Significance and Specific Aims:

The National Cancer Institute (NCI) estimates that 10-20% of all cancer patients are prescribed cisplatin. It is a first-line therapy for many solid-organ cancers, including testicular, lung, bladder, and cervical cancer, despite a panel of severe side effects (1). Cisplatin is known to induce gastrotoxicity, ototoxicity, myelosuppression, and dose-limiting nephrotoxicity (2). The most commonly observed nephrotoxicity is acute kidney injury, which presents in 20-30% of all patients treated with cisplatin though toxicity is unpredictable (3). Conventional treatment to reduce cisplatin-induced nephrotoxicity includes active hydration via saline drip supplemented with mannitol, however evidence of reduced renal damage remains unclear (4,5). Current cisplatin-induced nephrotoxicity research is focused on reducing the activation of pro-inflammatory cytokine, tumor necrosis factor α (TNF-α), among other cell signaling mediators (6). Alternative routes exist for cisplatin toxicity, including formation of a reactive thiol-cisplatin metabolite that is more cytotoxic than the parent compound. This metabolite is formed after conjugation to glutathione through the action of glutathione S-transferase P1 (GSTP1) and further metabolism by the mercapturic acid pathway (7). The cisplatin-mercapturic acid conjugate then undergoes β-elimination, catalyzed by cysteine S-conjugate β-lyase, releasing thiol-cisplatin (Figure 1) (8-10). The severity of cisplatin-induced nephrotoxicity in mice was directly correlated to the expression and activity of murine GSTP1 but not with total kidney platinum concentrations (11), suggesting that formation of thiol-cisplatin contributes more to nephrotoxicity than overall cisplatin exposure. A better understanding of this toxicity pathway will allow future research into the pharmacogenetics of cisplatin sensitivity.

Thiol-cisplatin is extremely cytotoxic and a substrate for intracellular methyltransferases, but it is unclear which enzymes catalyze the methylation reaction. It is thought that genetic variation in drug-metabolizing methyltransferases may be responsible for the variable cisplatin sensitivity observed in the general population. Polymorphisms in thiopurine methyltransferase (TPMT) and catechol O-methyltransferase (COMT) are associated with extremely high odds ratios of cisplatin-induced toxicities and can predict cisplatin toxicity in half of patient populations, supporting genetic control of cisplatin sensitivity (12-14). Unfortunately, prior genetic screening was limited to known polymorphisms meaning that human thiol-methyltransferase (TMT) was not investigated as the gene is unidentified. TMT catalyzes the methylation of aliphatic thiol compounds and shows highly variable, yet heritable, inter-individual activity (15). Our lab has discovered that methyltransferase-like protein 7B (METTL7B) catalyzes the TMT-specific methylation of captopril. We believe that METTL7B will methylate thiol-cisplatin and that inter-individual differences in METTL7B activity can account for the unexplained variability in cisplatin sensitivity. In vitro validation of TPMT-, COMT-, and METTL7B-mediated methylation of thiol-cisplatin will enable further pharmacogenetic research, more accurate screening protocols, and safer cisplatin dosing regimen.

We hypothesize that thiol-cisplatin is primarily cleared by methylation to a methyl thioether metabolite and that this reaction reduces cisplatin nephrotoxicity. We further hypothesize that COMT, TPMT, and METTL7B are responsible for this reaction. Preliminary studies in our lab have shown that METTL7B catalyzes methylation of exogenous aliphatic thiols and is expected to methylate thiol-cisplatin as well. We will test our hypothesis through the specific aims below.

Aim 1. Determine kinetic parameters of COMT-, TPMT-, and METTL7B-dependent thiol-cisplatin methylation in vitro. A liquid chromatography-mass spectrometry (LC-MS/MS) method will be developed for the sensitive and selective quantification of thiol-cisplatin and its methylated metabolite. This method will be used to determine pertinent kinetic parameters for the methylation of thiol-cisplatin by recombinant COMT, TPMT, and METTL7B.

Aim 2. Examine the effects of altered COMT, TPMT, and METTL7B expression on cisplatin toxicity in HEK293T and primary human renal cells. Overexpression plasmids, lentiviral transduction kits, siRNA, and lipofection reagents are available commercially to facilitate modulation of gene expression in mammalian cells. Effects of gene expression changes on cisplatin LD₅₀ will be measured and compared to kinetic parameters collected in Aim 1.
Innovation:  
This proposal represents novel in vitro validation of the importance of thiol-cisplatin on nephrotoxicity. The data collected could lead to new therapeutic and screening approaches by better defining modulators of cisplatin toxicity for future pharmacogenetic research. Additionally, this project represents further characterization of the substrate specificity and kinetic parameters of METTL7B. As this enzyme has only recently been shown to be involved in thiol drug metabolism, it is important to determine the extent to which it impacts the metabolism of commonly used pharmaceuticals.

Research Plan:

Aim 1.  
1a. Measurement of thiol-cisplatin methylation by LC-MS/MS. Standards of thiol-cisplatin and the methylated metabolite will be synthesized and purified or bought from a commercial source prior to assay development. If possible, a deuterated form of the methylated metabolite will be procured to act as an internal standard. Initial mass spectrometric and chromatographic parameters will be sourced from relevant literature (16) and optimized for use on the instruments in the University of Washington School of Pharmacy Mass Spectrometry core facility. We will also create a standard curve for the methylated metabolite to allow quantitation. If LC-MS/MS is not suitable for measuring the analyte of interest, alternative methodologies include UPLC-Vis which may be more suitable for unstable compounds than ionization.

1b. Determination of enzyme activity assay parameters. Recombinant COMT and TPMT will be purchased from commercial sources and assayed for activity using known substrates. Recombinant METTL7B will be expressed and purified from induced E. coli lysates using protocols developed in-house. Assay conditions will be optimized for each enzyme to ensure linear metabolite formation with respect to protein concentration and time. Additionally, multiple quench and extraction conditions will be tested to maximize assay sensitivity. Each experiment will be conducted in triplicate and on multiple days to account for inter-day variability.

1c. Measurement of kinetic parameters. Using the assay parameters developed in Aim 1b, Michaelis-Menten kinetic curves will be developed for all three enzymes of interest. Thiol-cisplatin concentration ranges will be chosen to ensure equal coverage above and below K_m and saturation at V_max. The data will be fit to a square hyperbolic curve and analyzed using GraphPad to obtain K_m and V_max values. Potential substrate inhibition or allosteric effects will be determined via Eadie-Hofstee plots and additional experiments will be conducted if needed. A buffer control will be included at all thiol-cisplatin concentrations to account for possible non-enzymatic methylation as this is a known phenomenon with SAM-dependent methyltransferases.

Aim 2.  
2a. Development of overexpression cell lines. Commercial lentiviral overexpression particles will be purchased from Origene for COMT, TPMT, and METTL7B. HEK293T cells will be transduced with the lentiviral particles and stable cell lines will be generated using published protocols (14). Upon development of stable cell lines, cell stocks will be prepared and kept in liquid nitrogen for future use. All future experiments will be conducted between three and five passages post-reconstitution to minimize expression changes.

2b. Optimization and validation of gene expression modulation. Primary renal proximal tubule cells will be purchased from a single ATCC lot and gene-specific siRNA will be purchased from Origene. Gene knockdown protocols will be optimized for time and siRNA concentration using the primary cells. Gene and protein expression knockdown will be analyzed via RT-PCR and western blot. Similarly, all overexpressing cell lines created in Aim 2a will be assayed for overexpression of the gene and enzyme of choice by RT-PCR and western blot. All experiments will be conducted in triplicate and on multiple days.

2c. Measurement of changes in cisplatin toxicity. Initial cisplatin survival curves will be created using untreated HEK293T and primary renal cells to determine baseline LD_50 values. Subsequently, the overexpression cell lines created in Aim 2a and siRNA-treated primary renal cells will be treated with cisplatin to determine experimental LD_50 values. Shifts in LD_50 values will be compared to changes in expression of COMT, TPMT, and METTL7B. Cell viability will be assayed using the MTT assay. All experiments will be conducted in triplicate and on multiple days.

Expected Outcomes: This project will confirm in vitro enzymatic methylation of thiol-cisplatin by COMT, TPMT, and METTL7B. Based on known substrate specificity data, we expect the relative rates of methylation in the following order: METTL7B>>TPMT>>COMT. The amount of protection observed in overexpressing cells should follow a similar order, assuming equal levels of expression. Overall, the information gained from this project will help reduce rates of nephrotoxicity in cancer patients and allow new research on the underlying mechanisms of cisplatin toxicity.
Works Cited:


