Assessing *in vivo* and *in vitro* stability of a pretargeted, bioorthogonal anti-sense oligonucleotide using click chemistry tools, a one-step SPE, and LC-MS/MS

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Purpose

By exploiting the rapid inverse electron-demand Diels-Alder (IEDDA) reaction between *trans*-cyclooctene (TCO) and tetrazine (Tz), Biogen has used a TCO-modified antisense oligonucleotide (ASO) in a pretargeted imaging strategy to visualize the distribution of ASO in CNS tissue. However, TCO groups are known to convert to cis-cyclooctene (CCO), an isomer that does not react with Tz; in this vein, the *in vivo* stability of ASO-TCO is unknown. Due to the nature of cis-trans isomerism and the inherent challenges of ASO bioanalysis, LC separation is not practical in stability assessments. In this poster, we employ methyltetrazine dibenzocyclooctyne (MeTz-DBCO) as a clickable probe to tag ASO-TCO and distinguish it from ASO-CCO. Both the unreacted ASO-CCO and the reacted conjugate can be extracted from samples using a one-step SPE and analyzed via LC-MS/MS.

Methods

ASO-TCO and ASO-TCO-MeTz-DBCO conjugates were evaluated for charge-state distribution with a precursor ion scan in a tuning-by-injection approach; the same approach was applied to optimize MRM parameters. The molar ratio of reactants was determined by titration. Completeness of the reaction was confirmed by comparing against removal of ASO-TCO from solution using magnetic bead immobilization. The SPE method was optimized across a range of wash and elution conditions using Phenomenex Clarity OTX plates. Extracted samples were separated on an AB Sciex ExionLC with a Phenomenex Clarity Oligo-MS column. Detection was carried out on an AB Sciex QTRAP 6500+. Data were processed using Analyst software.

Results

Our work began with optimization of the LC-MS/MS conditions for separation and detection of ASO-TCO. The compound was injected onto an ion-pair reversed-phase system and analyzed for charge state distribution using a precursor ion scan of 94.9 Da, which represents the phosphorothioate backbone. The most abundant charge states were selected and selection of product ions provided MRMs, which were tuned-by-injection and gave robust signal concurrent with good specificity. The optimized SPE method required a more basic elution buffer than we had used historically, owing to the properties imparted by the TCO group. After adjusting the pH of the elution buffer, recovery increased from 2 – 7% to 28 – 35%. The completeness of the click reaction was tested by titration of ASO-TCO against increasing concentrations of MeTz-DBCO; the result was confirmed by a similar titration against MeTz immobilized to biotinylated magnetic beads. We found that a 10-fold molar excess of MeTz groups was sufficient to react with ASO-TCO in solution, and that approximately 30% of ASO related material in our standards was not reactable. This procedure was applied to samples from *in vivo* and *in vitro* studies to assess the stability of ASO-TCO in mouse brain, artificial CSF, FBS, and PBS. As expected, ASO-TCO had a shorter half-life than ASO-CCO under all test conditions. Notably, in PBS and artificial CSF, in which total ASO is stable, the disappearance of ASO-TCO aligned with appearance of ASO-CCO. In the brains of live mice, ASO-TCO and ASO-CCO had half-life values of 3.3 and 4.7 days, respectively.

Conclusions

By modifying the pH of the SPE elution buffer, a TCO-modified ASO can be extracted from biological samples in one step. The eluent sample can then be reacted with an excess of MeTz-DBCO to form ASO-TCO-MeTz-DBCO conjugate while leaving ASO-CCO intact. The signal intensities of conjugate and ASO-CCO can be compared across time points to determine rate constants for ASO-TCO and ASO-CCO, allowing for a half-life calculation.