Identification of reversible and irreversible covalent inhibitors using a novel GSH reactivity assay

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Introduction

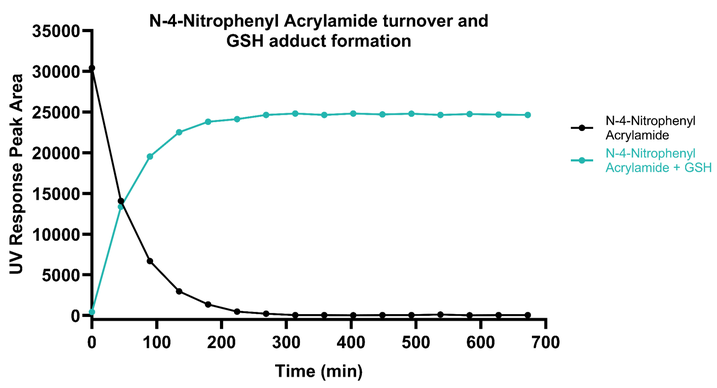
Covalent inhibitors have recently gained increased attention in drug discovery. Glutathione (GSH) can be used to assess the reactivity of cysteine-targeting covalent inhibitors, measuring the reactivity between an inhibitors' electrophilic "warhead" and nucleophilic cysteine residues, aiding the fine-tuning of inhibitors' reactivity. Most covalent inhibitors are irreversible, forming a permanent covalent bond with their target, whereas reversible covalent inhibitors form temporary covalent bonds with their targets.

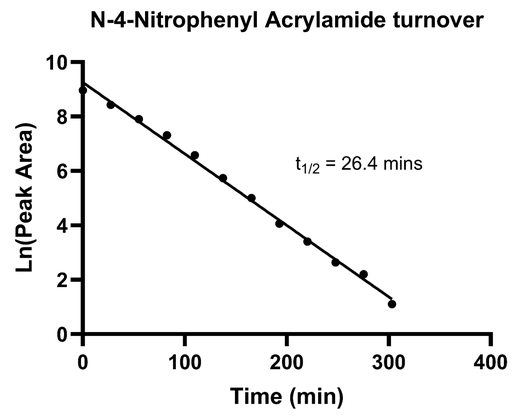
Here we present our novel GSH reactivity assay, which is optimised to identify a reversible reaction between GSH and a covalent inhibitor by monitoring the equilibrium between parent and adduct, and assessing adduct depletion and parent re-formation due to the release of GSH.

Routine GSH Reactivity Assay

Compound concentration	1.67 µM
Final solvent concentration	<0.2% DMSO
Positive control	N-nitrophenyl acrylamide
Incubation duration	8 hours*
Analysis	LC-MS with UV-DAD
Compound requirements	50 µL of 10 mM DMSO stock or 1 mg of solid material
Deliverables	half-life ($t_{1/2}$), presence of GSH adduct (Y/N), PBS stability (%)

- Our well-established GSH reactivity assay monitors for parent compound depletion and adduct formation in real time, allowing the determination of compound half-life and an assessment of compound stability.
- Compounds are incubated with GSH at 37 ºC and real-time kinetics data of multiple injections generated using LC-MS with UV-DAD.

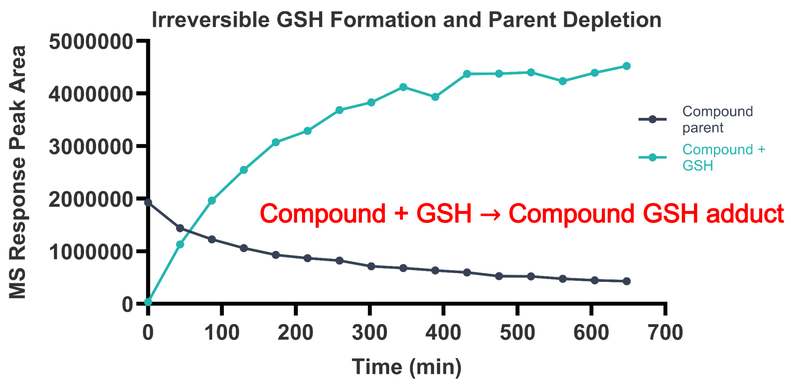




Exemplary real-time kinetics data generated for the assay control compound N-4-Nitrophenyl Acrylamide using the UV response to determine the area under the curve. Formation of the GSH adduct and depletion of the compound parent over time (left). Turnover of N-nitrophenyl acrylamide parent over time determining the half-life of the compound parent (right).

Irreversible Covalent Inhibitors

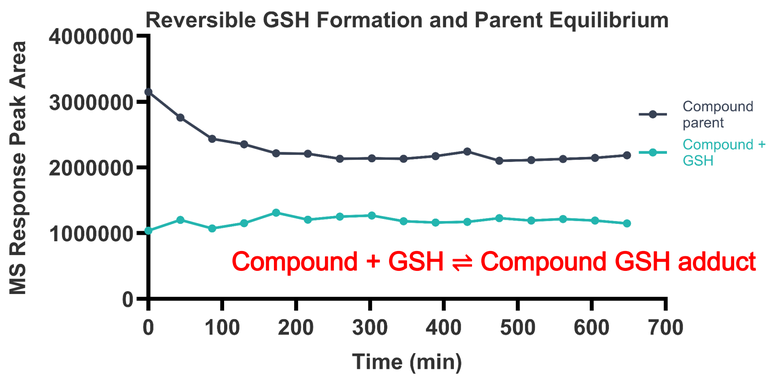
- Irreversible covalent inhibitors form permanent covalent bonds with their targets leading to desired effects such as long-lasting inhibition. However, they also pose significant issues such as off-target toxicity.
- GSH adduct formation will be permanent, with adduct formation increasing simultaneously to compound parent depletion.



Exemplary data for an irreversible covalent inhibitor incubated with GSH, resulting in formation of GSH adduct with a permanent covalent bond as compound parent depletes.

Reversible Covalent Inhibitors

- Reversible covalent inhibitors form temporary bonds allowing for the release from off-target proteins, therefore limiting the potential toxic effects.
- Due to the reversible covalent bonds formed, an equilibrium between GSH adduct and parent compound forms using the routine GSH reactivity assay.

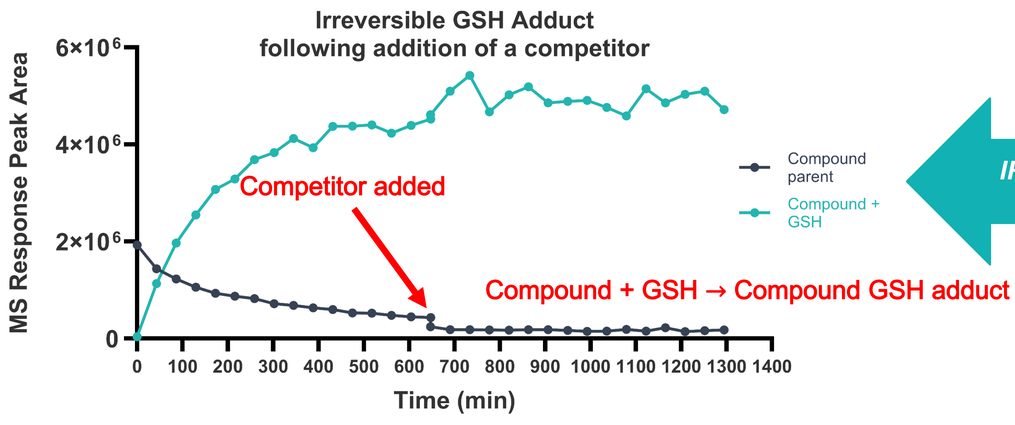


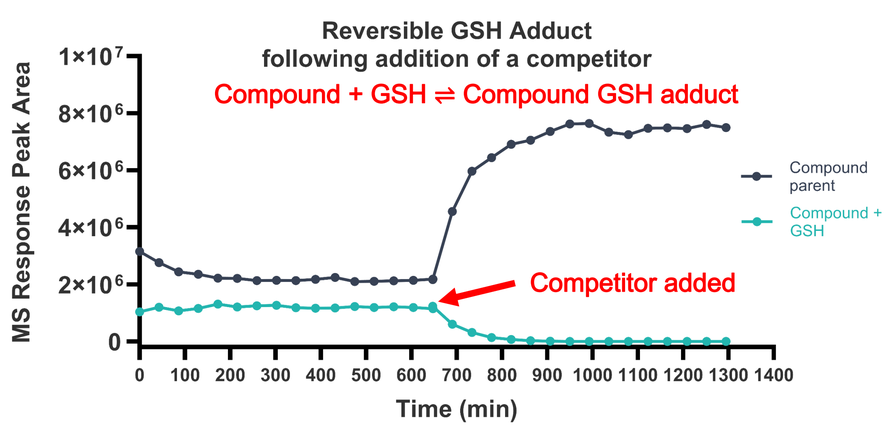
Exemplary data for a reversible covalent inhibitor incubated with GSH, resulting in an equilibrium forming between GSH adduct and compound parent as a temporary covalent bond is formed between inhibitor and GSH.

IRREVERSIBLE vs REVERSIBLE

Novel GSH Assay to Confirm Reversible Covalent Inhibitors

- Following the incubation of compound with GSH, an irreversible covalent inhibitor can be added to the reaction mixture as a competitor to assess the presence of a reversible reaction. This will scavenge any excess GSH as well as any GSH released from a reversible reaction.
- For an irreversible covalent inhibitor, the addition of a competitor does not change the profile, with the levels of GSH adduct and parent compound remaining the same.
- For a reversible covalent inhibitor, GSH will be released from the inhibitor due to the temporary bond, resulting in depletion of GSH adduct and an increase in compound parent.





Exemplary real-time kinetics data for the novel GSH assay. At the start of the reaction compound is incubated with GSH, leading to formation of GSH adduct and depletion of compound parent for the irreversible inhibitor (left) and an equilibrium forming between GSH adduct and compound parent for the reversible inhibitor (right). Addition of a competitor leads to no change to the levels of GSH adduct or compound parent for the irreversible inhibitor (left), however results in the depletion of GSH adduct and re-formation of compound parent for the reversible inhibitor (right) due to the temporary covalent bond. Due to selectivity, mass spec had to be used, resulting in variable responses for compound parent and adduct, therefore data must be treated with caution and should only be used qualitatively.

Conclusions and Next Steps

- Our well-established GSH reactivity assay monitors parent depletion and adduct formation, determining compound half-life and compound stability as well as identifying between reversible and irreversible covalent inhibitors.
- Our novel GSH reactivity assay provides an invaluable tool to assess both a compound's reactivity and the reversibility of the reaction.
- Further development is required to be able to quantify kinetic parameters for the reversible reaction between GSH and covalent inhibitors.

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