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Chemoprotective activity of boldine: modulation of drug-metabolizing enzymes

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Possible chemoprotective effects of the naturally occuring alkaloid boldine, a major alkaloid of boldo (*Peumus boldus* Mol.) leaves and bark, including *in vitro* modulations of drug-metabolizing enzymes in mouse hepatoma Hepa-1 cell line and mouse hepatic microsomes, were investigated. Boldine manifested inhibition activity on hepatic microsomal CYP1A-dependent 7-ethoxyresorufin *O*-deethylase and CYP3A-dependent testosterone 6β-hydroxylase activities and stimulated glutathione *S*-transferase activity in Hepa-1 cells. In addition to the known antioxidant activity, boldine could decrease the metabolic activation of other xenobiotics including chemical mutagens.

1. Introduction

Boldo (*Peumus boldus* Mol.) has been traditionally used in folk medicine of South America for its hepatoprotective, anti-inflammatory, choleretic, and cholagogic effects, as well as for the treatment of gastrointestinal disorders [1, 2]. Boldo leaves and bark contain about 2% of essential oil, 1.2% of tannins, flavonoids (e.g. quercetin), alkaloids and the glucoside boldoglucine. The alkaloids are assumed to be the active components of boldo. There is evidence for the presence of at least 17 alkaloids in the plant (e.g. isocorydine, *N*-methyl laurotetanine); boldine is the major alkaloid [2] present in leaves and bark.

Series of *in vitro* model systems which have been used to identify the chemoprotective properties include the detection of one or more of the following mechanisms [3–6]:

1) inhibition of the enzymes which bioactivate promutagens and procarcinogens, especially hepatic microsomal cytochromes P4501A (CYP1A) and P4503A (CYP3A); the metabolic bioactivation of promutagens and procarcinogens by CYP1A and CYP3A leads to the formation of reactive intermediates which are the ultimate mutagens and/or carcinogens;

2) induction of glutathione *S*-transferase activity and other enzymes detoxifying reactive metabolites of xenobiotics;

3) antioxidant activity (inhibition of peroxidation of key cellular components and scavenging of free radicals);

4) other effects, such as anti-tumour-promoting action and inhibition of 5-lipoxygenase.

Boldine was found to be weakly toxic and did not show genotoxic activity *in vitro* and *in vivo* [7, 8]. The antioxidant activity of boldine was assessed in both biological and abiotic models. Boldine acted as an efficient hydroxyl radical scavenger and *in vitro* inhibitor of carbon tetrachloride- or *tert*-butyl-hydroperoxide-stimulated lipid peroxidation [9–11]. Boldine even manifested protective activity against carbon tetrachloride-induced hepatitis in mice.

Considering the known antioxidant properties of boldine, modulations of drug-metabolizing enzymes were measured in the present study. The following parameters were tested to screen for the chemoprotective activities of boldine: 1) *in vitro* inhibition of the CYP1A-dependent 7-ethoxyresorufin *O*-deethylase (EROD) and of the CYP3A-dependent testosterone 6β-hydroxylase activities in rat hepatic microsomes; 2) *in vitro* stimulation of glutathione *S*-transferase in the Hepa-1 cell line following a 24-h treatment with various doses of boldine.

2. Investigations, results and discussion

2.1. In vitro inhibition of CYP1A and CYP3A activities in mouse hepatic microsomes

This study was designed to evaluate the modulation by boldine of drug-metabolizing enzymes involved in the metabolic activation and detoxication of xenobiotics. The *in vitro* inhibition by the alkaloid of the CYP1A-dependent EROD activity is shown in Fig. 1. The EROD activity was suppressed by boldine down to 55% at the highest concentration of 25 μM. To date, a number of compounds, such as both synthetic and natural flavonoids, coumarins or chalcones including α-naphtoflavone and quercetin, have been shown to selectively inhibit CYP1A [12]. In our study, α-naphtoflavone and quercetin were used as reference inhibitors; at the highest concentration of 25 μM, they inhibited the EROD activity completely.

Boldine also reduced effectively the hepatic microsomal CYP3A-dependent testosterone 6β-hydroxylase activity. An inhibition of 60% was found at the final concentration of 0.1 μM (Fig. 2). Like the EROD inhibition potency of boldine, the inhibition of CYP3A was not complete up to 50 μM; however, IC_{50} concentration was obtained already at a much lower concentration.

CYP1A and partly also CYP3A are the major oxidative enzymes involved in the metabolic activation of many xenobiotics, such as polycyclic aromatic hydrocarbons, or afla-

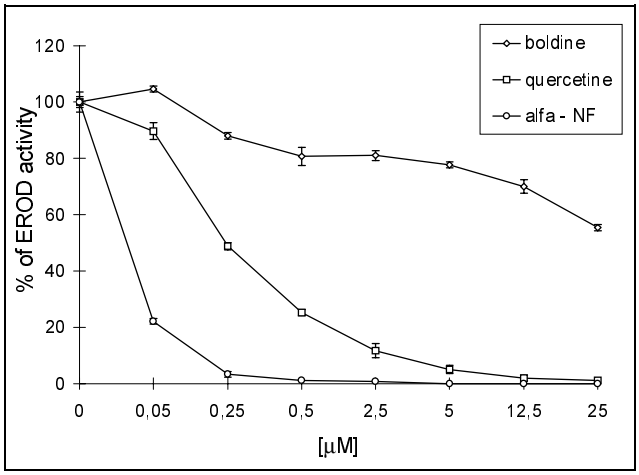

Fig. 1: *In vitro* inhibition of the CYP1A-dependent EROD activity by boldine; EROD, 7-ethoxyresorufin *O*-deethylase activity in hepatic microsomes. The results were expressed as mean values ± s. e. m, n = 4

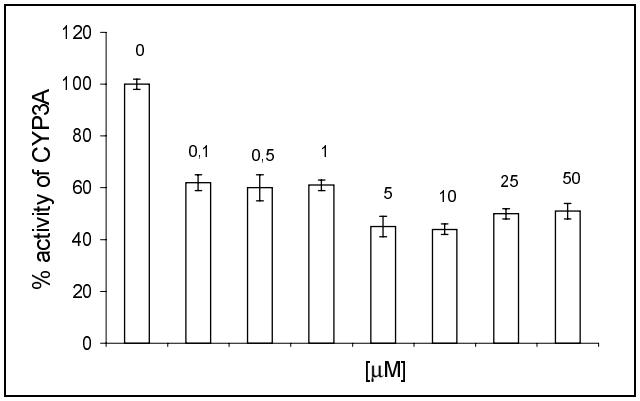

Fig. 2: In vitro inhibition of the CYP3A-dependent 6β-OH-T-activity by boldine. The results were expressed as mean values ± s. e. m, n = 4

toxin B1 [13]. It is evident that modulation of these enzymes can dramatically affect toxic, mutagenic and carcinogenic processes resulting from an exposure to xenobiotics [4].
No effect of boldine on another CYP isoenzyme, CYP2E1 was found [14]. In our study, boldine was only a moderate CYP inhibitor. Nevertheless, one of the modes of its chemoprotective action may be a reduction of the rise of reactive intermediates of xenobiotics by inhibition of bioactivating CYP1A and CYP3A enzymes.

2.2. In vitro modulation of glutathione S-transferase activity

The induction of GST activity was determined in Hepa-1 cells after a 24-h treatment with various concentrations of boldine (Fig. 3). The chemoprotective effect of the novel and promising agent boldine can be also based on the induction of GST enzymes. It has been recognized that many anticarcinogens shared the ability to induce Phase II enzymes [15]. The measurement may also show realistic ratios between the rates of monooxygenase activities leading to electrophilic intermediates and GST activities detoxifying these hazardous metabolites. At low concentrations (1 μM), boldine significantly induced the GST activity in hepatoma cells. This *in vitro* screening experiment showed that, as a Phase II enzyme(s) inducer, boldine may be a candidate anticarcinogen to be evaluated *in vivo*. In conclusion, our data show that the aporphine alkaloid boldine has a strong chemoprotective potential. Besides its antioxidative activity and low toxicity, boldine inhibited the CYP isoenzymes bioactivating promutagens and procarcinogens and induced GST enzyme(s) detoxifying reactive toxic intermediates. Boldine is a promising chemoprotective drug.

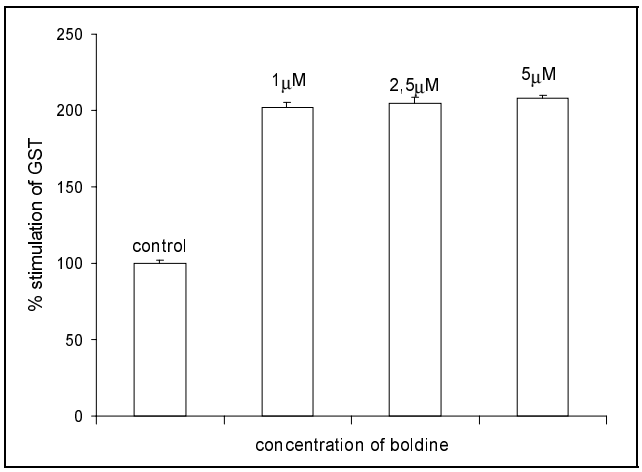

Fig. 3: In vitro modulation of the GST activity by boldine. The results were expressed as mean values ± s. e. m, n = 4

3. Experimental

3.1. Materials

7-Ethoxyresorufin was purchased from Molecular Probes Co. (Eugene, MI, U.S.A.), testosterone, 6β-hydroxytestosterone, NADPH, reduced glutathione, butylated hydroxytoluene (BHT), α-naphtoflavone, quercetin, thiobarbituric acid, bicinchonic acid and 1-chloro-2,4-dinitrobenzene were supplied by Sigma Chem. Co. (Prague, Czech Republic). Boldine was isolated from the plant *Peumus boldus* Mol., purified by conventional chromatographic methods on a series of silica columns, and identified by spectral methods at the Faculty of Pharmacy, Brno, Czech Republic.

3.2. Preparation of subcellular fractions

The hepatic microsomal fraction was isolated from C57Bl/6 mouse liver tissue by homogenization and differential centrifugation. The microsomes were washed once, resuspended in 0.05 M Tris-HCl buffer, pH 7.5 containing 20% of glycerol and 0.1 mM EDTA, and stored at −80 °C until used.

3.3. Hepa-1 cells

Mouse hepatoma Hepa-1c1c7 cells were grown in minimal essential medium (α-MEM, Sigma Chem. Co., Prague, Czech Republic) without antibiotics, supplemented with 10% of heat-inactivated foetal bovine serum for 24 h prior to experimental treatment. The cells were then exposed to various concentrations of boldine or the dimethyl sulfoxide (DMSO) vehicle for another 24 h. Then the cells were collected and resuspended in PBS and the suspension was centrifuged and stored at −80 °C until used.

3.4. Determination of enzymatic activities

The inhibition of the mouse hepatic microsomal 7-ethoxyresorufin *O*-deethylase activity (EROD) was determined by direct fluorimetry [16]. The reaction mixture contained microsomes, 2 μM 7-ethoxyresorufin, 0.1 M Tris-HCl buffer, pH 7.6, 0.3 mM NADPH, and 0.5% (v/v) of DMSO or boldine in DMSO.
The inhibition of the microsomal activity of CYP3A isoenzymes was determined as testosterone 6β-hydroxylase by the reverse-phase HPLC [17] after 10 min of incubation with 2 mM testosterone.
The glutathione *S*-transferase (GST) activity was assayed in sonicated Hepa-1 cell suspensions by the spectrophotometric method with 1.25 mM 1-chloro-2,4-dinitrobenzene as the substrate [18].
Protein concentrations were determined by the bicinchonic acid assay [19].

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