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Characterization of the activity of KTX-1001, a small molecule inhibitor of multiple myeloma SET domain using surface plasmon resonance

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KTX-1001 is a small molecule inhibitor of NSD2 in clinical development for multiple myeloma. It was identified as a potent and selective inhibitor of NSD2 (also known as MMSET) using a high-throughput biochemical assay with LC/MS-MS detection of SAH production as the endpoint. Subsequent evaluation of the binding of KTX-1001 to its target was conducted using surface plasmon resonance to quantify on-rate, off-rate, and equilibrium dissociation constant utilizing the SET domain as the immobilized target. In this format, no saturable or specific binding could be observed, despite the potent activity in the biochemical assay using full length NSD2 and radiolabeled SAM. To interrogate the discordance between potent activity and the lack of detectable binding, a series of experiments were designed in which KTX-1001 with a biotin-PEG tether (KTX-1001-3) was immobilized to the chip, with target (NSD2 SET domain) in-flow, with nucleosomes and with and without cofactor. These experiments demonstrated that KTX-1001-3 bound to the SET domain in a specific and saturable manner, with an affinity comparable to the IC_{50} determined in the enzymatic assay. Further, these studies confirmed unique binding properties of KTX-1001 in the presence of nucleosomes, cofactor, and in combination. These data identify the utility of surface plasmon resonance in a "reverse" format, where immobilization of KTX-1001 allowed for the interrogation of binding to a protein target that may be challenging in the conventional SPR format. Collectively, this analysis demonstrates the specific potent biochemical activity of KTX-1001 against MMSET and supports the ongoing evaluation of KTX-1001 in the clinic.

Nuclear receptor–binding SET domain protein 2 (NSD2), also known as multiple myeloma SET domain–containing protein (MMSET) is a histone lysine-methyl transferase that specifically produces monomethylated and dimethylated histone 3 at lysine 36 (H3K36me1, H3K36me2 respectively) and regulates gene transcription (1, 2). NSD2 is a 1365 amino acid protein that is comprised of a catalytically functional SET domain that is flanked by associated with SET and post-SET domains and contains additional proline-tryptophan-tryptophan-proline (PWWP), plant homeodomain and high mobility box domains. NSD2 is of particular interest as a druggable target due to chromosomal translocation (4;14) resulting in overexpression which is associated with a subset of multiple myeloma with poor prognosis (3, 4), and the associations of gain of function mutations with acute lymphocytic leukemia (5). Most recently, NSD2 has been described to be an important regulator of epithelial-to-mesenchymal transition, a process that is critical for metastasis of solid tumors (6–8). While the human gain of function and translocation data point directly to a driver role in blood and bone marrow cancers, the translational work *in vitro* in cell systems or animal models have largely relied on its deletion (KO). Small molecule tools would be an ideal orthogonal approach to support the observations with genetic KO, but the generation of small molecule inhibitors of NSD2 has remained elusive (9).

A major challenge with developing small molecule inhibitors of epigenetic targets, including NSD2, is the competition for the cofactor SAM-binding pocket in the presence of high intracellular SAM concentrations (10). SAM competitive small molecule tools have been described (11), but with biochemical IC_{50} values in the micromolar (μM) range. Small molecules with potencies in the nanomolar (nM) range show lack of selectivity for NSD2, and while these are used as tools to understand the biology of the target, the low affinity or lack of selectivity is nonoptimal for a drug candidate.

An alternate strategy has been to target the PWWP (named based upon PWWP core) domain of NSD2 to either degrade the target or to impede the association of the NSD2 PWWP domain with H3K36me2 to propagate the demethylation in specific regions (12–14). Targeting the PWWP domain specifically would likely not impact the biochemical activity measured in a radiolabeled assay, and binding events in SPR have been demonstrated (14).

KTX-1001, a small molecule inhibitor of NSD2, was evaluated in biochemical assays and determined to have a high level of specificity for NSD2. To further describe the binding of KTX-1001 to NSD2, surface plasmon resonance (SPR) was conducted, according to the standard assay format of immobilization of a truncated form of NSD2 (aa 934–1241,

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containing the SET domain) onto a chip *via* amine coupling, followed by passing of the ligand of interest (KTX-1001) in solution phase over the immobilized NSD2. While the biochemical activity was quantified using full-length NSD2 (aa 2–1365), the size of the NSD2 protein relative to the size of KTX-1001 precluded SPR binding studies with the full-length protein and required the truncated form of the protein. This truncated form contains the both the SAM binding and histone-interacting domains and is known to bind small molecules that target the SAM-binding pocket (11, 15, 16). The natural ligand, SAM, and its product SAH showed saturable and specific binding in SPR assays, confirming that the cofactor-binding pocket is maintained following immobilization onto the sensor chip. Therefore, we predicted that a small molecule with potent activity in the biochemical assay would also exhibit binding to the SET domain in SPR.

KTX-1001 did not demonstrate specific binding to immobilized SET domain in the standard SPR format, an observation that was unexpected based on its biochemical potency. To interrogate this observation further, a modified SPR assay was developed, in which the small molecule was tethered and immobilized to the chip surface, and the protein target, NSD2 SET domain, was passed in solution over the chip. In this "reverse" format, the tethered analogue of KTX-1001 (KTX-1001-3) showed specific and saturable binding to the SET domain. The addition of SAM, nucleosomes, or the combination of both in preincubation with SET domain further confirmed the binding of KTX-1001-3.

Results

KTX-1001 inhibits NSD2 in a biochemical assay with radiolabeled cofactor SAM

Biochemical activity of KTX-1001 was determined using full-length NSD2 (aa 2–1365) in a multiwell format that measures the incorporation of ^{3}H from tritium-labeled SAM onto filter captured nucleosomes (see Experimental Procedures). To confirm biochemical activity towards the SET domain, KTX-1001 was also evaluated in a similar assay format, using the SET domain (aa 934–1241) as the enzyme source. In the same assay format, KTX-1001 showed comparable potency against full-length NSD2 or the SET domain (Table 1). KTX-1001 was also selective against nine other methyltransferase targets in the same multiwell format (Table S1, S2).

Table 1
Inhibition of enzymatic activity of full-length NSD2 and SET domain in the biochemical assay with SAH as a reference control agent

	IC_{50}	
Molecule	NSD2 (aa 2–1365)	SET domain (aa 934–1241)
KTX-1001	0.460 (0.040) nM	2.32 (0.47) nM
SAH	3.83 μM	10.8 μM

Values represent the mean of technical triplicates (SD) for KTX-1001. SAH was evaluated as a reference control as a single point determination.
NSD2, nuclear receptor–binding SET domain protein 2; SPR, surface plasmon resonance.

KTX-1001 inhibits NSD2 and is not competitive with SAM or nucleosome

To describe the nature of the inhibition observed, experiments were designed that utilized the assay format with full-length NSD2, and preincubation of increasing concentrations of nucleosomes to the reaction conditions, or addition of increasing concentrations of SAM to initiate the reaction. In these experiments, the activity of KTX-1001 was considered noncompetitive (or mixed inhibition) with regard to SAM (Fig. 1*A*) and nucleosome (Fig. 1*B*). The SAM concentration was varied from 0.25 to 4.0 μM resulting in a calculated IC_{50} of KTX-1001 with a range of 0.72 to 2.17 nM (Table 2). Similarly, the nucleosomes concentration was varied from 0.00625 to 0.1 mg/ml resulting in a calculated IC_{50} range of KTX-1001 of 0.50 to 1.28 nM (Table 2). These data suggest that unlike the majority of NSD2 inhibitors previously described (11, 15, 16), KTX-1001 was not cofactor competitive, which could allow for high potency in the presence of high intracellular SAM concentrations. However, interpretation of the nature of competition may be challenging regarding SAM, as the assay format includes preincubation of KTX-1001 with enzyme and nucleosome, and so true competition with SAM may be impacted if KTX-1001 is a slow "off" binder. Reversible binding, however, should allow for competition with SAM, even under the condition of SAM initiating the reaction, when the reaction is monitored over a 60-min time course.

The activity observed with SET domain only, comparable to full length (Table 1), also supported that the binding event localized within the SET domain.

KTX-1001 lacks saturable and specific binding to immobilized SET in SPR

Once it was established in biochemical assays that KTX-1001 was potent against NSD2 using either the full length or truncated form of the enzyme, immobilized NSD2 SET domain (aa 934–1241) was used to interrogate binding of KTX-1001, and to quantify on-rate, off-rate, and K_D, using SPR. In this format, several small molecule inhibitors of SET domain have been characterized (11), and both SAM and SAH have been routinely measured as control molecules.

Initial experiments at concentrations ranging from low nanomolar through to the low micromolar range demonstrated no specific or saturable binding of KTX-1001 to the immobilized SET domain with a lack of saturation. The concentration range was then expanded to include up to 200 μM, where dimethyl sulfoxide (DMSO) concentration becomes limiting (biochemical potency was in nanomolar range, Fig. 2, *A* and *B*). Although binding was observed at these high concentrations, no saturation was evident and signal was high relative to the theoretical maximum of 137.5 RU. These observations, together with the great disparity with the biochemical potency, were indicative of nonspecific binding (Fig. 2*A*). SAM and SAH demonstrated binding (Fig. 2, *C* and *D*) that was consistent with historical data and the literature (17), establishing that the immobilized SET domain was capable of binding cofactor.

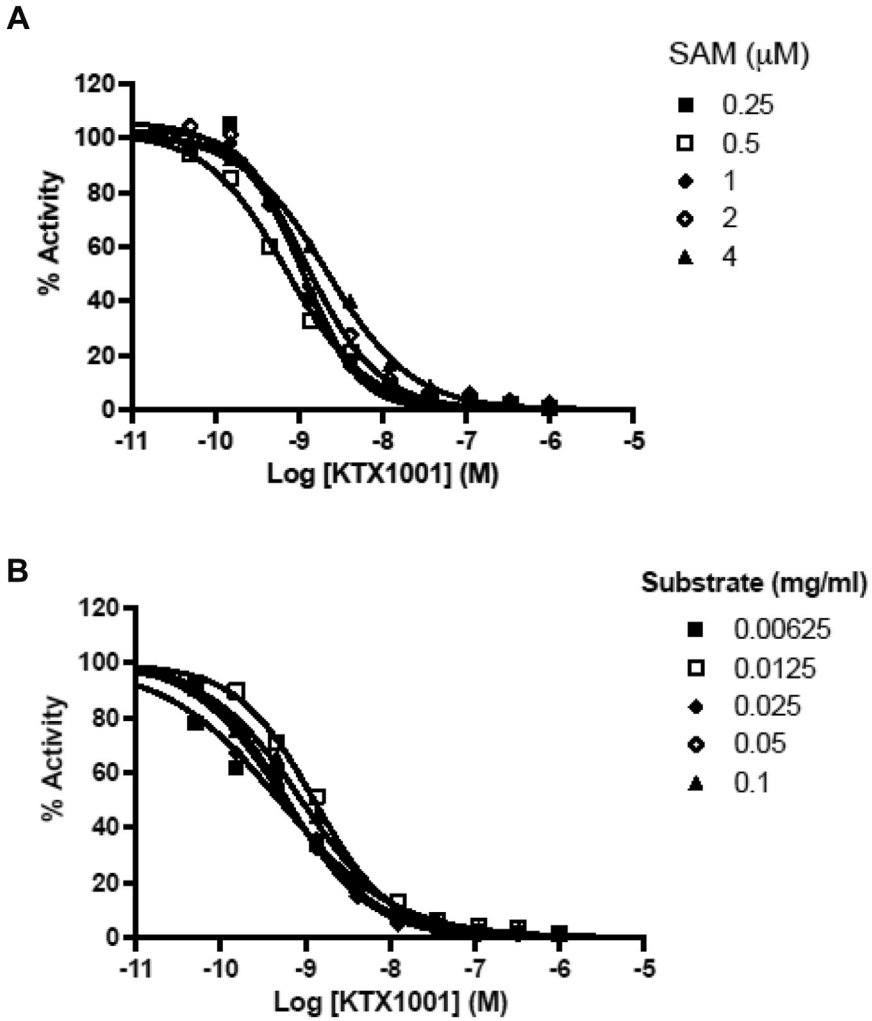

Figure 1. Mode of inhibition by KTX-1001. Mode of inhibition of NSD2 (aa 2–1365) by KTX-1001 with regard to cofactor SAM (*A*), and nucleosomes (*B*). The slope of the progress curves (0–60 min) at each KTX concentration, over the range of SAM or nucleosome concentration was calculated, and the % inhibition of enzymatic activity (represented by each symbol) by KTX-1001 as compared to DMSO, at each SAM concentration, and each nucleosome concentration were determined by comparing the slopes of the progress curves for each KTX-1001 concentration relative to the DMSO control. Curve fitting of the individual % inhibition values over the range of KTX-1001 concentrations was performed by GraphPad Prism. For SAM concentrations of 0.25, 0.5, 1, 2, and 4 uM, R^2 values of the fitted curves were 0.991, 0.997, 0.995, 0.991, and 0.997, respectively. For nucleosome concentrations of 0.00625, 0.0125, 0.025, 0.05, and 0.1 ug/ml, the R2 values of the fitted curves were 0.987, 0.995, 0.993, 1.0, and 0.996, respectively. DMSO, dimethyl sulfoxide; NSD2, nuclear receptor–binding SET domain protein 2.

Two main hypotheses emerged: (1) KTX 1001 did not bind to the SET domain, but exerted activity at a site in the full-length protein that could inhibit activity by an allosteric or indirect mechanism; (2) the immobilization negatively impacted the KTX-1001 binding site. The latter scenario could theoretically be explained by the exposure to the low pH required for immobilization to the chip (pH 4.5) altering the protein fold, the immobilization of the domain to the chip through lysine residues sterically blocking access to the binding site due to orientation on the chip surface, or contributions from both the pH and the orientation due to immobilization.

To address these hypotheses, a series of experiments were designed in which KTX-1001 was modified to include a biotin-PEG "tether" of differing lengths and immobilized to the sensor

Table 2
Inhibition of NSD2 activity by KTX-1001 is not impacted by increasing concentrations of SAM or nucleosome

NSD2 (aa 2–1365)	SAM (μM)	IC_{50}[a] (nM)	Nucleosome (mg/ml)	IC_{50}[a] (nM)
	0.25	1.09 (0.82–1.47)	0.00625	0.50 (0.28–0.90)
	0.5	0.72 (0.57–0.90)	0.0125	1.28 (0.99–1.66)
	1.0	1.12 (0.90–1.40)	0.025	0.56 (0.39–0.80)
	2.0	1.24 (0.88–1.75)	0.05	0.60 (0.54–0.64)
	4.0	2.17 (1.88–2.51)	0.1	0.91 (0.71–1.19)

Values in parentheses represent the 95% confidence interval of the curve fit for each SAM and nucleosome concentration.
NSD2, nuclear receptor–binding SET domain protein 2.
[a] IC50 values calculated from the curves in Figure 1 for SAM and nucleosomes, respectively.

chip, in a "reverse" SPR approach (Fig. 3). In a sequential manner, the characteristics of solution phase SET domain binding were interrogated for each "tether length" and a KTX-1001 analog with a PEG chain of 3 units, KTX-1001-3, was chosen for further study. Once saturable and specific binding of solution SET domain was established, the SET domain with nucleosomes (of the same source used for biochemical assays) were coincubated and passed over the surface of immobilized KTX-1001-3. SAM cofactor was evaluated with SET domain in a similar manner, as were all three (SET domain, nucleosomes, and SAM) to determine if binding was impacted by the presence of substrate (nucleosome) and cofactor.

Tethered KTX-1001 as immobilized target

Efforts to understand structure-activity relationship for KTX-1001 identified regions of the molecule that could be modified with little perturbation of biochemical potency. Particularly, one site was modified to include a tether of biotin-PEG such that different linker lengths could be used to separate the molecule from the streptavidin functionalized chip surface for SPR evaluation. KTX-1001-3, KTX-1001-5, and KTX-1001-8 (Fig. 3), contained three, five, and eight PEG units, respectively. To assure that these tethered molecules retained activity, they were evaluated in the biochemical assay format using the SET domain as the enzyme source. KTX-1001-3, KTX-1001-5, and KTX-1001-8 retained activity of 44.2, 95.3, and 121 nM IC_{50}, respectively, and were immobilized for initial evaluation in SPR. As a first pass experiment to aid in choosing a molecule for further study, binding of SET domain in flow to the immobilized KTX molecules at a single 1 μM concentration was evaluated. Binding was evident, with estimated K_D values of 1.17, 767, and 368 nM for KTX-1001-3, KTX-1001-5, and KTX-1001-8, respectively (Fig. S-1 and Table S3). Deviation from the fit to KTX-1001-3, especially in the association phase, suggested that 1 nM was not an accurate K_D due to the 1 μM dose being well above K_D, but as this effect was absent from the other two molecules, it was suggestive of a tighter affinity for KTX-1001-3. An additional measurement of a single 100 nM concentration of SET domain, with a K_D of 9.00 nM, appeared to be more representative of the binding behavior. Based on review of these data, KTX-1001-3, with a PEG linker length of three, was chosen for continued evaluation. This molecule had the smallest modification (by molecular weight), as compared to the parent KTX-1001, and was the most potent in binding affinity of the three linker molecules tested.

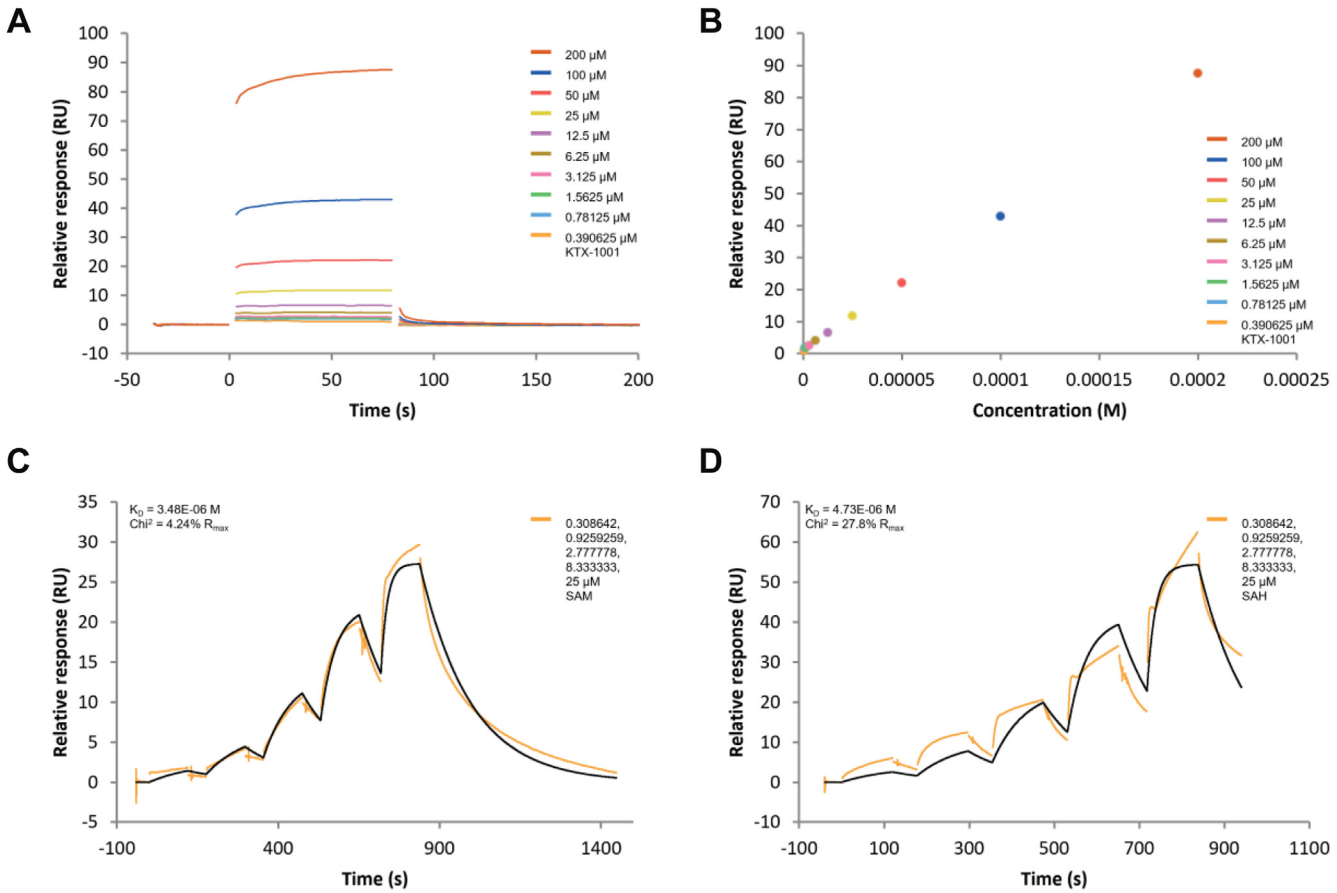

Figure 2. KTX-1001 binding to immobilized NSD2 SET domain by SPR. *A,B,* KTX-1001 shows dose-dependent binding to NSD2 SET, but without saturation and with high signal relative to the theoretical maximum, indicating nonspecific binding to the target. *C,* SAM and (*D*) SAH exhibit binding to immobilized SET domain as expected and consistent with published K_D. *Black lines* represent 1:1 Langmuir model fit. All data are double referenced and solvent corrected. Concentrations in figures are expected values from a dilution series. NSD2, nuclear receptor–binding SET domain protein 2; SPR, surface plasmon resonance.

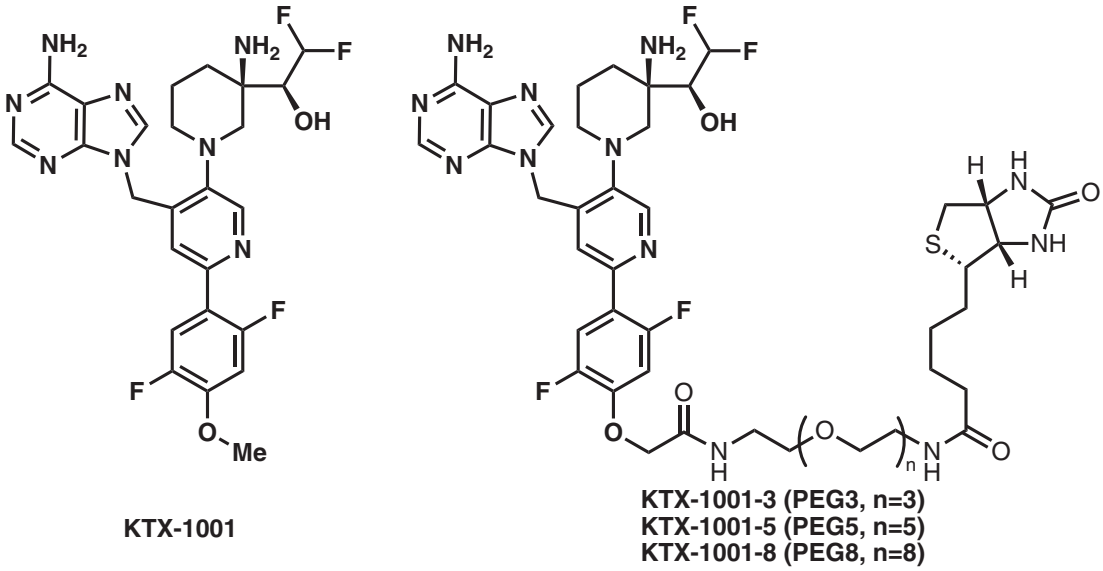

Figure 3. KTX-1001 and biotin-PEG analogues. KTX-1001 and KTX-1001-3/KTX-1001-5/KTX-1001-8.

Immobilized KTX as an approach to understand binding to cofactor and substrate

A stepwise approach to the binding characteristics was employed, based on review of each of the solution phase components flowed over the immobilized KTX molecule. SET domain binding was observed with KTX-1001-3 sensorgram consistent with specific binding (Fig. 4, *A* and *B*). Binding parameters were determined from 1:1 kinetic binding and steady-state affinity fits and the former are summarized (Table 3, Entry 1).

Once SET domain binding was confirmed, chicken nucleosomes of the same lot used for the radiolabeled biochemical assays were added to the SET domain and preincubated for 30 min and then applied in solution over the chip with the immobilized KTX-1001-3.

The addition of nucleosomes markedly increased the on-rate of SET domain binding to KTX-1001-3 but did not impact the dissociation phase (Fig. 4, *C* and *D*). The overall impact on the K_D was a decrease from ∼70 nM with SET alone to ∼15 nM in the presence of preincubated nucleosomes and SET (Table 3, Entry 2). To confirm that this effect was specific to binding to the SET domain, nucleosomes were applied to the chip in solution phase without the addition of SET domain. In these experiments, no measurable binding was observed, supporting that the interaction of KTX-1001-3 was with SET domain, and that the nucleosome addition was mediated by interaction with the SET domain at a site that was not the binding site for KTX-1001-3 (Fig. S-2).

Similarly to the preincubation with nucleosome, SAM was incubated with the SET domain for 30 min prior to the flow over the immobilized KTX-1001-3. Based on our observation in the competition experiments, it was anticipated that SAM would not impede the binding of SET with KTX-1001-3. Addition of SAM to the SET domain in preincubation slowed the association of KTX-1001-3 with SET domain (the on-rate), as is often seen with tighter binding events, and also resulted in a significant decrease in the off-rate (Fig. 4*E*). The result was a striking prolongation of the residence time (approximately 500 *versus* 20 s with SET alone) and a marked enhancement of the K_D [as compared to SET alone, but comparable to SET + nucleosomes (Table 3, Entry 3)]. In this experimental condition, where SAM is preincubated with SET domain, it is possible that SAM is converted to SAH. However, both cofactors bind the SET domain with similar affinity and kinetics, suggesting that the SET domain would be bound to cofactor at the time of flow over the immobilized KTX-1001-3.

The remaining experiment was to coincubate the SET domain with its cofactor (SAM) and its substrate (nucleosome) prior to exposure to KTX-1001-3 on the SPR chip. This experiment may reflect the most biologically relevant condition that can be established in an *in vitro* format, to identify if the complex was capable of binding KTX-1001-3 and if the binding parameters were modulated from the SET only condition. The preincubation of all components with subsequent flow over the chip with KTX-1001-3 showed the association observed was similar to incubations with SET domain only, but the residence time was prolonged (∼200 s), intermediate between SET coincubated with nucleosomes (∼20 s) and SET and SAM coincubation (∼500 s) (Fig. 4*F*). Though *Chi*-square for the fit in the lattermost condition is reasonable, there is some visible deviation from the fit lines during the dissociation phase that suggest some deviation for a straight 1:1 binding model and might also point toward an underestimate of residence time. The resultant overall K_D was 6.3 nM (Table 3, Entry 4), the most potent of all of the conditions, which was comparable to the biochemical potency of the unmodified parent molecule KTX-1001 in the biochemical assay using SET domain as the enzyme source.

Discussion

NSD2, either by mutation in its catalytic domain, or by overexpression mediated by translocation of chromosomes 4

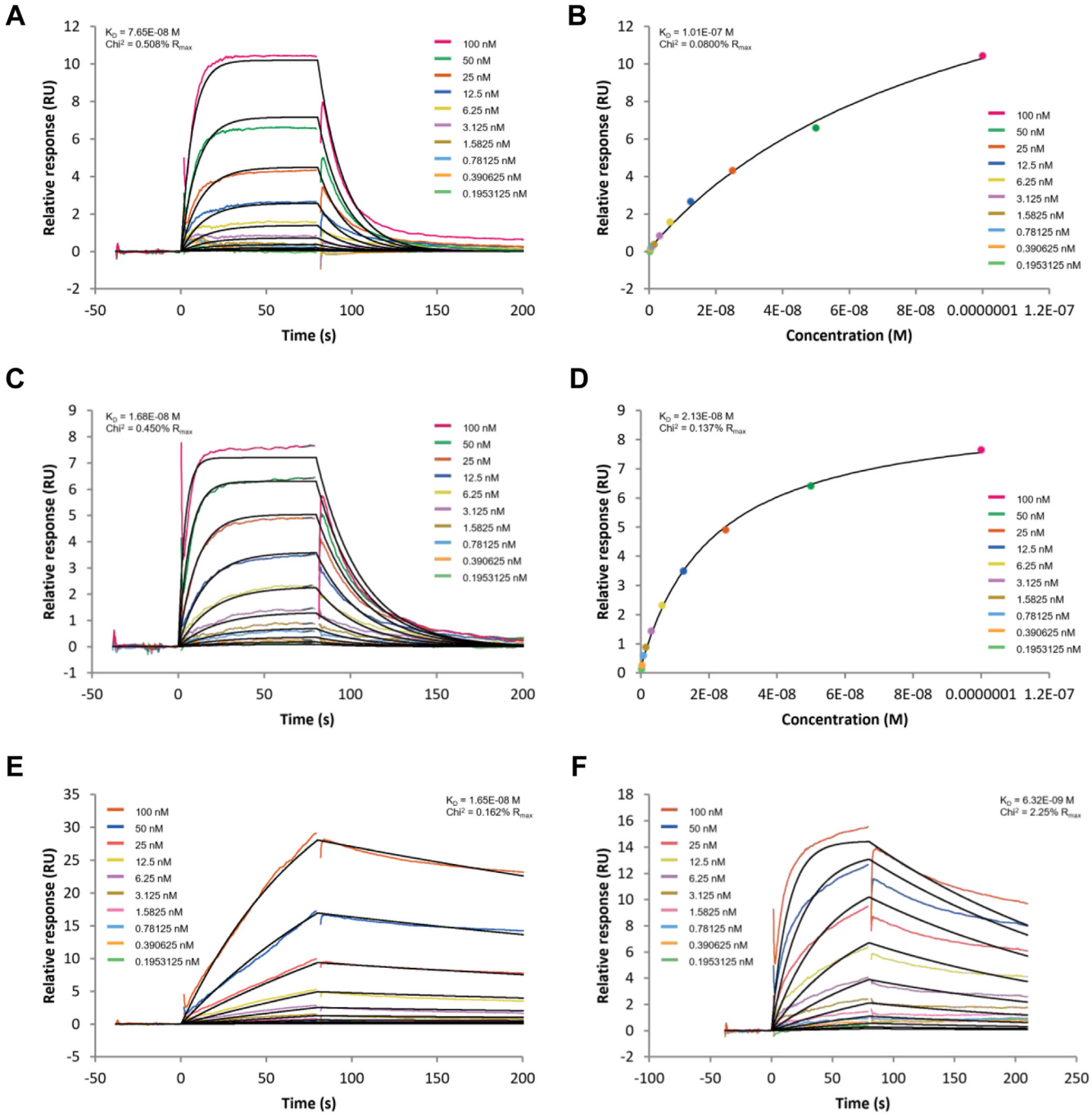

Figure 4. Immobilized KTX-1001-3 binding to NSD2 SET domain by SPR. SET domain was evaluated in flow over the concentration range reflected in each figure (0.195 nM–100 nM). *A* and *B*, binding of immobilized KTX-1001-3 to NSD2 SET domain in flow is specific and consistent with the biochemical IC_{50}. *C* and *D*, preincubation of NSD2 SET with nucleosomes augments KTX-1001-3 affinity due to faster on-rate. *E*, preincubation of NSD2 SET with SAM dramatically slows kinetics, increasing affinity compared to SET alone. *F*, preincubation of NSD2 SET with nucleosomes and SAM maintains an on-rate comparable to SET alone but shows a much slower off-rate, resulting in the tightest affinity of any tested condition. Data are representative replicates of triplicate (*A-B*) or duplicate (*C–F*) measurements. *Black lines* represent 1:1 Langmuir model fit. Steady-state fits in *panels B* and *D* were derived from the kinetic data in *panels A and C*, respectively. All data are double referenced and solvent corrected. Concentrations in figures are expected values from a dilution series. NSD2, nuclear receptor–binding SET domain protein 2; SPR, surface plasmon resonance.

and 14 (4;14) which place NSD2 under the influence of the strong promoters of the immunoglobulin heavy chain locus resulting in overexpression, is an established driver of acute lymphocytic leukemia and multiple myeloma, respectively (3–5). Targeting NSD2 has been a challenge for small molecule drug discovery for multiple reasons. The enzyme is large (152 kDa) and exists in complex with nucleosomal DNA. Furthermore, SAM, the cofactor utilized for transfer of a methyl group to histone protein, is found in abundance intracellularly, estimated to be present in micromolar concentrations. A competitive inhibitor to the SAM cofactor-binding pocket must overcome high levels of competing

Table 3
Derived binding parameters of NSD2 SET domain to KTX-1001-3, a PEG modified immobilized analog of KTX-1001

Entry	Condition[a]	Mean k_a (1/M·s)	Mean k_d (1/s)	Mean residence time (s)[b]	Mean 1:1 kinetic binding K_D (nM)[c]	Fold reduction in K_D over SET alone
1	SET alone	7.56E+05 (1.05E+05)	5.32E-02 (9.9E-03)	19.2 (3.4)	70.4 (8.0)	NA
2	SET + nucleosomes	3.31E+06 (1.43E+06)	4.63E-02 (1.11E-02)	22.2 (5.3)	14.6 (3.0)	4.81
3	SET + SAM	1.20E+05 (1.6E+04)	1.87E-03 (1.1E-04)	535 (31)	15.6 (1.2)	4.50
4	SET + SAM + nucleosomes	6.69E+05 (6.0E+04)	4.19E-03 (4.3E-04)	240 (25)	6.3 (0.1)	11.2

[a] Values represent the mean (SD) of duplicate (SET + nucleosomes, SET + SAM, SET + SAM + nucleosomes) or triplicate (SET only) determinations.
[b] Mean residence time is the mean of the inverse of the off-rates (k_d) representing the lifetime of the complex.
[c] Note that K_Ds differ by < 2-fold between replicates in each condition.

SAM, ultimately requiring nanomolar potency to be established. Assays to quantify NSD2 activity have been described in detail and have been used successfully in the lead identification of several SAM competitive tool molecules (11). Confirmation of biochemical activity through orthogonal assays has also been successful, however the ability to quantify binding by methods traditionally employed have been primarily focused on SAM competitive inhibitors that can bind in a specific and saturable manner in SPR format. The main issue is the full-length NSD2 molecule is too large to immobilize sufficient protein for reliable measurement of small molecule binding by SPR, limiting the applicability of this format to screening for molecules that bind the smaller catalytic SET domain once this domain is immobilized onto the chip.

KTX-1001 is a small molecule inhibitor of NSD2 with nanomolar potency in biochemical assays (Table 1) but showed noncompetition with regard to SAM and nucleosomes (Fig. 1). Interpretation of these enzyme inhibition studies needs to be performed in the context of the experimental design. Specifically, preincubation of the full-length enzyme with nucleosomes and small molecule, prior to initiation of the reaction with SAM, may elicit highly potent inhibition if the molecules have rapid on-rates and slow off-rates, or covalent interactions that would make off-rates negligible. For both the screening biochemical assay and the mode of enzyme inhibition studies, the cofactor was added to a preincubation mixture of enzyme, nucleosome (range of nucleosome concentrations in the mode of action studies), and KTX-1001. This format gives the small molecule time to bind the enzyme, especially at the SAM-binding pocket, without any competition from SAM. For a slow off molecule, this may be reflected in enzyme inhibition studies, as "noncompetitive." Alternately, this may reflect that the site of interaction of KTX-1001 with full-length NSD2 is not at the SAM or nucleosome binding sites, but distal to those that could still impact enzymatic activity. This latter possibility was tested experimentally by using the SET domain in the biochemical assay, where KTX-1001 was equipotent. The biochemical assay data supported the hypothesis that KTX-1001 was inhibitory in the SET domain.

SPR using SET domain as the immobilized target, however, demonstrated no specific or saturable binding of KTX-1001. The endogenous ligand, SAM, and its product, SAH, both bound to the SET domain with K_D values consistent with historical data at the laboratory conducting the experiments (Reaction Biology Corporation) and reported in the literature (17). This suggested that the SAM-binding pocket was not impacted following low pH exposure required for SET immobilization or altered or made inaccessible due to orientation on the chip surface as a result of coupled lysine residues. The lack of specific binding in SPR with KTX-1001, and the biochemical enzyme inhibition studies both support that the location of the binding event is not identical to the SAM-binding pocket.

Our approach was to quantify the binding event of KTX-1001 to SET domain by incorporating a tether linker to KTX-1001 and immobilizing the small molecule, rather than the target (NSD2 SET) to the SPR chip. Tethers of differing lengths were synthesized to vary the distance of the ligand from the chip into the solution space in an attempt to test binding of SET passed over in flow. The shortest tether link was chosen for all subsequent experiments, given that it represented the least modified analog of KTX-1001 and the tightest initial binding K_D when SET was used at a 1 μM concentration.

The results of the immobilized KTX tethered molecule experiments (Fig. 4) were consistent with the biochemical assay data, that KTX-1001-3 (and KTX-1001-5 and KTX-1001-8 to a lesser extent) was a potent interactor with NSD2, and that the site of that interaction was within the SET domain. The initial condition of SET alone binding showed an on-rate and a residence time that was reversible and not prolonged, or covalent. When nucleosomes were added to the SET domain, the on-rate was markedly enhanced (Table 3) but there was no change in the residence time. It has been described (16) that the binding of DNA promotes the relaxation of the auto-inhibitory loop that allows for the opening of the H3K36 binding site into the catalytic pocket (16). Nucleosomes, without SET domain did not show binding to KTX-1001-3, confirming that the interaction of the molecule was not direct with nucleosomes (Fig. S-2).

SAM was included with the SET domain to understand if KTX-1001-3 binding was SAM-competitive. SAM inclusion resulted in slower association of KTX-1001-3 (~6-fold), but more strikingly a significantly slower dissociation (~28-fold). This had a dramatic effect on the residence time, which was prolonged from approximately 20 s in the absence of SAM to greater than 500 s with SAM preincubation. The impact of this on the K_D value was an overall increase in affinity of 4-fold over SET domain alone (Table 3). It was anticipated that the

addition of SAM would have eliminated or reduced binding of KTX-1001-3 should the nature of the binding be colocated with SAM, but this was not observed.

Addition of SET with cofactor SAM and substrate nucleosomes demonstrated binding consistent with a specific saturable event in SPR. This combination appeared to mimic the most biologically relevant condition, in which all components are present to participate in the enzyme reaction, or in this case, binding event. The association phase was most comparable to that observed with the SET only condition, and the residence time was intermediate between the nucleosomes only and the SAM only addition to SET. The overall K_D showed the highest affinity binding of all conditions tested at 6.3 nM. This value is also very aligned with KTX-1001 enzymatic activity in the biochemical assay using SET domain as the enzyme source (2.32 nM). Taken together, these data support the conclusion that KTX-1001 is a potent inhibitor of NSD2, at a binding site not directly competitive for cofactor SAM or substrate nucleosomal DNA. The strategy to identify allosteric inhibitors has been described for other histone methyltransferases (18).

There are multiple approaches to characterize binding of small molecules with NSD2, however each is limited by challenges presented by its molecular weight, and its association with even larger histones with accompanying DNA. Crystal structures for NSD2-SET domain have been a challenge, although recent work has described the SAM-binding pocket and the interactions with DNA (16). Demonstration of binding of small molecules to the SET domain in the presence of cofactor and DNA, especially if a small molecule requires both cofactor and DNA to properly engage the enzyme, may be difficult to visualize by modelling of the binding sites. Insights from these SPR approaches will be instrumental to guide further efforts to define the binding site for KTX-1001.

Collectively, the data presented here demonstrate that binding of SET domain to KTX-1001-3 (the tethered analog of KTX-1001) was specific and saturable when evaluated with SET domain in flow, as compared to immobilized SET domain. This was unique from other reported NSD2 small molecule tools that demonstrate binding to the SET domain and have reported on-rate, off-rate, and K_D in the original SPR format. What was also differentiating for KTX-1001-3 was the binding characteristics in the presence of nucleosomes and cofactor. Specifically, the addition of nucleosomes enhanced the "on-rate" of binding of KTX-1001-3 to SET domain, while in the absence of SET domain, nucleosomes showed no binding on its own. SAM preincubation also modulated both the on-rate and off-rate of SET domain binding to KTX-1001-3, maintaining and even enhancing potent saturable and specific binding. The coincubation of nucleosome, SET, and cofactor (SAM) exhibited potent binding between SET and KTX-1001-3 with K_D approaching the IC_{50} observed in biochemical assay format.

These data describe the differentiated binding characteristics of KTX-1001-3 to the NSD2 SET domain, which was unique from other small molecule SAM competitive tools, and dependent on the SET domain in solution, *versus* immobilized format. The differentiated profile of KTX-1001 suggests that potency towards this target can be attained even within the context of high intracellular cofactor concentrations. Evidence for biologic activity in human clinical trials is underway (NCT05651932).

Experimental Procedures

Chemicals and reagents

KTX-1001, KTX-1001-3, KTX-1001-5, and KTX-1001-8 were provided to Reaction Biology Corporation, (RBC) for all biochemical and SPR assays. Synthesis and characterization of KTX-1001-3, KTX-1001-5, and KTX-1001-8 (the tethered KTX-1001 molecules of length three, five and eight PEG units) is available (Supporting Information). Human recombinant NSD2 (residues 2–1365; NM_001042424) was expressed with an N-terminal polyhistidine tag in an insect cell/baculovirus expression system. Human recombinant NSD2-SET domain (residues 934–1241; NM_001042424) was expressed with an N-terminal polyhistidine tag in *Escherichia coli*. Purity for each was determined by Coomassie Blue staining of SDS-PAGE, and were as follows: NSD2 (residues 2–1365) was approximately 90% pure, NSD2 SET domain utilized for KTX-1001 biochemical assay was approximately 95% pure, and NSD2 SET domain used for biochemical activity of KTX-1001-3, KTX-1001-5, and KTX-1001-8 was 80% pure. NSD2 SET used for SPR evaluations was also determined to be 80% pure. All other reagents for biochemical assays and SPR were provided by RBC and include Series S CM5 and Series S SA sensor chips (Cytiva).

Histone methyltransferase biochemical assay

The biochemical reaction was performed (16) in a buffer containing 50 mM Tris–HCl (pH 8.5), 5 mM $MgCl_2$, 50 mM NaCl, 0.01% Brij35, 1 mM DTT, 1% DMSO, and chicken nucleosomes (0.05 mg/ml) as the substrate for the enzyme. Full-length NSD2 (aa 2–1365) was added to a final concentration of (2 nM) and the reaction mixture was incubated for 20 min at room temperature. Radiolabeled SAM (^3H) at a final concentration of 1 μM was added to initiate the reaction, which proceeded for 60 min at 30 °C (19). Activity toward the SET domain was evaluated using SET domain (aa 934–1241) produced as a reagent by Reaction Biology at 2 nM, with all other reaction conditions the same as for full-length NSD2. KTX molecules were evaluated in ten-point dose response in technical triplicates. Data were analyzed by GraphPad Prism software (https://www.graphpad.com/features) for curve fit and IC_{50} values are reported.

Mode of enzyme inhibition studies were performed using the biochemical assay format, with the exception that each reaction was done as a single measurement. A range of concentrations of SAM was used to initiate the reactions and to determine SAM cofactor competition with KTX-1001. Likewise, for substrate competition, a range of concentrations of nucleosomes were preincubated with enzyme in the reaction buffer for 20 min prior to the addition of a fixed concentration (1 μM) SAM to initiate the reaction. The slope of the progress curves (0–60 min) at each KTX concentration, over the range

of SAM or nucleosome concentration was calculated, and the percent inhibition of enzymatic activity by KTX-1001 as compared to DMSO, at each SAM concentration, and each nucleosome concentration were determined by comparing the slopes of the progress curves for each KTX-1001 concentration relative to the DMSO control. Curve fitting of the individual percent inhibition values over the range of KTX-1001 concentrations was performed by GraphPad Prism.

SPR of immobilized SET domain

All SPR experiments using immobilized NSD2 SET were performed on a Biacore 8K+ (Cytiva) using a Series S CM5 sensor chip (Cytiva, cat# 29149603). NSD2 SET was immobilized as previously described (11). Following immobilization, the active flow cell was deactivated with ethanolamine. The reference cell was activated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide and deactivated with ethanolamine. Control and test samples were diluted in DMSO to 50X the highest concentration to be measured (200 μM in final experiments). The 50X stocks were diluted in running buffer to obtain 1X solutions in running buffer with 2% DMSO. A 3-fold serial dilution in running buffer with 2% DMSO was used to generate five doses (control measurements) or a 2-fold serial dilution in running buffer with 2% DMSO was used to generate 10 doses (KTX-1001). These were injected from lowest to highest concentration using a contact time of 80 s, a dissociation time of 120 s, and a flow rate of 30 μl/min. Data for each sample were collected in at least duplicate to ensure reproducibility. Data were analyzed using Biacore Insight Evaluation Software (Cytiva). All data were solvent corrected and double referenced (blank- and reference-subtracted) using a reference flow cell with immobilized biotin and 1:1 kinetic binding fits were employed as applicable to determine binding parameters.

SPR of immobilized KTX molecules

All SPR experiments using immobilized KTX molecules were performed on a Biacore 8K+ (Cytiva) using a Series S SA sensor chip (Cytiva, cat# 29699621). KTX-1001-3, KTX-1001-5, and KTX-1001-8 were immobilized to the sensor chip surface to levels of ∼30 RU. Following immobilization, the active and reference flow cells were blocked with biotin. NSD2 SET (±20 μM SAM, ± 0.02 mg/ml nucleosomes) was diluted in running buffer to 102 nM (1.02X the highest concentration to be measured). DMSO addition followed to generate a final solution of 1X NSD2 SET (100 nM) in running buffer with 2% DMSO. This was measured either as a single dose or as 10 doses generated from a 2-fold serial dilution in running buffer with 2% DMSO. When SAM and/or nucleosomes were included a 30 min incubation at room temperature followed. Doses were injected from lowest to highest concentration using a contact time of 80 s, a dissociation time of 120 s, and a flow rate of 30 μl/min. Ten dose data for each sample were collected in at least duplicate to ensure reproducibility. Data were analyzed using Biacore Insight Evaluation Software (Cytiva). All data were solvent corrected and double referenced (blank- and reference-subtracted) using a reference cell with immobilized biotin. Steady-state affinity (10 dose only) fits using data 5 s before injection end with a 5 s window and 1:1 kinetic binding fits (single and 10 dose) were employed to determine binding parameters.

Data availability

The data supporting this article are contained within the article, with the exception of the data describing the impact of varying SAM and nucleosome concentrations on the inhibition of enzymatic activity by KTX-1001. The figures shown are derived from progress curves at each concentration of SAM, nucleosome, and KTX-1001, and the progress curve raw data is stored at Reaction Biology Corporation, Malvern, PA. Charles Schmidt is an employee of Reaction Biology Corporation and the corresponding author to whom requests for data should be addressed.

Supporting information—This article contains supporting information.

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Conflict of interest—T. J. C. is an employer and holds stock ownership in K36 Therapeutics, Inc. C. A. L. is an employee of K36 Therapeutics Inc. The other author declares that there is no conflict of interest with the contents of this article. L. B. is a paid consultant for K36 Therapeutics. C. S. is an employee of Reaction Biology Corporation.

Abbreviations—The abbreviations used are: MMSET, multiple myeloma SET domain–containing protein; NSD2, nuclear receptor–binding SET domain protein 2; SPR, surface plasmon resonance.

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