K17 Covalent Protein Adduction by Drugs of Abuse

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After attending this presentation, attendees will glean some of the principles of covalent protein adduct formation, previous advances in generating and detecting protein adducts, how drugs of abuse form protein adducts, and the potential importance of protein adducts in the fields of clinical and forensic toxicology.

This presentation will impact the forensic science community by opening a new subset of toxicological analysis for clinical and forensic inquiries. It will allow for the development of new biomarkers of exposure to detect the use of addictive and illegal substances even after the parent compounds and major metabolites have been eliminated from biological samples. These persistent adducts can be employed to expand the range of time a substance can be detected in biological samples; a beneficial advance for both clinical and forensic applications.

Introduction: Protein adducts are formed by the covalent binding of an electrophilic, metabolically activated xenobiotic to nucleophilic sites on endogenous proteins or protein precursors. These permanent covalent bonds remain for the lifetime of the protein. However, not all nucleophilic sites on a given protein are equally reactive to activated xenobiotics. Certain amino acid residues are more susceptible to electrophilic attack, due to both steric and electronic factors. Each xenobiotic will interact with a given protein in a characteristic way determined by the combination of these factors.

While only a few studies have examined protein adduction by drugs of abuse, results suggest that this is a valuable issue to explore further. In this report, initial data is presented on such adducts by evaluating the relative binding affinity of several common drugs of abuse to amino acids known to be reactive under biological conditions. Initial research using a controlled *in vitro* exposure system is required prior to expanding studies into complex matrices and case studies involving biological samples from drug users. This initial step is germane to the understanding of how and where these drugs of abuse covalently bind to endogenous proteins and is imperative to the understanding of *in vivo* formation of protein adducts. This approach involved utilizing HPLC- MS, which allows for the sensitive and discriminating analysis of adducted peptide structure.

Methods: In order to generate protein adducts with drugs of abuse, an *in vitro* metabolic system was used. This system has previously been employed to assess hepatic metabolism of xenobiotics under controlled conditions. The system consisted of purified human cytochrome P450 3A4, human cytochrome b5, and human NADPH cytochrome P450 reductase in conjunction with required lipid cofactors (1,2-dilauroyl-sn- glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphocholine, and 1,2-dilauroyl-sn-glycero-3-phospho-L-serine). To each mixture, one of three test peptides was added: Ac-PAAHAA-OH, Ac-PAAKAA-OH, and Ac-PAACAA-OH. These short peptides allowed for the analysis of modifications of the reactive amino acid residue (His, Lys, and Cys, respectively). Each peptide was tested with each of three common substances of abuse: cocaine, morphine, and methamphetamine (each at 200 µM final xenobiotic concentration). Peptides were incubated at 37°C in 200 mM phosphate buffered saline (pH 7.4) for 15 - 60 min to allow for metabolic activation of the xenobiotics and adduction to the peptides. Following incubation, 10 µL aliquots of the mixtures were acidified with TFA and introduced into a Varian 1000 LC-ion trap MS equipped with Polaris C18 column and optimized for the analysis of the individual peptides.

Results: Stable, time-dependent covalent adduction of all three model peptides at varying efficiencies was noted for each drug. Adduct formation was confirmed by the appearance of new peptide peaks with MS molecular ion and fragmentation data consistent with covalently bound drug. MS/MS with *de novo* peptide sequencing results confirmed the location of adduct at the putative reactive site for each peptide. Additional MS studies are currently ongoing to identify the reactive metabolite of each drug and the chemical structure of peptide-bound drug moiety. Data will be presented detailing the relative binding affinities for each tested drug of abuse and peptide.

Conclusions: The data presented in this study demonstrates the ability of metabolically activated cocaine, morphine, and methamphetamine to form viable adducts with nucleophilic residues of model peptides. The analytical detection of these adducts in *in vitro* studies provides the groundwork for further studies of *in vivo* production and analytical detection of these adducts in biological specimens.

Protein, Adduct, Toxicology

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