

Abstract

Fluorescence lifetime technology (FLT) is considered an attractive assay technology due to minimized assay interference caused by compounds or assay reagents. Recently the field was largely improved by the introduction of fluorophores with appropriately long lifetimes. This has led to the development of new approaches to measure enzyme activity through changes in fluorescence lifetime. Together with the availability of new FLT readers, the setting for new kinase, phosphatase, protease, and most recently histone methyl transferase (HMT) FLT assays (Almac FLEXYTE™ assay platform) are now feasible.

Here we present

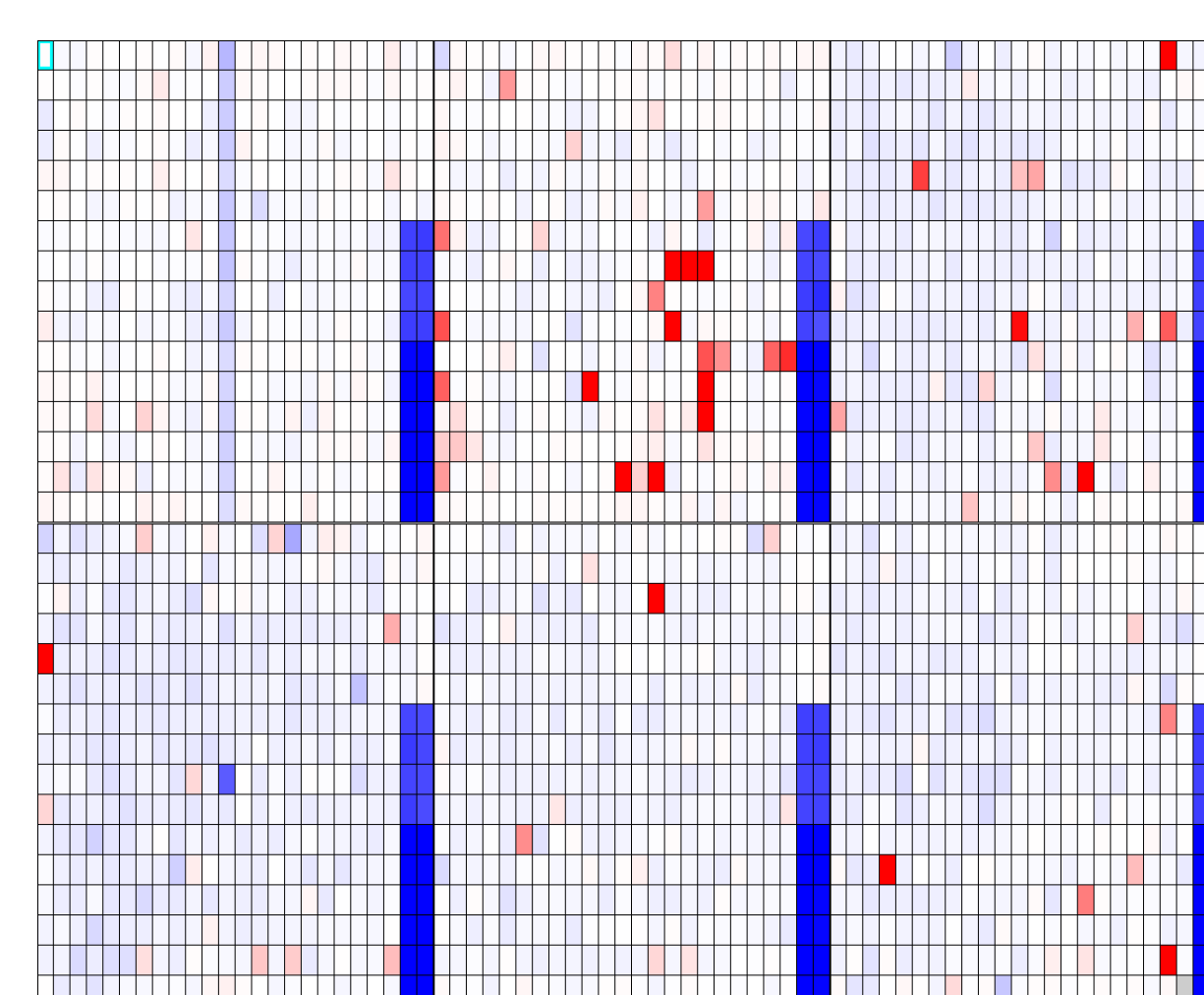
- assay development of a FLEXYTE FLT assay for the HMT G9a
- assay validation by potency determination of SAM (Sinefungin and SAH) and histone substrate binding competitors (BIX-01294)
- the impact of different histone substrates on assay sensitivity

A subset of 2,112 compounds from the BioFocus compound library was specifically selected by virtual screening approaches against the HMT G9a. The compound set was screened against G9a in the FLEXYTE HMT FLT assay. Compound activities were compared to screening data that were obtained by orthogonal assay technologies (Caliper LC3000 mobility shift assay (MSA) and a radioactive FlashPlate (FP) assay). Although the overall hit rate was lower in the LC3000 MSA assay and the FLT assay when compared to the FlashPlate assay, overlapping hits were identified and initial MOA studies were performed. In summary, the results show FLT to be a highly attractive assay technology for drug discovery in the epigenetic field.

Compound selection and G9a Mini screen

- 2,112 compounds out of the entire BioFocus compound collection comprising ~870,000 compounds were selected using complementary virtual screening (VS) approaches
- Structure-based virtual screening (SBVS)
 - G9a X-ray crystal structure (PDB 3nni) was used
 - Docking (using Glide; Schödinger Inc.) in SAM binding site + post processing
- Ligand-based virtual screening (LBVS)
 - Cheminformatics-based hit expansion methods:
 - Pharmacophore tree (using Discngine)
 - SAH and two reference compounds (BIX-01294 and UNC0224) were used as query structures for the LBVS approaches

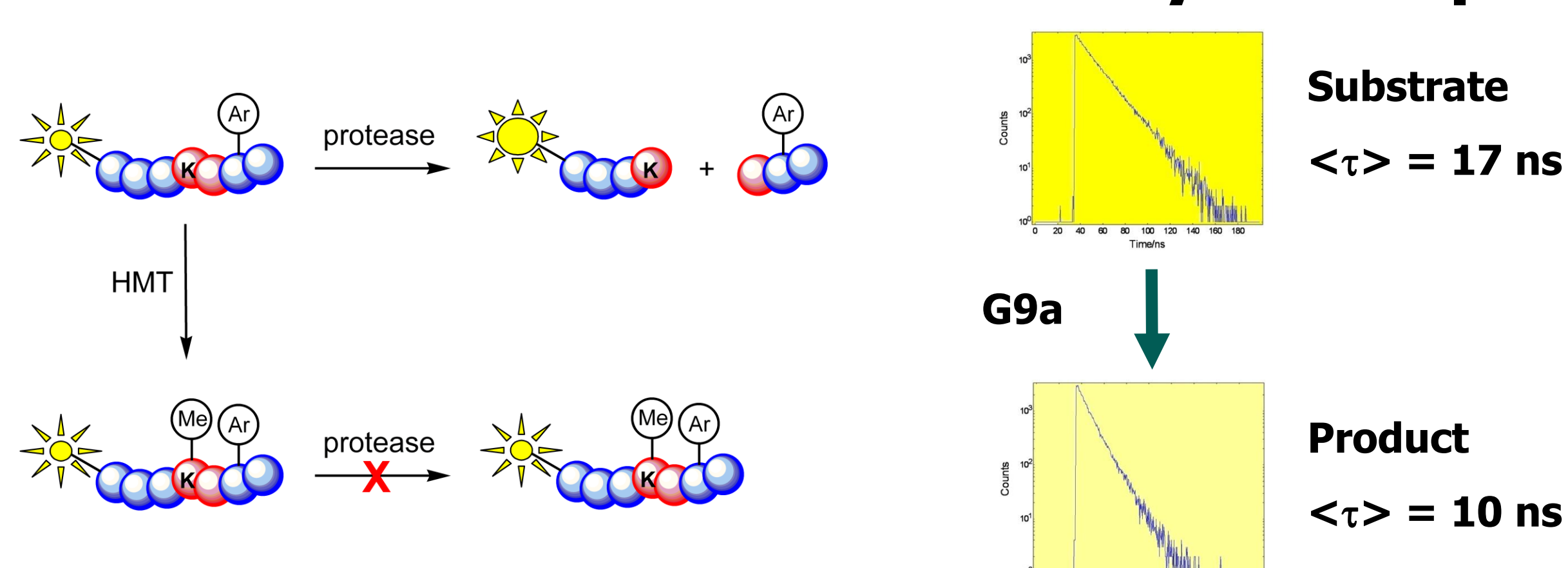
Testing of a subset of the BioFocus library



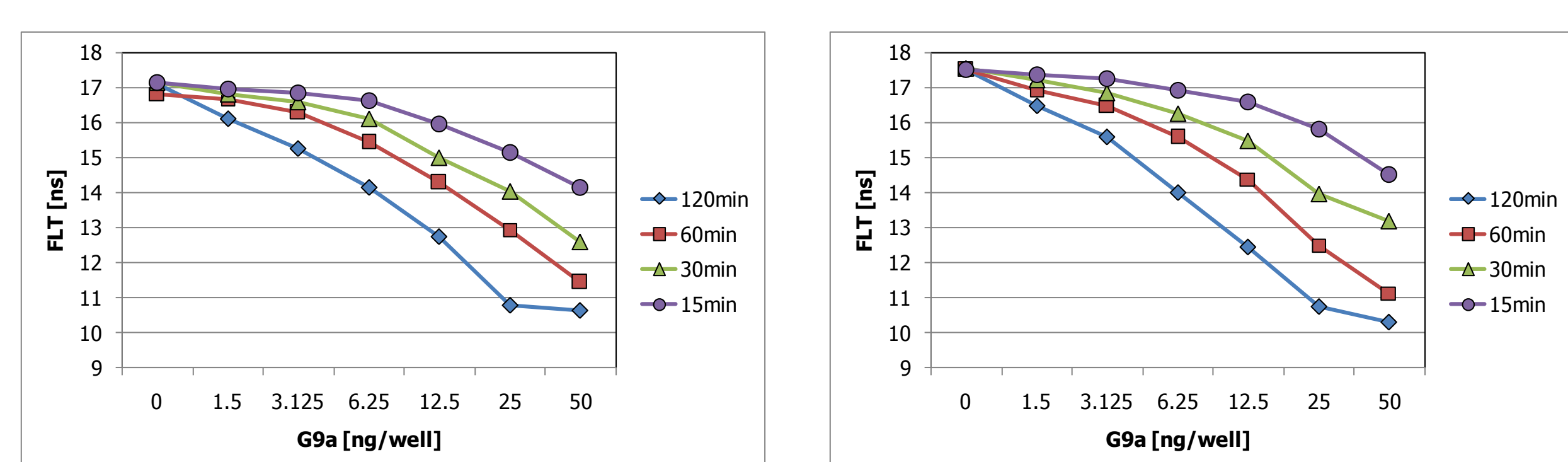
| Plate # | Z' | Mean T (100% Refs.) | Mean T (0% Refs.) | Mean T SAH controls | C.V. (100% Refs.) | C.V. (0% Refs.) | C.V. SAH controls |
|----------|------|---------------------|-------------------|---------------------|-------------------|-----------------|-------------------|
| 84181001 | 0.91 | 14.14 | 17.08 | 16.33 | 0.36 | 0.23 | 0.26 |
| 84181002 | 0.81 | 14.19 | 17.13 | 16.39 | 1.01 | 0.24 | 0.74 |
| 84181003 | 0.86 | 14.24 | 17.13 | 16.38 | 0.53 | 0.37 | 0.46 |
| 84181004 | 0.86 | 14.21 | 17.13 | 16.33 | 0.64 | 0.28 | 0.45 |
| 84181005 | 0.88 | 14.10 | 17.08 | 16.30 | 0.46 | 0.34 | 0.22 |
| 84181006 | 0.85 | 14.18 | 17.09 | 16.33 | 0.50 | 0.43 | 0.26 |

Compound activity heat maps (left) and assay plate statistics (Table) of G9a FLT screen. Final screening conditions were 10 μM compound concentration, 15 ng/well G9a (8 nM), 1 μM Pep G9a-1, 3.5 μM SAM and 120 min reaction time. 5 μM SAH were used as sensitivity control on each assay plate.

Almac FLEXYTE G9a HMT assay development

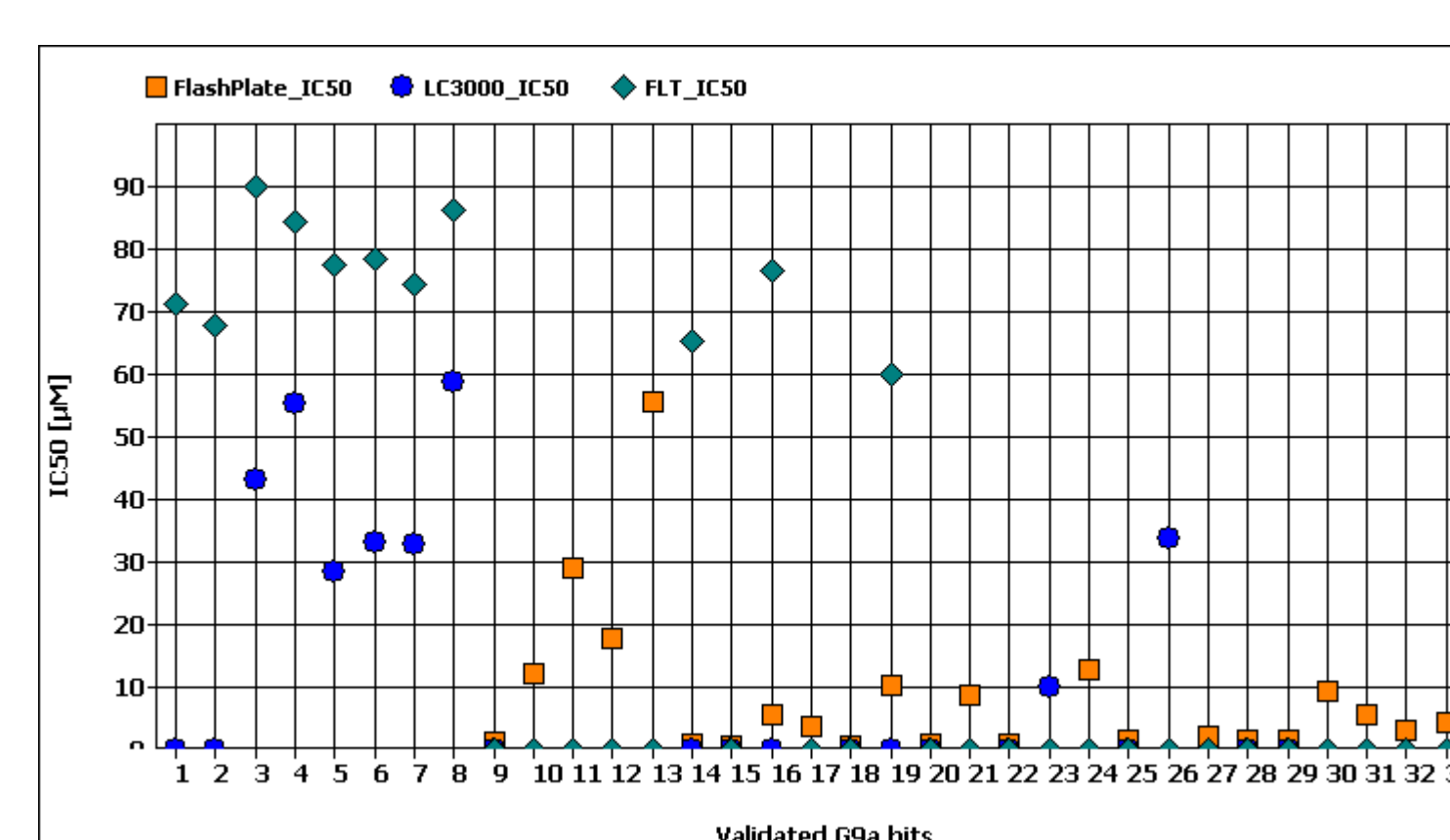


FLEXYTE FLT assay principle for G9a assay: 9-aminoacridine (9AA) lifetime is modulated by presence of aromatic moiety in the uncleaved peptide substrate. Histone H3Lys9 methylation prevents recognition and cleavage by protease EndoLysC. G9a activity is reported by a decrease in the measured fluorescence lifetime.



Reaction linearity of FLEXYTE G9a HMT assay. G9a (BPS Bioscience) was titrated in the presence of 1 μM peptide G9a-1 (peptide substrate based on H3 residues 6-12; left) and peptide G9a-2 (peptide substrate based on H3 residues 1-13; right) and 5 μM SAM. The reaction was measured at several time points using the NanoTaurus™ FLT reader (Edinburgh instruments).

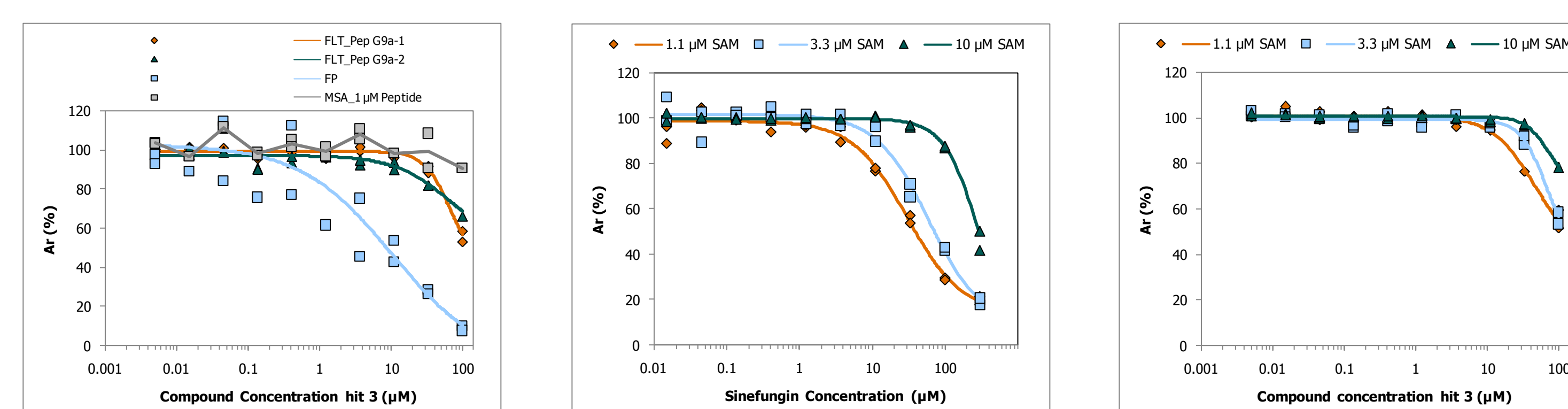
G9a mini screen hit validation



| Validated G9a hits | |
|-------------------------------|----|
| FlashPlate hits | 23 |
| FLT hits | 11 |
| LC3000 hits | 8 |
| Overlap | |
| FLT and LC3000 | 6 |
| FlashPlate and FLT | 3 |
| LC3000 and FlashPlate | 0 |
| FLT and LC3000 and FlashPlate | 0 |

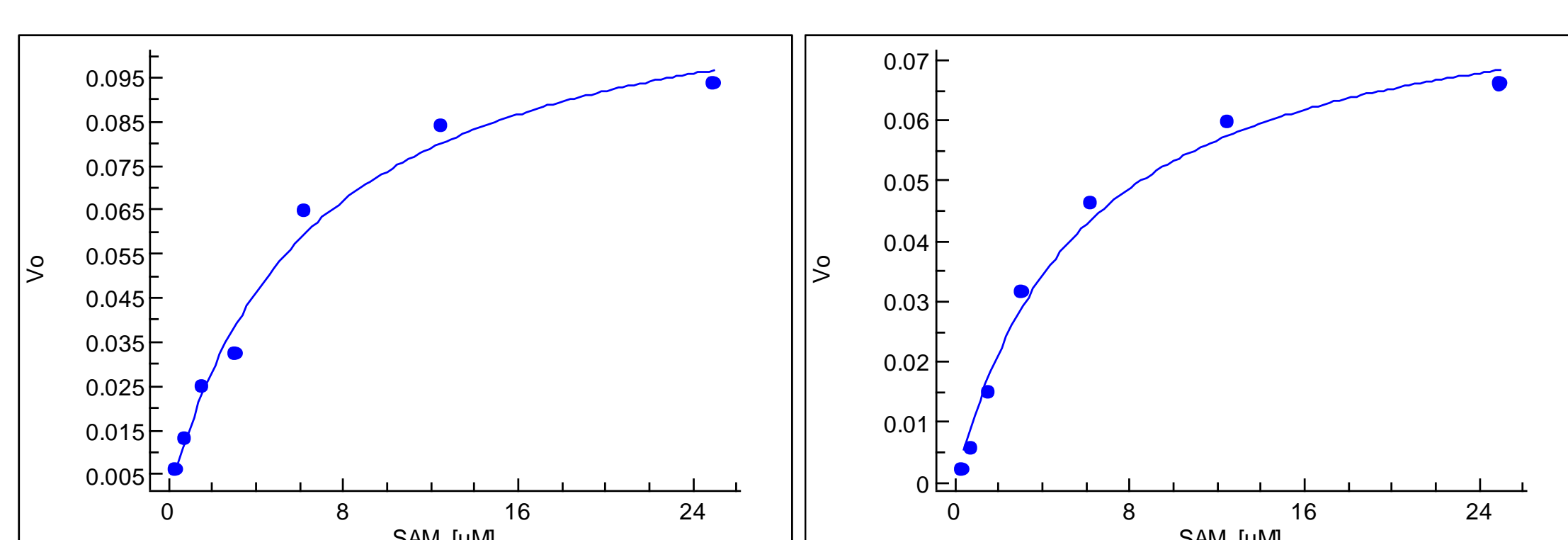
IC50 values (left) and overlap (Table) of validated hits from the FLT, LC3000 MSA and FlashPlate mini screens were compared. The overlap of active compounds between orthogonal assay technologies was small. Potencies of newly identified inhibitors in the LC3000 and FLT G9a assays all were in the μM range.

G9a hit characterization: SAM competitive compounds

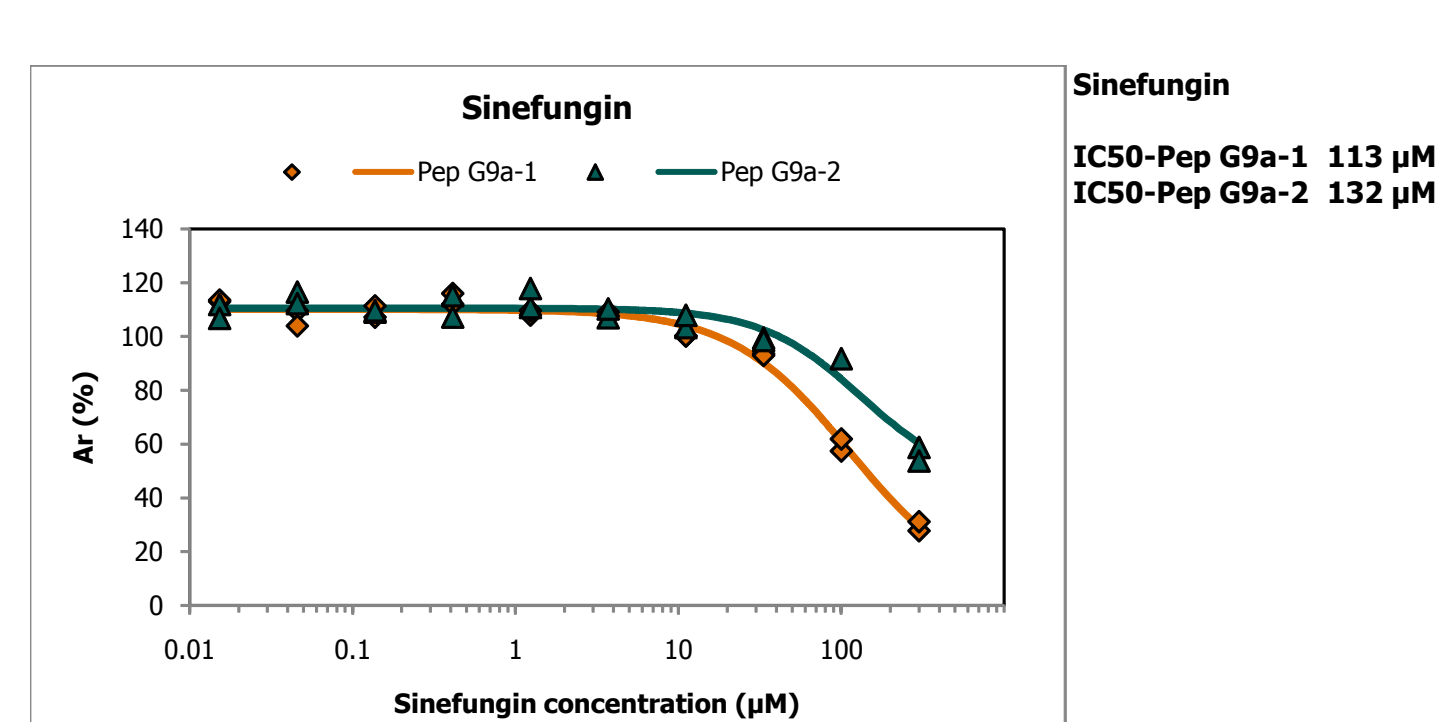
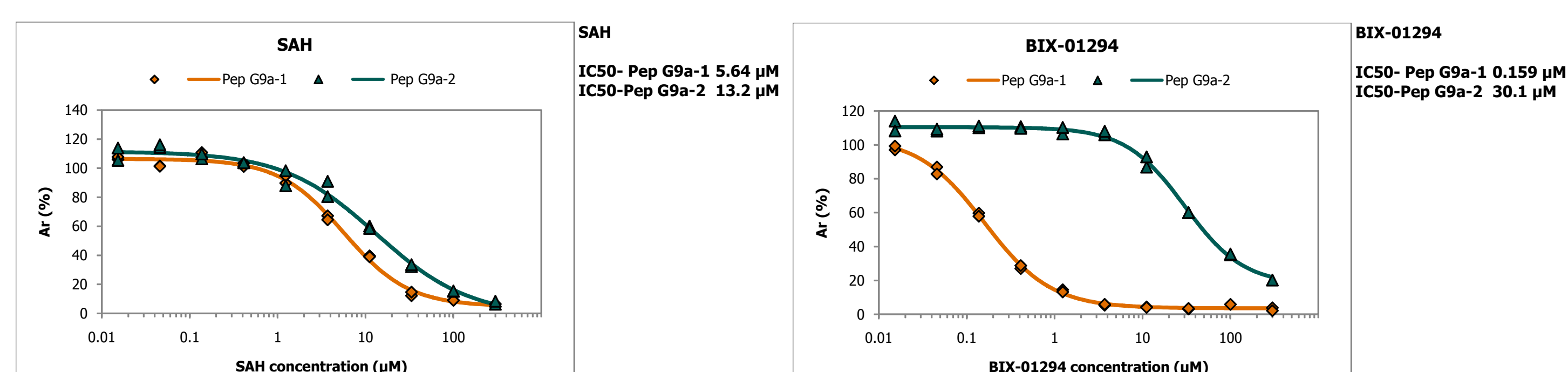


G9a inhibitors in the FlashPlate miniscreen (light blue, left graph) showed better potencies when compared to FLT and MSA assays. Binding MOA was assessed for Sinefungin in the FLT assay (middle). Potencies of hit compound 3 decreased in the FLT assay with increasing SAM concentrations (right) indicating potential binding to the G9a SAM binding site.

Almac FLEXYTE G9a HMT assay development

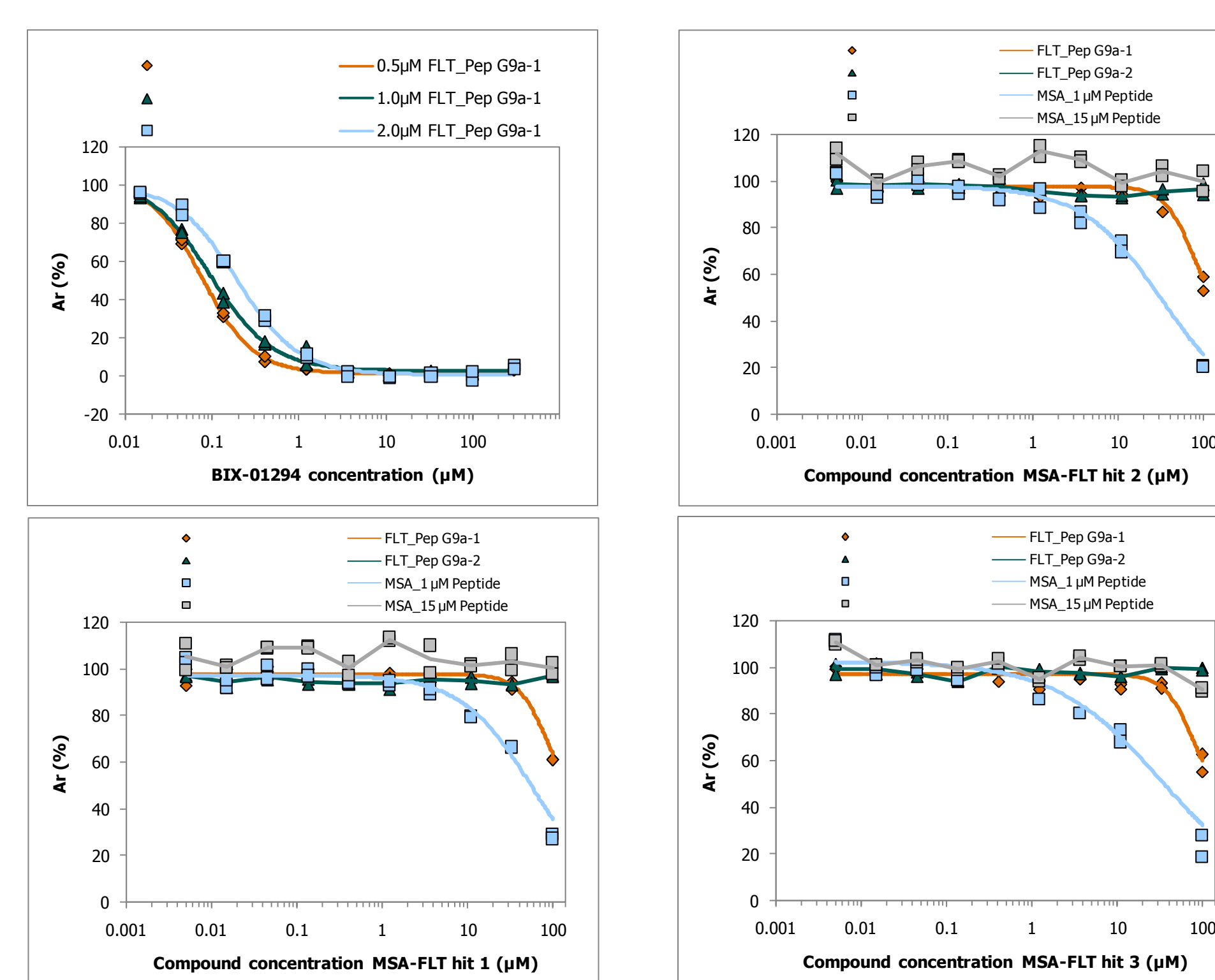


SAM $K_{m,app}$: 15 ng/well G9a per well and 1 μM peptide substrates were used. SAM $K_{m,app}$ of 6.5 μM for histone H3 peptide G9a-1 (left) and of 5.8 μM using histone H3 peptide G9a-2 (right) were determined.



Validation of FLEXYTE G9a HMT assay by IC50 determination of known inhibitors BIX-01294, Sinefungin, and SAH. Potency of BIX-01294 (Lit. values are between 100 nM and 2 μM) shifted depending on the histone H3 peptide used whereas IC50 for Sinefungin and SAH were in the same range. Binding site specificity of reference compounds was also addressed by substrate competition experiments.

G9a hit characterization: peptide substrate competitive compound series



Inhibitory potencies of one compound hit series active in MSA and FLT assays were modulated by histone peptide titration or FLT histone peptide used. For control, binding MOA to the G9a histone binding pocket was confirmed for BIX-01294 in the FLT assay (top left)

Summary

- A FLEXYTE FLT assay for the HMT G9a, assay validation by potency determination of SAM (Sinefungin and SAH) and histone substrate binding competitors (BIX-01294), and screening of 2,112 selected BioFocus compounds was successfully performed
- A small subset of validated G9a hit compounds showed an overlap between FLT and orthogonal assay technologies
- Initial MOA experiments with G9a hit compounds indicated inhibition through different binding sites