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Screening of potential chemopreventive compounds from *Poncirus trifoliata* Raf.

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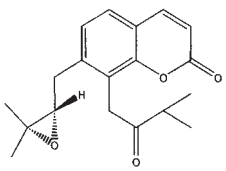
Chemopreventive agents induce a battery of genes whose protein products can protect cells from chemical-induced carcinogenesis. In this study, we isolated three different coumarins compounds (**1**; poncimarin, **2**; heraclenol 3′-methyl ester and **3**; oxypeucedanin methanolate) from *Poncirus trifoliata* Raf., and studied whether these compounds increase glutathione *S*-transferase (GST) expression and activity in the H4IIE cell-line (a rat hepatocyte cell line). CDNB (1-chloro-2,4-dinitrobenzene; GST subtype-nonspecific) and NBD (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; GSTα-type-specific) assays revealed that compound **1** most potently increased GST enzyme activities. Western blot analysis using subtype-specific antibodies confirmed that these three coumarins also selectively increased GSTα-protein expression, and that compound **1** most actively induced GSTα. In contrast, the expressions of the GSTμ and GSTπ subtypes were not altered by these three coumarins. Reporter gene analysis using an antioxidant response element (ARE) containing construct and subcellular fractionation assays, revealed that GSTα-induction by compound **1** might be associated with Nrf2/ARE activation. These results suggest that these three coumarin compounds from *Poncirus trifoliata* Raf possess phase II enzyme inducible functions, and in particular, that poncimarin has chemopreventive potential.

1. Introduction

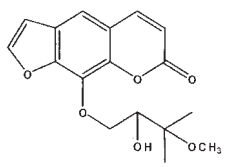
Cancer chemopreventative treatment is defined as the use of naturally occurring compounds to prevent, or reverse the process of carcinogenesis. Diverse phytochemicals can act as chemopreventive agents, these include diallyl sulfide from garlic and dithiolthiones from cruciferous plants, which are generally recognized to protect tissues and prevent carcinogenesis (Wargovich et al. 1988; Wilkinson et al. 1997). These natural compounds have also been extensively studied as potential chemopreventive agents, because of their outstanding protective effects against cancer and cytotoxicity (Wargovich et al. 1988; Kensler et al. 2004).

One key mechanism of chemoprevention by plant compounds involves the induction of phase II detoxifying enzymes, such as glutathione *S*-transferase (GST) (Kensler et al. 1986). In fact, the long-term consumption of chemopreventive agents increases hepatic GST activity by upregulating transcription (Primiano et al. 1995; Guyonnet et al. 1999). Hence, one of the efficient ways of screening for new potential chemopreventives in medicinal plants is to determine their abilities to induce GST.

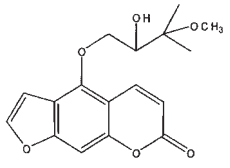
The dried immature fruit of *Poncirus trifoliata* Raf. (Rutaceae), Poncirus fructus, is widely used as a traditional medicine in Eastern Asia, especially as a means of treating inflammation, ulcers, or gastritis. It has been reported that crude extracts and a coumarin compound from Poncirus fructus have anti-inflammatory, anti-bacterial and anti-mucin releasing activities (Kim et al. 1999a, 1999b; Lee et al. 2004). Recently, Yi et al. (2004) also demonstrated that high concentrations (500 μg/ml) of Poncirus fructus extract caused cancer cell-specific apoptosis in HL-60 cells, a human leukemia cell-line. During our program to screen for potential chemopreventive compounds from medicinal plants, we isolated three different coumar-



Compound 1 (Poncimarin)



Compound 2 (Heraclenol 3'-methylester)



Compound 3 (Oxypeucedanin methanolate)

ins (**1**; poncimarin, **2**; heraclenol 3′-methyl ester and **3**; oxypeucedanin methanolate) from the dried immature fruits of *Poncirus trifoliata* Raf, and studied the effects of these coumarins on GST activities and on the expressions of GST sybtypes in a H4IIE cells, a rat hepatocyte-derived cell line. Furthermore, we monitored NF-E2 related factor2 (Nrf2)/antioxidant response element (ARE) activation in order to investigate the mechanistic basis underlying the induction of GSTα by these coumarins.

2. Investigations, results and discussion

We isolated the three coumarins from the immature fruits of *Poncirus trifoliata* Raf. Conjugation between xenobiotics and glutathione (GSH) catalyzed by hepatic phase II enzymes functions as a critical detoxifying event in the human body and this is viewed as an efficient chemoprotective mechanism. GSH-conjugation with xenobiotics can be accelerated by GST, and increased GST activity can potentiate this conjugative reaction and lead to the detoxification of many xenobiotics (Kensler 1997). Moreover, the chemopreventive effects of plant compounds may be due to their abilities to elevate GST. Hence, the induction of GST is believed to be an important determinant of the characteristics of chemopreventive agents. In order to determine whether the coumarins affect the enzyme activities of GST, we performed CDNB (1-chloro-2,4-dinitrobenzene) and NBD (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) assays using cell lysates obtained from H4IIE cells pretreated with each compound (30 μM, 24 h incubation). Compounds **1** and **3** significantly increased CDNB (GST subtype nonspecific) enzyme activity, which represents the catalytic activity of the GSTα subtype (Ricci et al. 1994), whereas compound **2** did not (Fig. 1). However, all three coumarins significantly enhanced NBD enzyme activity (Fig. 1). In particular, compound **2** (poncimarin) was found to be most active and to increase the activities of both enzymes.

Next, we determined the levels of the GST subtypes in H4IIE cells after treating them with the three coumarins. Exposure of cells to any of the three for 18 h significantly increased the level of GSTα, but the protein levels of other GST subtypes, i.e., GSTμ and GSTπ, were unchanged by the three compounds (Fig. 2). In particular, the expression of GSTα subtype in cells treated with 30 μM poncimarin was increased 4 fold (Fig. 2). Moreover, this result is consistent with our NBD enzyme activity assay. Hence, these results show that the selective induction of GSTα by these coumarins seems to be associated with increased GST activity.

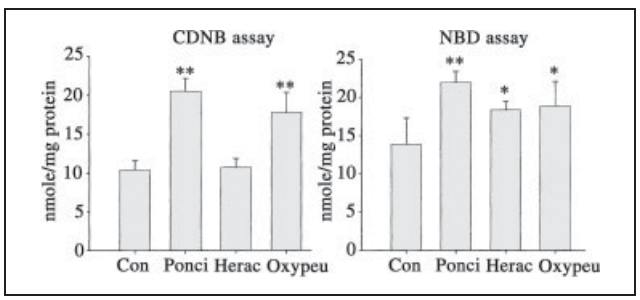


Fig. 1: The effects of the three coumarins on glutathione *S*-transferase (GST) activity. Cells were serum-starved and incubated in the presence or absence of each coumarin (30 μM) for 24 h, and cell lysates were used to determine GST activities. The CDNB (1-chloro-2,4-dinitrobenzene) assay represents subtype-nonspecific GST activity and the NBD (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) assay represents GSTα subtype-specific enzyme activities. Data represent the means ± SD of 6 different samples (significant as compared to the untreated control, $^{*}p < 0.05$; $^{**}p < 0.01$)

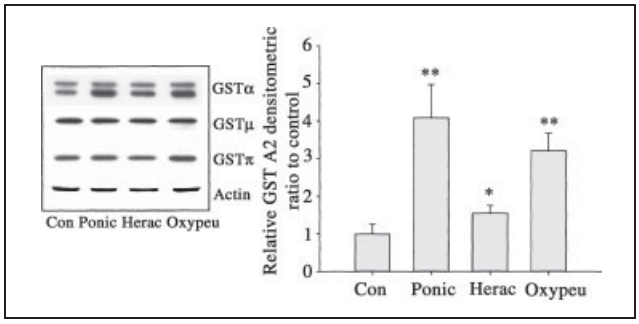


Fig. 2: Effects of the three coumarins isolated from *Poncirus trifoliata* Rafin. on GST protein levels. Protein expression of each GST subunit was monitored 18h after treating cells with each coumarin (30 μM). Lanes were loaded with 10 μg of cytosolic protein. Data represent the means ± SD of 3 separate experiments (significant as compared to the untreated control, $^{*}p < 0.05$; $^{**}p < 0.01$; control level = 1)

Nrf2 is a key transcription factor that binds to ARE sequences and which is implicated in the regulation of GSTα expression (Kang et al. 2001, 2002; Kwak et al. 2001; Ikeda et al. 2004). The role of ARE in the inducible expression of phase II enzymes by several antioxidants and chemopreventive agents has been extensively studied (Kang et al. 2001, 2003). The incidences of gastric neoplasia and urinary bladder carcinoma in response to carcinogens was found to be significantly increased in Nrf2 (−/−) mice, which was demonstrated to be closely associated with a reduced expression of phase II enzymes, including GST (Ramos-Gomez et al. 2001; Iida et al. 2004). Hence, the activation of Nrf2, which controls the constitutive and inducible expression of GSTα, is a protective mechanism against carcinogens. To determine whether the induction of GSTα by the coumarins of *Poncirus trifoliata* Raf is mediated via the activation of Nrf2/ARE, a reporter gene assay was performed using H4IIE cells transfected with the mammalian expression vector pGL-797 containing the luciferase structural gene and the ARE sequence

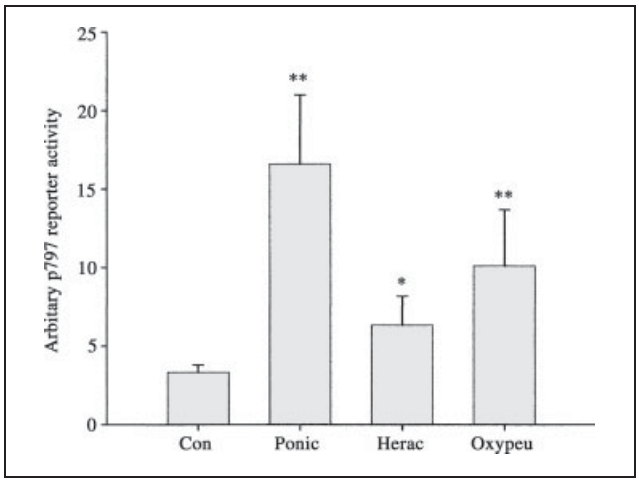


Fig. 3: Induction of luciferase activity by the coumarins in H4IIE cells transiently transfected with the GSTA2 chimeric gene construct pGL-797 containing the ARE element. Dual luciferase reporter assays were performed on lysed H4IIE cells co-transfected with pGL-797 (firefly luciferase) and pRL-SV (*Renilla* luciferase) (in the ratio 50 : 1) after exposure to each compound (30 μM) for 18 h. Reporter gene activations (firefly luciferase activity) were expressed as ratios relative to *Renilla* luciferase activity. Data represent the means ± SD of 4 separate experiments (significant as compared to the control, $^{*}p < 0.05$; $^{**}p < 0.01$)

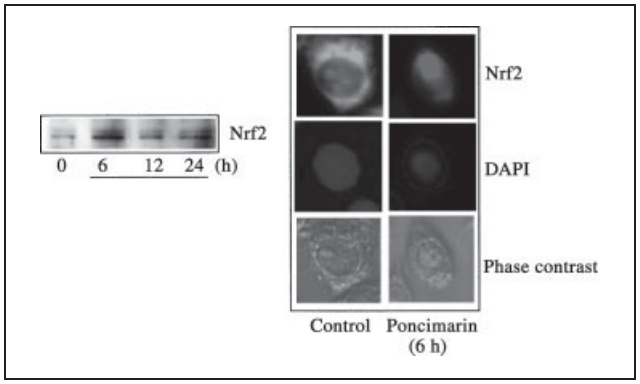


Fig. 4: Nuclear translocation of Nrf2 by poncimarin. The subcellular localizations of Nrf2 were assessed/detected immunochemically (left panel) by fluorescence microscopy (right panel) in cells treated with poncimarin (30 μM). All lanes contained 20 μg of nuclear extracts. Equal protein loadings were verified by Ponceau-S staining

of GSTA2 promoter (Kang et al. 2003). Exposure of H4IIE cells transiently transfected with pGL-797 to the three coumarins significantly increased luciferase activities (Fig. 3). In particular, poncimarin at 30 μM increased ARE-dependent reporter activity >5-fold (Fig. 3). To confirm this result, we also examined whether poncimarin stimulates the translocation of Nrf2 to the nucleus, which is essentially required for the binding of Nrf2 to the ARE consensus sequence (Huang et al. 2000; Kang et al. 2002). Subcellular fractionation and immunoblot blot analyses revealed that poncimarin increased Nrf2 level in nuclear fractions at 3–6 h (Fig. 4, left panel), and immunocytochemistry showed that poncimarin stimulated the nuclear distribution of Nrf2 in paraformaldehyde-fixed H4IIE cells (Fig. 4, right panel). These data suggest that the induction of GSTα by the three coumarins from *Poncirus trifoliata* Raf is associated with Nrf2-mediated ARE activation.

In conclusion, treating H4IIE cells respectively with the three coumarins of *Poncirus trifoliata* Raf, namely, poncimarin, heraclenol 3′-methyl ester, or oxypeucedanin methanolate, increased GST activity via up-regulating GSTα transcription. The nuclear translocation of Nrf2 and its subsequent activation of ARE appear to be responsible for the induction of GSTα by these coumarins. Our results suggest that *Poncirus trifoliata* Raf. extracts and Poncirus fructus may possess anti-hepatocarcinogenic effects, and they raise the issue of their usefulnesses as chemopreventives.

3. Experimental

Repeated column chromatography of the methylene chloride soluble fraction of the dried immature fruits of *Poncirus trifoliata* Rafin afforded compounds **1**–**3**. Physical and chemical data, including UV, IR, ^{1}H NMR, ^{13}C NMR, HSQC, and HMBC, of compounds **1**–**3** were identical with those previously reported (Gray 1981; Harkar et al. 1984; Bergendorff et al. 1997). H4IIE cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. CDNB and DNB assays were performed as previously described (Habig et al. 1974; Ricci et al. 1994). Cytosolic and nuclear fraction isolations, immunoblot analysis, and reporter gene assays were performed as described in our previous report (Kang et al. 2003). The paired Student's t-test was used to assess significant differences between the different treatment groups. The criterion for statistical significance was set at either $p < 0.05$ or $p < 0.01$.

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