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HIGHLIGHT

Cryptic pockets in proteins: Harnessing conformational dynamics for rational drug design

KEY WORDS

Cryptic pockets;
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Allosteric regulation

A longstanding challenge in drug discovery is the "undruggable" designation of many promising therapeutic targets, which stems from the apparent absence of well-defined binding sites in their ground-state structures. In contrast to conventional strategies targeting the well-defined, static pockets found in experimentally determined protein structures, the discovery of cryptic pockets represents a paradigm shift in engaging such challenging proteins. Harnessing transient binding sites has therefore become a cutting-edge research frontier for expanding the druggable human proteome[1,2]. Cryptic pockets are inherently dynamic and short-lived sites that are usually absent or occluded in *apo* protein structures. Lacking a universally recognized energetic or structural threshold, a cryptic pocket is defined operationally by its ligand-induced formation, a process driven by conformational rearrangements that can range from the displacement of flexible loops and side chains to the movement of entire secondary structural elements[3]. It is important to differentiate these conformation-dependent sites from allosteric pockets. The "allosteric" designation is location-based, referring to any site distal to the orthosteric site, which can be constitutively present. The "cryptic" designation, however, is mechanism-based, describing a pocket formed only through protein dynamics and thus absent in the *apo* state. The two concepts are not mutually exclusive, and the identification of cryptic allosteric sites offers a powerful strategy for modulating protein function[4]. Consequently, cryptic pockets represent a vast, underexplored druggable landscape arising from the intrinsic structural dynamics of proteins. Targeting these sites can render previously "undruggable" targets amenable to therapeutic intervention, thereby opening new avenues for modern drug discovery and development.

Recently, Rowlands and colleagues reported a novel strategy for allosterically inhibiting HECT E3 ligases by targeting cryptic pockets in Cell[5]. E3 ligases have long presented a significant challenge to small-molecule drug discovery because catalytic sites are typically surface-exposed and lack the deep, well-defined pockets required for high-affinity ligand binding[6]. To address these challenges, the team developed a time-resolved fluorescence resonance energy transfer (TR-FRET)-based assay to monitor SMURF1 auto-ubiquitylation. Subsequently, an unbiased high-throughput screen (HTS) of 1.1 million compounds identified three distinct inhibitor chemotypes, with the pyrrole derivative Cpd-8 exhibiting potent and selective inhibition of SMURF1. Structural studies revealed that Cpd-8 binds to a cryptic allosteric pocket within the N-lobe. Crucially, the binding stabilizes an extended conformation of α-helix 10 (αH10), elongating it by approximately 1.5 turns and repositioning it over the conserved glycine hinge (G634). The conformational rearrangement substantially shortens the effective hinge length (from 27.0 to 15.4 Å). The extension of the α-helix incorporates the flexible G634 into a rigid secondary structure, which functionally repositions the hinge's pivot point to K637. This shift of the pivot point from a flexible glycine to a site near a more rigid lysine residue restricts the hinge's dihedral angle flexibility (Fig. 1A). As a consequence, the catalytic motion of the glycine hinge is impaired, abolishing SMURF1's ubiquitin transfer activity. The hinge-dependent

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inhibitory mechanism was corroborated by the validation of engineered SMURF1 escape mutants.

To assess whether this cryptic pocket represents a conserved, druggable site across the HECT E3 ligase family, the researchers used the inhibitor-bound SMURF1 structure as a template to build a homology model of E6-associated protein (E6AP), a prototypical member of the family. The model similarly predicted inhibitor-induced αH10 elongation, shortening of the glycine hinge (G738), and opening of an analogous allosteric pocket in the N-lobe. A machine learning-based virtual screen was then employed to efficiently screen a library of approximately 8 million compounds to identify potential E6AP allosteric inhibitors. *In vitro* experiments confirmed that selected compounds, such as compound i-27, inhibited E6AP activity in a dose-dependent manner. Importantly, the inhibitory mechanism was also validated to be dependent on allosteric regulation of the glycine hinge, as demonstrated by the construction of E6AP escape mutants.

The study represents a landmark advance by revealing a key allosteric cryptic pocket in HECT E3 ligases and defining a unique mechanism of action: inhibition of enzymatic activity by locking a flexible glycine hinge. While this finding establishes a broadly applicable strategy for targeting cryptic pockets, detecting such pockets through conventional approaches like HTS remains challenging. MD simulations serve as a powerful complementary approach, delivering atomistic-level insights into protein dynamics. Exploration of the full conformational landscape enables prediction of the location, opening frequency, and dynamic properties of transient cryptic pockets, thereby providing precise structural blueprints to unlock previously "undruggable" targets for rational drug design.

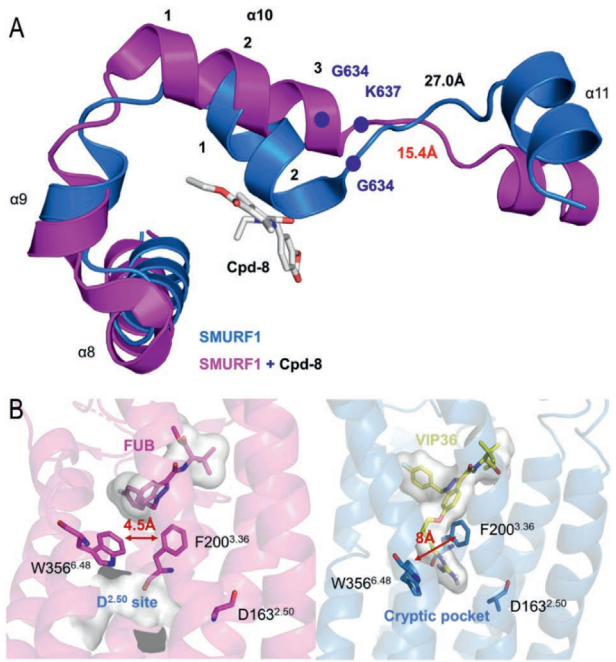

Figure 1 Structural mechanisms of allosteric modulation by targeting cryptic pockets in proteins. (A) Superimposition of the *apo* structure (PDB code: 9FSK; blue) with the Cpd-8-bound structure (PDB code: 9FSJ; pink) of SMURF1 reveals that the allosteric inhibitor Cpd-8 occupies a cryptic pocket. This binding induces extension of the αH10 helix, constrains the glycine 634 hinge, and thereby blocks ubiquitin transfer activity. (B) Molecular dynamics simulations of the FUB-bound CB1 receptor (PDB code: 6N4B; left) displayed a transient cryptic pocket extending toward residue $D163^{2.50}$. Guided by the insight, the agonist VIP36 was rationally designed and subsequently confirmed by Cryo-EM (PDB code: 9B54; right) to engage the cryptic pocket, stabilizing an "active-open" conformation associated with functional selectivity.

Targeting cryptic pockets also holds breakthrough potential for classically challenging protein families such as GPCRs. In the search for safer, non-opioid analgesics, a recent *Nature* study by Majumdar and colleagues employed MD simulations to identify a transient cryptic pocket in CB1[7]. The clinical utility of synthetic cannabinoid receptor agonists (SCRAs) has been hampered by centrally mediated adverse effects and the rapid onset of tolerance, the latter being strongly associated with β-arrestin pathway activation[8]. To overcome these hurdles, the researchers designed novel CB1 agonists that maintain analgesic efficacy while minimizing the central nervous system (CNS) exposure and β-arrestin recruitment. Their strategy was two-pronged: (i) incorporating a positively charged group to restrict CNS penetrance and (ii) targeting the conserved $D163^{2.50}$ residue to attenuate β-arrestin signaling (Fig. 1B). A major structural barrier was that in all previously determined CB1 conformations, the $D163^{2.50}$ residue is sterically occluded by the "toggle switch" residues $F200^{3.36}$ and $W356^{6.48}$. MD simulations addressed this challenge by uncovering a cryptic pocket extending from the orthosteric site to $D163^{2.50}$. The pocket, sampled in only $\sim$8% of trajectories, becomes accessible through an outward rotation of $W356^{6.48}$. Identification of this dynamic feature enabled structure-guided development of CB1 agonists with improved neurological safety, demonstrating how cryptic pocket discovery can expand opportunities in GPCR-targeted drug discovery.

Building on MD-derived insights, the team designed novel CB1 agonists using the MDMB-Fubinaca (FUB) scaffold as a starting point. A positively charged guanidino head group was introduced and tethered *via* an optimized alkyl linker to extend into the cryptic pocket. Iterative computational modeling combined with medicinal chemistry optimization yielded the lead compound VIP36. *In vitro* characterization demonstrated that VIP36 substantially attenuated β-arrestin recruitment (E_{max} = 47% *vs.* 178% for FUB) while maintaining high binding affinity (K_i = 22 *vs.* 75 nmol/L for FUB) and potent G-protein activation, establishing a pronounced G-protein bias. Structural validation by Cryo-EM confirmed that the guanidino moiety of VIP36 occupies the cryptic pocket and induces the outward rotation of $W356^{6.48}$, thereby stabilizing an "active-open" receptor conformation. *In vivo* pharmacokinetic profiles revealed that VIP36 is peripherally restricted, effectively limiting CNS exposure. The compound produced dose- and time-dependent analgesia in inflammatory, neuropathic, and migraine pain models, with efficacy dependent on peripheral CB1 activation. Notably, repeated dosing over 9 days sustained anti-allodynic efficacy without significant tolerance, validating the therapeutic advantage of G-protein bias. VIP36 exhibited >100-fold separation between analgesic doses and those eliciting CNS side effects, highlighting an outstanding therapeutic window.

This work not only delivers a novel therapeutic candidate but also provides deep mechanistic insights into CB1 biased signaling. The strategy is poised to transform the design of functionally or peripherally selective drugs for other GPCRs. While MD simulations provide valuable information, their prohibitive computational cost, coupled with the inherent difficulty of sampling rare and transient conformational states within a feasible simulation

time, restricts their application to systematic, large-scale screening for cryptic pockets. Methodological advancements are crucial to overcoming these sampling challenges. A synergistic approach is often employed, particularly for difficult targets like GPCRs. Computationally, enhanced sampling simulations and Markov state models (MSMs) are used to predict transient states. These predictions are subsequently validated experimentally through methods like site-directed mutagenesis and BRET-based functional assays, which serve to confirm the pocket's allosteric role[9-12]. To augment these approaches, deep generative models such as BioEmu have been developed[13]. By emulating protein equilibrium ensembles with high efficiency, BioEmu generates thousands of conformations at a fraction of the cost of traditional MD simulations. It has demonstrated strong performance in identifying experimentally validated cryptic pockets, thus reducing a major computational burden and accelerating the exploration of this dynamic druggable landscape.

Beyond BioEmu, AI is emerging as a broader suite of powerful tools for discovering new cryptic sites. PocketMiner[14], a graph neural network, can rapidly and accurately predict cryptic pocket locations from a single static protein structure, achieving more than a 1000-fold acceleration over conventional simulation-based methods. Such transformative speed supports systematic, proteome-wide screening pipelines. Complementing this predictive approach, DynamicBind[15] applies a deep learning-based "dynamic docking" strategy that refines an *apo* conformation into a *holo*-like state, capturing large-scale, ligand-induced rearrangements that reveal cryptic pockets hidden in static structures. Collectively, these AI-powered methods establish a robust computational framework for rapid, large-scale discovery and characterization of cryptic pockets.

Given the inherently dynamic and conformationally rare nature of cryptic pockets, no single computational or experimental method can independently provide a complete discovery pipeline. The transient nature of cryptic pockets presents a fundamental challenge to achieving high ligand affinity, as a site's druggability is strongly coupled to the timescale of its opening. Consequently, pockets revealed by slower, large-scale motions are more amenable to high-affinity binding than those formed by rapid, local fluctuations, as the former remain accessible for longer periods, increasing the probability of ligand capture[16]. Their full potential can be achieved only through synergistic integration within a head-to-tail loop framework characterized by dynamic iteration and bidirectional feedback (Fig. 2). The framework is organized around two interconnected functional sides. On one side, computational approaches like MD simulations and AI models generate hypotheses by exploring a conformational landscape of the target to predict the location and properties of potential cryptic pockets. The resulting *in silico* predictions inform the rational design of chemical probes for experimental validation. On the other side, high-sensitivity biophysical and biological techniques, including X-ray crystallography, NMR spectroscopy, and target-based enzyme assays are employed to empirically verify predictions, with high-resolution structural data providing definitive evidence of binding events. The experimental outcomes are then fed back into computational models, creating a data-driven loop that progressively improves predictive accuracy and drives further discovery. Iteration across the two sides helps overcome the limitations of individual methods, such as computational sampling gaps and the stochastic nature of experimental screening.

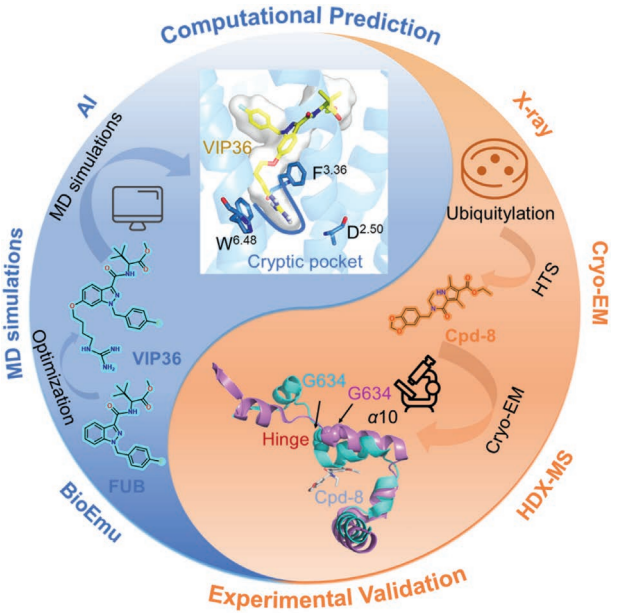

Figure 2 The synergy of computation and experiment in targeting cryptic pockets.

Cryptic pockets represent a new frontier in drug discovery, offering opportunities to target "undruggable" proteins, address drug resistance, and improve selectivity[17,18]. Progress depends on the integration of advanced computational and experimental approaches. MD and AI accelerate pocket prediction and ligand design, while X-ray crystallography, Cryo-EM, and Hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) provide definitive validation of these transient states. Future priorities include the development of algorithms to capture rare conformational events and the establishment of workflows that more tightly couple prediction with experimental testing. By uniting computational innovation with experimental rigor, this field is poised to deliver next-generation therapeutics with greater efficacy and precision.

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Author contributions

Feiyue Ma: Writing - original draft, review & editing. Shuo Wang: Writing - review & editing. Chunkyu Ko: Funding acquisition, review & editing. Meehyein Kim: Funding acquisition, review & editing. Peng Zhan: Writing-review & editing, supervision, funding acquisition, conceptualization.

Conflicts of interest

The authors declare no conflicts of interest.

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