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# INHIBITION OF *IN VITRO* MICROSOMAL LIPID PEROXIDATION BY ISOFLAVONOIDS

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**Abstract**—In a comparative study the inhibition of microsomal lipid peroxidation induced by an $Fe^{2+}$–ADP-complex and NADPH by naturally occurring isoflavones and their reduced derivatives (isoflavanones and isoflavans) has been examined. It is found that the isoflavanones are more active than the parent isoflavones and the isoflavans are by far the most potent inhibitors. In our *in vitro* test system 6,7,4′-trihydroxy- and 6,7-dihydroxy-4′-methoxyisoflavans ($IC_{50}$ values $1.3 \times 10^{-6}$ and $1.1 \times 10^{-6}$ mol/l respectively) surpass the inhibitory effect of α-tocopherol, (+)-cyanidanol-3 and BHA (butylated hydroxyanisole). In order to establish a structure–activity relationship, a few more isoflavans have been included in the investigation.

Several indications suggest that the lipid peroxidation might play an important role in cell ageing, in some forms of liver injury and in oxygen toxicity in general [1]. It is initiated by reactive oxygen species like hydrogen peroxide, superoxide radical anion, hydroxyl radical and singlet-oxygen [2, 3]. These oxygen species attack polyunsaturated fatty acids (PUFA) in cell membranes. The PUFAs are destroyed by radical propagation and peroxidation, which possibly lead to destabilization and disintegration of cell membranes [4].

Cellular defence mechanisms against toxic oxygen effects in *in vitro* systems comprise enzymatic inactivation of reactive $O_2$ species by superoxide-dismutase, GSH-peroxidase and catalase as well as nonenzymatic protection of PUFA by radical scavengers and antioxidants like α-tocopherol, ascorbic acid and β-carotene [3]. *In vitro* lipid peroxidation of liver microsomal membranes can also be inhibited by phenols like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), catechols and pyrogallols [5–7]. Recent studies show that the use of these synthetic antioxidants are accompanied by several toxic effects [8–10]. For this reason, an intensive search for novel natural as well as synthetic antioxidants is being very seriously pursued.

The flavonoids, a class of natural products, have been found to exhibit strong antioxidative properties. The wide range of biochemical and pharmacological activities of these compounds have been recently reviewed [11]. The sub-group of isoflavonoids has remained comparatively unexplored till now, although they are also attributed with several physiological properties [12]. In the present study the most common natural isoflavones of food plants and their reduced derivatives have been examined for inhibition of liver microsomal lipid peroxidation induced by an $Fe^{2+}$–ADP-complex and NADPH.

Determination of NADPH dependent *in vitro* lipid peroxidation is an established method to study the inhibitory power of antioxidants against radical-induced oxidative destruction of membrane lipids [1, 4]. Enhancement of lipid peroxidation by complex-bound $Fe^{2+}$ ion was first described by Hochstein and Ernster in 1963 [13].

## MATERIALS AND METHODS

All standard chemicals were obtained from Merck (Darmstadt, F.R.G.) and were of analytical reagent grade. The biochemicals ADP and NADPH of the purest grade available were obtained from Boehringer (Mannheim, F.R.G.). Isoflavones, isoflavanones and isoflavans were synthesized in our laboratory and the identity of the products was established by NMR- and mass spectrometry, α-Tocopherol and butylated hydroxyanisole (BHA) were of reagent grade and supplied by Merck. (+)-Cyanidanol-3 was a kind gift by Zyma laboratories (Munich, F.R.G.).

### *Preparation of microsomes*

Wistar rats, weight 200–250 g, were decapitated and the liver was removed and homogenized. Microsomes were gained by differential centrifugation according to Kornbrust and Mavis [14]. Microsomal protein was determined by the method of Hartree [15] and amounted to 50 mg protein/ml. This stock solution was stored at −40°. For experimental purposes fresh solutions were made every time by diluting 150 μl of it with 8.85 ml of Tris–HCl buffer so that 300 μl of this solution contained 250 μg microsomal protein. The following other solutions were prepared for the estimation of lipid peroxidation and its inhibition.

*Inhibitor solution*. A definite quantity of the inhibitors was dissolved in a particular volume of ethanol or DMSO (dimethyl sulfoxide) and diluted with water so that the final concentration of organic solvent did not exceed 1%.

*Initiator solution* was prepared afresh by dissolving $FeSO_4$, 100 μmol, ADP 2.5 mmol, NADPH 5 mmol in a litre of distilled water.

*Stopper solution* contained 15% (w/v) TCA, 0.375% (w/v) TBA, 0.25 mol/l HCl, 0.01% BHA (after Buege and Aust [4]).

*Microsomal lipid peroxidation*

All incubations were carried out in glass tubes at 37° under free access of air. 300 μl of diluted microsomal solution and 100 μl of inhibitor solution (containing the required concentration) were pre-incubated for 5 min. 100 μl of initiator solution were added to the mixture and incubated for 10 min. Addition of 500 μl of stopper solution and heating at 80° for 15 min produced a pink colouration. The solution was cooled down at room temperature and centrifuged at 1000 *g* for 10 min. Absorption of the pink supernatant was determined spectrophotometrically at 535 nm. The amount of formed malondialdehyde–thiobarbituric acid complex was calculated using an absorption coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. At least eight different concentrations of inhibitors were used. Values obtained without inhibitor were taken for 100% activation, i.e. 0% inhibition. Mean values of three estimations were used for calculation.

*Determination of NADPH consumption*

500 μl of buffered microsomal solution containing $Fe^{2+}$–ADP-complex and NADPH as described above was incubated at 37°. With addition of the NADPH the absorption at 340 nm was registered continuously for 20 min. The oxidation rate of NADPH was calculated using an extinction coefficient of $6.2 \times 10^3\ M^{-1}\ cm^{-1}$.

## RESULTS AND DISCUSSION

Incubation of rat liver microsomes with $Fe^{2+}$–ADP-complex in the presence of NADPH produced 20 nmole/mg microsomal protein. The amount of MDA formed in the absence of NADPH (auto-oxidation) was below 30%. In tubes containing no inhibitor the rate of NADPH consumption was 3.55 nmole $min^{-1}$ (mg protein)$^{-1}$.

With increasing concentrations of inhibitors MDA formation was decreased to zero. Half-maximal inhibition concentration ($IC_{50}$) was determined from the curves obtained by drawing the per cent inhibition against different concentrations of the isoflavonoids.

Almost all isoflavonoids tested are able to inhibit microsomal lipid peroxidation. The 6,7-dihydroxylated isoflavones texasin and György isoflavone [16, 17] and their reduced derivatives show the strongest inhibition. The hydroxyl groups in meta-positions of ring A (e.g. 5,7-dihydroxy-) or a single hydroxyl group at $C_7$ are less favourable for inhibitory action. The progressive reduction of the pyrone ring (ring C) leads to a large enhancement of inhibitory power. The $IC_{50}$ values of 6,7-dihydroxylated isoflavans are in the range of $10^{-6}$ mol/l. Methylation of 4′-hydroxyl group generally improves the inhibitory effect slightly, which could be explained by a greater lipophilicity of these compounds.

For the sake of comparison three well known antioxidants, butylated hydroxyanisole (BHA,

Table 1. Half-maximal inhibition concentrations ($IC_{50}$) of isoflavonoids against lipid peroxidation induced by $Fe^{2+}$–ADP complex

| Inhibitors with substituents | Isoflavone | Isoflavanone | Isoflavan |
|---|---|---|---|
| | $IC_{50}$ in moles/l | | |
| 6,7,4′-OH (György isoflavone) | $3.2 \times 10^{-5}$ | $3.1 \times 10^{-5}$ | $1.3 \times 10^{-6}$ |
| 6,7-OH, 4′-$OCH_3$ (Texasin) | $2.9 \times 10^{-5}$ | $2.1 \times 10^{-6}$ | $1.1 \times 10^{-6}$ |
| 7,4′-OH, 6-$OCH_3$ (Glycitein) | $1.6 \times 10^{-3}$ | $6.0 \times 10^{-4}$ | $6.0 \times 10^{-6}$ |
| 5,7-OH, 4′-$OCH_3$ (Biochanin A) | $1.5 \times 10^{-4}$ | $8.0 \times 10^{-4}$ | $2.2 \times 10^{-5}$ |
| 5,7,4′-OH (Genistein) | $1.8 \times 10^{-4}$ | $5.0 \times 10^{-4}$ | $3.0 \times 10^{-5}$ |
| 7-OH, 4′-$OCH_3$ (Formononetin) | $> 10^{-3}$ | $3.0 \times 10^{-4}$ | $2.4 \times 10^{-5}$ |
| 7,4′-OH (Daidzein) | $6.0 \times 10^{-4}$ | $6.1 \times 10^{-5}$ | $3.8 \times 10^{-5}$ |
| 6,7-OH-isoflavan | — | — | $2.1 \times 10^{-6}$ |
| 6-OH, 7-$OCH_3$-isoflavan | — | — | $1.7 \times 10^{-6}$ |
| 6-OH, 7′,4′-$OCH_3$-isoflavan | — | — | $1.3 \times 10^{-6}$ |
| 6,7-$OCH_3$, 4′-OH-isoflavan | — | — | $2.6 \times 10^{-4}$ |

$IC_{50}$ $1.7 \times 10^{-6}$ moles/l), (+)-cyanidanol-3 ($IC_{50}$ $1.2 \times 10^{-5}$ moles/l), and α-tocopherol ($IC_{50}$ $8.0 \times 10^{-5}$ moles/l), were also tested. Compared with these antioxidants, the 6,7-dihydroxylated isoflavans show a stronger inhibitory effect than BHA and are found to possess 80 times stronger activity than α-tocopherol in our *in vitro* system. Half-maximal inhibition values of flavonoids like Quercetin and rutin and of a number of catechols and pyrogallols against lipid peroxidation lie in the region of $10^{-6}$ moles/l [6, 18]. A common feature of these substances is an ortho-dihydroxybenzene or catechol structure, which is considered to be important for antioxidative effectiveness [6]. This corroborates our finding that the ortho-dihydroxy-isoflavonoids are most potent inhibitors.

α-Tocopherol acts as an antioxidant by cleavage of the chroman ring system to a quinone [19]. The position of the hydroxyl group in the chroman ring of α-tocopherol corresponds to the 6-hydroxyl group of the isoflavonoids. To evaluate the importance of the 6-hydroxyl group in 6,7-dihydroxylated isoflavones, we blocked the 6- and 7-positions alternately by methylation. Methylation of $C_7$-OH did not reduce the inhibitory effect, while methylation of $C_6$-OH or of both hydroxyl groups resulted in lowering the inhibitory power.

The tested antioxidants did not inhibit enzymatic oxidation of NADPH in our test system. NADPH consumption was even slightly enhanced by BHA and by 6,7,4′-trihydroxyisoflavan.

The low $IC_{50}$ values of the inhibitors indicate that a free radical scavenging mechanism is more plausible here and not an inhibition of the electron transfer process through the cytochrome P-450 chain.

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