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The use of urinary and kidney SILAM proteomics to monitor kidney response to high dose morpholino oligonucleotides in the mdx mouse

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ABSTRACT

Phosphorodiamidate morpholino oligonucleotides (PMO) are used as a promising exon-skipping gene therapy for Duchenne muscular dystrophy (DMD). One potential complication of high dose PMO therapy is its transient accumulation in the kidneys. Therefore new urinary biomarkers are needed to monitor this treatment. Here, we carried out a pilot proteomic profiling study using stable isotope labeling in mammals (SILAM) strategy to identify new biomarkers to monitor the effect of PMO on the kidneys of the dystrophin deficient mouse model for DMD (mdx-23). We first assessed the baseline renal status of the mdx-23 mouse compared to the wild type (C57BL10) mouse, and then followed the renal outcome of mdx-23 mouse treated with a single high dose intravenous PMO injection (800 mg/kg). Surprisingly, untreated mdx-23 mice showed evidence of renal injury at baseline, which was manifested by albuminuria, increased urine output, and changes in established urinary biomarker of acute kidney injury (AKI). The PMO treatment induced further transient renal injury, which peaked at 7 days, and returned to almost the baseline status at 30 days post-treatment. In the kidney, the SILAM approach followed by western blot validation identified changes in Meprin A subunit alpha at day 2, then returned to normal levels at days 7 and 30 after PMO injection. In the urine, SILAM approach identified an increase in Clusterin and γ-glutamyl transpeptidase 1 as potential candidates to monitor the transient renal accumulation of PMO. These results, which were confirmed by Western blots or ELISA, demonstrate the value of the SILAM approach to identify new candidate biomarkers of renal injury in mdx-23 mice treated with high dose PMO.

Abbreviations: 5-OPase, 5-oxoprolinase; Aass, alpha-aminoadipic semialdehyde synthase; AKI, acute kidney injury; AMY2, pancreatic amylase α2; Anx2, annexin 2; AP-A, glutamyl aminopeptidase; AP-N, aminopeptidase N; CI-B22, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9; Cytcox, cytochrome c oxidase subunit 2; DMD, Duchenne muscular dystrophy; EGF, pro-epidermal growth factor; FABPH, fatty acid binding protein heart type; PK, pharmacokinetics; GGT1, gamma glutamyltransferase 1; H&E, hematoxylin and eosin; IP2, integrated proteomics pipeline; KIM-1, kidney injury molecule-1; mAspAT, mitochondrial aspartate aminotransferase; Mep-1, Meprin A subunit alpha; NGAL, neutrophil gelatinase-associated lipocalin; OPN, osteopontin; PAS, periodic acid shift; PCB, mitochondrial pyruvate carboxylase; PMO, phosphorodiamidate morpholino oligonucleotide; PSs, phosphorothioates; SILAM, stable isotope labeling in mammals; SUn, serum urea nitrogen.

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1. Introduction

Therapeutic oligonucleotides have received sustained interest due to the high specificity to their mRNA or microRNA targets, and effectiveness in inhibiting their target [1,2]. Multiple chemistries are under development with goals of optimizing cellular delivery, affinity and specificity for the target. As with most drugs, toxicity findings may limit the doses of the oligonucleotides that can be safely administered. Acute inflammatory reactions can be a dose-limiting toxicity, but are observed with some chemistries such as phosphorothioates (PSs) [3–5], but not others such as phosphorodiamidate morpholino oligonucleotides (PMO) [6]. Target organs for oligonucleotide drugs typically include the liver and kidney, where these drugs accumulate causing cellular toxicity prior to being metabolized and/or excreted by these tissues [7–9].

The large majority of oligonucleotide drugs utilize a PSs chemical linkage between adjacent nucleotides. This linkage has been shown to improve stability by reducing cleavage by nucleases. PSs drugs also show strong pharmacokinetic and pharmacodynamic profiles [10]. However, PSs drugs may also show off-target protein binding and induction of complement or other inflammatory pathways. The PMO chemistry seems entirely resistant to nuclease cleavage, and shows no induction of inflammatory pathways at very high doses [6,11,12].

Clinical development of PMO chemistry is most advanced in applications to exon-skipping in Duchenne muscular dystrophy (DMD) [13–15]. DMD is caused by dystrophin deficiency in myofibers, leading to plasma membrane instability. Overt breaches of the dystrophin-deficient myofiber membranes appear to provide a delivery route for the PMO drugs, thus bypassing the otherwise rapid clearance [16,17]. Pre-clinical studies conducted in murine and canine models for DMD showed that systemic IV injection of PMO drug successfully restored the missing dystrophin protein in skeletal muscle with concomitant improved functional outcomes [18–21]. Both biochemical and clinical efficacy is dose responsive, with high doses (100–300 mg/kg/wk.) showing the most pronounced long-term therapeutic effects, a finding likely related to the presumed bulk flow process of drug delivery across unstable myofiber membranes.

Previous toxicity studies performed on the dystrophin deficient mouse model for DMD (mdx-23) and monkeys [12], showed that weekly injections of high doses of PMO (960 mg/kg/wk in mice; 320 mg/kg/wk in monkeys), can induce transient renal tubular injury. PMO is accumulated in renal proximal tubular cells, in a similar manner to other oligonucleotide drugs [6]. From the renal histological view, the accumulation of PMO in renal tubules is associated with flattened tubular epithelium, basophilic granules, and mild tubular vacuolation [6,12]. These lesions, however, are considered dose-dependent and potentially reversible [12]. Therefore, because PMO appears a promising treatment for patients with DMD, it is necessary to further evaluate the effect of PMO on kidneys and identify new urine biomarkers to monitor the renal PMO load and outcome for DMD patients [22].

Urine is a readily accessible body fluid and useful source for biomarker discovery and assessment of the renal effects of cytotoxic drugs [23–27]. Here, we carried out a pilot proteome profiling study to follow the renal outcome of the mdx-23 mouse after a single intravenous injection of high dose PMO (800 mg/kg). More specifically, we analyzed kidney and urine proteomes collected from PMO and saline treated mdx-23 mice using SILAM strategy in combination with high precision LC–MS/MS analysis of peptides, and bioinformatics mapping to parent proteins (supplemental Fig. S4). Subsequently, we verified the positive findings by Western blots or ELISA assay using all mouse groups. This approach identified an alterations in the levels Meprin A subunit alpha (Mep-1) in the kidney and an increase of two urine accessible candidate biomarkers clusterin, and gamma glutamytransferase 1 (GGT1) (Fig. 1), that may become a useful biomarker profile to monitor the renal outcome of patients treated with PMO and other oligonucleotides.

2. Material and methods

2.1. Animal experiments

All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Children's National Medical Center (protocol # 199-01-07 for ^{15}N labeled SILAM [Stable isotope labeling by amino acids in mouse]; protocol # 304-13-04 for toxicity test of PMO oligonucleotide therapy).

The animal models used in this study included the mdx-23 mouse model having a spontaneous *DMD* gene mutation causing a splice site mutation in exon 23 on the C57BL/10 background (C57BL/10ScSn-Dmdmdx/J). Wild-type background strain (C57BL/10) was used as baseline control. All mice were purchased from Jackson Laboratory, Bar Harbor, Maine.

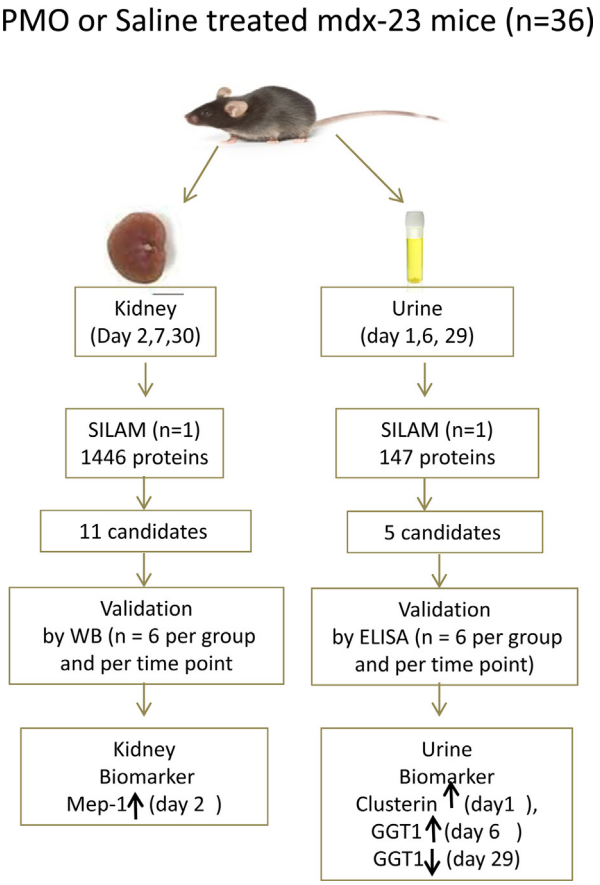

Fig. 1. Overall study design. Mdx23 (n = 36) randomly divided two groups, one group (n = 18) was treated with a single high dose (800 mg/kg) and the second group (n = 18) was treated with the same volume of saline vehicle. Urine samples at days 1, 6, and 29 urine while kidneys were collected at days 2, 7 and 30 following treatment (n = 6 per group and per time point). For SILAM proteome profiling of urine and kidney samples one mouse was randomly selected from PMO and saline treated group and at each time point totaling 6 urine samples and 6 kidney samples for LC–MS/MS analysis. Protein that were found differentially altered in kidney and urine samples were further validated by western blotting for kidney extract and ELISA for urine samples (samples size n = 6 per group per time point); also urine candidates validated by ELISA (samples size n = 6 per group per time point). Mep-1 increased day 2 as a, potential biomarker in kidney and clusterin increased day 1 and GGT1 increased day 6, decreased at day 29 as potential urinary biomarkers which associated with PMO kidney loading and clearance.