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Exposure profiling of reactive compounds in complex mixtures

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ABSTRACT

Humans are constantly exposed to mixtures, such as tobacco smoke, exhaust from diesel, gasoline or new bio-fuels, containing several 1000 compounds, including many known human carcinogens. Covalent binding of reactive compounds or their metabolites to DNA and formation of stable adducts is believed to be the causal link between exposure and carcinogenesis. DNA and protein adducts are well established biomarkers for the internal dose of reactive compounds or their metabolites and are an integral part of science-based risk assessment. However, technical limitations have prevented comprehensive detection of a broad spectrum of adducts simultaneously. Therefore, most studies have focused on measurement of abundant individual adducts. These studies have produced valuable insight into the metabolism of individual carcinogens, but they are insufficient for risk assessment of exposure to complex mixtures. To overcome this limitation, we present herein proof-of-principle for comprehensive exposure assessment, using N-terminal valine adduct profiles as a biomarker. The reported method is based on our previously established immunoaffinity liquid chromatography–tandem mass spectrometry (LC–MS/MS) method with modification to enrich all N-terminal valine alkylated peptides. The method was evaluated using alkylated peptide standards and globin reacted *in vitro* with alkylating agents (1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, propylene oxide, styrene oxide, N-ethyl-N-nitrosourea and methyl methanesulfonate), known to form N-terminal valine adducts. To demonstrate proof-of-principle, the method was successfully applied to globin from mice treated with four model compounds. The results suggest that this novel approach might be suitable for *in vivo* biomonitoring.

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1. Introduction

Environmental and occupational exposures have been associated with increased risk for the development of numerous cancers (Davis et al., 2010; Swenberg et al., 2008). Accurate exposure assessment in humans, however, is challenging because humans are exposed to a variety of mixtures, such as tobacco smoke, exhaust from diesel, gasoline or new bio-fuels. Such exposures contain several 1000 compounds, including many known or suspected human carcinogens. Covalent binding of reactive metabolites to DNA and the formation of stable adducts is believed to be the causal link between exposure and carcinogenesis. DNA adducts are well established biomarkers for the internal dose of reactive compounds or their metabolites and are an integral part of science-based risk assessment. Reactive compounds that form DNA adducts often also form protein adducts, such as with albumin and hemoglobin. The corresponding protein adducts are commonly used as surrogate biomarkers for DNA adducts and are well established biomarkers of exposures (Swenberg et al., 2008; Törnqvist et al., 2002; Wild, 2009; Wild and Pisani, 1998).

While globin adducts are not causally linked to mutagenesis, they have several advantages over DNA adducts: (i) protein adducts are recognized as good surrogate markers for the internal formation of the activated metabolites; (ii) in molecular epidemiology studies, blood samples are easier to obtain than tissue specimens; (iii) stable hemoglobin (Hb) adducts accumulate over the lifespan of the erythrocytes, which is about 30 days for mice; (iv) they are not removed by enzymatic repair systems like DNA adducts; and (v) due to their stability, protein adducts represent the cumulative exposure prior to sampling, which makes the timing of sample

Abbreviations: BD, 1,3-butadiene; DEB, 1,2:3,4-diepoxybutane; EB, 1,2 epoxy-3-butene; EB-diol, 3,4-epoxy-1,2-butanediol; ENU, N-ethyl-N-nitrosourea; ENU-Val, carbamoylated-valine; Et-Val, ethyl-valine; FA, formic acid; HB-Val, N-(2-hydroxy-3-buten-1-yl)-valine; Hb, hemoglobin; HP-Val, 1-hydroxy (or 2-hydroxy)-propyl-valine; IA, immunoaffinity; LC–MS/MS, liquid chromatography–tandem mass spectrometry; Me-Val, methyl-valine; MMS, methyl-methanesulfonate; H_2N-Val, non-alkylated-valine; PO, propylene oxide; *pyr*-Val, N,N-(2,3-dihydroxy-1,4-butadiyl)-valine; SO, styrene oxide; SO-Val, 1-phenyl-2-hydroxyethyl-valine or 2-phenyl-2-hydroxyethyl-valine; THB-Val, 2,3,4-trihydroxybutyl-valine.

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collection less critical. (vi) Lastly, they are excellent biomarkers that integrate *in vivo* metabolism over time and do not require invasive or time sensitive sampling (Törnqvist et al., 2002).

Extensive efforts have been made to quantitate adducts in albumin and hemoglobin. Of these, utilization of the modified Edman degradation method for analysis of N-terminal valine adducts is the most common (Boysen et al., 2007; Boysen and Hecht, 2003; Osterman-Golkar et al., 2003; Törnqvist et al., 1986, 2002). A comprehensive review of mass spectrometry study of protein adducts was recently published by Rubino et al. (2009). Unfortunately, technical limitations, such as low recovery, sensitivity and the need of adduct specific immunoaffinity chromatography, have prevented comprehensive exposure profiling of complex mixtures ("exposure-omics"), and the majority of studies report measurements of a single adduct or selected few adducts.

More recently Rappaport and colleagues reported progress in simultaneous monitoring of multiple adducts on cysteine in human serum albumin (Funk et al., 2010; Li et al., 2011). Further modifications of the Edman procedure for analysis of N-terminal valine adducts seem promising for future adduct profiling studies (Von Stedingk et al., 2010, 2011). These new approaches are aimed to establish a tool for multi-adduct profiling of reactive, and potentially genotoxic, compounds in mixtures. Such technology is expected to enable determination of the internal dose of numerous carcinogens simultaneously to (a) better understand the effects and fate of individual carcinogens in mixtures; (b) identify novel, until now, unknown adducts; and (c) investigate potential compound–compound interactions.

We report herein a novel proof-of-principle for a sensitive and specific method for qualitative profiling of exposure to a broad spectrum of reactive compounds or their metabolites using N-terminal valine adducts of hemoglobin as biomarkers. The reported method is based on our previously established immunoaffinity liquid chromatography–tandem mass spectrometry (LC–MS/MS) method (Boysen et al., 2004, 2012; Georgieva et al., 2007, 2010). To enable multi-adduct profiling, the adduct enrichment step has been modified to selectively isolate all alkylated N-terminal peptides of the globin α-chain, independent of adduct structure or chemical properties, prior to analysis by LC–MS/MS (Fig. 1).

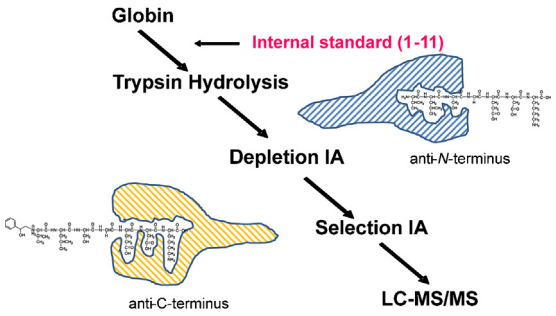

Fig. 1. Scheme of adduct profiling assay. The alkylated N-terminal peptides of the α-chain are isolated from trypsin hydrolyzed globin by series of depletion and selection IA chromatography, prior to analysis by LC–MS/MS as described in Section 2.

2. Materials and methods

2.1. Materials

Trypsin (biotin agarose, from bovine pancreas) was purchased from Sigma–Aldrich (St. Louis, MO). All reagents and solvents used were ACS grade or higher. Amicon 3 filters were obtained from Amicon Inc. (Beverly, MA) and Microspin filter tubes (regenerated cellulose, 0.2 μm) were from Altech Associates Inc. (Dearfield, IL). The non-alkylated (1–11) and methylated (1–11) peptide standards and [$^{13}C_5$] valine labeled non-alkylated (1–11) peptide used for synthesis of internal standards were purchased from Neo-Peptide, a subdivision of Neo-Group, Inc. (Cambridge, MA). The antibodies against the N-terminus (depletion) and C-terminus (selection) of the mouse α-Hb (1–7) peptides were raised by Open Biosystems Inc. (Huntsville, AL). Immunoaffinity (IA) depletion and selection columns were built using the respective antibody with the highest ELISA titers according to the procedure described in our earlier publication (Boysen et al., 2004).

2.2. Synthesis of standard peptides

The syntheses of analytical standard (AST) and internal standard (IST) peptides were performed by direct reaction of the non-alkylated (1–11) peptide with the reagents of interest in 0.1 M NH_4HCO_3 buffer for 72 h at the optimal molar ratio of 1:50 and pH 6.5. Full scan MS and MS/MS experiments of the products were used to confirm peptide sequence and site of alkylation.

2.3. In vitro alkylation of mouse (1–11) peptide or control globin

Globin was extracted from a female 12 week old B6C3F1 mouse as described previously (Mowrer et al., 1986). The reactions were performed at a molar ratio of peptide (globin):reagent = 1:1 in 0.1 M NH_4HCO_3 buffer for 72 h at pH 6.5. Reagents, 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB), propylene oxide (PO), styrene oxide (SO), N-ethyl-N-nitrosourea (ENU) and methyl methanesulfonate (MMS), were used alone or in a mixture (Fig. 2). After trypsin hydrolysis, the samples were processed over depletion and selection IA columns, and elutes were analyzed as described below.

2.4. Animal exposures

Female B6C3F1 mice were exposed in the UAMS Animal Facility to MMS, ENU, EB, and SO. MMS and ENU were given in saline by gavage once a day for 4 days and EB and SO were given once by i.p. injection on the fourth day. Mice were sacrificed on the fifth day and blood and tissues were harvested for further analyses. Globin from 2 samples of highly exposed mice (25 mg/kg body weight MMS and 100 mg/kg body weight ENU, plus 500 μmol/kg body weight both EB and SO) was extracted and analyzed for the expected adducts as described below.

2.5. Immunoaffinity enrichment and purification of in vitro reaction mixtures or globin samples from mice

Globin was first trypsinized as described previously (Boysen et al., 2004). After filtration on Amicon 3 columns and concentration in a speed-vac to about 0.3 mL, samples were loaded to the depletion IA columns, containing antibodies specific

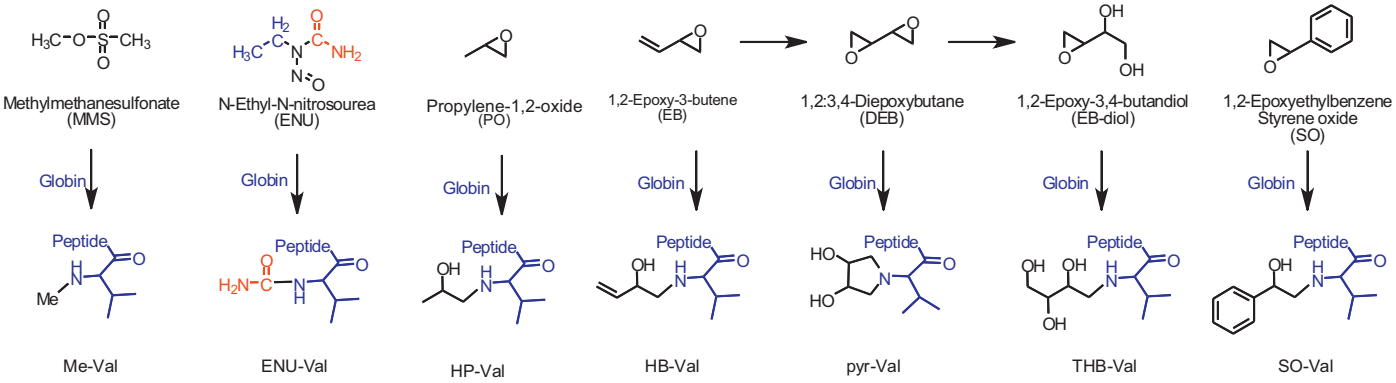

Fig. 2. Overview of selected model compounds and their corresponding N-terminal valine adducts.

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