Chemico-Biological Interactions 296 (2018) 117–123

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Phloretin cytoprotection and toxicity☆

Brian C. Geohagen[a], Boris Korsharskyy[a], Amaresh Vydyanatha[a], Lars Nordstroem[b], Richard M. LoPachin[a,*]

[a] Albert Einstein College of Medicine, Department of Anesthesiology, Montefiore Medical Center, Bronx, NY, USA
[b] The Chemical Synthesis & Biology Core Facility, Albert Einstein College of Medicine, Bronx, NY, USA

ARTICLE INFO

Keywords:
Phytopolyphenol
Hepatoprotection
Acetaminophen overdose
Drug-induced toxicity
Enol-based cytoprotectants

ABSTRACT

Phloretin (Phl) is a dihydrochalcone flavonoid with significant cytoprotective properties; e.g., free radical trapping, electrophile scavenging. Based on this, it has been suggested that Phl might be useful in the treatment of pathogenic processes and prevention of drug toxicities. Therefore, we determined the ability of Phl to provide route- and dose-dependent hepatoprotection in a mouse model of acetaminophen (APAP) overdose. Intraperitoneal (i.p.) administration of Phl produced a bimodal effect; i.e., the highest dose (2.40 mmol/kg) did not prevent APAP-induced lethality, whereas lower doses (0.2–0.4 mmol/kg) afforded modest hepatoprotection. When given alone, the highest i.p. Phl dose was lethal within 24 h, whereas the lower doses were not toxic. Oral Phl (0.40–2.40 mmol/kg) did not prevent APAP-induced hepatotoxicity. The highest oral dose given alone (2.4 mmol/kg) produced 64% lethality, whereas lower doses were not lethal. This toxicity profile was reflected in a study using APAP-exposed isolated mouse hepatocytes, which showed that the Phl pharmacophores, 1,3,5-trihydroxyacetophenone (PG) and 2′,4′,6′-trihydroxyacetophenone (THA) where protective. Corroborative cell free studies showed that polyphenol protectants prevented glutathione loss mediated by the APAP metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Thus, in spite of possessing cytoprotective attributes, Phl was generally toxic in our APAP models. These and earlier findings suggest that Phl is not a candidate for drug design. In contrast, we have found that the enol-forming pharmacophores, THA and PG, are potential platforms for pharmacotherapeutic development.

1. Introduction

Phloretin (Phl) is a dihydrochalcone flavonoid that is found in apple skins. This phytopolyphenol has been shown to have significant antioxidant properties and might, therefore, be useful in treating pathogenic conditions that have oxidative stress as a common mechanism [1,2]. Indeed, some evidence suggests that Phl is a pharmacotherapeutic approach to cardiovascular disease [3], cancer [4], ischemia-reperfusion injury [5] and neurodegeneration [6]. In this regard, we have previously shown that Phl prevented electrophile (e.g., acrolein)-induced nerve terminal toxicity [7]. We also found that the Phl pharmacophores, 2′,4′,6′-trihydroxyacetophenone (THA) and 1,3,5-trihydroxyacetophenone (phloroglucinol), could prevent hepatotoxicity as indicated by prevention of cell lethality and mitochondrial dysfunction in an isolated mouse hepatocyte model of acetaminophen (APAP) overdose [8]. Results indicated that these derivatives were hepatoprotective and that the corresponding levels of protection were significantly greater than that provided by either 2-acetylcyclopentanone (2-ACP), a 1,3-dicarbonyl enol derivative of curcumin, or N-acetylcysteine (NAC; Mucomyst™), the thiol-based acetaminophen (APAP) antidote [8,9].

The apparent broad protective abilities of Phl are consistent with certain chemical attributes of this polyphenol. Specifically, Phl is an aromatic flavonoid that can act as an antioxidant and trap toxic free oxygen/nitrogen radicals [10]. In addition, Phl has several enolizable sites (e.g., C-3 enol on the A ring) that can ionize to form enolates with modest nucleophilicity [7]. Therefore, Phl cytoprotection could also involve scavenging electrophiles such *N*-acetyl-*p*-benzoquinone imine

Abbreviations: APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinonimine; PBS, phosphate buffered saline; 2-ACP, 2-acetylcyclopentanone; NAC, N-acetylcysteine; GSH, glutathione; DMSO, Dimethyl sulfoxide; THA, 2′,4′,6′-trihydroxyacetophenone; PG, 1,3,5-trihydroxyacetophenone; PEG, polyethylene glycol; LDH, lactate dehydrogenase; ALT, alinine aminotransferase; pBQ, para-benzoquinone; MDA, malondialdehyde; HSAB, Hard and Soft, Acids and Bases; E_{LUMO}, Lowest Unoccupied Molecular Orbital energy; E_{HOMO}, Highest Occupied Molecular Orbital energy

☆ The research described in this manuscript was supported by an NIH grant from the National Institute of Environmental Health Sciences (RO1 ES003830-30).

* Corresponding author. Department of Anesthesiology, Montefiore Medical Center, 111 E.210th St, Bronx, NY, 10467, USA.
E-mail address: lopachin@einstein.yu.edu (R.M. LoPachin).

https://doi.org/10.1016/j.cbi.2018.09.020
Received 24 July 2018; Received in revised form 10 September 2018; Accepted 28 September 2018
Available online 01 October 2018
0009-2797/

(NAPQI) and unsaturated carbonyl species (e.g., acrolein) that mediate APAP hepatotoxicity [7,8,11,12]; reviewed in Refs. [13,14]. These chemical properties suggest that Phl could treat pathogenic conditions and drug toxicities that involve electrophiles. Electrophilic species cause toxicity by forming covalent adducts with biological nucleophiles, which subsequently produces mitochondrial damage, glutathione (GSH) depletion, protein inactivation and secondary oxidative stress [13,15]. Therefore, to further our understanding of enolate-based cytoprotection, the route- and dose-dependent effects of Phl were evaluated in a mouse model of acute APAP overdose [9]. During the course of these studies, we found that Phl produced dose-dependent toxicity. To evaluate this bimodal effect, corroborative studies were conducted in freshly isolated mouse hepatocytes. Results were compared to findings from previous pharmacological studies involving enol- and thiol-based cytoprotectants [7,8,9]. Research has provided evidence that electrophile-based GSH depletion was a significant index of cytotoxicity (reviewed in Ref. [13]). Therefore, as a measure of enolate scavenging kinetics, we conducted cell-free *in chemico* studies to quantify polyphenol prevention of NAPQI-induced GSH loss.

2. Materials and methods

2.1. Reagents

All chemicals, reagents and phytopolyphenols were of the highest grade commercially available and were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals and treatments

All aspects of animal use in this study were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Montefiore Medical Center Animal Care Committee. Three month old male C57BL/6N mice (mean weight 27 gm) were purchased from Charles River Laboratory (Wilmington, MA). Mice were housed individually in polycarbonate boxes, and filtered drinking water and Purina Rodent Laboratory Chow (Purina Mills, Inc., St. Louis, MO) were available *ad libitum*. The animal room was maintained at approximately 22 °C and 50% humidity with a 12 h light/dark cycle. Prior to each experiment, mice were fasted overnight and treatments began at 0800 the following morning. Food was returned 1 h post-treatment. APAP and all experimental compounds were administered in phosphate buffered polyethylene glycol (PEG). Preliminary studies demonstrated that this vehicle did not affect the experimental outcome [9]. As a general protocol, groups of mice (n = 15) were pretreated by i.p. (10 ml/kg) injection or oral (4 ml/kg) administration of putative hepatoprotectant followed 20 min later by oral (4 ml/kg) APAP (500 mg/kg). Initial research indicated that the onset and development of enol hepatoprotection was similar regardless of APAP administration time; i.e., 20 min before, 20 min after or simultaneously [9]. A separate group of animals were given vehicle by i.p. injection or oral administration followed twenty minutes later by oral APAP. Control mice received an i.p./oral sequence of vehicle injections. Separate groups (n = 15) of animals were administered a cytoprotectant by either the oral or i.p. route. An assessment of general toxicity was conducted at two day intervals over a 7 day post-intoxication period. Specifically, animals were weighed and assessed by a blinded observer for changes in grooming, nest building, open field behavior, recumbency and gait [9]. Kaplan-Meier survival curves were used to illustrate the cumulative percent daily lethality of mice in different experimental groups and were generated in Prism 6.0 (Graphpad software). In previous studies, we (e.g., see Refs. [8,9]) fully characterized the molecular pathogenesis of APAP-induced hepatotoxicity and enol-based hepatoprotection using temporal measurements of histopathology, liver-specific enzymes, GSH/GSSG ratio, mitochondrial function and unsaturated aldehyde production.

2.3. Hepatocyte isolation procedures and incubations

Hepatocytes were prepared from anesthetized mice (isoflurane inhalation) using the collagenase perfusion method of Geohagen et al. [8]. Briefly, to separate dead hepatocytes, isolated cells were centrifuged (140 g × 8 min) in a Percoll gradient and then washed in media (140 g × 3 min) to remove Percoll. The isolation procedure yielded ~30–40 million cells with 80–90% viability as determined by trypan blue exclusion. Hepatocytes (100,000 cells/ml) were incubated in covered 35 mm plastic dishes containing supplemented RPMI-1640 media at 37 °C in a humidified atmosphere of 95% O_2/5% CO_2. The concentration-dependent cytoprotection (0.01–3.0 mM) of individual test compounds was determined in isolated hepatocytes exposed to APAP (e.g., 1 mM × 4 h incubation). Control conditions included: vehicle alone (0.1% DMSO in media), vehicle plus APAP (e.g., 1 mM × 4 h incubation) and cytoprotectants alone (3.0 mM × 4 h). As sensitive indices of hepatocyte injury [8,9], we measured the respective activities of lactate dehydrogenase (LDH) and the liver specific enzyme, alanine aminotransferase (ALT) in hepatocyte medium. The concentration-response data were fitted by nonlinear regression analyses [8]. In all studies, hepatocyte viability and other toxic measures (see ahead) were determined in at least n = 4–6 independent experiments.

2.4. In chemico studies – effects of cytoprotectants on electrophile-induced GSH loss

Graded concentrations of NAPQI (2–128 μM) were pre-incubated (15 min) in phosphate buffered saline (PBS; pH 7.4, 25 °C) with selected cytoprotectants (50 μM) from different chemical classes (thiol and phytopolyphenols) or vehicle (PEG). Following pre-incubation, GSH (30 μM) was added and remaining sulfhydryl content was measured after 15 min by the DTNB method of LoPachin et al. [16]. For each hepatoprotectant, respective sulfhydryl data were fitted by nonlinear regression analyses (r^2 for all curves ≥ 0.90) and electrophile concentrations that produced 50% thiol loss (IC_{50}'s) and their 95% confidence intervals were calculated by the Cheng-Prusoff equation [17].

2.5. Calculations of hard and soft, acids and bases (HSAB) parameters

The Lowest Unoccupied Molecular Orbital (LUMO) energy (E_{LUMO}) and Highest Occupied Molecular Orbital (HOMO) energy (E_{HOMO}), were determined using Spartan 14 (version 1.1.8) software (Wavefunction Inc., Irvine CA). For each structure, ground state equilibrium geometries were calculated with Density Functional B3LYP 6-31G* in water starting from 6 to 31G* geometries. Global (whole molecule) hardness (η) was calculated as $\eta = (E_{LUMO}\text{-}E_{HOMO})/2$ and softness (σ) was calculated as the inverse of hardness (i.e., $\sigma = 1/\eta$). The electrophilicity index (ω) was calculated as $\omega = \mu^2/2\eta$, where μ is chemical potential of the electrophile ($\mu = (E_{LUMO} + E_{HOMO})/2$). An index of nucleophilicity (ω^-, see Ref. [18] for more detailed discussion) was calculated as $\omega^- = \eta_A(\mu_A - \mu_B)^2/2(\eta_A+\eta_B)^2$, where A = reacting nucleophile and B = NAPQI ($\mu = -5.235$ ev, $\eta = 2.005$ ev).

2.6. Statistical analyses

All statistical analyses were conducted using Prism 6.0 (GraphPad software; San Diego, CA) with significance set at the 0.05 level of probability. In studies evaluating the relative abilities of potential hepatoprotectants to modify APAP lethality in experimental groups of mice, the Mantel-Cox log-rank test was used to compare survival rates among the experimental groups.