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REVIEW ARTICLE

Identification of Drug Binding Sites and Action Mechanisms with Molecular Dynamics Simulations

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Abstract: Identifying drug binding sites and elucidating drug action mechanisms are important components in a drug discovery process. In this review, we briefly compared three different approaches (sequence-based methods, structure-based methods and probe-based molecular dynamics (MD) methods) to identifying drug binding sites, and concluded that probe-based MD methods are much more advantageous in dealing with flexible target macromolecules and digging out druggable macromolecule conformations for subsequent drug screening. The applications of MD simulation to studying drug-target interactions were demonstrated with different types of target molecules, including lipid membrane, protein and DNA. The results indicate that MD simulations with enhanced sampling methods provide a powerful tool to determine free energy profiles/surfaces and identify important intermediate states, which are essential for the elucidation of drug action mechanisms. The future development of methods in MD simulations will benefit and speed up the drug discovery processes.

Keywords: Drug action mechanisms, drug binding sites, molecular dynamics simulations.

1. INTRODUCTION

In living organisms, proteins are versatile and flexible organic macromolecules, playing important roles in maintaining various biological functions (ion channels, transporters, enzymes, *etc*.). When a molecule binds to a specific site of the target protein, it may modulate protein dynamics and functions. Such specific sites are named as drug binding sites, and understanding the binding process is the basis of the structure-based drug design. Over a hundred years ago, Emil Fischer first formulated a lock-and-key model for drugs and protein, which offers a static point of view for drug-protein interactions [1]. In this model, the binding process is driven by shape complementarity between the ligand and the fixed protein. However, since this model was proposed, more and more evidences have revealed that proteins exhibit remarkable conformational flexibility upon ligand binding, which makes this simple model inadequate to describe the association and dissociation processes of drugs.

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Essentially, protein flexibility enables the exploitation of more possible high-affinity interactions. Proteins are dynamic entities and their conformations can be modulated by not only physiological factors such as temperature or pH, but also direct factors such as interactions with other entities (proteins, peptides, DNA, membrane, hormone molecules, *etc*.) [2-3]. The intrinsic flexibility of proteins may arise from the whole structure domain, or just side chains of a few amino acids, which results in large-scale movements through transition intermediates [4-5]. Hence, the energetic quantification of the structure and dynamics of interest will be very important for the design and development of new drugs and also contribute to our knowledge of biological processes [6-9].

It has been widely shown that protein flexibility can also affect drug's binding kinetics and thermodynamics, which are of great importance and should be taken into account in structure-based drug design strategies [10-11]. Hence, understanding these structural changes related to the association and/or dissociation of drug molecules is crucial and can provide considerable insights for further optimization of drugs [12-13]. In addition to traditional approaches such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, considerable efforts have been made to develop new experimental methods to investigate the dynamics and flexibility of biomolecules. Although time-resolved studies using solution X-ray scattering or cryo-electron mi-

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croscopy (Cryo-EM) have been applied to investigate the dynamics of some flexible molecular systems, these methods still remain expensive and time-consuming. Besides, due to the complexity of biomolecules, it is difficult to directly measure the conformational changes in experiments for many biological systems of interest [14]. On the other hand, computational methods, in particular, the molecular dynamics (MD) simulation, may provide an alternative way to study the protein dynamics, even for large biomolecular systems. Starting from an experimentally determined structure or from an *in silico* predicted model, classical MD (cMD) simulations can sample the confirmation states around the given structure. However, due to the presence of high energetic barriers on the whole free energy surface of the system, cMD may fail to sample all states within limited simulation time. To improve the efficiency of conformational sampling, several enhanced MD sampling techniques have been developed. These new sampling approaches, combined with advanced computing hardware, allow the study of biological processes from the perspective of the structure, kinetics and thermodynamics in an appropriate biological environment [15-16].

In this review, we first introduce the types of drug binding sites and then discuss the methods for drug binding site identification in detail. The discussion is mainly focused on the applications of cMD and several widely used enhanced sampling methods including accelerated molecular dynamics (aMD) simulation, and Gaussian accelerated molecular dynamics (GaMD) simulation. The advantages and limitations of each MD method as well as their application examples are also summarized. Finally, the drug action mechanism and future prospective on the identification of drug binding sites are discussed.

2. TYPES OF DRUG BINDING SITES

The drug binding sites can be generally classified into three major types. The first type is called “active site” Fig. (**1A**), which includes catalytic sites or enzymatic binding sites [17]. These binding sites usually possess specific catalytic functions, allowing it to interact with a substrate and execute chemical reactions, transforming the substrate into a new product. The second type comprises “allosteric site” Fig. (**1B**) [18-21], which do not induce any catalytic activity [22]. However, when a drug interacts with this site of a target protein, whose conformation can be changed to allow further interactions with other proteins. In other words, drugs can indirectly affect the activity of a target protein by interacting with these sites. The third and the most complicated type of binding sites is usually termed as “cryptic site” Fig. (**1C**) [23]. The binding sites of this type are almost hidden and rarely can appear on the surface of the protein. They usually occupy a small portion of the conformational ensemble of the target protein and are only partly detectable in the unbound target. Identifying cryptic sites may require a great deal of structural and conformational analysis of the protein.

3. IDENTIFICATION METHODS OF DRUG BINDING SITES

There are many computational methods available that can be used to identify drug binding sites. The methods are generally classified into the following three groups: sequence-based, structure-based and probe-based methods, respectively.

3.1. Sequence-based Methods

Sequence-based prediction methods operate under the assumption that the protein residues involved in ligand binding are conserved through evolution [24-25]. With methods of this kind, the protein sequence is firstly scanned and conserved residues are identified as potential binding sites. The fundamental limitation of sequence-based methods is that sequence conservation alone is not a specific criterion to identify binding residues, as many non-binding residues can have a high degree of conservation. The sequence-based methods also cannot account for the specific structural and physicochemical attributes of the binding sites.

3.2. Structure-based Methods

Historically, the estimation of the druggability and binding sites was solely based on prior data from known drugs and drug targets. Current structure-based methods have been

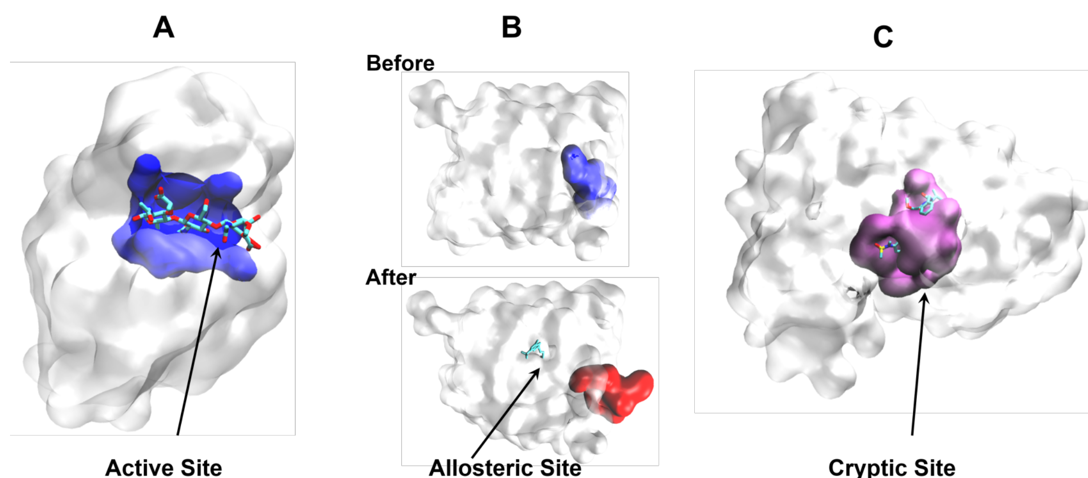

Fig. (1). Three major types of binding sites: (**A**) active site, (**B**) allosteric site, which can change their conformation before (blue) and after (red) binding with allosteric modular (cyan), and (**C**) cryptic site, which can occasionally open (purple).

designed to overcome this shortcoming and allows to identify new drug binding sites of various sizes, shapes, and chemical characters without a prior knowledge of the drug binding target and with much higher accuracy. The basis of the structure-based methods, three-dimensional (3D) structural models of proteins, can be determined directly from experimental methods such as X-ray crystallography, NMR spectroscopy and Cryo-EM, or predicted by computational methods such as *ab initio* and homology modeling. The increasing availability of structures from both experimental and computational methods have greatly advanced drug binding site identification.

Structure-based methods can be further classified into template-based and pocket-based methods. Template-based methods as the name suggested rely on the construction of local structure motifs or patterns from a predefined portion of a structure or substructure that known to be able to define and characterize a functional site. Fuzzy Functional Forms [26] and PHUNCTIONER [27], are two examples of template-based methods that use the evolutionary information among proteins to identify binding sites. On the other hand, pocket-based methods focus mainly on the 3D structures of the target proteins themselves rather than their evolutionary information. In most of these programs, a binding site is identified either as the largest pocket on the protein surface or a small cavity that a molecule or a molecular group can fit inside, which employ the geometrical identification methods or probes such as water or energetics metrics (*e.g.* hydrophobicity) [28-29]. Currently, most of the frequently used structure-based methods fall in this group. The representative packages are shown in Table **1**.

However, widely used classical structure-based methodologies have a common limitation: their accuracy depends on the rigidity of the binding site. However, binding sites cannot always be approximated to be rigid since proteins may change their conformations in response to the ligand binding. In some cases, binding sites are visible only when a protein changes its conformation [46]. Hence, in case of the identification of elusive cryptic sites, or allosteric sites, these aforementioned structure-based methods are inadequate. To offset this, methods such as MD simulations with normal mode analysis [47] to quantify the dynamics of the drug binding sites are rapidly being developed.

3.3. Probe-based MD Methods

The probe-based MD simulation method is a new way to detect drug binding sites by flooding the protein structure with different probes during MD simulations, which properly takes into account their flexibility. In this approach, the protein structure is soaked in the aqueous solution with different explicit solvents at certain concentrations, allowing these solvent molecules to diffuse and fully interact with hot spots on the protein surface. In this way, probe-based MD simulations have two major advantages over the sequence-based or structure-based methods as described previously. First, probe-based MD simulations do not require a training set , so that the method can be applied to all types of protein structures without a prior knowledge about similar protein structures or potential binding sites [48]. Secondly, the most important feature of probe-based MD simulation is that it fully accommodates protein flexibility, solvent effects and many other parameters during simulations.

The organic solvents used as probes can have different chemical properties and shapes, allowing the study of different possible interactions with the protein and thus helping in predicting the maximum binding affinity for any identified binding site [49-52]. During a typical simulation, the different solvent molecules are spontaneously distributed and concentrated around possible binding sites. The elapsed time for solvent molecules to occupy the binding site is directly related to its druggability [51]. In this context, the identified binding sites are ranked by the occupation time and the increase in the local density of the interacting organic molecules. The druggability is also assessed by the maximum binding affinity as predicted [51, 53].

Probe-based MD simulations were applied across a series of protein-protein interaction (PPI) targets to detect and characterize the interfaces where small molecules bind. The visual inspection of the probe-based MD maps for the presence or absence of hotspots provides valuable insights into the druggability of a PPI interface. In the work by Ghanakota *et al*. [54], several probes (acetonitrile, isopropanol, and pyrimidine) were used to capture a range of interactions such as hydrophilic/hydrophobic, hydrogen bonding, and aromatic interactions. Probe-based MD simulations for each protein were carried out with these three different probes separately. To set up the simulations, a layer of probe molecules was firstly placed around the surface of the protein, and then the whole system was solvated with water molecules. In these simulations, the ratio of probe molecules to all the solvent molecules was kept at 5%. PPI targets with the available ligand-bound crystal structures are selected for probe-based MD simulations based on the following two reasons: (1) the presence of small molecules binding at the interface confirms that it is possible for small molecules to bind with at least some amount of detectable affinity, thereby providing us with a dataset to assess if probe-based MD simulations can detect binding hotspots; (2) the ligand-bound crystal structure allows a direct comparison of the hotspot locations from probe-based MD with the ligands from crystal structures. Compared to a grid-based rigid receptor approach (SiteMap), probe-based MD simulation method has better performance in identifying the ligand binding hotspots Fig. (**2**). In addition to small probes mentioned above to capture the specific interactions, ligand molecules can also be used as probes to detect binding sites of the protein. For example, the ColDock method was recently proposed [55], which used multiple ligands as probes to quantify the most representative ligand poses in their MD simulations and further predicted the complex structure.

Although probe-based MD method shows advantages in detecting drug binding sites, it also has its own limitations. For example, probe-based MD simulations seldom pick up the metal interaction sites as hotspots, which might be caused by the lack of proper probes or the accuracy of force fields in dealing with the interactions between probes and metal ions. Besides, sufficient sampling is always a key point for the accuracy of the drug binding site identification using probe-based MD simulations. The development in force fields and computer hardware will help overcome these

Table 1. Examples of available structure-based tools for drug binding site identification.

Software	Developer	Year	Website
LIGSITE[30]	M. Hendlich	1997	none
LigandFit[31]	C.M. Venkatachalam, *et.al.*	2003	none
POCKET[32]	D. G. Levitt & L. J. Banaszak	1992	none
VOIDOO[33]	G. Kleywegt &T. A Jones	1994	xray.bmc.uu.se/usf/voidoo.html
PocketDepth[34]	Y. Kalidas & N. Chandra	2008	proline.physics.iisc.ernet.in/pocketdepth
SURFNET[35]	R. Laskowski	1995	ebi.ac.uk/thornton-srv/software/SURFNET/
Cavity Search[36]	C. M. Ho & G. R. Marshall	1990	none
APROPOS[37]	K. Peters	1996	csb.yale.edu/userguides/datamanip/apropos
Sitemap[38]	*Schrödinger, LLC*	2009	schrodinger.com/sitemap
bSiteFinder[39]	J. Gao, Q. Zhang *et al.*	2016	binfo.shmtu.edu.cn/bsitefinder/
POCASA[40]	J. Yu, *et al*.	2010	altair.sci.hokudai.ac.jp/g6/service/pocasa
Q-SiteFinder[41]	A. T. R. Laurie & R. M. Jackson	2005	bioinformatics.leeds.ac.uk/qsitefinder
LIGSITEcsc[42]	B. Huang & M. Schroeder	2006	projects.biotec.tu-dresden.de/pocket/
FTMAP[43]	C. H. Ngan *et al*	2012	ftmap.bu.edu/param
AlloFinder[44]	M. Huang	2018	mdl.shsmu.edu.cn/ALF/
GRAIL[45]	D. A Schuetz, T. Seidel, *et al.*	2018	none

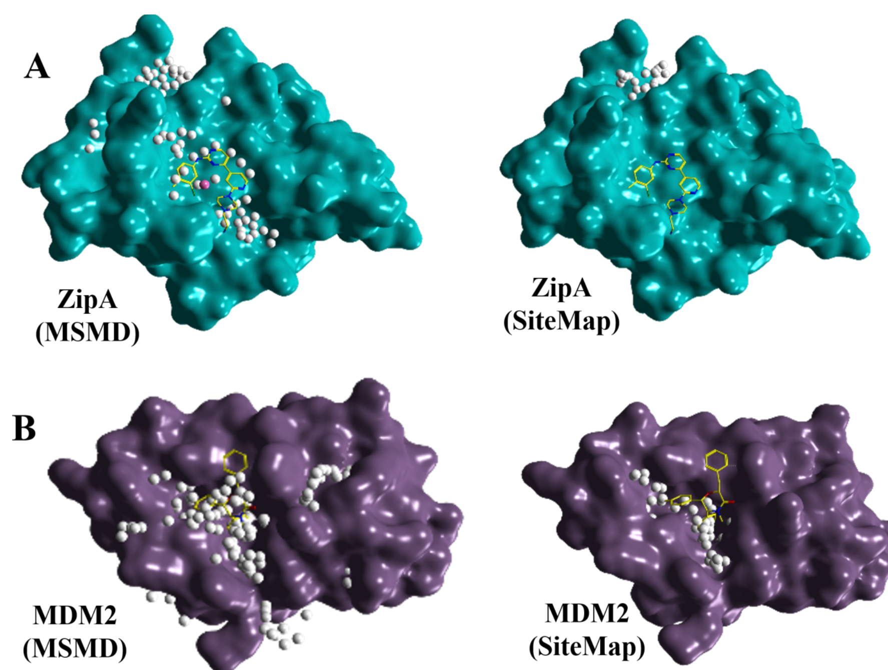

Fig. (2). Comparison between the probe-based MD method (mixed-solvent molecular dynamic simulations, MSMD) and structure-based method (SiteMap). Hotspots are represented in white spheres and are in strong agreement with the location of small molecules in crystal structures. The small molecules disrupting the protein-protein interaction (PPI) interface are rendered in yellow sticks. All probe-based MD maps are contoured at 20σ (σ: the standard deviation of the grid). (A) Probe-based MD simulations detected strong hotspot activity on protein ZipA (PDB ID: 1Y2F) rather than SiteMap. (B) Both methods detect the PPI interface in MDM2 (PDB ID: 4JV7), while probe-based MD shows a more comprehensive coverage of the sub-pockets occupied by the ligand. The figures schematically indicate the binding sites based on the original results by Ghanakota *et al*. [54].

limitations and promote the applications of probe-based MD simulations in identifying drug binding sites.

3.4. Enhanced Sampling Method in Molecular Dynamics Simulations

As mentioned above, sufficient sampling in configuration space is essential for probe-based MD simulations in detecting drug binding sites. However, in many cases, the binding site of the target protein that is not obvious in a crystal structure, and it may take a very long simulation time for the binding site to expose to the probe molecules, making it difficult to access with conventional MD (cMD) simulations. This is mainly because the protein is trapped in a local minimum state and it is very difficult to cross the high energy barriers separating these minima [56]. In order to overcome this shortcoming, one can extend the simulations to a longer time (much longer than hundreds of nanoseconds), which is not effective in most cases. An alternative is to incorporate enhanced sampling methods in the simulations. The enhanced sampling methods, such as accelerated molecular dynamics (aMD) [57], metadynamics [58-60], can provide much better sampling of the configuration space of the target protein, which is important to identify the hidden binding sites [61-63]. In this section, we focus on aMD and metadynamics as well as their applications in drug binding site identification.

3.4.1. Accelerated Molecular Dynamics Method (aMD)

aMD simulation [57] has attracted growing attentions in a series of MD simulation work. Compared to the cMD simulation, aMD can achieve much longer time scale with the same computing resources. The enhanced sampling capability of aMD is accomplished by elevating the energy minima in the potential energy landscape to reduce the transitional energy barriers that separate different states [64-65]. Therefore, aMD allows more sufficient sampling of conformational spaces that are not readily accessible in a cMD scenario. As mentioned above, this method modifies the potential energy landscape by raising energy wells that are below a certain threshold, while leaving those above this threshold unchanged. As a result, energy barriers separating adjacent energy basins are reduced, allowing the system to transit among different conformational states much easier, which is almost impossible in a cMD simulation. In the original form of aMD [64-65], when the system's potential energy $V(r)$ falls below the threshold energy, *E*, a boost potential $\Delta V(r)$ is added. Hence, the modified potential, $V^*(r)$, can be expressed as:

$$V^*(r) = V(r) + \Delta V(r) \quad (1)$$

When $V(r) \geq E, \Delta V(r) = 0$; otherwise,

$$\Delta V(r) = \frac{\left(E - V(r)\right)^2}{\alpha + E - V(r)}, \;\; V(r) < E \quad (2)$$

where α is a tuning parameter that determines the depth of the modified potential energy basin.

It is worth mentioning that this approach multiplies each configuration by the strength of the bias (ΔV) to reweight the phase space of the modified potential. By doing so, aMD achieves the enhanced sampling of the conformational space while accurately converges to the correct canonical probability distribution.

Here, we take Bcl-x_L protein as an example to demonstrate the application of aMD in drug binding site identification. The Bcl-2 family proteins play key roles in the initiation of the apoptosis process and the regulation of programmed cell death by controlling the outer mitochondrial membrane integrity [66]. However, PPI interface is highly adaptable according to the comparative analysis of the Bcl-x_L crystal structures, which means that the binding pocket may easily change its 3D configuration in response to the perturbations exerted by different binding partners. This high flexibility of Bcl-x_L protein makes it inadequate to design drugs solely on the available crystal structures. Therefore, Guo *et al*. [66] performed extensive aMD simulations to investigate the conformational flexibility of the prototypical protein-protein interaction system Bcl-x_L, whose effective timescales are equivalent to several-thousand folds of the previous cMD investigations of several nanoseconds. With aMD simulations, the conformational transitions between the *apo* and *holo* states resolved by crystallographic experiments were investigated. Combined with the variational implicit solvent model [67], the dynamic changes in the topological and physicochemical properties of the BH3 binding pocket on the surface of the Bcl-x_L protein were studied in a systematic manner. Associated with the highly flexible nature of the Bcl-x_L protein, the druggability of the binding pocket changes with the time. Besides, the conformations similar to the ligand binding states can be attained by simulating the *apo* protein system. The results showed that aMD can be applied to identify the more druggable conformations and effectively study them for proper drug design, contributing to the success of drug discovery.

3.4.2. Gaussian Accelerated Molecular Dynamics Method (GaMD)

As mentioned above, aMD has shown advantages in revealing druggable conformations and details of drug binding sites. In the original aMD model, the boost potential $\Delta V(r)$ (Equation 2) was introduced and the subsequent reweighting was integrated in the algorithm for correct convergences on the probability distribution. Hence, different kinds of new $\Delta V(r)$ have been proposed to further increase the efficiency of this method. Among them, Gaussian aMD has attracted lots of attentions [68-69]. This method has simplified the original $\Delta V(r)$ into a harmonic boost potential: $\Delta V(r) = \frac{1}{2}\frac{k_0(E-V(r))^2}{V_{max}-V_{min}}$, which can smooth the potential energy surface, reducing the energy barriers between energy minima. In the modified potential energy formulation, k_0 is the parameter that determines the magnitude of the boost potential. Because the new boost potential follows a Gaussian distribution, the original free energy profiles of biomolecules can be recovered through cumulant expansion to the second order for characterizing biomolecular dynamics, such as ligand binding and activation of G protein–coupled receptors.

3.4.3. Metadynamics

Metadynamics is another powerful enhanced sampling approach in MD simulations, which promotes the quantification of free energy surface as a function of several preselected degrees of freedom or collective variables (CVs). [58-60] In a metadynamics simulation, a history-dependent biased potential is added to the system in the real space to accelerate the evolution of the system states. The general idea of metadynamics is to enhance the system sampling by discouraging revisiting of sampled states. It is achieved by augmenting the system potential $V(r)$ with a bias potential $V_{bias}(r)$. Similar to the Equation (1), the modified potential term $V^*(r)$ can be expressed as:

$$V^*(r) = V(r) + V_{bias}(r) \tag{3}$$

The bias potential $V_{bias}(r)$ is a function of collective variables, which are relevant to particle positions. The bias potential is continuously updated by adding the bias with the rate ω. Then the biased potential can be written as:

$$V_r = \tau \sum_{j=0}^{\left|\frac{t_{sim}}{\tau}\right|} \omega exp^{-\frac{(r-r_j)^2}{2\sigma^2}} \tag{4}$$

Where the parameters τ, ω, σ are determined a priori and kept constant during the simulation. The finite size of the kernel makes the bias potential to fluctuate around a mean value. A converged free energy can be obtained by averaging the bias potential.

The metadynamics-based methods are especially useful for exploring the systems that do not require an initial estimation of the energy landscape [70]. Taking the widely studied G-protein coupled receptors (GPCR) systems as an example [71], the common and effective CV has been defined using the ideally placed and highly conserved residue Trp$^{6.48}$ as a reference point for ligand-GPCR distance measurement and the common orientation of GPCRs in the cell membrane. Using this single CV, a well-tempered multiple-walker metadynamics method [72] with a funnel-like boundary allows an efficient exploration of the entire ligand-binding path from the extracellular medium to the orthosteric binding site. The protocol can be used with X-ray structures or high-quality homology models for the receptor and is universally applicable to agonists, antagonists, partial and reverse agonists. In a recent work, this protocol is also proved to be effective in finding the mixed agnostic and positive allosteric modulators of the cannabinoid CB1 receptor [73]. On the other hand, for complex systems, it is essential to find a proper CVs for the application of metadynamics-based methods. To meet this end, a series of methods such as essential coordinates [74], Sk*etc*h-Map [75] and non-linear data-driven CVs [76] have been developed. Besides, independent metadynamics simulations can be coupled together to improve usability and parallel performance. There are several metadynamics-based methods proposed in this case: the parallel tempering metadynamics [77], the bias-exchange metadynamics [78], and the collective-variable tempering metadynamics [79].

4. DRUG ACTION MECHANISM

As discussed, MD simulations play important roles in identifying drug binding sites especially for those proteins with high flexibility. The detailed information of dynamic interaction processes provided by all-atom MD simulations can also shed light on the exact drug action mechanism at molecular levels. In order to conduct the drug-protein interaction simulations, one needs to parameterize drug molecules, which are mainly proteins or small molecules. For the former, the general parameterization procedure for proteins is sufficient. For small molecules, common force field compatible parameterization toolsets have been well developed [80-82], which enables the direct investigations of the interactions between drug molecules and target macromolecules. With the help of MD simulations, we can reveal the atomic-level information of interactions between drug molecules and their targets.

4.1. Drug-Membrane Interactions

In order to regulate cellular activities, drug molecules will target the key components of cells such as proteins, DNAs, membranes, *etc*. Plasma membrane is the first biological barrier of a mammalian cell, and membrane proteins account for more than 60% of protein targets [83]. Drug molecules either interact with the membrane (or membrane proteins) directly or cross the membrane to access their target molecules. Thus, it is important to understand the drug-membrane interactions for the elucidation of drug action mechanisms. MD simulations can provide detailed dynamic information for understanding drug-membrane interactions.

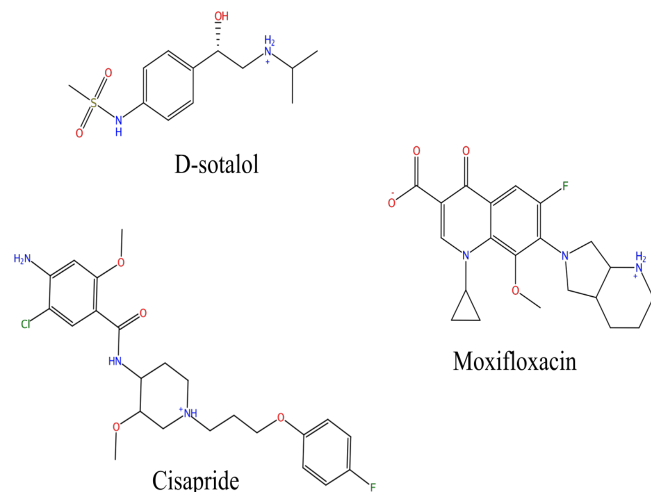

Fig. (3). Chemical structures of three different ionized drugs: d-sotalol, cisapride and moxifloxacin.

For example, a recent MD work focused on interactions of the cardiac ion channel blocker drugs with model membranes [84]. By combining cMD and umbrella sampling simulations, the authors showed the detailed partitioning dynamics and free energy profiles of different drugs Fig. (**3**) or the same drugs (d-sotalol) with different protonation states. Based on free energy profiles, the calculated drug permeability further revealed that only the neutral form of d-sotalol accumulated in the membrane interior and could relatively easily translocate across the lipid bilayer, which thus

was more relevant to the lipophilic channel access and also shared the similarity with other small molecule drugs [85] (*e.g.* aliphatic amines and carboxylic acids). Moreover, the water-membrane partitioning coefficient of this form calculated in all-atom MD simulations was very consistent with the experimental data [86], which validated the feasibility of all-atom MD simulations in studying drug-membrane interactions at the atomic level.

The protein dynamics can also be regulated by its surrounding membrane environment. For example, Janosi *et al*. performed a series of MD simulations, systematically varied the properties of membrane domains and observed the corresponding changes of the nanocluster stability of membrane-bound Ras proteins [87-89]. MD simulations have revealed that the membrane domain stability could regulate the preferred membrane localization of transmembrane peptides [90], where the physicochemical properties of transmembrane peptides also played important roles in their membrane partitioning [91]. Different localization may result in different clustering dynamics. In other words, membrane domains participate in the regulations of the protein dynamics and the subsequent protein-mediated signaling pathways [91].

4.2. Drug-Protein Interactions

MD simulations can be used to study the dynamic binding processes of designed drugs on the target proteins and related regulation pathways. The time scales of these dynamic regulation processes are often beyond the grasp of conventional MD simulations, so enhanced sampling methods are utilized in this kind of studies. Hence, we will discuss how MD simulations help study the drug-protein interactions. Recently, Miao *et al*. has applied GaMD simulations to probe the interactions of G-protein mimetic nanobody Nb9-8 or the agonist iperoxo (IXO) with the M_2 receptor Fig. (**4A**) [92]. Starting from the X-ray structure of the agonist M_2 receptor-nanobody complex [PDB ID: 4MQS], they first placed nanobody Nb9-8 and IXO at least 2nm away from the M_2 receptor and then performed multiple 4.5μs GaMD simulations. Although the calculated two-dimensional (2D) free energy surface was not well converged, GaMD simulations did reproduce the experimental binding modes with a minimum RMSD of 2.48 Å compared with the complex crystal structures, where nanobody Nb9-8 and IXO preferred the intracellular and extracellular regions of the M_2 receptor correspondingly. Moreover, their simulations revealed important low-energy intermediate states for the nanobody-receptor binding and provided possible conformation-regulating pathways for the M_2 receptor after the binding of the nanobody Nb9-8.

As discussed, the protein conformational changes (*e.g.* “open”, “intermediate”, “close” states) are critical to their activation/deactivation regulations. Besides, protein orientation relative to membrane also affects their biological functions. For example, the orientation of RAS proteins may affect the binding of the protein with their downstream effector proteins [93] and the formation of the protein clustering [94]. MD simulation is a powerful tool to quantify the distribution of preferred membrane orientations [95-96]. The insights from MD simulations indicated that the regulation of protein-membrane orientations may be another drug action mechanism. Ryckbosch *et al*. performed all-atom MD simulations (400-500μs) focusing on the impacts of bryostatin (a compound in clinical trials for Alzheimer’s disease) on membrane-bound protein kinase C. [97]. They found that bryostatin’s unique activity is correlated to the distinctive orientation of bryostatin-kinase complex.

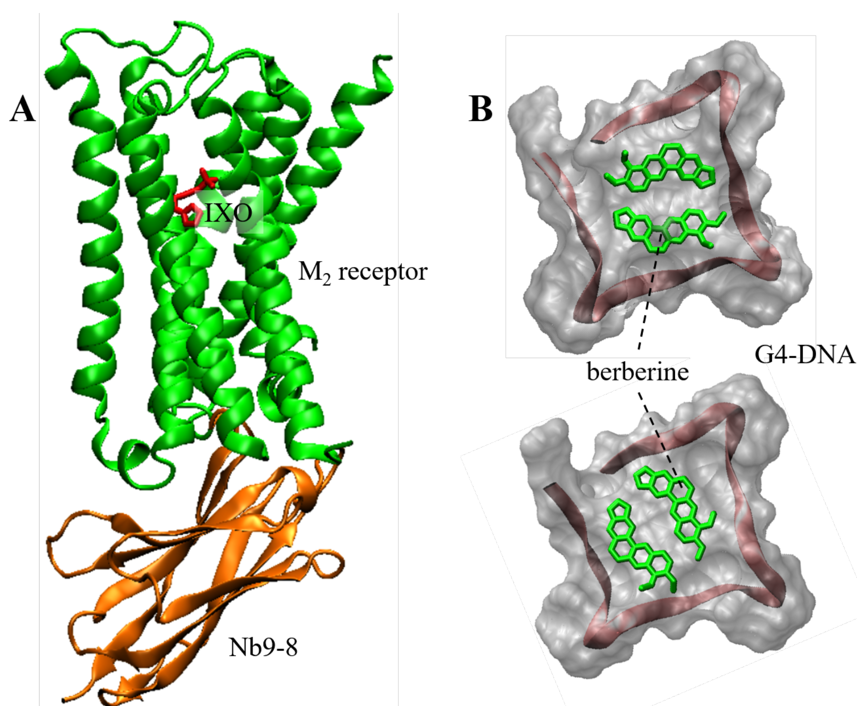

Fig. (4). **(A)** The snapshot of the interactions between the nanobody Nb9-8 and M2 receptor with the agonist IXO (PDB ID: 4MQS); **(B)** Two binding modes of the anticancer alkaloid berberine on the surface of G4-DNA (PDB ID: 3R6R).

4.3. Drug-DNA Interactions

DNA is a molecule composed of two nucleotide chains which coil around each other to form a double helix carrying the genetic instructions used in the growth, development, functioning and reproduction of the living organisms. Given its significance, DNA has always been another important drug targ*et al*most all the available MD force fields have systematic parameterizations for DNA molecules, which make it possible to investigate the DNA dynamics using MD simulations. However, most biological processes related to DNA molecules occur at large time scales. Hence, MD simulations with enhanced sampling methods are necessary to study Drug-DNA interactions. Here, we will take the G-quadruplexes (G4s) as an example to discuss the usefulness of MD simulations to drug-G4 interactions.

As a higher-order DNA structure, G4-DNA usually exits in promoter regions of genes and telomeres, which regulates several important cellular functions including gene transcription and mitotic clock. Therefore, many compounds including the anticancer alkaloid berberine have been proposed as G4-DNA binders. Using a recently developed enhanced sampling method (funnel-metadynamics) [98], Moraca *et al*. successfully revealed the binding mechanism of berberine onto human G4-DNA with all-atom MD simulations Fig. (**4B**) [99]. Funnel-metadynamics is a method which greatly enhances the sampling of the ligand binding process while reduces the over-exploration of the unbound state. Starting from the crystal structure of the berberine-G4-DNA complex (PDB ID: 3R6R), 0.8μs funnel-metadynamics simulations help capture the transition between different binding modes of berberine on G4-DNA. The obtained well-characterized free-energy landscape identified the most preferred ligand binding modes (a parallel orientation at the $3'$-end), a less preferred ligand binding modes (an antiparallel orientation at the $3'$-end) and their corresponding higher energy pre-binding states, which were critical to understand the overall binding dynamics. Further steady-state fluorescence experiments validated the results in the simulations. This indicates the feasibility of MD simulations to help elucidate the drug-DNA binding mechanisms.

CONCLUSIONS

In this review, we focus on the applications of MD simulations in the identifications of drug binding sites and action mechanisms. Generally, drug binding site identification methods can be classified into three categories: sequence-based method, classical structure-based method and probe-based MD method. In the sequence-based method, the conserved sequence may be not sufficient to predict drug binding sites. Although the structure-based method is much more reasonable, it fails to capture the flexibility of target macromolecules. To overcome this shortcoming, MD simulations may provide a suitable way to deal with the flexibility of target macromolecules. By performing MD simulations with enhanced sampling methods, more druggable conformations of target macromolecules and possible drug binding sites can be identified using standard probes of different physiochemical properties. The enhanced sampling MD simulation also provides a way to estimate the overall free energy profiles/surfaces that can be used to identify low-energy intermediate/minimum states and dynamic pathways for the drug binding, and thus helps elucidate drug action mechanisms.

Although MD simulations show great promises in the identifications of drug binding sites and action mechanism, sufficient sampling is always essential to achieve a solid conclusion. In the future, the continued development of more efficient enhanced sampling algorithms and higher computing ability will be important to promote broader applications of MD simulations in drug discovery. In addition, a series of current enhanced sampling methods still depend on predefined collective variables, which may limit the application of the methods and bring bias to the results. Hence, developing algorithms for unbiased efficient sampling could be a research focus in the future.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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