Proteomic analysis of the insoluble protein adducts induced by reactive metabolites

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Abstract

Covalent binding of reactive metabolites of drugs to proteins has been a predominant hypothesis for the mechanism of toxicity caused by numerous drugs. The development of efficient and sensitive analytical methods for the separation, identification, quantification of drug–protein adducts have important clinical and toxicological implications. For example idiosyncratic drug toxicity (IDT) is a specific type of drug toxicity characterized by reactive metabolite formation. IDT is unpredictable and often leads to the post marketing failure, for example; troglitazone, tolcapone and felbamate. Two dimensional gel electrophoresis (2-DE) separation is utilized to investigate the covalent adduct formation with soluble proteins. But it could not be applicable to the insoluble proteins including P450s. The P450 enzymes are the major catalysts involved in the metabolism of drugs.

We applied ***"two-step pre-fractionation"*** of the insoluble membrane proteins and storage phosphor analysis of SDS-PAGE gel band to detect the insoluble protein adducts. The insoluble proteins including P450s were extracted by 2 step solubility based fractionation from ^{14}C-naphthalene incubated microsome. The fractionated insoluble proteins were separated by SDS-PAGE and radioactive proteins were localized by 10-40 days exposure to phosphorscreen. The bands and spots of adducts were identified with originally developed ***"spot array"*** analysis of storage phosphor. Furthurmore the amount of protein adducts were estimated from spot volume of internal standard.

Introduction

Several low molecular weight aromatic hydrocarbons including naphthalene, 2-ethylnaphthalene, and 1-nitronaphthalene appear to initiate toxicity primarily in pulmonary epithelial Clara cells, by virtue of reactive metabolite generation in that tissue. The work reported here was conducted to identify proteins adducted by reactive metabolites of naphthalene generated by hepatic tissue.

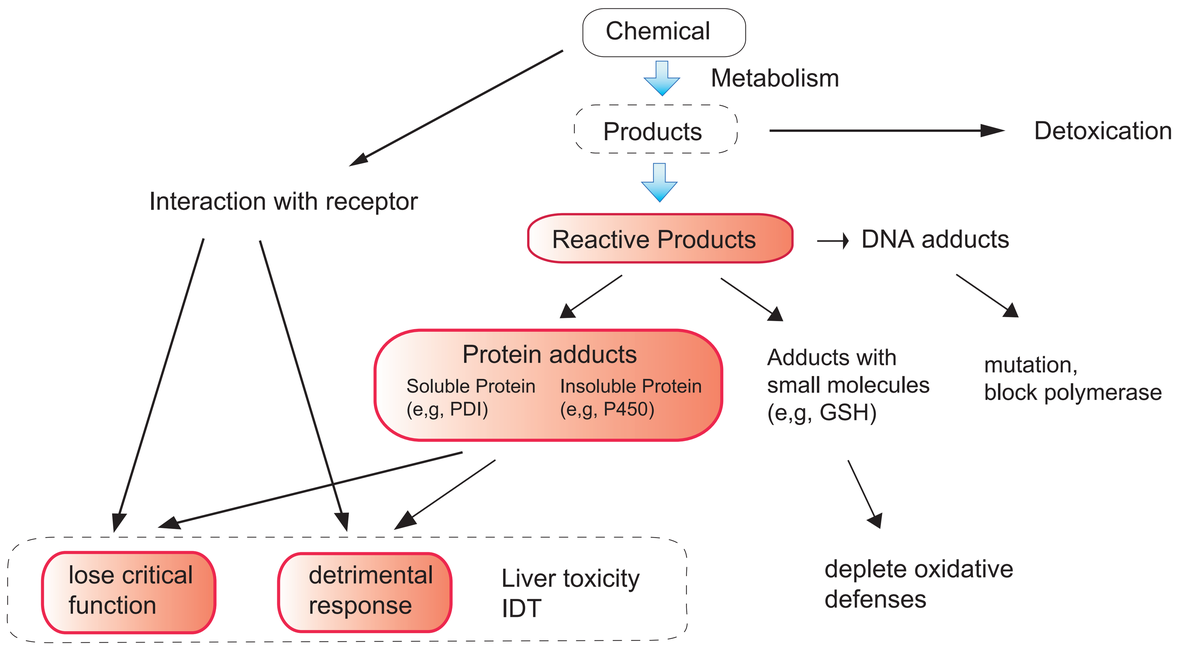
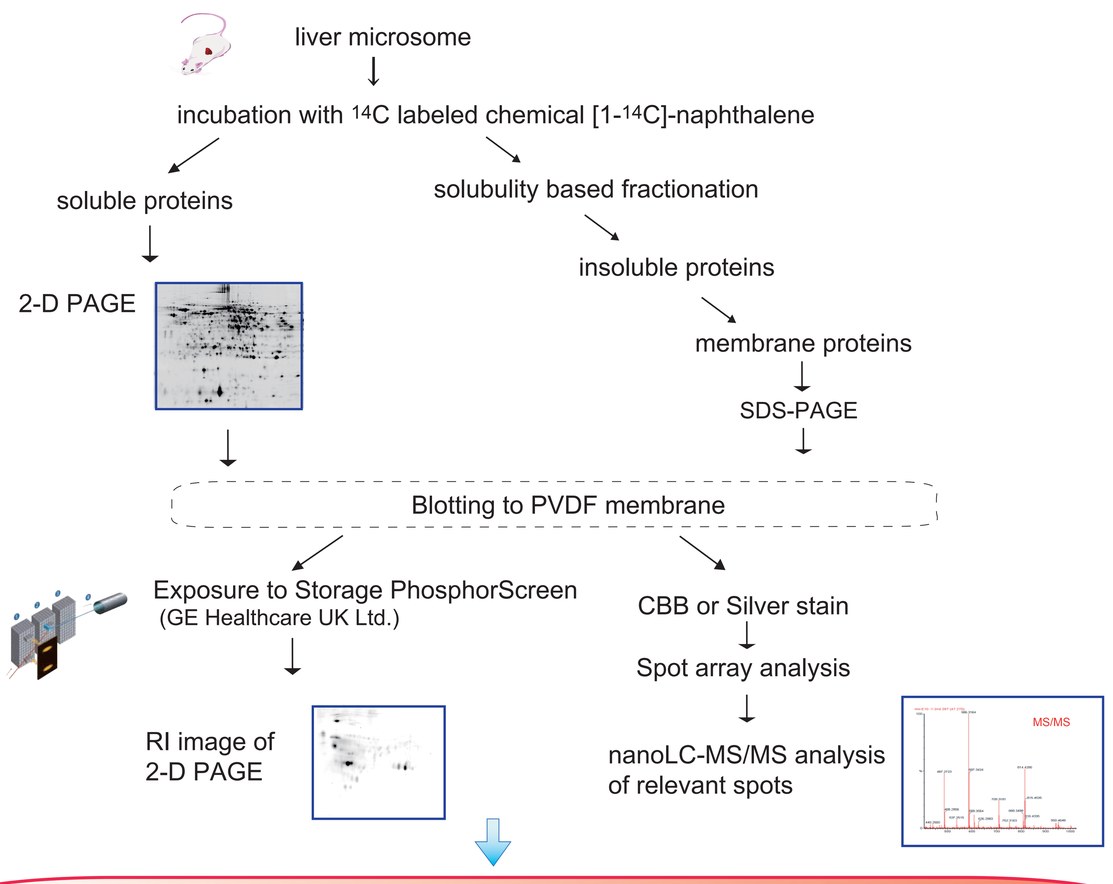


Fig 1. Biological events possibly relevant to chemical toxicity

Experimental

Incubations were prepared on ice in a final volume of 2mL using 0.1 M sodium phosphate buffer (pH 7.4). Incubations consisted of: either 0.5mM unlabeled naphthalene or 0.5mM [1–^{14}C]- naphthalene (specific activity 1.14 x 10 dpm/nmole) added in no more than 2 mL methanol, microsomes (4mg protein), and NADPH generating system (15mM $MgCl_2$, 60mM glucose-6-phosphate, 2mM NADP,and 15 iu/mL glucose-6-phosphate dehydrogenase). The incubation was permitted to proceed for 20 min with shaking at 37℃.
Soluble proteins were analyzed by 2-D DIGE. The insoluble proteins were fractionated with solubility. Furthurmore, membrane protein extraction and purification was executed with Triton X114 based fractionation (Ready prep membrane protein extraction kit; Bio-Rad).



Reliable identification of total proteins (soluble and insoluble) adducted by reactive metabolites

Results

Insoluble protein (membrane protein) enrichment by ***"2 step solubility-based fractionation"***

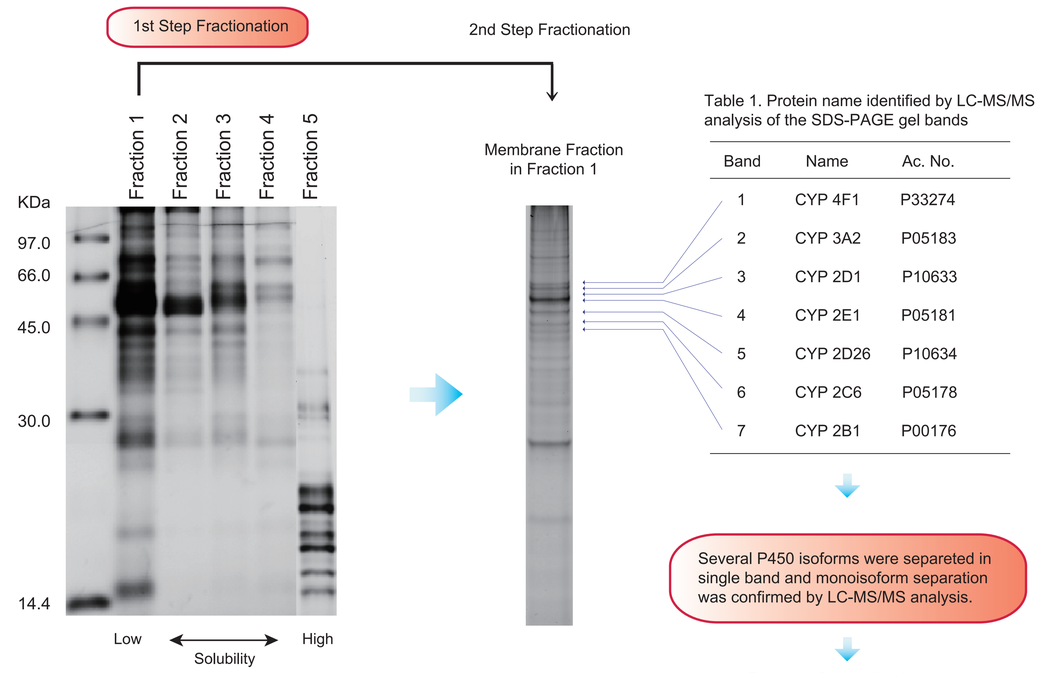


Table 1. Protein name identified by LC-MS/MS analysis of the SDS-PAGE gel bands

Band	Name	Ac. No.
1	CYP 4F1	P33274
2	CYP 3A2	P05183
3	CYP 2D1	P10633
4	CYP 2E1	P05181
5	CYP 2D26	P10634
6	CYP 2C6	P05178
7	CYP 2B1	P00176

Fig 2. Two step solubility-based fractionation and identification of rat liver microsomal proteins separated by 1-DE

"Spot array" analysis for reliable identification of protein adducts

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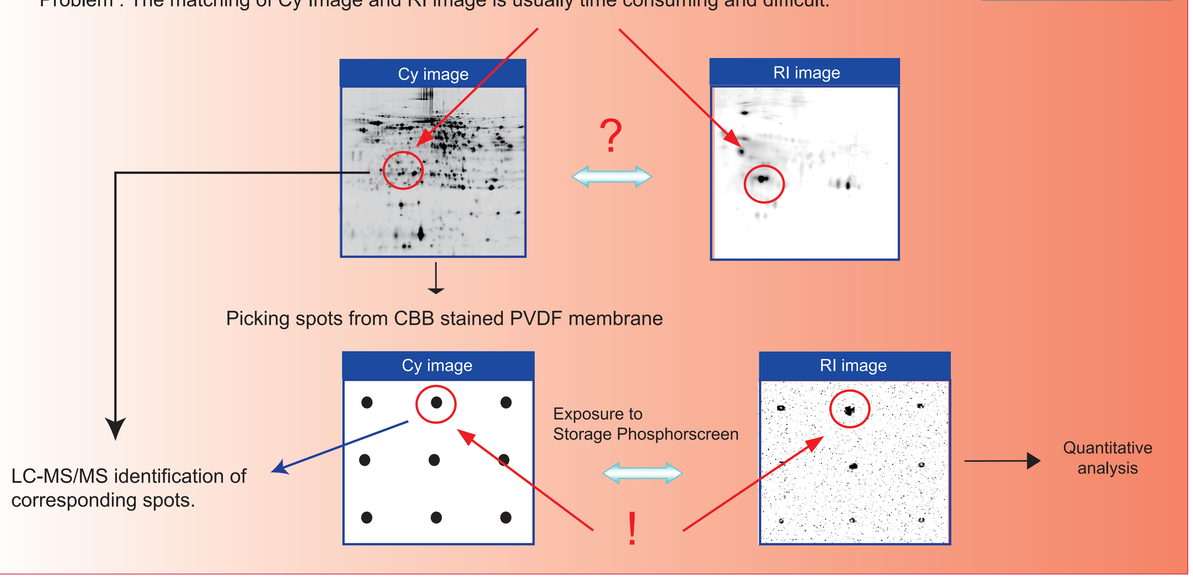
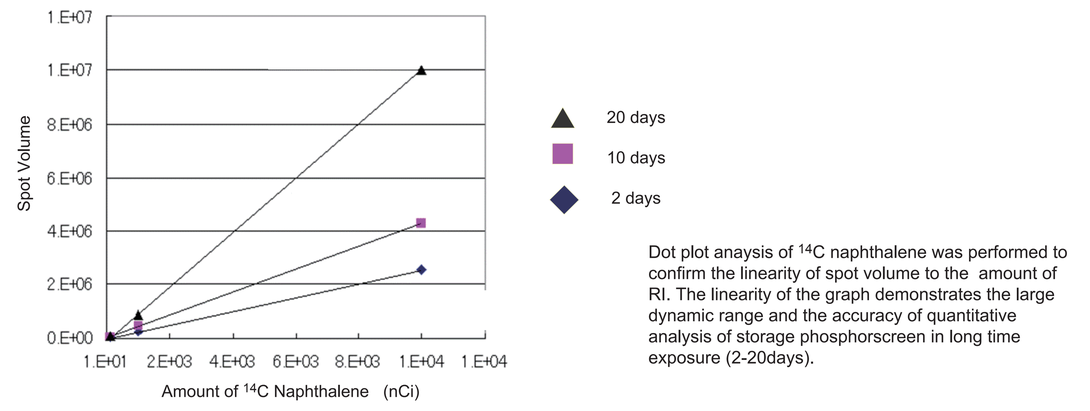


Quantitative analysis of Storage Phosphorscreen image of RI compounds



Dot plot anaysis of ^{14}C naphthalene was performed to confirm the linearity of spot volume to the amount of RI. The linearity of the graph demonstrates the large dynamic range and the accuracy of quantitative analysis of storage phosphorscreen in long time exposure (2-20days).

Naphthalene adducted soluble and insoluble proteins

Table 2. Soluble protein adducts identified by LC-MS/MS and spot array analysis

Ac.No.	Protein Name	Mw	RI量(Bq)	
P14659	Heat shock-related 70 kDa protein 2	69642	1.54	
P04762	Catalase	59757	3.21	
P63039	60 kDa heat shock protein, mitochondrial	60955	3.51	
P11598	Protein disulfide-isomerase A3	56623	1.26	
P04785	Protein disulfide-isomerase	56951	12.7	
O55239	Nicotinamide N-methyltransferase	29,598	2.42	✻

Table 3. Insoluble protein adducts identified by LC-MS/MS and spot array analysis

Ac.No.	Protein Name	Mw	RI量(Bq)	
P05183	Cytochrome P450 3A2	57,732	2.56	✻
P05178	Cytochrome P450 2C6	56,003	1.22	✻

✻ ***Newly identified adducts***

Conclusions

We developed the quantitative analysis system for total (soluble and insoluble) protein adducts formed with reactive metabolites. The system includes newly developed ***"2 step fractionation method"*** for the separation of P450 isoforms and ***"spot array"*** for reliable identification of adducted proteins. For proof of principles, we executed the identification and quantitative analysis of naphthalene adducts. In this study, we identified and analyzed 6 soluble proteins and 2 P450 isoforms. These P450 isoforms and 1 soluble protein (Nicotinamide N-methyltransferase) have never reported as naphthalene adducts.
This newly developed quantitative analysis of protein adducts promises to provide new insights into understanding drug–protein adducts induced toxic effects including IDT.