CHAPTER IV
RESULTS AND DISCUSSION

4.1 Part I: *In vitro* study

4.1.1 Effect of andrographolide on CYP1A1 expression in primary mouse hepatoctyes

Andrographolide induced CYP1A1 mRNA expression by itself in a concentration-dependent pattern in primary mouse hepatocytes (Figure 9). In the presence of β-NF, the synergistic CYP1A1 induction was observed after the andrographolide treatment.

Lactate dehydrogenase (LDH) activity was examined to indicate non-toxic compound at those concentrations of the treatments (Figure 10). The medium from the cultured dish was measured for cytotoxicity after treatments at 24 h, compared to 0.3% Tween 20 (100% cytotoxicity). All treatments showed non-toxicity, % cytotoxicity was less than 20%. However, andrographolide showed slight cytotoxicity at concentration 50 μM. Hence, we selected the concentration of andrographolide at 25 μM, which had a synergistic effect without cytotoxicity, for the further studies.

4.1.2 Time-dependent induction of CYP1A1 mRNA expression by andrographolide

The expression of CYP1A1 mRNA in primary mouse hepatocytes after treatment with either 10 μM β-NF, 25 μM andrographolide, or β-NF+andrographolide were observed (Figure 11). The effect of β-NF reached a maximum level at 9 h after which the expression decreased. Andrographolide itself had a little effect, but β-NF+andrographolide had a synergistic effect at 24 h, however, considerably suppressed β-NF-induced CYP1A1 mRNA expression at 9 h. These results revealed a bimodal influence of andrographolide on β-NF-inducible CYP1A1 mRNA expression, namely suppression early on (9 h) and enhancement later (24 h).

Jaruchotikamol et al. (2007) reported a synergistic enhancement of CYP1A1 expression by andrographolide with several CYP1A inducers, according with our results. However, the present investigation demonstrated that andrographolide suppressed β-NF-inducible CYP1A1 mRNA expression at around its peak, and that the synergism was most prominent during the decreasing phase. Therefore, it is necessary to reveal the mechanism or key factors of the conflicting effects of andrographolide on CYP1A1 mRNA expression. In addition, microarray analysis was used to determine how andrographolide influenced the expression of analogous or other CYP genes in mouse hepatocytes.

4.1.3 Effect of andrographolide on whole hepatic mRNA expression

The present microarray system (GeneChip® Mouse Exon 1.0 ST array) was useful for the analysis of 28,853 genes. Treatment with andrographolide up-regulated 18 genes, while it down-regulated 5 genes, if the change in expression was taken as more than two-fold (Figure 12). Almost all the up-regulated genes were related to metabolism/oxidation/reduction such as *Cyp1a1*, *Cyp2a4, Gstm3, Gstm4, Ugt2b35,* and *Nqo1*. The down-regulated genes were protein binding or calcium ion binding-related genes (*Thbs1*, *Cdh17,* and *Sbp1*). β-NF up-regulated 4 genes, *Cyp1a1, Cyp1a2, Nqo1*, and *Olfr1029* (Figure 13). Interestingly, the combination of β-NF and andrographolide had a greater impact (Figure 14). The number of modified genes was 118, with 78 up-regulated and 40 down-regulated. Correspondingly, most of the up-regulated genes were involved in metabolism/oxidation/reduction such as *Cyps, Gstm*, *Ddc*, *Cbr*, *Aox3*, *Ugt*, and *Akr*, while the down-regulated genes belonged to several groups including oxidation/reduction/ion binding-related genes, i.e., *Plscr1, Steap4, Bhmt, Vil1, Pdzrn3, Ngb*, and *Cyp17a1*, protein binding-related genes, i.e., *Vim, Cish, Hp, Ctgf, Cdh17,* and *Vcam1*, and chemokine/cytokine-related genes, *i.e., Cxcl13, Ccl2,* and *Spp1*. Comparison of gene expression between the combined treatment of β-NF with andrographolide and the single treatment of β-NF (Figure 15), demonstrated that 27 genes were up-regulated whereas 18 genes were down-regulated by the combined treatment. Again, most genes up-regulated by the combined treatment were involved in metabolism and oxidation-reduction, such as *Cyp1a1,*

Cyp2a4, *Adh7*, *Ddc*, *Gstm*, and *Eid3*, while the down-regulated genes were similar to those down-regulated by the combined treatment in comparison with the control.

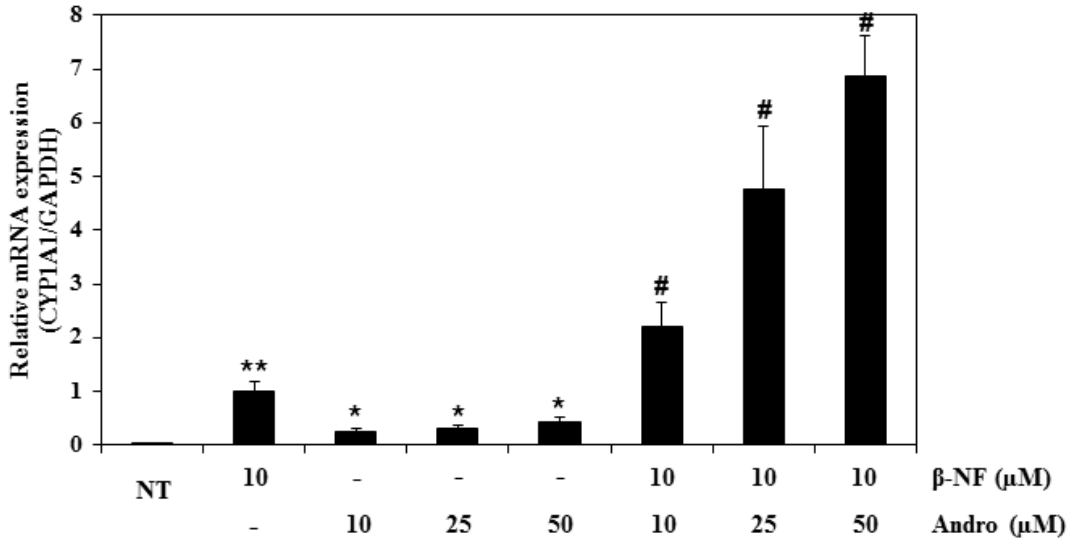

Figure 9 Andrographolide concentration-dependently affected expression of CYP1A1 mRNA

Primary mouse hepatocytes were treated with 0.2% DMSO (non-treatment, NT), 10 μM beta-naphthoflavone (β-NF), 10, 25, 50 μM andrographolide (Andro), or combination of β-NF and Andro. Total RNA was prepared after starting treatments at 24 h. The expression of CYP1A1 mRNA was determined by quantitative real-time RT-PCR after normalization with that of GAPDH mRNA. Each column represented the means ± SD (n=4). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and ** represented significantly differences from the NT group at $p<0.05$ and $p<0.001$, respectively; # represented a significantly difference from the β-NF group at $p<0.001$.

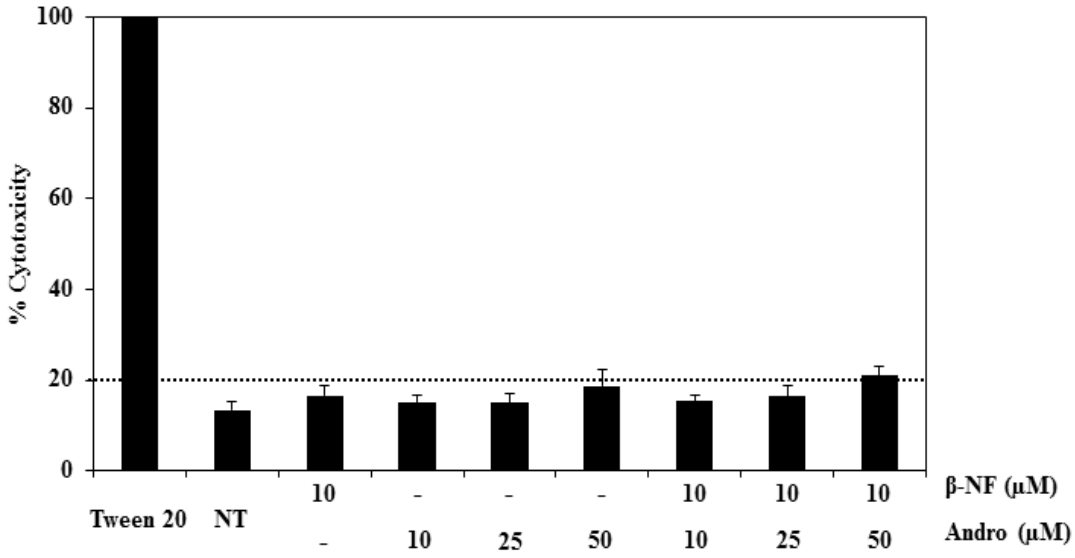

Figure 10 LDH cytotoxicity test of andrographolide

Primary mouse hepatocytes were treated with 0.2% DMSO (non-treatment, NT), 10 μM beta-naphthoflavone (β-NF), 10, 25, 50 μM andrographolide (Andro), or combination of β-NF and Andro. The medium from cultured dishes were collected after starting treatments at 24 h. The LDH cytotoxicity was determined, compared to 0.3% Tween 20, as 100% cytotoxicity. Each column represented the means ± SD (n=4).

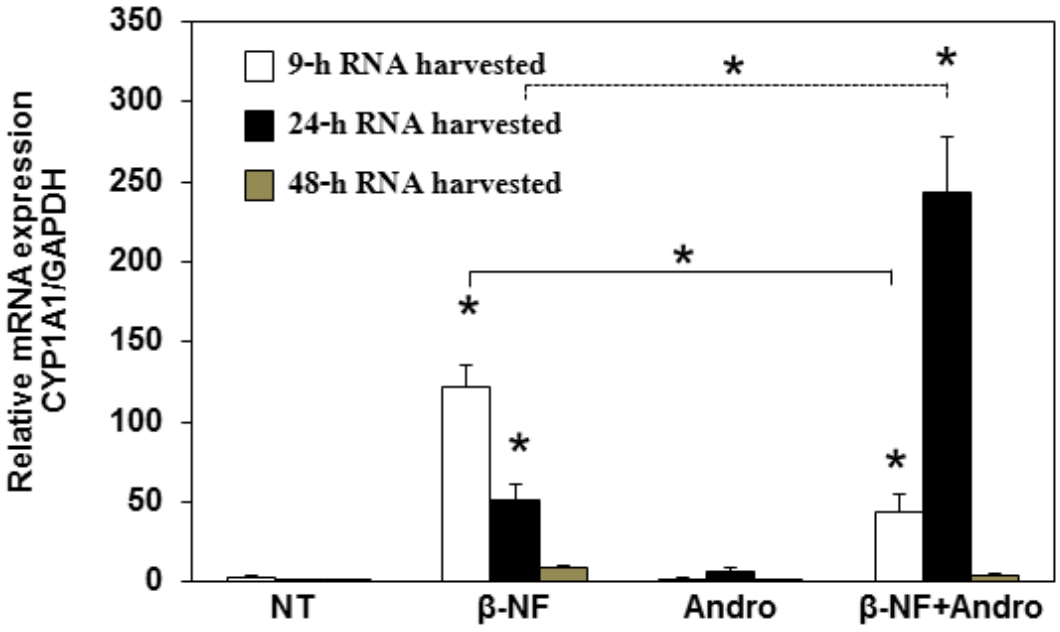

Figure 11 Effect of andrographolide on CYP1A1 mRNA expression at 9, 24, 48 h

Primary mouse hepatocytes were treated with 0.2% DMSO (non-treatment, NT), 10 μM beta-naphthoflavone (β-NF), 25 μM andrographolide (Andro), or combination of β-NF and Andro (β-NF+Andro). Total RNA was prepared at the indicated times. The expression of CYP1A1 mRNA was determined by quantitative real-time RT-PCR after normalization with that of GAPDH mRNA. Each column represented the means ± SD (n=4). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test.

* represented a significantly difference from the NT group at $p<0.05$.

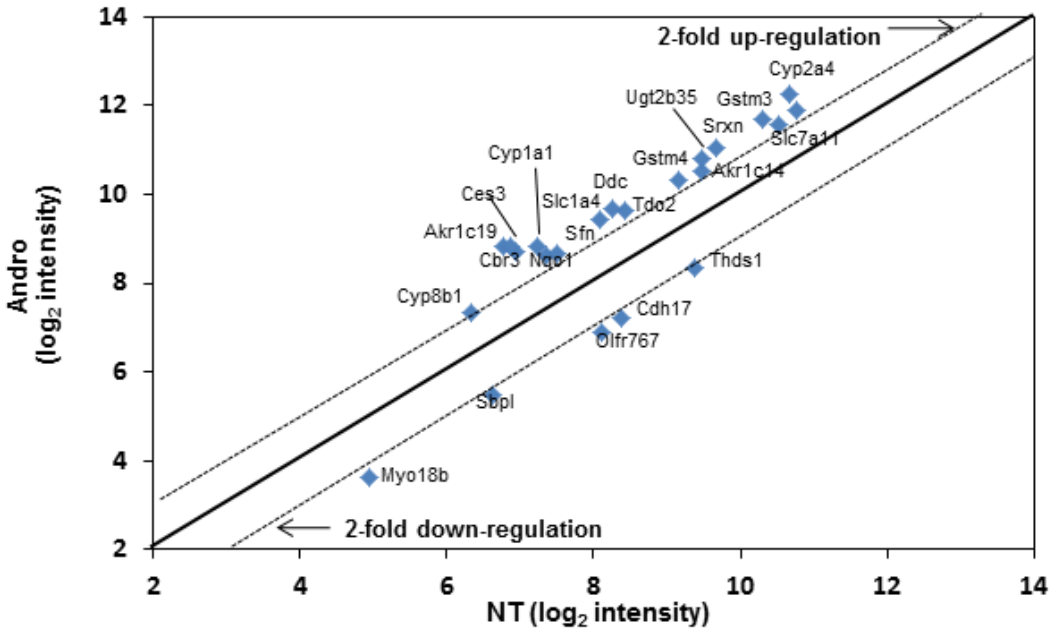

Figure 12 Effect of andrographolide on expression of genes in mouse hepatocytes

Primary mouse hepatocytes were treated with 0.2% DMSO (non-treatment, NT) and 25 μM andrographolide (Andro). Total mRNA was harvested at 24 h and re-purified for microarray analysis. The lines indicate a change in gene expression compared to the control of higher or lower than 2-fold. Abbreviations in the figure: *Akr*, aldo-keto reductase; *Cdh17*, cadherin 17; *Ces3*, carboxylesterase 3; *Cyp*, cytochrome P450; *Ddc*, dopa decarboxylase; *Gstm*, glutathione S-transferase (GST) μ class; *Ugt*, UDP glucuronosyltransferase; *Myo18b*, myosin XVIIIb; *Nqo1*, NAD(P)H dehydrogenase quinine; *Olfr767*, olfactory receptor 767; *Cbr3*, carbonyl reductase 3; *Sbpl*, spermine binding protein-like; *Sfn*, stratifin; *Slc*, solute carrier (glutamate/neutral amino acid transporter); *Srxn*, sulfiredoxin 1 homolog; *Tdo2*, tryptophan 2,3-dioxygenase; *Thbs1*, thrombospondin 1.

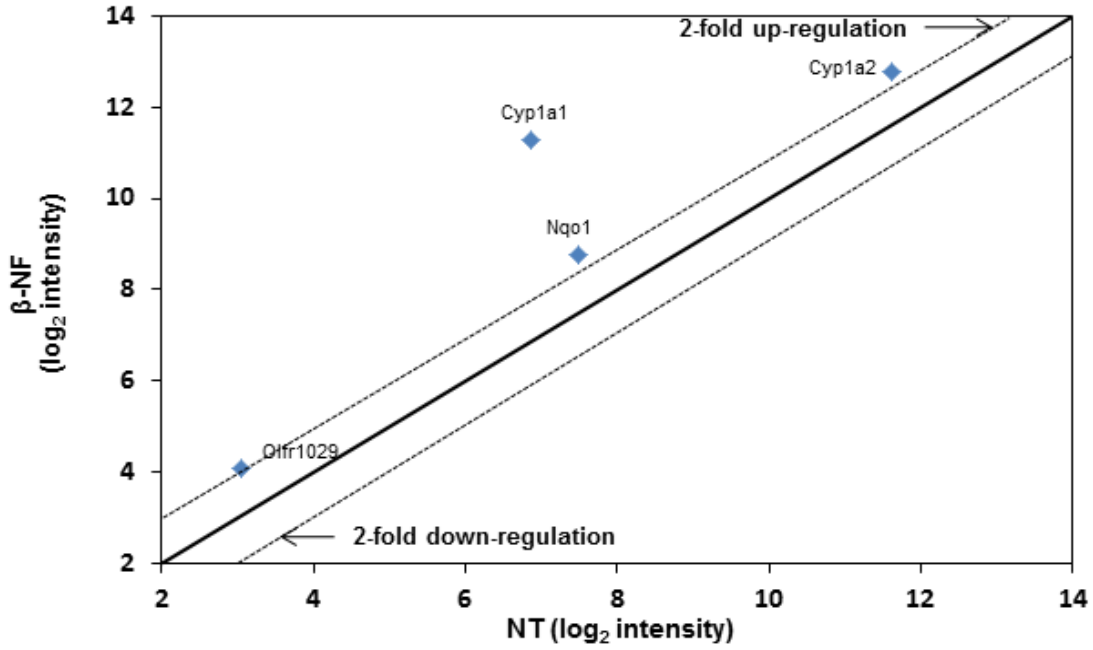

Figure 13 Effect of beta-naphthoflavone on expression of genes in mouse hepatocytes Primary mouse hepatocytes were treated with 0.2% DMSO (non-treatment, NT) and 10 μM beta-naphthoflavone (β-NF). Total mRNA was harvested at 24 h and re-purified for microarray analysis. The lines indicate a change in gene expression compared to the control of higher or lower than 2-fold. Abbreviations in the figure: *Cyp*, cytochrome P450; *Nqo1*, NAD(P)H dehydrogenase quinine; *Olfr1029*, olfactory receptor 1029.

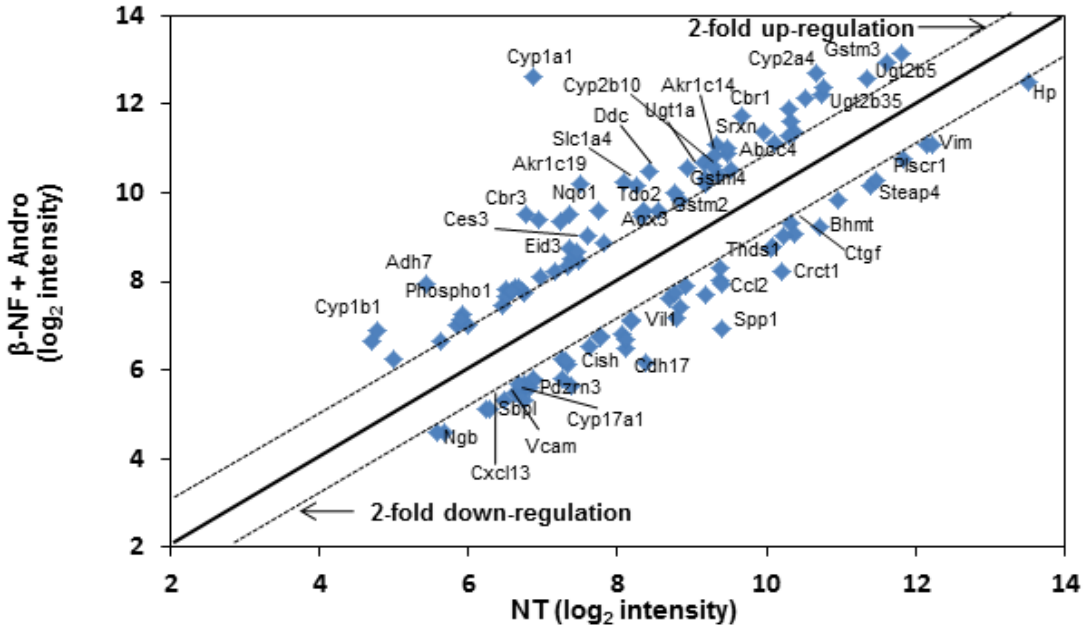

Figure 14 Effect of combination of andrographolide and beta-naphthoflavone on expression of genes in mouse hepatocytes

Mouse hepatocytes in primary culture after were treated with 0.2% DMSO (non-treatment, NT) and 10 μM beta-naphthoflavone (β-NF) plus 25 μM andrographolide (Andro). Total mRNA was harvested at 24 h for microarray analysis. The lines indicate a change in gene expression compared to the control of higher or lower than 2-fold. Abbreviations in the figure: *Abcc*, ATP-binding cassette; *Adh7*, alcohol dehydrogenase 7; *Akr*, aldo-keto reductase; *Aox3*, aldehyde oxidase 3; *Bhmt*, betaine-homocysteine methyltransferase; *Cbr*, carbonyl reductase; *Ccl*, chemokine (C-C motif) ligand; *Cdh17*, cadherin 17; *Ces3*, carboxylesterase 3; *Cish*, cytokine inducible SH2-containing protein; *Cyp*, cytochrome P450; *Ctgf*, connective tissue growth factor; *Cxcl*, chemokine (C-X-C motif) ligand; *Ddc*, dopa decarboxylase; *Eid3*, EP300 interacting inhibitor of differentiation 3; *Gstm*, glutathione S-transferase (GST) μ class; *Hp*, haptoglobin; *Myo18b*, myosin XVIIIb; *Ngb*, neuroglobin; *Nqo1*, NAD(P)H dehydrogenase quinine; *Pdzrn3*, PDZ domain containing RING finger 3; *Phospho1*, phosphatase, orphan 1; *Plscr1*, phospholipid scramblase 1; *Sbpl*, spermine binding protein-like; *Sfn*, stratifin; *Ugt*, UDP glucuronosyltransferase.

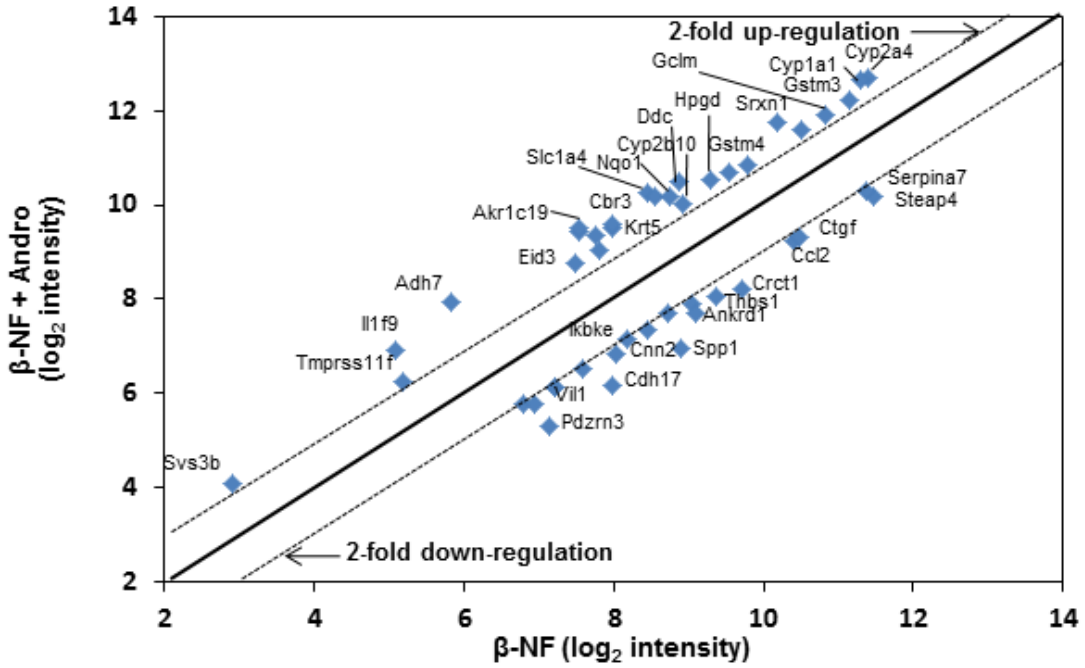

Figure 15 Effect of combination of beta-naphthoflavone and andrographolide and on expression of genes compared to the single treatment of beta-naphthoflavone

The lines indicate a change in gene expression compared to the control of higher or lower than 2-fold. Abbreviations in the figure: *Adh7*, alcohol dehydrogenase 7; *Akr*, aldo-keto reductase; *Ankrd1*, ankyrin repeat domain 1, *Cbr*, carbonyl reductase; *Ccl*, chemokine (C-C motif) ligand; *Cdh17*, cadherin 17; *Cnn2*, calponin 2; *Cyp*, cytochrome P450; *Crct1*, cysteine-rich C-terminal 1; *Ctgf*, connective tissue growth factor; *Ddc*, dopa decarboxylase; *Eid3*, EP300 interacting inhibitor of differentiation 3; *Gclm*, glutamate-cysteine ligase, *Gstm*, glutathione S-transferase (GST) μ class; *Hpgd*, hydroxyprostaglandin dehydrogenase 15 (NAD); *Ikbke*, inhibitor of kappaB kinase epsilon; *Ilf9*, interleukin 1 family, member 9; *Krt5*, keratin 5; *Nqo1*, NAD(P)H dehydrogenase quinine; *Pdzrn3*, PDZ domain containing RING finger 3; *Slc1a4*, solute carrier family 1 (glutamate/neutral amino acid transporter; *Spp1*, secreted phosphoprotein 1; *Srxn*, sulfiredoxin 1 homolog; *Steap4*, STEAP family member 4; *Svs3b*, seminal vesicle secretory protein 3B; *Thbs1*, thrombospondin 1; *Tmprss11f*, transmembrane protease, serine 11f; *Vil1*, villin 1.

Table 3 summarized the cytochrome P450-related genes, the expression of which increased at least two-fold after either treatment. The numbers of modified genes were 14 for the drug metabolism group and 13 for the oxidoreductase group. *Cyp1a1* was the highest up-regulated gene among increased CYPs, like *Cyp1a2*, *Cyp1b1*, *Cyp2a4/Cyp2a5*, *Cyp2b9*, *Cyp2b10*, and *Cyp8b1*. In addition, *Ugt1a*, *Ugt2b5*, and *Ugt2b35*, members of UDP glucuronosyltransferase, were up-regulated. Glutathione *S*-transferase (GST) μ class, *Gstm2*, *Gstm3*, and *Gstm4*, were up-regulated. For these genes, the combined treatment with β-NF and andrographolide had more of an effect than the single treatments. Among oxidoreductase genes, *Adh7*, *Cbr3*, and *Nqo1* were highly induced by the combined treatment (Table 4).

A. paniculata extract has long been employed as a folk remedy or health supplement. The genes involved in responsible anti-oxidation, which were up-regulated or down-regulated more than 20 % by andrographolide treatment, are listed in Table 5. The expression of 7 anti-oxidation-related genes was found to be slightly increased, namely NADPH oxidase, peroxiredoxin, catalase, glutathione peroxidase, glutathione *S*-transferase, and glutathione reductase.

4.1.4 Effect of andrographolide on cytochrome P450 family 1, 2, and 3, and UGT1A6 mRNA expression

To confirm the expression of the CYP family 1, 2, and 3, and UGT1A6, quantitative real-time RT-PCR was carried out (Figure 16). Changing ratios of the expression were generally higher among the results of RT-PCR than those of microarray. Significant difference ($p<0.05$) between the combination of β-NF and andrographolide and the single treatment of β-NF was clear in the expression of CYP1A1, CYP1B1, CYP2A4, CYP3A11, CYP3A41, and UGT1A6 mRNAs. In the case of CYP1A1 and CYP1B1, the combined treatment had a synergistic effect, whereas the expression of CYP3A11 and CYP3A41 was higher on the combined treatment but not the single treatment of andrographolide. CYP2A4, CYP2B9, and CYP2B10 were significantly induced by andrographolide, but the combined treatment with β-NF further increased the expression of CYP2A4.

Table 3 Effect of beta-naphthoflavone and/or andrographolide on cytochrome P450 related genes

Accession No.	Gene	Gene symbol	Fold up-regulation*		
			β-NF	Andro	β-NF+Andro
U03283	cytochrome P450, family 1, subfamily b, polypeptide 1	*Cyp1b1*	1.92	1.07	3.80
BC044927	glutathione S-transferase, mu 3	*Gstm3*	1.44	2.65	3.05
J04696	glutathione S-transferase, mu 2	*Gstm2*	1.24	1.88	2.21
BC030444	glutathione S-transferase, mu 4	*Gstm4*	1.29	2.25	2.89
BC003240	P450 (cytochrome) oxidoreductase	*Por*	1.35	1.23	2.01
BC011233	cytochrome P450, family 2, subfamily a, polypeptide 4, 5	*Cyp2a4|Cyp2a5*	1.63	3.01	4.11
BC028262	UDP glucuronosyltransferase 2 family, polypeptide B5	*Ugt2b5*	1.13	1.87	2.01
BC113789	UDP glucuronosyltransferase 2 family, polypeptide B35	*Ugt2b35*	1.44	2.50	2.87
BC026561	UDP glucuronosyltransferase 1 family, polypeptide A	*Ugt1a*	1.67	1.35	2.06
M21855	cytochrome P450, family 2, subfamily b, polypeptide 9	*Cyp2b9*	1.13	1.45	2.00
BC060973	cytochrome P450, family 2, subfamily b, polypeptide 10	*Cyp2b10*	1.11	1.68	2.33
Y00071	cytochrome P450, family 1, subfamily a, polypeptide 1	*Cyp1a1*	21.52	3.90	54.59
BC018298	cytochrome P450, family 1, subfamily a, polypeptide 2	*Cyp1a2*	2.25	1.22	2.54
BC010973	cytochrome P450, family 8, subfamily b, polypeptide 1	*Cyp8b1*	1.25	2.02	1.61

Note:* The expression of each gene was compared to non-treatment (0.2% DMSO) treated group; β-NF, 10 μM beta-naphthoflavone; Andro, 25 μM andrographolide.

Table 4 Effect of beta-naphthoflavone and/or andrographolide on oxidoreductase related genes

Accession No.	Gene	Gene symbol	Fold up-regulation*		
			β-NF	**Andro**	**β-NF + Andro**
AF172276	aldehyde oxidase 3	*Aox3*	1.29	1.77	2.09
BC013482	aldo-keto reductase family 1, member C14	*Akr1c14*	1.35	2.05	2.70
U31966	carbonyl reductase 1	*Cbr1*	1.26	1.78	2.05
BC028763	carbonyl reductase 3	*Cbr3*	1.69	4.21	6.71
BC049957	sulfiredoxin 1 homolog (S. cerevisiae)	*Srxn1*	1.45	2.64	4.22
U95053	glutamate-cysteine ligase , modifier subunit	*Gclm*	1.14	1.68	2.43
AF331802	alcohol dehydrogenase 7 (class IV), mu or sigma	*Adh7*	1.32	1.50	5.71
BC018390	tryptophan 2,3-dioxygenase	*Tdo2*	1.24	2.68	3.79
BC067055	N-terminal EF-hand calcium binding protein 1	*Necab1*	1.24	1.86	2.25
BC014865	leukotriene B4 12-hydroxydehydrogenase	*Ltb4dh*	1.40	1.92	2.91
BC006617	biliverdin reductase B (flavin reductase (NADPH))	*Blvrb*	1.24	1.74	2.21
BC021157	hydroxyprostaglandin dehydrogenase 15 (NAD)	*Hpgd*	1.27	1.57	3.05
BC004579	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	2.39	2.24	6.46

Note:* The expression of each gene was compared to non-treatment (0.2% DMSO) treated group;
β-NF, 10 μM beta-naphthoflavone; Andro, 25 μM andrographolide.

Table 5 Effect of andrographolide on anti-oxidation related genes

Accession No.	Gene	Gene symbol	Fold change*
Anti-oxidation			
AB042745	NADPH oxidase 4	*Nox4*	0.80
D16142	peroxiredoxin 1	*Prdx1*	1.20
AF093852	peroxiredoxin 6	*Prdx6*	1.45
BC013447	catalase	*Cat*	1.33
BC010823	glutathione peroxidase 2	*Gpx2*	1.34
AY279096	glutathione S-transferase kappa 1	*Gstk1*	1.23
BC057325	glutathione reductase	*Gsr*	1.25

Note:*Hepatocytes were treated with 25 μM andrographolide for 24 h. Expression of the respective genes was shown as fold change, compared with that of non-treatment (0.2% DMSO treated).

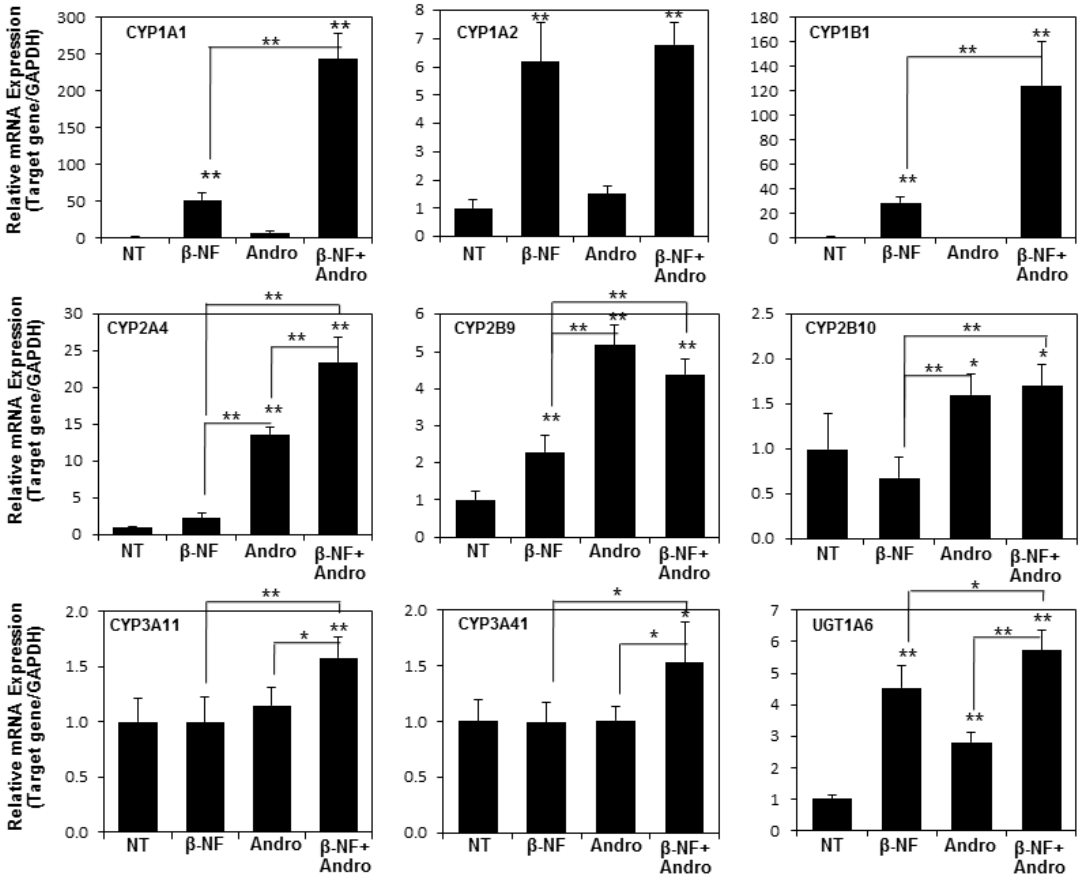

Figure 16 Effect of beta-naphthoflavone and/or andrographolide on mRNA expression in mouse hepatocyes using real-time RT-PCR

Primary mouse hepatocytes were treated with 0.2% DMSO (non-treatment, NT), 10 μM beta-naphthoflavone (β-NF), 25 μM andrographolide (Andro), or combination of β-NF and Andro (β-NF+Andro). Total RNA was prepared at 24 h after starting treatments. The expression of mRNAs was determined by quantitative real-time RT-PCR. All target genes were normalized to GAPDH. Each column represented the means ± SD (n=4). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and ** represented significantly differences from the NT group at $p< 0.05$ and $p<0.001$, respectively.

These results revealed that andrographolide had the potential to increase the expression of several genes involved in drug metabolism and oxido-reduction in mouse hepatocytes in primary culture. Furthermore, in combination with a typical CYP1A inducer, β-NF, andrographolide had a synergistic effect on CYP1A1 and CYP1B1 expression.

A previous study of Jarukamjorn et al. (2006) reported that the expression of CYP1A1 and CYP2B mRNA was enhanced by a crude extract of *A. paniculata* in mouse liver. Moreover, andrographolide synergistically enhanced typical CYP1A inducers-induced CYP1A1 expression in mouse hepatocytes in primary culture and the enhancement reflected the responsible marker enzyme activity, ethoxyresorufin *O*-deethylation (EROD) (Jaruchotikamol et al., 2007). The present study confirmed these findings and suggested that andrographolide influenced the expression of many metabolizing enzymes related-genes. Singh et al. (2001) reported that the high dose of *A. paniculata* extract significantly increased the expression of catalase, glutathione reductase, and glutathione peroxidase in mouse liver. In addition, an aqueous extract of *A. paniculata* significantly elevated catalase and glutathione *S*-transterase levels in mice (Verma & Vinayak, 2008). In the present study, we observed a tendency for the expression of catalase, glutathione reductase, glutathione peroxidase, and glutathione *S*-transterase to be increased by andrographolide from microarray analysis, suggesting its anti-oxidation activity. One reason for the slight increase might be that the total RNA samples were prepared at 24 h after the addition of andrographolide.

Although the single treatment with either β-NF or andrographolide changed the expression of small numbers of genes, the combination of these two compounds affected many genes. Most of the up-regulated genes were of oxidation or reduction related to the phase I and II enzymes of drug metabolism. Andrographolide by itself influenced the expression of these genes while synergism with β-NF was found only in limited numbers of genes, by which the synergistic expression of CYP1B1 and CYP1A1 mRNA was remarkable (Figure 16). These two CYP isoforms do not constitutively but inducibly express in the liver. The induction of both is mediated by the AhR pathway, suggesting that andrographolide might involve in this pathway. Though AhR-mediated activation is also involved in CYP1A2 and UGT1A6

mRNA expression, andrographolide did not synergistically enhance the expression of either CYP1A2 or UGT1A6, compared to single treatment of β-NF. Therefore, the synergistic expression of CYP1A1 and CYP1B1 was preferentially influenced by andrographolide. Proposed mechanism of the synergism might raise by anti-oxidation activity of andrographolide since down-regulated expression of CYP1A1 by oxidative stress has been reported (Morel & Barouki, 1998; Barouki & Morel, 2001; Barker et al., 1994). Although expression of anti-oxidation-related genes was not much affected by andrographolide in the present microarray observation, the activity was strongly suggested *in vivo* and *in vitro* experiments (Akowuah et al., 2009). Further study of effects of andrographolide on the expression of anti-oxidation related genes and oxidative stress condition in CYP1A1 induction are still required. Moreover, the expression of CYP1A1 induction at several time points after addition of andrographolide should be observed since the present protocol observed the expression only at 24 h.

Effect of *A. paniculata* extract or andrographolide on expression of CYPs *in vivo* and *in vitro* were recently reported by Pekthong et al. (2009) with both suppressive and promotive effects, depending on the CYPs isoform. These observations were not comparable to those of our findings due to differences in experimental protocol, i.e. cell type, culture conditions, time of sample harvest, etc. (LeCluyse et al., 2000). In general, there is a time-dependent expression of all major CYPs mRNA, CYP1A2, CYP2C9, CYP2E1, and CYP3A4, in human hepatocytes (George et al., 1997; Liddle et al., 1998). Therefore, it is necessary to establish a procedure for the risk assessment of *A. paniculata* extract or andrographolide.

We observed a tendency for the expression of catalase, glutathione reductase, glutathione peroxidase, and glutathione *S*-transterase to be increased by andrographolide. These effects suggested that andrographolide might affect oxidative stress in the primary hepatocytes. Oxidative stress has potency to down-regulate CYP1A1 expression. Therefore, a compound affects oxidative stress might influence CYP1A1 expression. Hence, it is worth further investigating the effect of reactive oxygen species (ROS) and glutathione (GSH) level on CYP1A1 expression.

4.1.5 Modification of reactive oxygen species after treatment with beta-naphthoflavone, andrographolide, or beta-naphthoflavone plus andrographolide

Since induction of CYP1A1 mRNA by AhR ligands was down-regulated by oxidative stress (Barouki & Morel, 2001; Morel & Barouki, 1998) and andrographolide caused oxidative stress (Li et al., 2007; Zhang et al., 2008), andrographolide might affect intracellular redox status, resulting in reduction of CYP1A1 mRNA expression early on. However, on an entirely opposite effect of andrographolide on oxidative stress, namely over-production of H_2O_2 (Li et al., 2007) on depletion of glutathione (GSH) (Zhang et al., 2008) or an increase in GSH (Woo et al., 2008), was reported. These findings suggested that andrographolide exerted different effects on oxidative stress depending on the cell lines. We observed a synergistic effect of andrographolide on the expression of CYP1A1 mRNA in primary cultures of mouse hepatocytes. Therefore, this primary mouse hepatocytes condition might be appropriate to reveal the effect of oxidative stress on CYP1A1 expression in the presence of andrographolide.

Because of anti-oxidation property of andrographolide, proposed mechanism of synergistic CYP1A1 induction by the co-treatment of β-NF and andrographolide via modification of oxidative stress might be raised. To investigate reactive oxygen species (ROS) status, mouse hepatocytes were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), 3′-O-Acetyl-6′-O-pentafluorobenzenesulfonyl-2′,7′-difluorofluorescein (BES-H_2O_2-Ac), BES-So-AM, hydroxyphenyl fluorescein (HPF), aminophenyl fluorescein (APF), or 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF-FM DA), fluorescence dyes for ROS analysis at 3 and 24 h after the treatments and then analyzed by flow cytometry (Figure 17). Marked production of ROS was observed in control hepatocytes, possibly responding to the preparation of primary cultures. Mouse hepatocytes in primary cultures were fragile, and mostly died during detaching from collagen-coated dishes with the treatment of trypsin and/or ethylenediaminetetraacetic acid (EDTA). Accutase, an enzyme cell detachment medium, was useful for detaching at a high percentage (60~70%) of viable mouse hepatocytes. The staining reagent permeates the cell membrane and the cell is

digested by the esterase. The digested form reacts with ROS, resulting in emission of fluorescence. Subsequently, ROS in the living cell is detected. Therefore, after staining cells with the above fluorescence dyes and detaching using Accutase, the cells were further stained with propidium iodide (PI). DCF is widely used to detect various kinds of ROS due to its high sensitivity (Figure 17A), but its specificity for individual ROS species is oppositely low. By contrast, BES-H_2O_2-Ac (Figure 17B), BES-So-AM (Figure 17C), HPF (Figure 17D), APF (Figure 17E) and DAF-FM (Figure 17F) are relatively specific, targeting H_2O_2, $O_2^{\bullet -}$, $^{\bullet}OH + ONOO^-$, $^{\bullet}OH + ONOO^- + OCl^-$, and NO, respectively.

At 3 h after the treatments, level of ROS detected by DCF was reduced in the cells treated with either β-NF, andrographolide, or β-NF+andrographolide (Figure 17A), whereas those by BES-H_2O_2-Ac, BES-So-AM, HPF, APF and DAF-FM were not (Figure 17B-F). On the other hand, the levels of ROS detected by all staining reagents were declined at 24 h after treatments in all groups. The patterns of change in ROS levels were varied by the staining reagents. No correlation between the effects of andrographolide on β-NF-induced CYP1A1 mRNA expression and ROS level was noted.

However, the patterns of ROS levels differed depending on the staining reagents. DCF-staining showed a decrease of ROS level in β-NF- and β-NF+andrographolide-treated cells at 3 h (Figure 17A), but BES-H_2O_2-AC, HPF and APF-staining showed an increase (Figure 17B, D, E). Moreover, a decrease of ROS level was observed at 24 h by almost staining reagents. The decrease in the ROS level was neither additive nor synergistic in the presence of the two compounds (β-NF+Andro). Therefore, the generation of ROS did not reflect the CYP1A1 expression in the present culture system.

4.1.6 Modification of glutathione level after treatment with beta-naphthoflavone, andrographolide, or beta-naphthoflavone plus andrographolide

After incubation for 24 h, levels of reduced glutathione (GSH) were higher and further raised in the presence of andrographolide (Figure 18), while the oxidized glutathione (GSSG) was undetectable in the control both at 6 and 24 h. Both level of GSH and GSSG were increased in the presence of β-NF at 6 h, but had returned to control values at 24 h. Andrographolide increased the GSSG levels and β-NF+andrographolide synergistically enhanced the levels of GSSG at both 6 and 24 h.

In the present study, an increase in the GSSG after treatment with andrographolide and/or β-NF suggested that the oxidative stress was generated. Although the treatments changed the proportion of ROS level (Figure 17), the relationship between ROS and GSH/GSSG level has not been found.

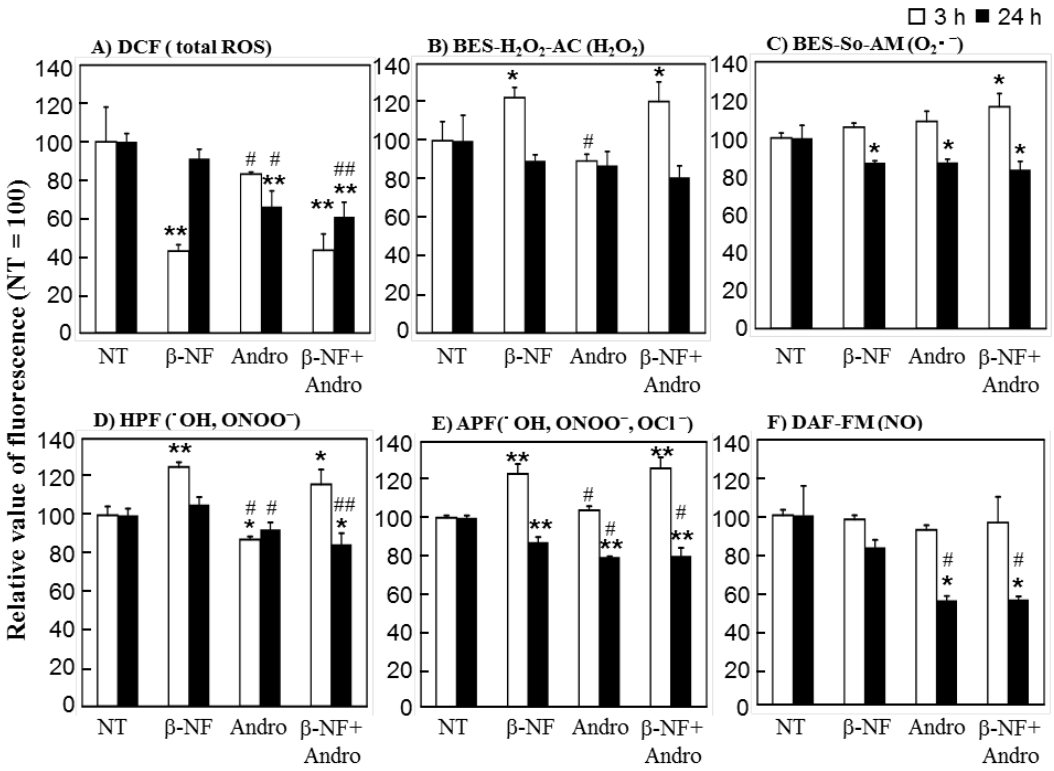

Figure 17 Level of reactive oxygen species in mouse hepatocytes treated with beta-naphthoflavone and/or andrographolide

Primary mouse hepatocytes were treated with 10 μM beta-naphthoflavone (β-NF) and/or 25 μM andrographolide (Andro) for 3 h (open column) or 24 h (closed column). Hepatocytes were treated with ROS staining reagent on culture dishes A) DCF-DA, B) BES-H_2O_2-Ac, C) BES-So-AM, D) HPF, E) APF, and F) DAF-FM DA, and then with propidium iodide (PI) after having detached. The fluorescence was detected using FACSCant II and analyzed with FACS Diva software. PI-negative and green fluorescence-positive cells were gated. The means fluorescent intensity in gated cells was calculated. Values are given relatively to that of the DMSO-treated group (NT=100). Each column represented the means ± SD (n=3-4). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and ** represented significantly differences from the NT group at the same time with $p<0.05$ and $p<0.01$, respectively. $^{\#}$ and $^{\#\#}$ represented significantly differences from the β-NF group at the same time at $p<0.05$ and $p<0.01$, respectively.

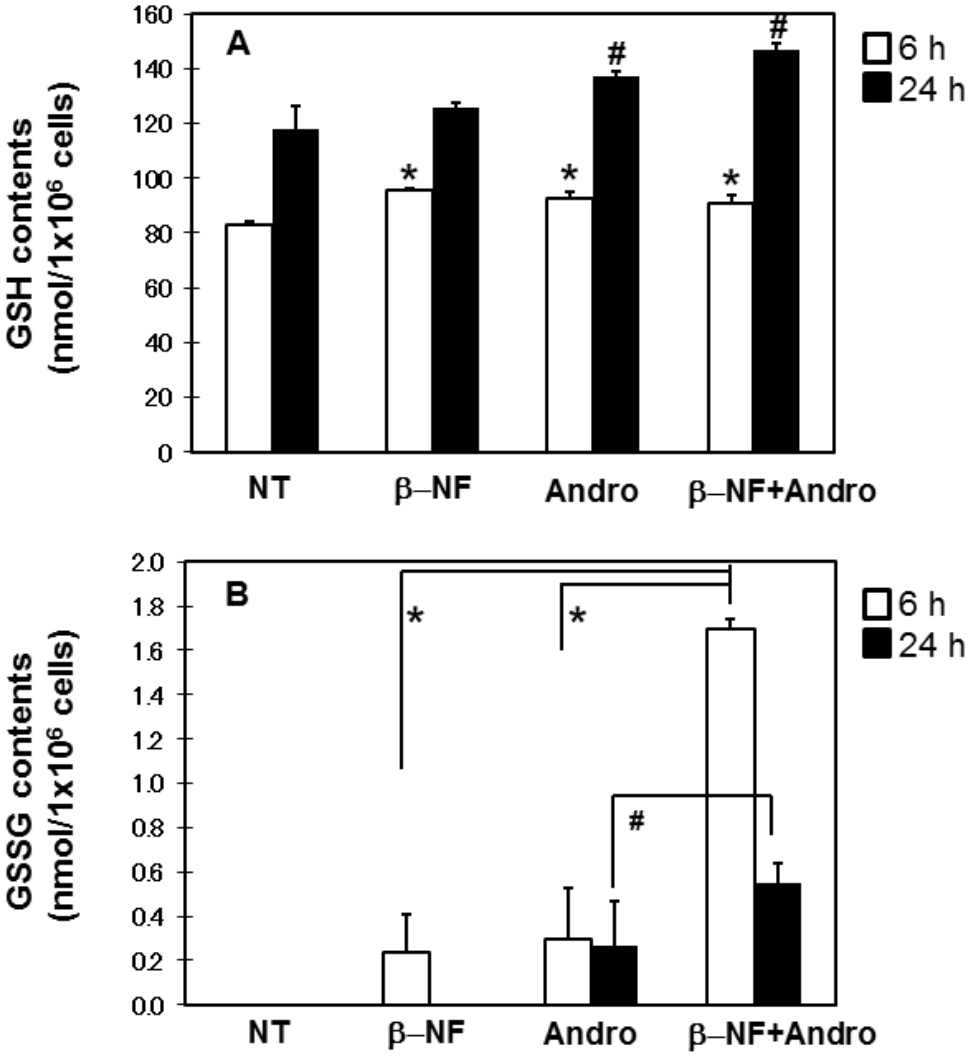

Figure 18 Glutathione level in mouse hepatocytes treated with beta-naphthoflavone and/or andrographolide

Primary mouse hepatocytes were treated with 10 μM beta-naphthoflavone

(β-NF) and/or 25 μM andrographolide (Andro). The intracellular level of reduced glutathione (GSH) and oxidized glutathione (GSSG) at 6 h (open column) and 24 h (closed column) were determined. Each column represented the means ± SD (n=3-4). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and $^{\#}$ represented significantly difference from the NT group at $p<0.05$ at 6 and 24 h, respectively.

Although there was no relationship between synergistic CYP1A1 induction by andrographolide and ROS status or glutathione level, the effect of other antioxidants or oxidative stress on synergism of CYP1A1 in the presence of andrographolide was worth further investigating.

4.1.7 Effects of hydrogen peroxide and antioxidants on modification of the beta-naphthoflavone-induced CYP1A1 expression by andrographolide

To observe the influence of oxidative stress on synergistic CYP1A1 induction by combination of β-NF and andrographolide, the primary mouse hepatocytes were treated with either hydrogen peroxide (H_2O_2), or antioxidants, including butylated hydroxytoluene (BHT) and ascorbic acid, in the presence of β-NF or combination of β-NF and andrographolide. H_2O_2 significantly up-regulated β-NF-induced and andrographolide -induced CYP1A1 mRNA expression at 24 h, but H_2O_2 did not modify these expressions at 9 h (Figure 19A). Both BHT and ascorbic acid suppressed β-NF-induced CYP1A1 expression at 9 h, but the synergism of β-NF-induced CYP1A1 by andrographolide was promoted by ascorbic acid at high concentration (1.0 mM) at 24 h (Figure 19B & 19C). BHT showed an increasing tendency of the synergism.

BHT and ascorbic acid further significantly suppressed the inducible expression of CYP1A1 mRNA at 9 h (Figure 19B & 19C). On the contrary, both of BHT and ascorbic acid demonstrated additive effect on the synergistic CYP1A1 induction by andrographolide and β-NF at 24 h. Study on the effect of these antioxidants on CYP1A1 expression was limited. BHT itself induced CYP1A1 mRNA and EROD activity in rat and mouse liver (Price et al., 2004; Sun & Fukuhara, 1997), while combination of BHT and flavones or flavanone did not modify the EROD activity (Sun & Fukuhara, 1997), without a feasible mechanism described. Ascorbic acid suppressed CYP1A1 expression in human HepG2 cells (Chang et al., 2009) and in sepsis rats (Kim & Lee, 2006). On the other hand, Ueta et al. (2001) observed that ascorbic acid at high dosage maintained CYP1A1 mRNA expression in the lung of rats inhaling cigarette smoke. Paolini et al. (1999) suggested that ascorbic acid possibly behaved as a potential pro-oxidant. Further investigation is required to

reveal an underlying mechanism of the additive modification of andrographolide on inducible CYP1A1 mRNA expression by antioxidants.

4.1.8 Modification of CYP1A1 expression by glutathione

Change in glutathione (GSH) content caused by either GSH, N-acetyl-L-cysteine (NAC), or L-buthionine-(S,R)-sulfoximine (BSO) against β-NF-induced CYP1A1 mRNA expression by andrographolide were evaluated at both early (9 h) and late (24 h) phases (Figure 20). NAC itself acts as an antioxidant and is a precursor of GSH. BSO is a potent and specific inhibitor of γ-glutamylcysteine synthetase, resulting in reduction of intracellular GSH levels. In the early phase, NAC slightly increased and GSH significantly increased β-NF-induced CYP1A1 mRNA expression, and both increased the expression in β-NF+andrographolide -treated cells (Figure 20A & 20B). BSO decreased the expression in the β-NF- or β-NF+andrographolide-treated cells (Figure 20C). In the late phase, BSO increased βNF-induced CYP1A1 mRNA expression (Figure 20C), but GSH and NAC had no effect. In the presence of andrographolide, BSO further increased the expression of β-NF-induced CYP1A1 mRNA, but GSH or NAC significantly attenuated the synergistic effect in a concentration-dependent pattern.

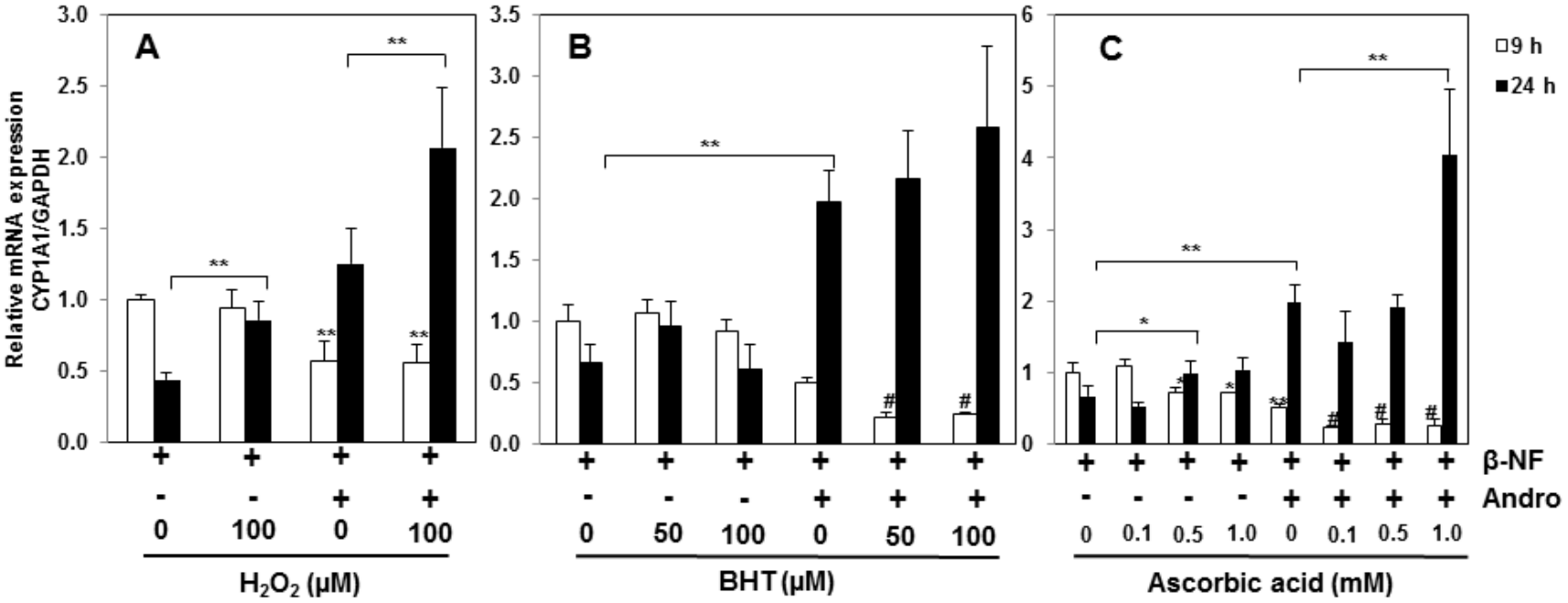

Figure 19 Expression of CYP1A1 mRNA in mouse hepatocytes in the presence of hydrgenperoxide or antioxidants

Primary mouse hepatocytes were treated with a combination of 10 μM beta-naphthoflavone (β-NF), 25 μM andrographolide (Andro), and hydrgenperoxide (H_2O_2), or butylated hydroxytoluene (BHT), or ascorbic acid at indicated concentrations. Total RNA was prepared 9 h (open column) or 24 h (closed column) later and the expression of CYP1A1 mRNA was analyzed by real-time RT-PCR. Each column represented the means ± SD (n=4). A) Effect of H_2O_2; B) Effect of BHT; C) Effect of ascorbic acid. Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and **, and $^{\#}$ and $^{\#\#}$ represented significantly differences from the β-NF or β-NF+Andro group at 9 and 24 h, at $p<0.05$ and $p<0.001$, respectively.

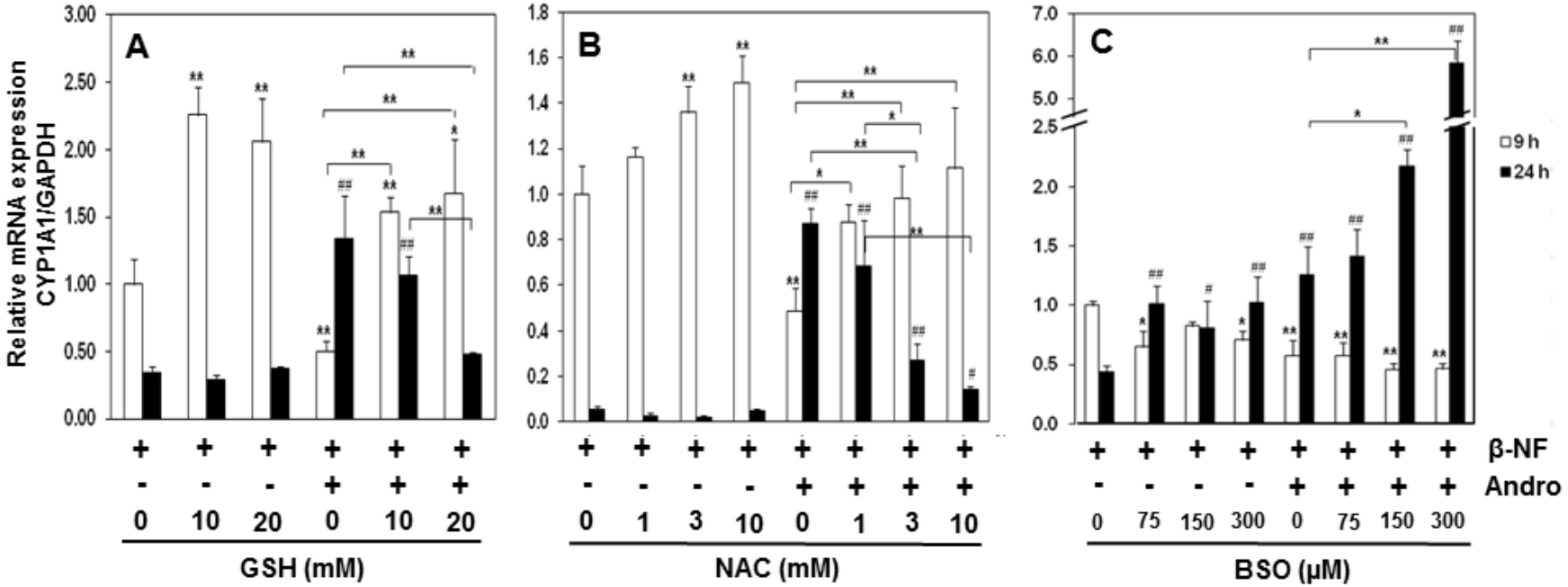

Figure 20 Expression of CYP1A1 mRNA in mouse hepatocytes in the presence of glutathione modulators

Primary mouse hepatocytes were treated with a combination of 10 μM beta-naphthoflavone (β-NF), 25 μM andrographolide (Andro), and glutathione (GSH) modulators. Total RNA was prepared 9 h (open column) or 24 h (closed column) later and the expression of CYP1A1 mRNA was analyzed by real-time RT-PCR. Each column represented the means ± SD (n=4). A) Effect of L-glutathione (GSH); B) Effect of N-acetyl-L-cysteine (NAC); C) Effect of L-buthionine-(S,R)-sulfoximine (BSO). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and **, and $^{\#}$ and $^{\#\#}$ represented significantly differences from the β-NF or β-NF+Andro group at 9 and 24 h, at $p<0.05$ and $p<0.001$, respectively.

GSH modulators extensively altered the effects of andrographolide and/or β-NF on CYP1A1 expression. When the GSH level was increased, the β-NF-induced CYP1A1 mRNA expression was increased at 9 h in the absence or presence of andrographolide (Figure 20A & 20B). The synergistic effect of andrographolide plus β-NF on CYP1A1 expression was reduced at 24 h in the presence of GSH and NAC, while the expression was further enhanced in the presence of BSO (Figure 20C). Therefore, an increase in the GSH content competed with the suppressive effect of andrographolide on CYP1A1 expression early on, and a decrease supported the synergism. Elimination of ROS by GSH in the present experimental system, were not in consistent with a report of Barker et al. (1994), that the expression of CYP1A1 was inhibited by ROS and recovered with the addition of NAC. A possible explanation was that andrographolide formed a complex with GSH (Zhang et al., 2008). Although andrographolide and a complex of andrographolide-GSH existed in certain proportion in the cell, andrographolide itself exerted the synergistic effect on CYP1A1 expression at 24 h. Synergistic CYP1A1 induction by andrographolide might be increased after production of andrographolide -GSH complex. These findings indicated that GSH was a key factor associated the synergism of inducible CYP1A1 expression by andrographolide. Addition of H_2O_2 may decrease free GSH level by interaction between them, resulting in elevation of expression CYP1A1 expression. Furthermore, GSH-related modification was entirely opposite between early and late phases of the CYP1A1 induction by andrographolide and/or β-NF. These observations suggested involvement of time-dependent regulatory mechanism in the induction.

The modification of inducible CYP1A1 mRNA expression by andrographolide was bimodal, time-dependent, and the synergism of CYP1A1 was retrieved by change of the intracellular GSH content. These results suggested that the GSH status or interaction of andrographolide and GSH might involve in the regulatory mechanism of CYP1A1 induction by andrographolide. A mechanism related modulation of inducible CYP1A1 expression by andrographolide is of interest. Since many environmental procarcinogens are CYP1A1 substrates, and CYP1A1, in turn, metabolizes these compounds to be reactive metabolites. Thus, the risk-benefit

assessment of using andrographolide or extract of *A. paniculata* containing product as a health supplement is recommended.

Therefore, it was of interest to study whether the synergistic effect on CYP1A1 induction of andrographolide plus a typical CYP1A inducer in cultured mouse hepatocytes occured *in vivo*, and if so, whether there were factor(s) influencing it.

4.2 Part II: *In vivo* study

4.2.1 Induction of hepatic CYP1A expression by andrographolide and/or 3-methylcholanthrene in C57BL/6 or DBA/2 mice

Andrographolide plus typical CYP1A inducers, including β-NF, TCDD, and benz[*a*]anthracene, synergistically induced CYP1A1 expression in mouse hepatocytes in primary culture, and the synergism was blocked by an AhR antagonist, resveratrol (Jaruchotikamol et al., 2007). The findings supported the involvement of an AhR-mediated pathway of transcriptional activation in the synergistic effect of andrographolide and the AhR ligands on CYP1A1 in mouse hepatocytes. Therefore, the synergistic effect on CYP1A expression by andrographolide plus a typical CYP1A inducer, 3-MC, and the involvement of AhR were examined in AhR-responsive, C57BL/6 (B6), and AhR-nonresponsive, DBA/2 (D2), mouse strains.

The effects of andrographolide and/or 3-MC on the expression of *Cyp1a1* gene was examined in livers of B6 and D2 mice (Figure 21), respectively.
3-MC significantly induced the expression of hepatic CYP1A1 in male B6 mice (Figure 21). Moreover, andrographolide plus 3-MC synergistically enhanced the expression of CYP1A1 mRNAs in the livers of B6 males. Neither the single treatment with 3-MC or andrographolide nor co-treatment significantly modified the expression of CYP1A1 mRNA in the male D2 mice.

This result demonstrated a synergistic effect on CYP1A1 expression by andrographolide plus 3-MC *in vivo*. In addition, the involvement of an
AhR-mediated pathway of transcription in regulating the synergistic effect was clearly affirmed that enhancement of CYP1A1 expression was found only in the liver of AhR-responsive B6 mice, and not in AhR-nonresponsive D2 mice.
Hence, AhR-responsive strain might be an appropriate strain for study the effect of andrographolide on synergism of CYP1A1. Not only hepatic CYP1A1 mRNA, CYP1A2 and CYP1B1 mRNA were also enhanced by andrographolide plus 3-MC (Figure 22), according with *in vitro* study (Figure 16).

Correspondingly, the levels of CYP1A1 and CYP1A2 protein in hepatic microsomes were markedly increased on treatment with 3-MC or andrographolide plus 3-MC in the B6 mice, but they were unchanged in the D2 mice (Figure 23). These observations supported the idea that the synergistic effect of

andrographolide plus 3-MC on the expression of CYP1A1 mRNA occurred in male B6 mice *in vivo* as well as in primary cultured mouse hepatocytes.

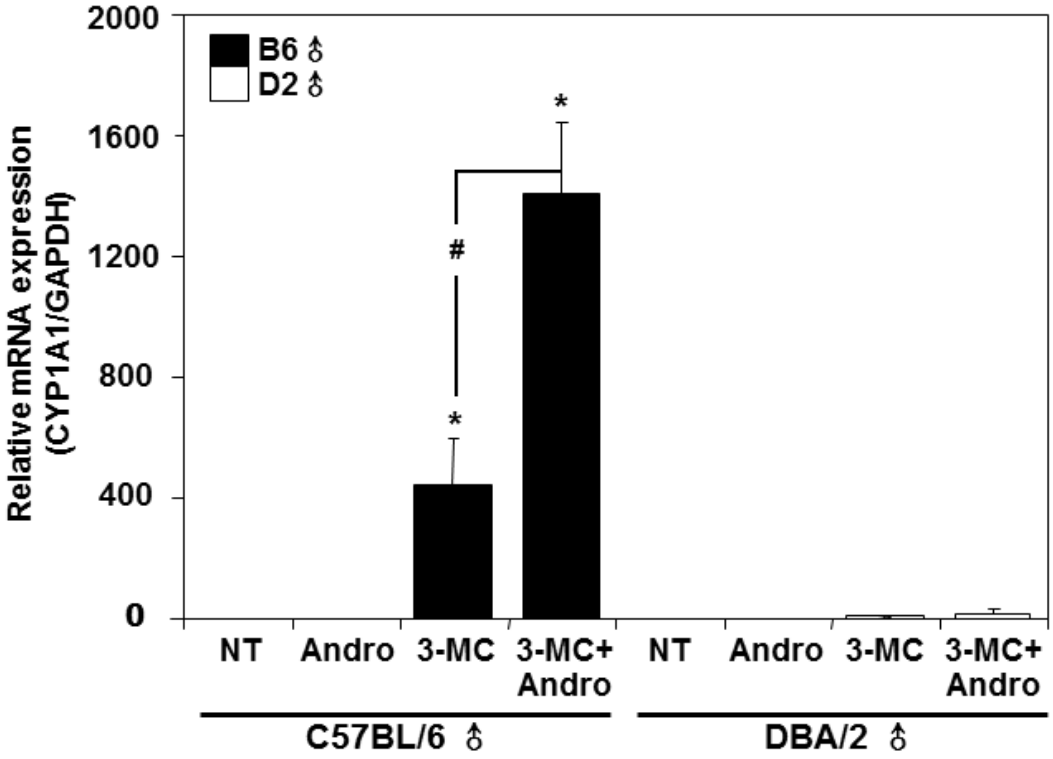

Figure 21 Hepatic CYP1A1 expression induced by andrographolide and/or 3-methylcholanthrene in male C57BL/6 or DBA/2 mice

Male C57BL/6 or DBA/2 mice were subcutaneously injected with corn oil 0.1 ml daily for 5 days (non-treatment-NT), andrographolide (Andro) (5 mg/kg/day) daily for 5 days, and/or intraperitoneally with 3-MC (20 mg/kg/day) for 3 days. Total RNA was prepared from the liver one day after the last treatment. Expression of CYP1A1 mRNA was normalized to that of GAPDH. Each column represented the means ± SD (n=4-5). Significance was calculated using the one-way ANOVA, followed by Tukey *post hoc* test. * and # represented significantly differences from the NT group at $p<0.001$ and from the 3-MC group at $p<0.01$, respectively.

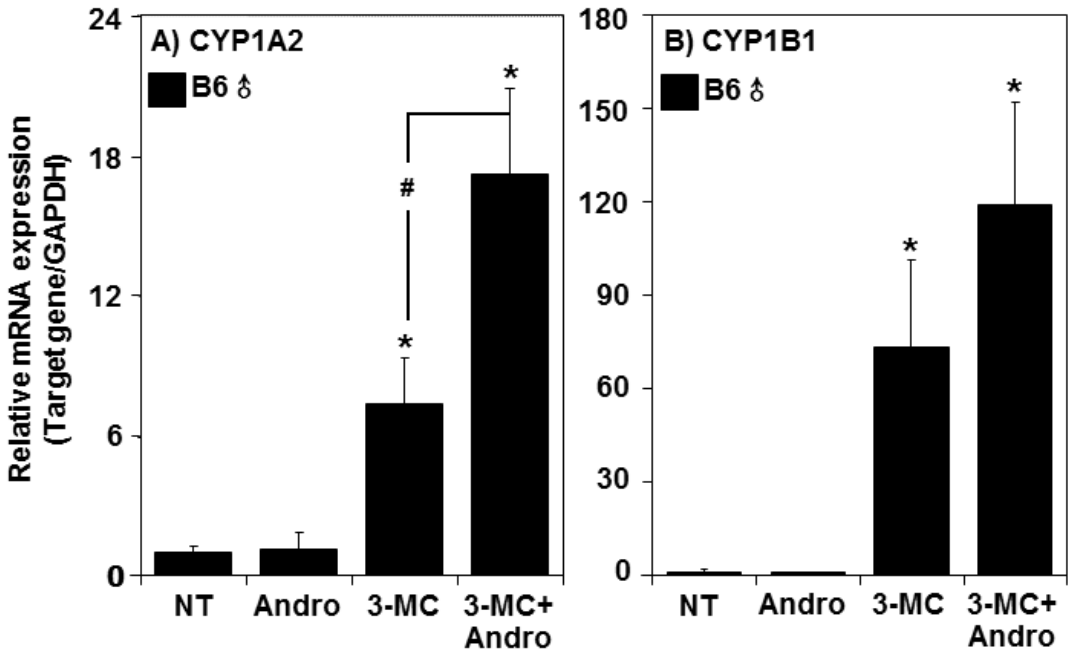

Figure 22 Hepatic CYP1A2 and CYP1B1 expression induced by andrographolide and/or 3-methylcholanthrene in male C57BL/6 mice

Male C57BL/6 (B6) mice were subcutaneously injected with corn oil 0.1 ml daily for 5 days (non-treatment-NT), andrographolide (Andro) (5 mg/kg/day) daily for 5 days, and/or intraperitoneally with 3-MC (20 mg/kg/day) for 3 days. Total RNA and a microsomal fraction were prepared from the liver one day after the last treatment. Expression of CYP1A1 mRNA was normalized to that of GAPDH. Each column represented the means ± SD (n=4-5). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and # represented significantly differences from the NT group at $p<0.001$ and from the 3-MC group at $p<0.01$, respectively.

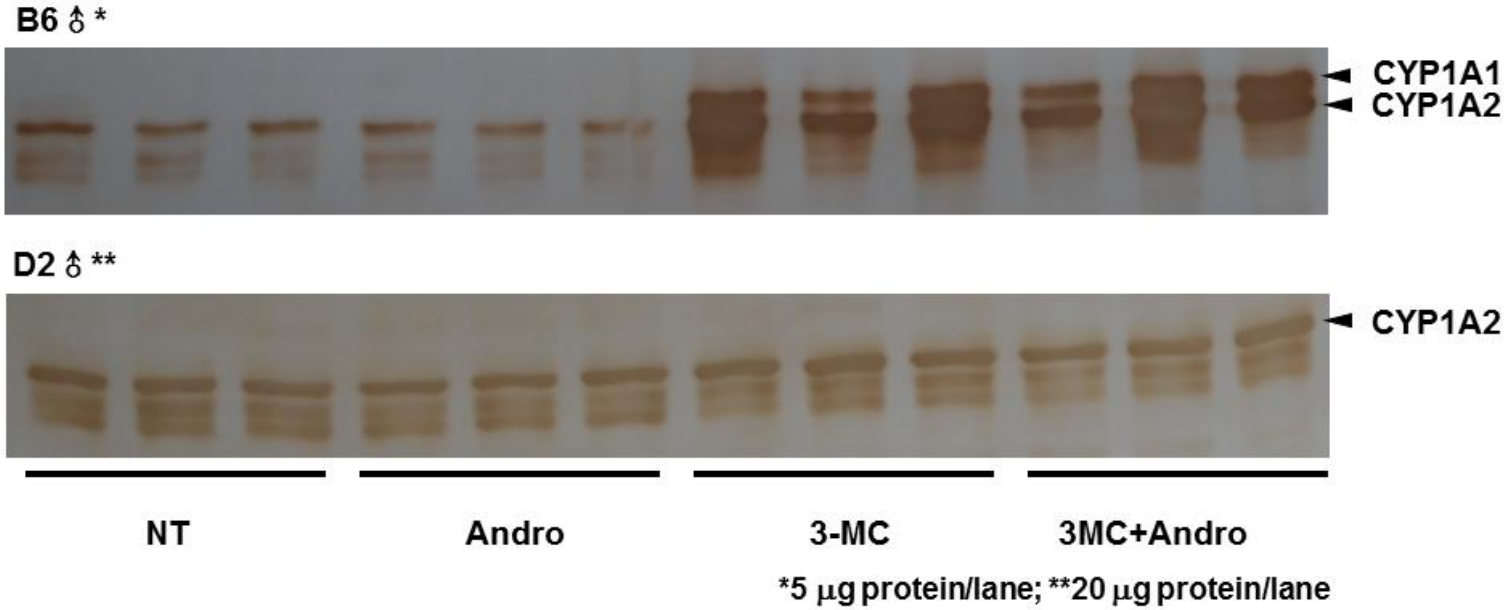

Figure 23 Expression of CYP1A1 and CYP1A2 protein induced by andrographolide and/or 3-methylcholanthrene in male C57BL/6 or DBA/2 mice

Male C57BL/6 (B6) or DBA/2 (D2) mice were subcutaneously injected with corn oil 0.1 ml daily for 5 days (non-treatment-NT), andrographolide (Andro) (5 mg/kg/day) daily for 5 days, and/or intraperitoneally with 3-MC (20 mg/kg/day) for 3 days. Hepatic microsomal protein was resolved using 10% SDS-PAGE and the expression of CYP1A1 and CYP1A2 proteins was detected using a rabbit polyclonal antibody against the rat CYP1A1 protein, which cross-reacted with CYP1A2. Besides CYP1A1 and CYP1A2, unidentified non-specific proteins beneath CYP1A were present in the preparations.

4.2.2 Expression of hepatic CYP1 mRNAs by phenobarbital after co-treatment with andrographolide

To further investigate whether the AhR-mediated pathway had an effect on the regulatory mechanism for the synergistic enhancement of CYP1 mRNA expression by andrographolide plus 3-MC in B6 males, 3-MC was replaced with phenobarbital (PB). PB significantly induced the expression of CYP1A1 mRNA (Figure 24). The expression of none of the CYP1 mRNA tested was increased by the co-treatment with PB and andrographolide (Figure 24).

From this experiment, no evidence of synergism after co-treatment with PB and andrographolide was obtained, though PB weakly induced CYP1A1 mRNA expression under the present experimental conditions (Figure 24A). PB did not show any effect on hepatic CYP1A2 and CYP1B1 mRNA expression (Figure 24B & 24C). Zaher et al. (1998) reported the induction of CYP1A2 mRNA and protein by PB in $Ahr^{-/-}$ mice, suggested that mechanism of PB-induced CYP1A2 was regulated independent of AhR. Therefore, these findings supported that andrographolide partially modified the activation pathway for CYP1A1 by β-NF or 3-MC via AhR.

4.2.3 Gender-related modulation of CYP1A1 mRNA expression by 3-methylcholanthrene after treatment with andrographolide

The expression of CYP1A1 induced by 3-MC after treatment with andrographolide was examined further to clarify whether other factors were involved. B6 mice of both sexes were administered 3-MC and andrographolide. Total hepatic RNA was prepared one day after the last treatment. CYP1A1 mRNA expression was determined in both sexes of B6 mice (Figure 25). Interestingly, the 3-MC-induced expression of CYP1A1 mRNAs in male mice was notably increased by the co-treatment with andrographolide. Although andrographolide did not increase the 3-MC-induced mRNA expression of CYP1A1 in female mice, the expression of CYP1A1 mRNA decreased significantly in females. These observations suggested that the enhancement of CYP1A1 expression on the co-treatment with 3-MC and andrographolide was gender dependent.

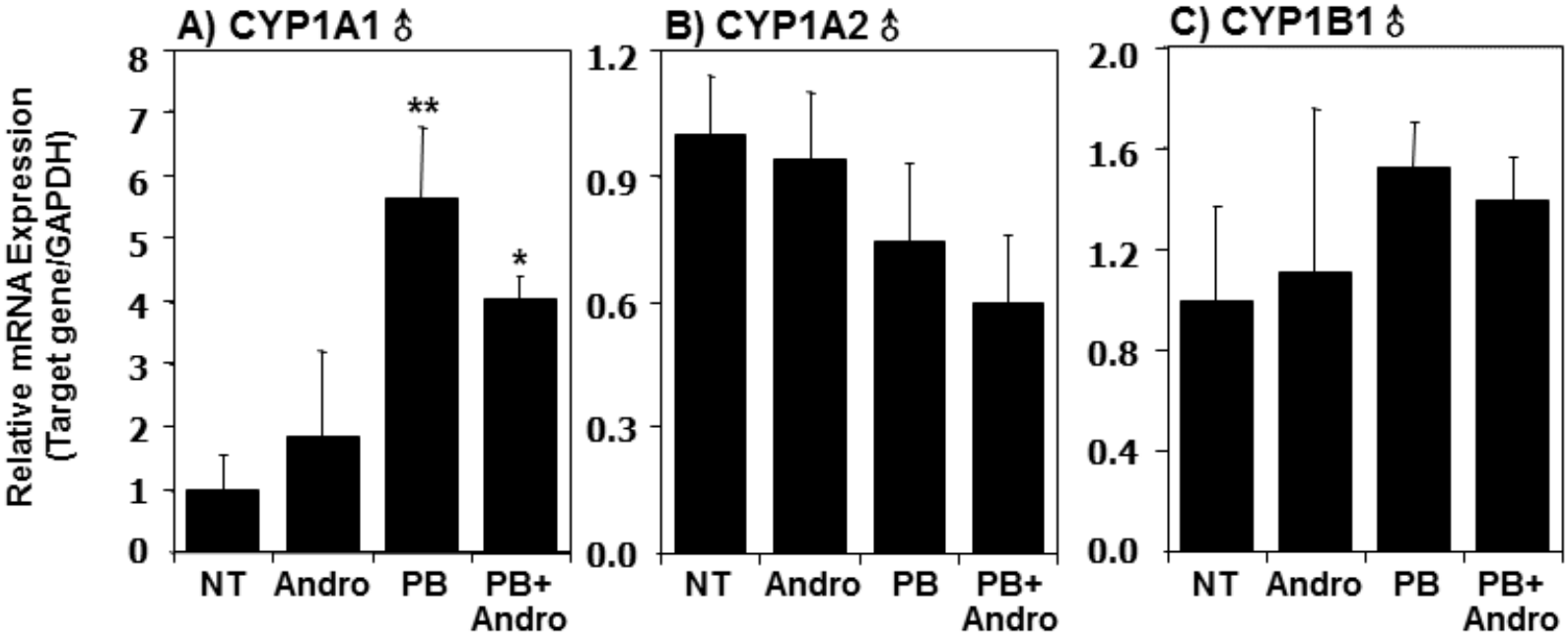

Figure 24 Expression of hepatic CYP1 mRNA by phenobarbital in the presence of andrographolide

Male C57BL/6 (B6) mice were subcutaneously injected with corn oil 0.1 ml daily for 5 days (non-treatment-NT), andrographolide (Andro) (5 mg/kg/day) daily for 5 days, and/or intraperitoneally with phenobarbital (PB) (100 mg/kg/day) for 3 days. Hepatic total RNA was reverse-transcribed and cDNA was amplified using a specific TaqMan® Gene Expression Assay or TaqMan® Gene Expression Detection Kit. Expression of the respective mRNA was normalized to that of GAPDH. Each column represented the means ± SD (n=4-5). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and ** represented significantly differences from the NT group at $p<0.01$ and $p<0.001$, respectively.

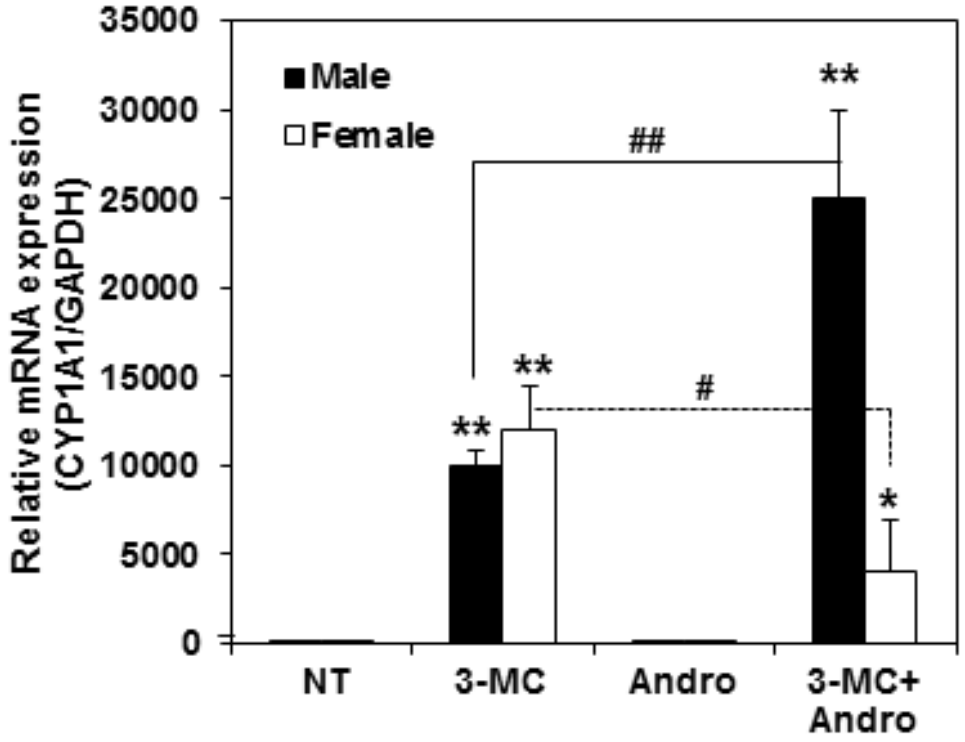

Figure 25 Expression of CYP1A1 mRNA induced by andrographolide and/or 3-methylcholanthrene in male and female C57BL/6 mice

Male (closed column) and female (opend column) C57BL/6 mice were subcutaneously injected with corn oil 0.1 ml daily for 5 days (non-treatment-NT), andrographolide (Andro) (5 mg/kg/day) daily for 5 days, and/or intraperitoneally with 3-MC (20 mg/kg/day) for 3 days. Hepatic total RNA was prepared at one day after the last treatment. Expression of CYP1A1 mRNA was normalized to that of GAPDH. Each column represented the means ± SD (n=4-5). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and $^{\#}$ represented significantly differences from the NT group at $p<0.001$ and from the 3-MC group at $p<0.01$, respectively.

4.2.4 Impact of testosterone on the expression of CYP1A1 mRNA in orchiectomized and ovariectomized C57BL/6 mice

Since the synergistic enhancement of CYP1A1 mRNA expression through the co-treatment with 3-MC and andrographolide was found to be sex-dependent (Figure 25), the expression of CYP1A1 mRNAs was examined in orchiectomized B6 mice under the same conditions. As expected, orchiectomy clearly diminished the synergistic effect on CYP1A1mRNA expression in male B6 mice (Figure 26).

These observations suggest that a male hormone or a male specific system is a crucial mediator of the synergistic effect on CYP1A1 activation of the co-treatment with 3-MC and andrographolide.

To further investigate whether a male sex hormone, testosterone, was an important factor in the synergistic promotion of CYP1A1 expression on treatment with andrographolide plus 3-MC in male mice, male and female B6 mice were orchiectomized and ovariectomized, respectively, before being treated with testosterone. Testosterone supplement clearly restored the synergism of CYP1A1 mRNA in B6 males (Figure 27A). Correspondingly, testosterone restored the expression of CYP1A1 mRNA in ovariectomized B6 females (Figure 27B) to a level comparable to that in orchiectomized B6 males. These findings suggested that testosterone was a crucial sex-related factor, involved in the synergistic effect on CYP1A1 by 3-MC and andrographolide in B6 mice.

The synergistic effect on CYP1A1 mRNA expression was noted in male, but not female mice (Figure 25). However, andrographolide did not enhance, but rather reduced, 3-MC-induced CYP1A1 mRNA expression in orchiectomized mice (Figure 26). The expression profile of CYP1A1 mRNA in orchiectomized males was almost the same as that in intact female mice (Figure 25 & 26). These observations suggested that the male hormone involved in the synergistic effect on CYP1A1 by andrographolide. To confirm this possibility, orchiectomized male and ovariectomized female B6 mice underwent testosterone supplementation simultaneously with the co-administration of 3-MC and andrographolide (Figure 27). The observations clearly demonstrated that the administration of testosterone to both orchiectomized and ovariectomized B6 mice restored the synergistic expression of

CYP1A1 to nearly the same level as that in the intact B6 male mice. The results revealed a male sex hormone-associated system to have a positive role in the synergistic effect on 3-MC-induced CYP1A1 expression by andrographolide.

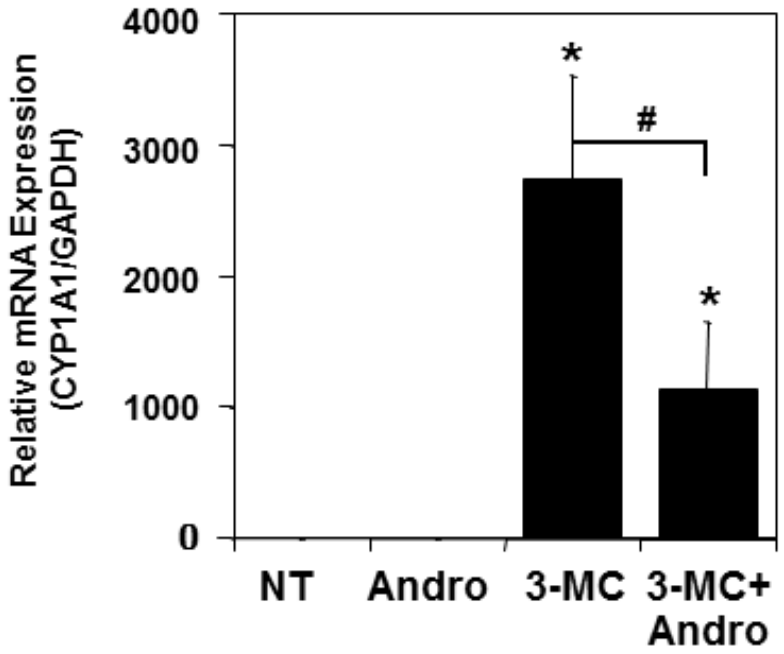

Figure 26 Expression of CYP1A1 mRNA induced by andrographolide and/or 3-methylcholanthrene in castrated male C57BL/6 mice

Male C57BL/6 mice were castrated at 5 weeks of age. Two weeks later, mice were subcutaneously injected with corn oil 0.1 ml daily for 5 days (non-treatment-NT), andrographolide (Andro) (5 mg/kg/day) daily for 5 days, and/or intraperitoneally with 3-MC (20 mg/kg/day) for 3 days. Hepatic total RNA was prepared at one day after the last treatment. Expression of CYP1A1 mRNA was normalized to that of GAPDH. Each column represented the means ± SD (n=4-5). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test. * and # represented significantly differences from the NT group at $p<0.001$ and from the 3-MC group at $p<0.01$, respectively.

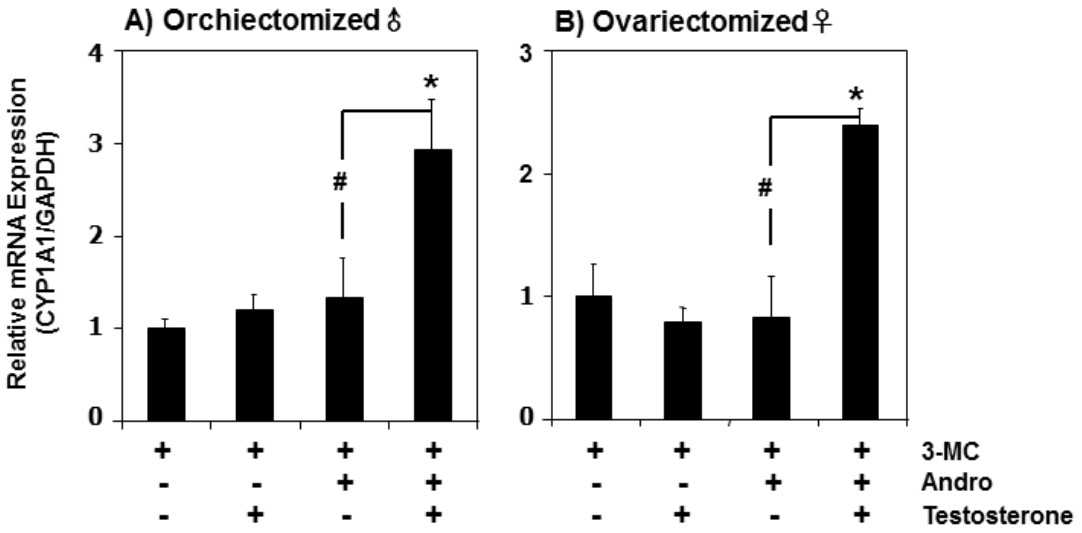

Figure 27 Impact of testosterone supplementation on the expression of CYP1A1 mRNA in gonadectomized C57BL/6 mice

Male and female C57BL/6 mice were orchiectomized or ovariectomized at 5 weeks of age. Two weeks later, the orchiectomized male and ovariectomized female B6 mice were subcutaneously injected with testosterone (5 mg/kg/day) daily for 7 days simultaneously with 3-MC plus andrographolide (Andro). The hormone treatment was started two days before that of 3-MC and andrographolide. Hepatic total RNA was reverse-transcribed and cDNA was amplified using a specific TaqMan® Gene Expression Assay or TaqMan® Gene Expression Detection Kit. Expression of the respective mRNAs was normalized to that of GAPDH. Each column represented the means ± SD (n=5-6). Significance was calculated using the one-way ANOVA followed by Tukey *post hoc* test.

* and # represented significantly differences from the 3-MC group at $p<0.001$ and from the 3-MC+Andro group at $p<0.001$, respectively.

The association between the regulatory pathway of CYP1A1 expression and condition of female sex hormone has been widely researched (Angus et al., 1999; Ricci et al., 1999) in term of the inhibitory effect of estradiol on CYP1A1 (Eugster et al., 1993; Lai et al., 2004). A role for estrogen receptor-α (ERα)-mediated transrepression of AhR-dependent regulation of CYP1A1 via direct interaction between AhR/ARNT and ERα has been mentioned (Beischlag & Perdew, 2005). AhR and ERα might be presented in the CYP1A1 enhancer region during transrepression. The estrogen-mediated suppression and the synthesis of protein factors were speculated to be due to blockage of the activated TCDD/AhR complex formation by estradiol, and subsequently binding to drug-responsive element (Safe & Wormke, 2003; Lai et al., 2004). The evidence that short hairpin RNA-mediated knockdown of ERα expression significantly decreased CYP1A1 expression suggested ERα as a promoter-specific modulator of AhR-dependent transcription, and demonstrated the complexity of the cross-talk between these two receptor pathways (Matthews & Gustafsson, 2006). In the other words, dioxins can mimic effect of estrogen through a mechanism that involves the activation of estrogen receptors by a transcriptional active AhR-ARNT complex (Brosens & Parker, 2003; Ohtake et al., 2003). Therefore, enhanced expression of CYP1A1 might be expected after ovariectomy, even if the evidence was noted.

Testosterone supplement restored the synergistic effect of CYP1A1 induction by andrographolide in both orchiectomized males and ovariectomized females. These observations indicated a male sex hormone-related system as a key mediator of the synergistic CYP1A1 expression by co-treatment of 3-MC and andrographolide. The proposed mechanism of synergistic CYP1A1 induction by andrographolide in the presence of testosterone was showed in Figure 28.

CYP1A1 expression markedly influences the activation of chemical carcinogens (Kimura et al., 1986; Nemoto et al., 1989; Iwanari et al., 2002). In the present study, the concentration of andrographolide presently employed (5 mg/kg/day for 5 days) was equivalent to that achieved in individuals ingesting *A. paniculata* (Calabrese et al., 2000; Panossian et al., 2000). Therefore, the risk of andrographolide to modify carcinogenesis via CYP1A1 regulation pathway should be strongly alerted.

Moreover, some risks associated with the use of this compound containing products might be of interest, and further evaluation of andrographolide analogs or other major constituents of *A. paniculata* should not be overlooked and further investigated.

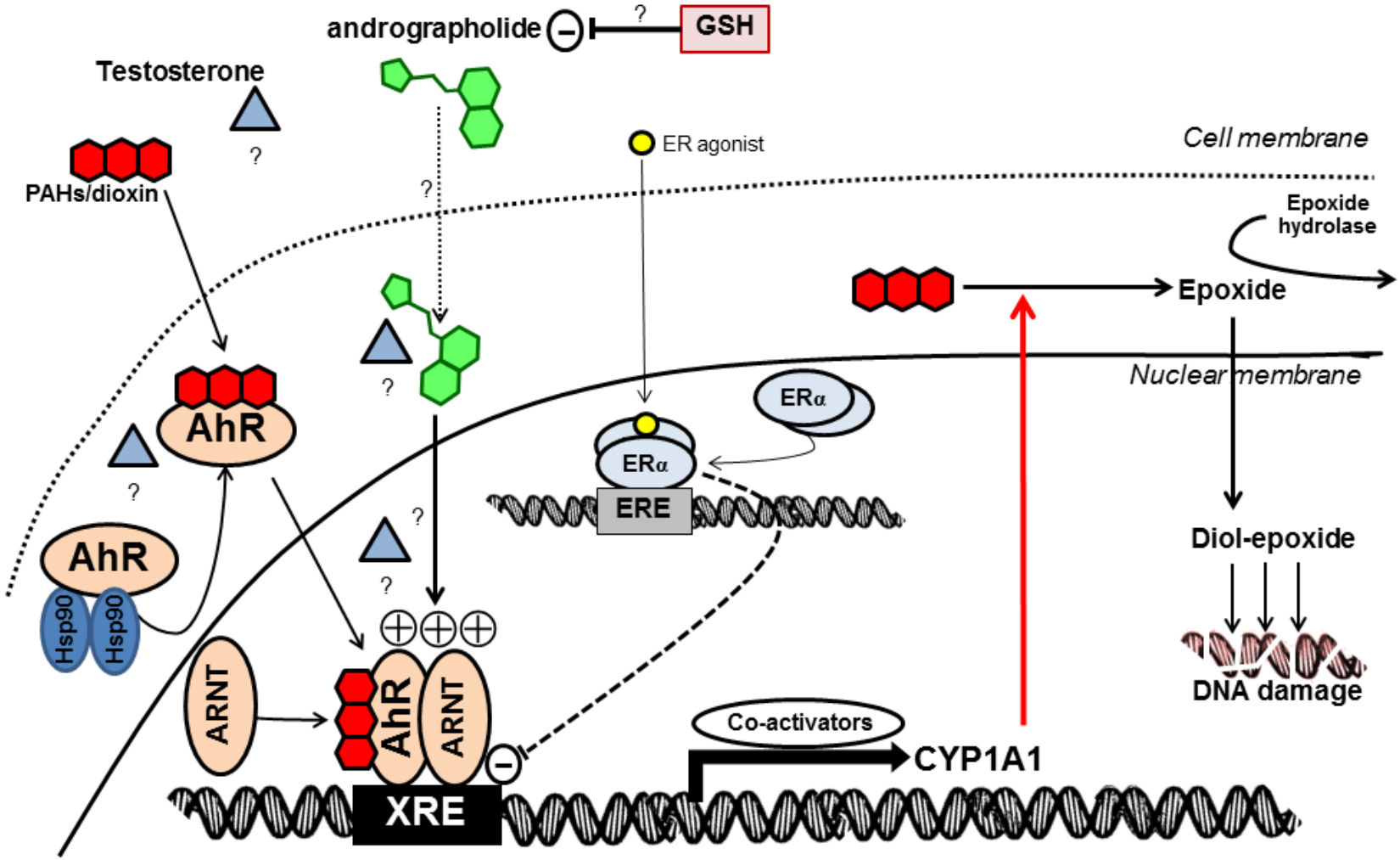

Figure 28 The proposed mechanism of CYP1A1 synergism by andrographolide in the presence of testosterone