2). In this communication, we report the isolation and characterization of glucuronide conjugates of three flavones, oroxylin-A, baicalein, and wogonin, in the methanol extract that are responsible for the inhibitory action of this herb on rat liver NAD(P)H:quinone acceptor oxidoreductase. We also present results obtained from kinetic studies of the oroxylin-A 7-*O*-glucuronide inhibition, to show that these compounds inhibit this quinone reductase in a fashion identical to that of dicoumarol.

Experimental Procedures

Materials. The methanol extract of scutellariae radix was prepared by a procedure described by Nikaido *et al.* (3). The NAD(P)H:quinone acceptor oxidoreductase was purified from livers of female Wistar rats injected daily for 3 days with 3-methylcholanthrene (4 mg/100 g of body weight), using a procedure described by Haniu *et al.* (4).

Enzymatic Assay. The NAD(P)H:quinone acceptor oxidoreductase activity was determined spectrophotometrically by measurement of the oxidation of NADH at 340 nm at 25°, when menadione was used as the substrate (or electron acceptor) (5). The assay mixture (1 ml) contained 50 mM sodium phosphate, pH 7.4, 197 μM NADH, and 160 μM menadione. The reaction was initiated upon addition of the enzyme. The enzymatic assays were always performed in duplicate, and good

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ABBREVIATIONS: FD, field desorption; TLC, thin layer chromatography.

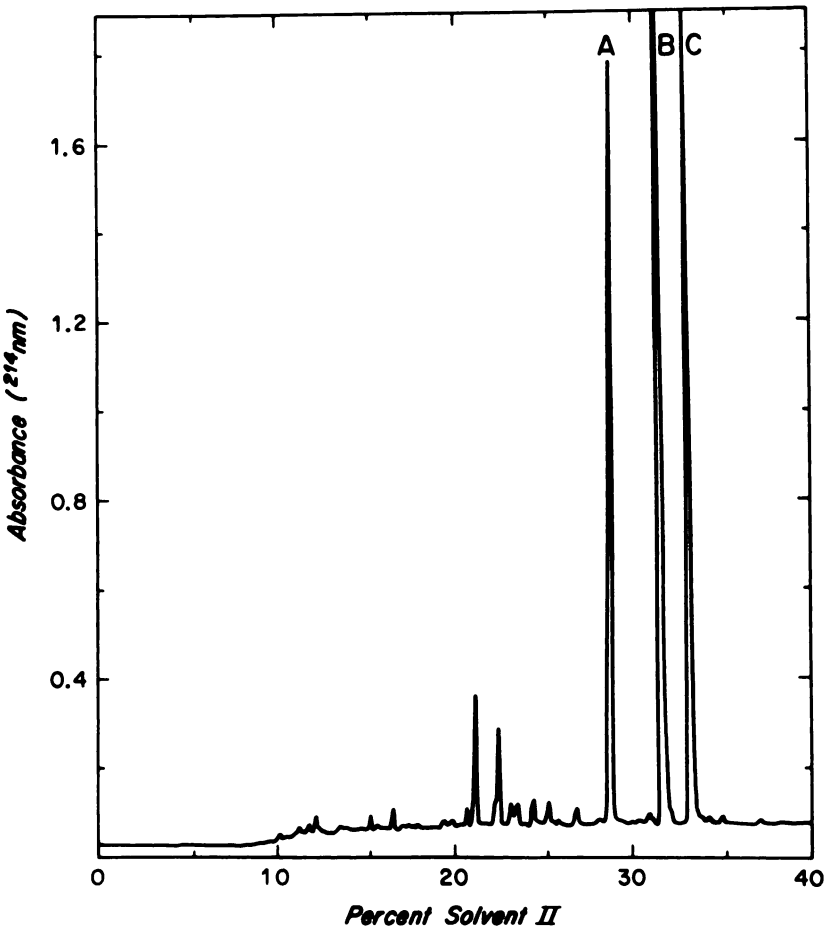

Fig. 1. Fractionation of NAD(P)H:quinone acceptor oxidoreductase inhibitors by reverse phase high performance liquid chromatography. The fractions containing quinone reductase inhibitors (from gel filtration chromatography) were concentrated and applied to a Vydac C-18 column (10 × 250 mm). A 60-min gradient program was run from 100% solvent I (0.1% trifluoroacetic acid) to 40% solvent II (trifluoroacetic acid/water/acetonitrile, 0.1:9.9:90, v/v/v) at a flow rate of 0.8 ml/min. Fractions were collected manually.

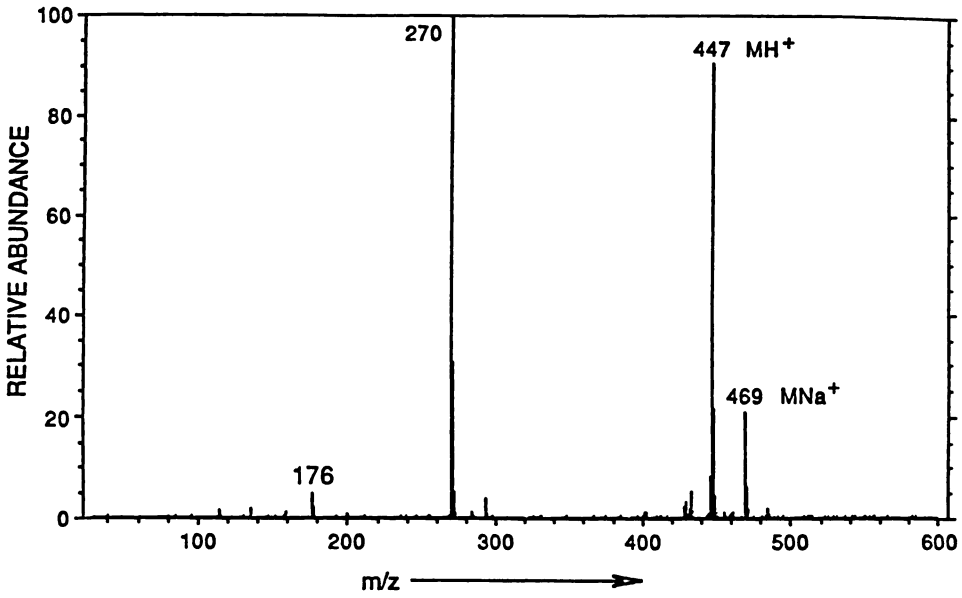

Fig. 2. Positive ion FD mass spectrum of fraction A from reverse phase high performance liquid chromatography.

agreement was always found between the two measurements. The specific activity of this enzyme preparation was 373 μmol of NADH oxidized/min/mg of protein. When potassium ferricyanide was used as the substrate, the activity was determined by measurement of the reduction of potassium ferricyanide at 420 nm [$\epsilon_{420\,nm} = 1.0 \times 10^3\ M^{-1}\ cm^{-1}$ (6)] at 25°. The assay mixture was identical to that described above, except 240 μM potassium ferricyanide was used instead of menadione.

Isolation and characterization of flavonoids from the methanol extract of scutellariae radix. The isolation of flavonoids is discussed in detail in Results and Discussion. The identification of these flavonoids was done by FD mass spectral analyses and TLC, using characterized flavonoids as standards. The FD mass spectral analyses were obtained using a JEOL HX100HF double-focusing magnetic sector mass spectrometer, operating at 5 KV accelerating voltage and at a nominal resolution of 1000. Samples were dissolved in meth-

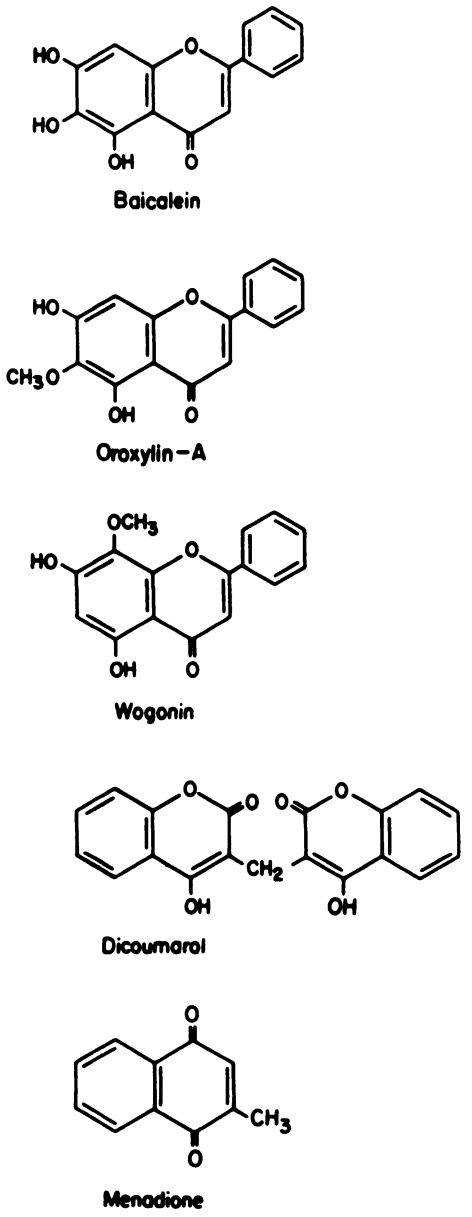

Fig. 3. Structures of baicalein, oroxylin-A, wogonin, dicoumarol, and menadione.

anol and applied to activated carbon emitters. Mass spectra were scanned and collected while the emitter heating current increased at a rate of 1 μA/min over the range of 0–20 μA. Mass values are reported as nominal mass. The TLC was performed using solvent systems described by Nikaido *et al.* (3). The standards, baicalin, oroxylin-A 7-*O*-glucuronide, and wogonin 7-*O*-glucuronide, were isolated as previously described (7, 8).

Results and Discussion

Inhibition of rat liver NAD(P)H:quinone acceptor oxidoreductase by three flavonoids isolated from the methanol extract of scutellariae radix (Huang Qin). A methanol extract of scutellariae radix inhibited rat NAD(P)H:quinone acceptor oxidoreductase. An IC_{50} value of 2.6×10^{-6} g/100 ml was determined under the assay conditions. The methanol extract was applied to a water-equilibrated Sephadex G25 column (2 × 20 cm). The column was washed with water, and the fractions having the inhibitory activity eluted between

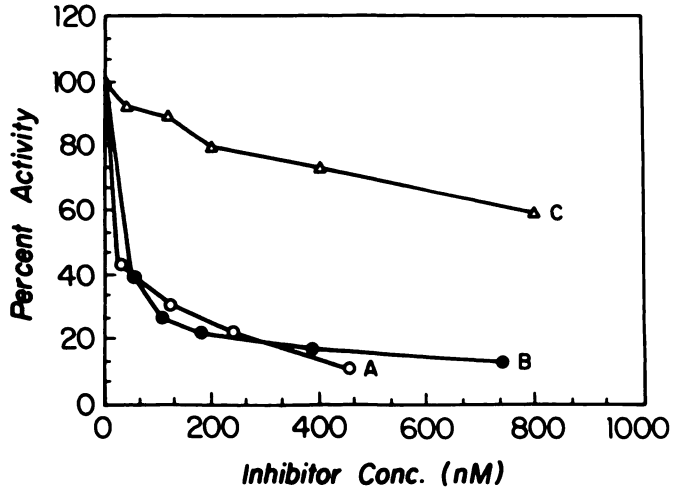

Fig. 4. Concentration dependency of the inhibition of rat liver NAD(P)H:quinone acceptor oxidoreductase by baicalin (A), oroxylin-A 7-*O*-glucuronide (B), and wogonin 7-*O*-glucuronide (C).

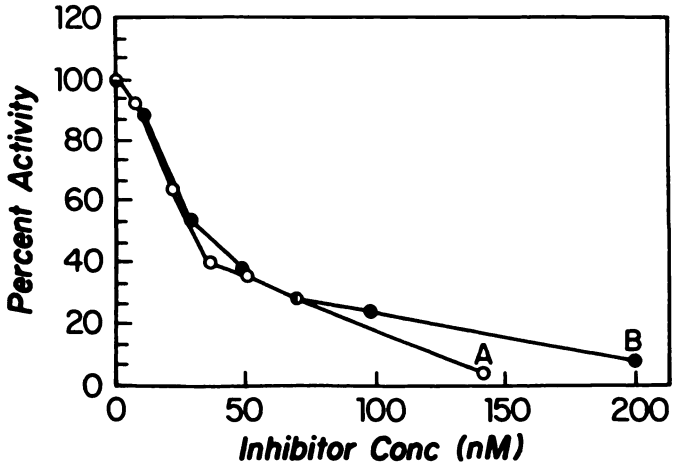

Fig. 5. Concentration dependency of the inhibition of rat liver NAD(P)H:quinone acceptor oxidoreductase by oroxylin-A (A) and oroxylin-A 7-*O*-glucuronide (B).

fractions that would contain NAD^+ (molecular weight, 663.4) and fluoroesceinamine (molecular weight, = 347.3). Therefore, the compounds inhibiting the quinone reductase were estimated as having molecular weights between 400 and 600. The active components were separated by reverse phase high performance liquid chromatography, using a Vydac C-18 column (10 × 250 mm, 5-μm particle size) with a 60-min linear gradient from 100% solvent I (0.1% aqueous trifluoroacetic acid) to 40% solvent II (trifluoroacetic acid/water/acetonitrile, 0.1:9.9:90, v/v/v). Three major UV (214 nm)-absorbing peaks were detected (Fig. 1), and all were found to inhibit the quinone reductase.

FD mass spectral analysis of fraction A gave two major ions, with *m/z* values of 270 and 447 (Fig. 2). The *m/z* 270 ion corresponds to the molecular weight of a flavone, baicalein (Fig. 3), previously identified from the same herb (7, 8). The *m/z* 447 ion is consistent with the protonated molecular ion of the glucuronic acid conjugate of baicalein, i.e., baicalin. The adduct with sodium was also observed (*m/z* 469). A low abundance ion was also observed in the spectrum at *m/z* 176, corresponding to glucuronic acid. Ions observed for baicalein and glucuronic acid are likely the result of thermal degradation of baicalin, which occurs as the FD emitter is heated. The identity of the

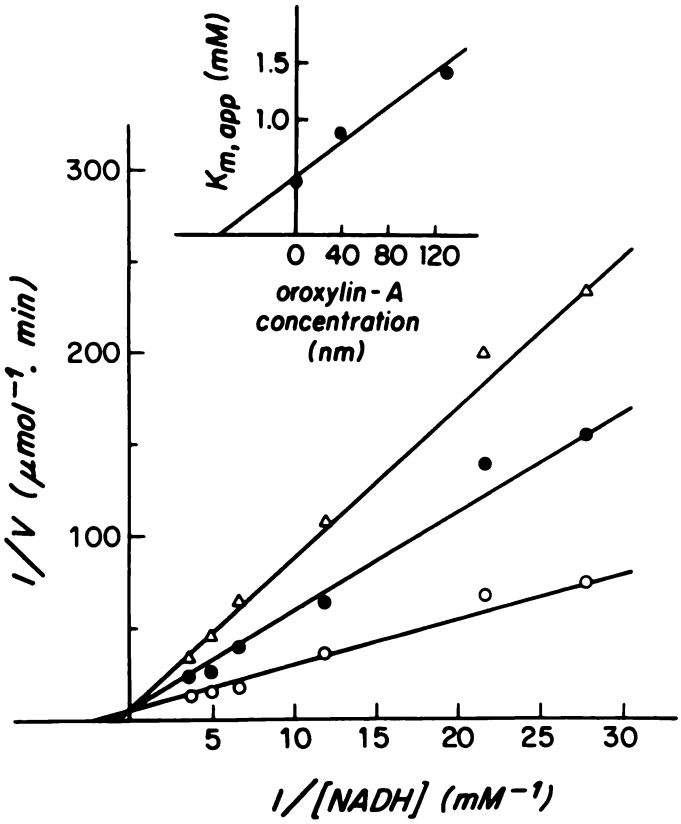

Fig. 6. Competitive inhibition of NADH oxidation of NAD(P)H:quinone acceptor oxidoreductase by oroxylin-A 7-*O*-glucuronide. The concentrations of oroxylin-A 7-*O*-glucuronide are 0 (○), 39 (●), and 130 nM (△). *Inset*, K_i determination.

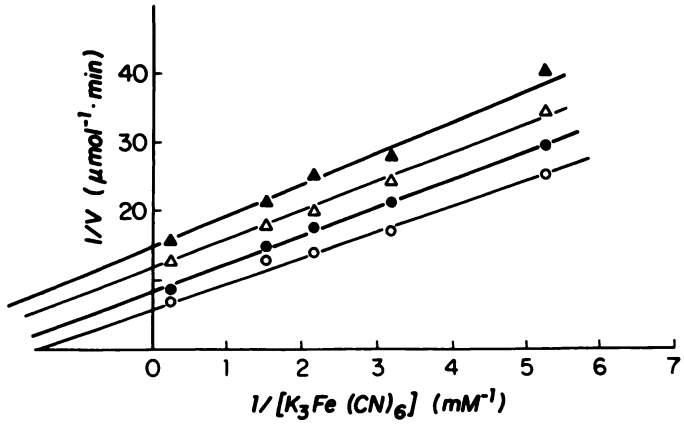

Fig. 7. Uncompetitive inhibition of potassium ferricyanide reduction of NAD(P)H:quinone acceptor oxidoreductase by oroxylin-A 7-*O*-glucuronide. The concentrations of oroxylin-A are 0 (○), 39 (●), 65 (△), and 130 nM (▲).

compound in fraction A was further confirmed by comparing its UV/visible spectral properties (λ_{max}, 280 and 315 nm) and TLC properties with those of an authentic baicalin standard.

In an analogous fashion, fraction B was identified as oroxylin-A 7-*O*-glucuronide (data not shown), and fraction C was identified as wogonin 7-*O*-glucuronide (data not shown). Oroxylin-A differs from baicalein by having a methoxyl group in the C-6 position instead of a hydroxyl group (Fig. 3). Wogonin had the same molecular weight as oroxylin-A but has the methoxyl group at the C-8 position instead of the C-6 position (Fig. 3).

Fig. 4 shows the concentration dependency of the inhibition of rat liver quinone reductase by the three flavonoids. As an example, Fig. 5 shows that oroxylin-A inhibits the quinone reductase with potency similar to that of oroxylin-A 7-*O*-glucuronide, suggesting that the flavone portion of the flavonoids (i.e., the aglycon) is the part providing inhibition of the enzyme. When the enzyme assays were performed using 200 μM NADH as the electron donor and 120 μM menadione as the electron acceptor, the IC_{50} values of the flavonoids were estimated to be 20 nM for baicalin and 30 nM for oroxylin-A 7-*O*-glucuronide. The results indicate that the interaction of these two flavonoids with the quinone reductase is not affected by there being either a hydroxyl or a methoxyl group at the C-6 position of the flavones. On the other hand, wogonin 7-*O*-glucuronide, up to 800 nM, inhibited only 40% of the enzyme activity. As mentioned previously, the only structural difference between oroxylin-A and wogonin is that oroxylin-A has the methoxyl group at the C-6 position and wogonin has the methoxyl group at the C-8 position. Apparently, such small structural differences have significant influence on the interaction with this enzyme.

Mechanism of the inhibition of NAD(P)H:quinone acceptor oxidoreductase by oroxylin-A 7-*O*-glucuronide. We have investigated further the inhibitory mechanism of these flavonoids, using oroxylin-A 7-*O*-glucuronide as the inhibitor. NAD(P)H:quinone acceptor oxidoreductase catalyzes the reduction of quinones, such as menadione, using NAD(P)H as the electron donor. Oroxylin-A 7-*O*-glucuronide was found to be a competitive inhibitor with respect to NADH, with a K_i value of 63 nM (Fig. 6). Because menadione provides substrate inhibition (9) (i.e., the enzyme exhibits lower NAD(P)H-menadione reductase activity at higher menadione concentration), it was not possible to investigate the nature of the oroxylin-A 7-*O*-glucuronide inhibition with respect to menadione by normal kinetic analysis. We have attempted to measure the quinone reductase at very low menadione concentrations using cytochrome *c* as a terminal electron acceptor. In such assays, menadione concentration remains constant because the product menadiol is rapidly and spontaneously oxidized to menadione by cytochrome *c*. Using this method, it was found that oroxylin-A 7-*O*-glucuronide inhibited the menadione reduction in a mixed-order fashion.

The NAD(P)H:quinone acceptor oxidoreductase can also use potassium ferricyanide as an electron acceptor. Oroxylin-A 7-*O*-glucuronide was shown to be a uncompetitive inhibitor with respect to potassium ferricyanide, suggesting that the inhibitor binds after potassium ferricyanide binds to the enzyme (Fig. 7). These kinetic studies revealed that oroxylin-A 7-*O*-glucuronide inhibits the quinone reductase in a fashion very similar to that of dicoumarol, which has been shown to be a competitive inhibitor of the quinone reductase with respect to NADH oxidation, with a K_i of 2–10 nM (10). Dicoumarol has a structure somewhat like a dimer of menadione (Fig. 3) and has similarities to the flavones identified in this study. It is our hypothesis that these flavonoids isolated from the roots of *S. baicalensis* inhibit the quinone reductase by binding to the same site that binds dicoumarol.

Scutellariae radix (Huang Qin) has been used as a remedy for arteriosclerosis and endotoxin-induced disseminated intravascular coagulation. Its effectiveness could be due in part to an anticoagulating effect mediated by the inhibition of this

quinone reductase or other vitamin K reductases by oroxylin-A 7-*O*-glucuronide and baicalin. Flavonoids are common components in these plants and have been found to be active in retarding thrombopoiesis (1), in affecting arachidonic acid metabolism (11), and in inhibiting cAMP phosphodiesterase (3) at a micromolar to millimolar concentration range. Flavonoids have also been shown to inhibit membrane-bound quinone reductases such as mitochondrial NADH-coenzyme Q reductase (12) and succinate-coenzyme Q reductase (13) at millimolar concentrations. In this communication, we indicate that the three flavonoids we have identified inhibit rat liver NAD(P)H:quinone acceptor oxidoreductase at nanomolar concentrations, indicating that these compounds bind this quinone reductase much more strongly than the other enzyme systems mentioned.

Oral anticoagulants are widely used in attempts to reduce the risk of recurrent myocardial infarction. The major pharmacological effect of oral anticoagulants is inhibition of blood clotting by interference with the hepatic posttranslational modification of the vitamin K hydroquinone-dependent clotting factors. Thus, most of the oral anticoagulants are antagonists of vitamin K, and they inhibit quinone reductases. NAD(P)H:quinone acceptor oxidoreductase reduces vitamin K; however, its role in the blood coagulation process remains controversial. Hildebrandt and Suttie (14), and recently Wallin and Martin (15), suggested that coumarin anticoagulant drugs, such as warfarin, inhibited mainly the microsomal dithiol-dependent vitamin K epoxide reductase. A microsomal membrane-bound dithiothreitol-dependent vitamin K reductase activity has been also shown to be more sensitive to warfarin treatment than NAD(P)H:quinone acceptor oxidoreductase, and this activity is less susceptible to warfarin inhibition in warfarin-resistant rats (16, 17). It has been further suggested that the microsomal dithiol-dependent vitamin K epoxide reduction and quinone reduction activities are catalyzed by the same enzyme (18). This latter enzyme has not yet been purified. Although these investigations suggest that NAD(P)H:quinone acceptor oxidoreductase may not play an important role in the blood coagulation process, this quinone reductase is a target for anticoagulants. Dicoumarol, a very potent inhibitor of the quinone reductase (K_i = 1–10 nM) (10), was the first oral anticoagulant but its effectiveness is limited by incomplete absorption. The most widely used oral anticoagulant is warfarin. It inhibits this enzyme in a fashion identical to that of dicoumarol, with a K_i of 1 μM (19), which is 100 to 1000 times less than the affinity of dicoumarol. Hildebrandt and Suttie (14) reported that warfarin inhibited the NAD(P)H:quinone acceptor oxidoreductase and vitamin K epoxide reductase in rat liver, with IC_{50} values of 33 and 4 μM, respectively. Because the NAD(P)H:quinone acceptor oxidoreductase is a comparatively well characterized vitamin K reductase and is inhibited by all known oral anticoagulants, it should be a useful model enzyme to investigate the mode of action of oral anticoagulants. In this study, oroxylin-A 7-*O*-glucuronide has been shown to inhibit the quinone reductase in the same manner as dicoumarol, with a K_i of 63 nM (16-fold higher affinity than warfarin). These flavonoids are soluble in water and the flavone moieties are lipophilic, facilitating passage through cell membranes. These results suggest that oroxylin-A 7-*O*-glucuronide and related flavonoids may be more effective oral anticoagulants. A detailed study of the degree of rat liver quinone reductase inhibition by different natural flavonoids is currently underway. The study of the structure-activity relationship of flavonoids should provide information useful in the development of new anticoagulants.

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