Towards the understanding of the activity of G9a inhibitors: an activity landscape and molecular modeling approach

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Received: 6 August 2019 / Accepted: 7 February 2020 / Published online: 14 February 2020
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Abstract
In this work, we analyze the structure–activity relationships (SAR) of epigenetic inhibitors (lysine mimetics) against lysine methyltransferase (G9a or EHMT2) using a combined activity landscape, molecular docking and molecular dynamics approach. The study was based on a set of 251 G9a inhibitors with reported experimental activity. The activity landscape analysis rapidly led to the identification of activity cliffs, scaffolds hops and other active and inactive molecules with distinct SAR. Structure-based analysis of activity cliffs, scaffold hops and other selected active and inactive G9a inhibitors by means of docking followed by molecular dynamics simulations led to the identification of interactions with key residues involved in activity against G9a, for instance with ASP 1083, LEU 1086, ASP 1088, TYR 1154 and PHE 1158. The outcome of this work is expected to further advance the development of G9a inhibitors.

Keywords Activity cliffs · Drug discovery · EHMT2 · Epi-pharmacology · Scaffold hops · Structure–activity relationships

Introduction
The study of diseases based on epigenetic approaches is crucial for the development of new therapeutic alternatives [1]. Protein lysine methyltransferases (PKMTs) have attracted much interest in the drug discovery field as their inhibition is believed to be specific at the functional level [2]. Particularly, G9a [also known as KMT1C (lysine methyltransferase 1C) or EHMT2 (euchromatic histone methyltransferase 2)] is a histone-lysine N-methyltransferase that catalyzes the transfer of one or two methyl groups to the ε-amino group of lysine 9 of histone H3 (H3K9me1 and H3K9me2), a hallmark associated with transcriptional gene silencing. Other protein targets of G9a include the tumor suppressor p53, whose methylation leads to its inactivation [3, 4]. G9a is upregulated in various cancers, and its overexpression has been associated with poor prognosis and metastasis [5–8]. In addition, a very recent study reveals that G9a expression is significantly associated with resistance to immunotherapy, programmed cell death protein 1 (PD1) inhibition, in a cohort of bladder cancer patients [9]. Moreover, G9a is involved in the maintenance of HIV-1 latency, cognitive disturbances (mental retardation, cocaine addiction, age-related cognitive decline), embryonic development, colitis, regulation of the cell cycle, and stem cell reprogramming, which has been used to produce inducible pluripotent stem cells (iPSCs) [10–15].

There is a large variety of compounds synthesized and evaluated against G9a [16]. Distinct structural classes are quinoline and indole derivatives (Fig. 1). Reports have shown the influence of substituents on the activity for each structural class. For instance, lysine mimetic substituents (positively charged) have been shown to have a higher affinity for the substrate pocket on G9a. Examples are the small molecules 23 m, CHEMBL3109631, and WO2017142947A1-4 (Fig. 1). Similarly, there are reports suggesting that the lysine mimetic substituents promote key interactions with residues involved in enzymatic inhibition, such as TYR 1067, LEU 1086, TYR 1154, and PHE 1152 [17–20]. However, it is necessary to compare the binding
mode and the molecular geometry of the inhibitors considering the different molecular scaffolds in the same study.

Recently, the structure–activity relationships (SAR) of G9a inhibitors has been explored using the concept of activity landscape modeling [21, 22]. In that work, the dual activity of inhibitors with a quinoline scaffold has been predominantly explored. These studies allowed the identification of key substituents in the selectivity and efficacy of compounds.

The objective of this paper was to further explore the SAR of G9a ligands using the established concept of the activity landscape model and molecular docking followed by molecular dynamics.

Materials and methods

Data set

This study is based on a data set of 251 compounds derived from aminoquinoline, indole and acridine core with reported activity against G9a (IC\textsubscript{50}) [21, 23, 24]. All compounds were retrieved from the public database ChEMBL, except for compounds based on the acridine core, that are reported in patents [19, 23–25]. The SMILES representation of the structures and pIC\textsubscript{50} (− log IC\textsubscript{50}) values are listed in Table S1 in the supplementary material. The pIC\textsubscript{50} values range from 9.30 to 5.0 [26].

Software and online resources

The activity landscape analysis was carried out with Activity Landscape Plotter, an open web tool (https://www.difacquim.com/d-tools/) that enables the analysis of SAR of screening data sets [27]. This tool facilitates a first and rapid exploration of the SAR of compound data sets with a common scaffold and rapid decomposition of R-groups [28]. Molecular docking was performed with the software Yasara (YASARA Biosciences GmbH, Vienna, AUT). Molecular dynamics (MD) studies were done with Desmond (Schrödinger, New York, NY, USA) [29].

Activity landscape modeling

A structure–activity similarity (SAS) map is a tool for SAR analysis of compound data sets tested against a molecular target. SAS maps are based on the concept of activity landscape and are suited for the rapid identification of activity cliffs (AC), defined as compounds with a high structure similarity but unexpected large activity difference [30, 31]. SAS maps also enable the identification of scaffold hops (SH), defined as compounds with low structural similarity due to differences in their scaffold but similar biological activity. This concept has been useful in medicinal chemistry to identify and develop novel and diverse chemical entities [32].

Since SAS maps are based on systematic pairwise comparisons of the compounds in a data set, the SAS maps generated in this work represented all 31,375 pairwise comparisons between the 251 compounds of the set. To generate the map, the structural similarity was calculated with the ECFP4 fingerprint and the Tanimoto coefficient and was represented on the X-axis. The activity difference (in logarithmic scale) was plotted on the Y-axis. To differentiate the four major regions in the SAS map, two thresholds were set along the X- and Y-axis, respectively. The criteria to select the threshold along the X-axis was the mean of the similarity values of all compounds in the set (0.667) (calculated with Tanimoto and the ECFP4 fingerprint). The threshold of the activity difference (Y-axis) was set to two logarithmic units [33].

The data points in the SAS map were further colored by the corresponding Structure–Activity Landscape Index (SALI) value [34]. This index, as implemented in Activity Landscape Plotter, quantifies AC using the equation proposed by Guha and Van Drie:

\[
SALI_i, j = \frac{(|A_i - A_j|)}{(1 - sim(i, j))}
\]

where \(A_i\) and \(A_j\) are the activities of the i\textsuperscript{th} and the j\textsuperscript{th} molecules, and \(sim(i, j)\) is the similarity coefficient between the two molecules (in this work computed with the ECFP4 fingerprint and the Tanimoto coefficient). The SALI values were mapped onto the SAS maps using a continuous color scale from the structurally most similar pairs (green) to the least similar pairs (red). For the quantitative analysis
of SAS maps, the structure similarity was also evaluated with MACCS keys (166-bits) and PubChem fingerprints as implemented in Activity Landscape Plotter [27].

**Scaffold content analysis**

For the study of scaffolds, we use the methodology implemented by Naveja et. al., generating the scaffolds of each compound based on Bemis and Murcko approach. Briefly, the method consists of a graph analysis for each compound, where a "scaffold" is defined as the union of ring systems and linkers in a molecule, and the side chains are removed (any non-ring, non-linker atoms). This was done with the RDKit Fragments node implemented in KNIME software v. 3.7.2. The chemical structures of the scaffolds are available in Table S1 of the supplementary material [35, 36].

**Molecular docking**

**Protein preparation**

The crystallographic structure of human G9a (PDB ID: 3RJW) was retrieved from the Protein Data Bank (https://www.rcsb.org/) [37]. The co-crystallized ligand was removed (UNC0638, a 4-aminoquinazoline). Missing loops and side-chains were added with YASARA software [38]. Finally, hydrogen atoms were added, followed by a minimization step with the AMBER99 forcefield in MOE software (Chemical Computing Group, Montreal, QC, Canada) [39].

**Ligand preparation**

The ligands were built and energy-minimized in MOE using the MMFF94x force field. The more stable protomers at physiological pH were identified [40].

**Yasara docking**

Yasara software was used to add the solvent model and assign the Gasteiger atomic charges to proteins and ligands [29]. The grid was centered on the binding site of the protein (PDB ID: 3RJW). Using the scoring function of AutoDock Vina, the binding compounds were subjected to 25 search steps and the default values for the other parameters. The clusters with an RMSD < 2 Å were visually explored. During the docking simulations the receptor was considered rigid and the ligands flexible. The conformations with the lowest binding energy and diverse scaffolds were selected for an additional MD analysis.

**Molecular dynamics**

MD studies of the protein–ligand complexes were performed using Desmond (version 2018-3, Schrödinger, New York, NY, USA) with the OPLS 2005 forcefield [41]. The most representative docking pose for each ligand was used as a starting point to initiate the MD simulations. The complexes were prepared with the System Builder Utility in a buffered orthorhombic box (10 × 10 × 10 Å), using the transferable intermolecular potential with 3-point model for water (TIP3P). The complexes were neutralized and NaCl was added in a 0.15 M concentration.

Complexes were minimized using the steep-descent (SD) algorithm followed by the Broyden-Fletcher-Goldfarb-Shanno (LBFGS) method in three stages. In the first stage water heavy atoms were restrained with a force constant of 1000 kcal mol⁻¹ Å⁻² for 5000 steps (1000 SD, 4000 LBFGS) with a convergence criterion of 50 kcal mol⁻¹ Å⁻²; for the second stage, backbones were constrained with a 10 kcal mol⁻¹ Å⁻² force constant using a convergence criterion of 10 kcal mol⁻¹ Å⁻² for 2000 steps (1000 SD, 1000 LBFGS); and for the third stage the systems were minimized with no restraints for 1000 steps (750 SD, 250 LBFGS) with a convergence criterion of 1 kcal mol⁻¹ Å⁻².

Equilibration was carried out in several steps. Beginning with Brownian Dynamics for 250 ps with the Berendsen thermostat. Followed by simulation on the NVT ensemble, slowly heating from 10 to 300 K over 3000 ps. At this stage, constraints were enforced on solute heavy atoms, using a constant of 50 kcal/mol.

Finally, equilibration on NPT ensemble used the Berendsen thermostat and Langevin barostat for additional 250 ps. Subsequently, the system was submitted to 300 ns of production runs, under NPT ensemble at 1 bar using the Martyna-Tuckerman-Klein (MTK) barostat and 300 K using the Nose–Hoover thermostat. Electrostatic forces were calculated with the smooth PME method using a 9 Å cut-off, while constraints were enforced with the M-SHAKE algorithm. Integration was done every 1.2 fs, with a recording interval of 50 ps. Finally, the first 30 ns of production runs were removed, this is due to system stabilization after this period [22].

The quality of the simulation and trajectory analyses were carried out with the tools implemented in the Maestro-GUI (Schrödinger, New York, NY, USA) (Fig. S2).

**Results and discussion**

First, we present and discuss the results of the activity landscape analysis that led to the rapid identification of AC and other related pairs of active-inactive compounds with distinct SAR. Then, we discuss a structure-based analysis of
selected compounds from the activity landscape analysis (focused on the most active compounds of the cliffs in the data set). The structure-based analysis was performed with docking followed by MD simulations.

**SAS map**

Figure 2-A shows the SAS map for the 251 G9a inhibitors. The graph contains 31,375 data points, each of which represents a pairwise comparison (vide supra). For each pair of compound the maps show the relationship between the difference in activity and the molecular similarity. As detailed in the Materials and methods section, the molecular similarity was quantified with the Tanimoto coefficient using the ECFP4 fingerprints. The data points are further distinguished by the SALI value, using a continuous color scale from a low value (green) to a high value (red). As discussed in the Materials and methods section, AC will have a high

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**Fig. 2** SAS map of 251 compounds with activity against G9a (31,375 data points or pairwise comparison). a Data points are colored by the SALI value using a continuous scale from low (green) to high (red) values. b Data points are colored by the maximum activity of the compound in each pair (Max. Activity) value using a continuous scale from low (green) to high (red)
SALI value. In the SAS map in Fig. 2-A most pairs are green and yellow, indicating a more continuous SAR i.e., similar structures with similar activity. This result can be partially explained by the fact that the compounds in the data set come from a lead-optimization process [21, 23, 24]. Figure 2b shows the SAS map colored by “Max.Activity” value, i.e., the activity of the most active compound in the pair.

As shown in Fig. 2, a significant proportion of pairs of compounds have activity differences larger than two log units. This suggests that this set of compounds explores in detail the SAR for G9a. However, the AC zone (upper right corner) is not so coarse, suggesting that the compounds are structurally similar, although we know of the overall scaffold variety in the set of compounds (Fig. 1). In the SH zone of the SAS maps (lower left corner) is possible to identify compounds with small activity difference, but with different scaffolds (low structural similarity), as shown below. We want to emphasize that the SAS maps in Fig. 2 were generated using structural fingerprints (ECFP4, as described in the Method section) and the AC rapidly identified in the SAS maps may have actually different molecular scaffolds. In other words, the fingerprint-based similarity may not be highly associated with the scaffold or sub-structure-similarity and it is not a straightforward approach to identify molecular matched pairs.

**Scaffold content analysis**

Figure 3 shows the frequency of Bemis-Murcko scaffolds (138 different scaffolds), along with the distribution of their biological activity (pIC\textsubscript{50}). It is noted that the set of compounds studied has several (more than 10) compounds with the same scaffold as 23 m (s17) and WO2017-7 (s62). However, there are compounds with unique scaffolds in the set such as CHEMBL3109631 (s59).

Specific examples of AC and SH identified in the activity landscape analysis are labeled in Fig. 2 and 3, respectively. The chemical structures are shown in Fig. 4. An AC for G9a is the compound pair 23 m/C32, differing in the methoxy group at the 6-position and the change of a pyrrole to furan substituent at the 2-position of the quinoline ring. Another example of a pair of compounds with similar structure
(structural changes at the substituent group at 4-position) but large activity difference is WO2017-4/WO2017-7 (Fig. 4). Examples of compounds associated with SH highlighted in Fig. 2a are 23 m, CHEMBL3109631 and WO2017-4 (Fig. 4a).

**Molecular docking**

Based on the results of the activity landscape analysis, molecular docking was used to generate representative binding poses with G9a of selected compounds. Docking was conducted with Yasara software, as detailed in the Materials and methods section.

Figure 5 shows one of the best ranked binding poses of representative active and inactive compounds. The compounds were selected because the SAS map identified them as the most representative active and inactive compounds per scaffold. The docking model suggested that the three most active compounds (23 m, CHEMBL3109631, and WO2017-4) establish hydrogen-bond contacts with the side chains of ASP 1083, and ASP 1088, as well as hydrogen-bond contacts between the positively charged pyrrolidine group of 23 m and WO2017-4 with the backbone of LEU 1086.

Of note, in the three active compounds in Fig. 5a the substituents that mimic lysine (marked in green in Fig. 1) make polar and stacking interactions with amino acids such as TYR 1067, TYR 1154, ARG 1157, and PHE 1158.

We emphasize that the binding poses of the active and inactive compounds are similar, however we consider that the flexibility of the mimetic substituents of lysine (in the inactive compounds) leads to overfit their binding poses. This hypothesis was tested using MD simulations (vide infra).

The docking was done with the crystallographic structure of G9a PDB ID: 3RJW. Of note, the structure of G9a PDB ID: 4NVQ is co-crystallized with the compound A-366, which is structurally similar to CHEMBL3109631. Interestingly, Fig. S1 in the Supplementary Material shows the results of cross-docking of the co-crystal ligands with the two crystallographic structures (PDB ID: 3RJW and 4NVQ, respectively). In cross-docking, the binding poses change significantly suggesting the relevance of the flexibility of the binding pocket. In this work, such flexibility was addressed with MD simulations (discussed in the next section). Overall, the results of the cross-docking further emphasize the importance of MD to perform structure-based interpretation of inhibitors of G9a.

**Molecular dynamics**

Based on the insights from activity landscape modeling and docking calculations, we performed MD simulations (100 ns) for selected and structurally related active and inactive compounds.

Figure 6 summarizes the interactions between the most active compounds (23 m, CHEMBL3109631, and WO2017-4) and G9a, according to the MD simulations. It is noteworthy the well-conserved interactions with ASP 1083, ASP 1088 (except CHEMBL3109631), and TYR 1154. However, interactions with LEU 1086 were replaced throughout the dynamics by interactions with the TYR 1154. We highlight the interaction of compound 23 m with ASP 1088, since it is generated through the pyrrole substituent in 2-position. Pyrrole substituent is particularly interesting given that it is found in the most active compounds of this series of compounds (derivatives of 4 amino quinoline).
Of the three compounds analyzed in MD, WO2017-4 showed more stable direct interactions with G9a during the simulation time. In contrast, the interactions of CHEMBL3109631 with the binding site are stabilized with structural waters. Of note, for the three most active compounds are predicted hydrogen-bond interactions between their positively charged scaffolds and ASP 1088. The lysine mimic substituents in the most active compounds make interactions with PHE 1158 (Pi-cation interaction dependent of pyrrole). Also 23 m and WO2017-4 make interactions with LEU 1086 (hydrophobic interaction dependent of aliphatic portion), and TYR 1154 (Pi-cation).

Figure 7-A describes the interactions of inactive compounds against G9a (C32 and WO2017-7), based on the MD simulations. As compared to the MD simulations of the active compounds it is remarkable that the inactive compounds do not make key interactions with the lysine-mimetic substituent: e.g. LEU 1086, TYR 1154, and PHE 1158 (Fig. 6). As per the interactions with their charged scaffolds, at least one of the key interactions observed for active compounds was lost (ASP 1083 and ASP 1088, Fig. 6). Moreover, as shown in the histograms of Fig. 6 it was also observed a decrease in the fraction of interaction with key residues during the MD simulations.

Figure 7 suggests non-specificity of interaction with respect to the most active compounds. This is further illustrated in Fig. S2 in the Supplementary Material, where the RMSD and RMSF values (per compound) are plotted, which show the conformational stability of G9a and the instability of the poses associated with inactive compounds (C32 and WO2017-7).

At the structural level, we have identified two possible conformational states of pharmacology importance in the SET domain of G9a (domain involved in the function of histone methylation) [42]. The first (closed state) that is associated with active compounds, and the second (open state) that is associated with inactive compounds. The compounds favor one or another conformational state as shown in Fig. S3A and B (Supplementary Material), respectively. The distance between ASP 1088 (carboxyl group carbon in the side chain) and ARG 1157 (amino group carbon in the side chain) (plotted in Fig. S3C) seems crucial in the formation of an open or closed conformational states. Inactive compounds tend to generate an open conformational state (e.g., distances > 10 Å), in contrast to active compounds that favor the closed state (Fig. S3 and S4) stabilizing the interaction between ASP 1088 and ARG 1157 (e.g., distances < 10 Å). These results are consistent with the two crystallographic
states reported for G9a (PDB IDs: 3RJW and 4NVQ respectively). Conformational changes in the SET domain have been described for other lysine methyltransferases, using computational and experimental methods, breaking the paradigm of the existence of a unique conformational state associated with methylating action in this type of targets [43–45]. Recently, Shin C. et al. demonstrated the existence of specific conformational changes in the SET domain associated with the type of substrate recognized [46].

**Conclusions and perspectives**

The concept of epigenetic pharmacology (epi-pharmacology) is increasingly relevant [47, 48]. In parallel, activity landscape modeling has contributed to the characterization of the epigenetic relevant chemical space [49, 50]. Therefore, this study that combines activity landscape modeling with molecular modeling augmented the SAR information for G9a inhibitors. Activity landscape modeling allowed the identification of structurally different compounds, but with similar biological activity (scaffold hops) against G9a, which will facilitate the generation of more robust SAR studies. Based on the activity landscape, the set of G9a compounds analyzed in this study are suitable for the generation of QSAR models [51]. The results of this work further supported the convenience of exploring different scaffolds for the molecular recognition of small molecules with G9a. The scaffold analysis revealed that the new approaches should consider exploring in more detail the influence of more and diverse scaffolds on molecular recognition. Thus far, three major chemical scaffolds have been explored (scaffold IDs S17, S62, and S87), however, one of the most promising and least studied scaffolds is S59.
Docking and MD studies further emphasized the importance of the interactions between lysine mimetic substituents with G9a (LEU 1086, TYR 1154 and PHE 1158). From the MD simulations it was concluded that, despite the fact the chemical structures of the ligands is different, they maintain key interactions with ASP 1083 and ASP 1088. At the structural level, hypothetical conformational states (open and closed) on SET domain of G9a were identified. These

Fig. 7 G9a–inactive compounds contacts analysis during the 100 ns MD simulations. a The figures show the fraction and type of interactions with representative ligands during the simulation. b Binding poses at the beginning (orange) and at the end (cyan) of the MD simulation. The end-structure of G9a is shown.
Authors contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by EL-L and OR. The first draft of the manuscript was written by EL-L and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This research was funded by the School of Chemistry of the Universidad Nacional Autónoma de México (UNAM), the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) grant number IA203718, UNAM and the Consejo Nacional de Ciencia y Tecnología (CONACyT) grant number 282785. We also thank the program Nuevas Alternativas para el Tratamiento de Enfermedades Infecciosas NUATEI-UNAM for funding. We thank the Foundation for Applied Medical Research, University of Navarra (Pamplona, Spain), as well as Fundación Fuentes Dutor, for financial support.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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