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Designed covalent allosteric modulators: an emerging paradigm in drug discovery

Shaoyong Lu[1] and Jian Zhang[1,2]

[1] Department of Pathophysiology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University, School of Medicine, Shanghai 200025, China
[2] Medicinal Bioinformatics Center, Shanghai Jiao Tong University, School of Medicine, Shanghai 200025, China

Covalent allosteric modulators possess the pharmacological advantages (high potency, extended duration of action and low drug resistance) of covalent ligands and the additional benefit of the higher specificity and lower toxicity of allosteric modulators. This approach is gaining increasing recognition as a valuable tool in drug discovery. Here, we review the recent advances in the design of covalent allosteric modulators with an emphasis on successful examples. A broad spectrum of protein targets capable of being modulated by them reflects the prevalence of this strategy. We also discuss the challenges and future directions in the development of covalent allosteric modulators.

Introduction

Drugs on the market can be categorized in two classes: covalent and noncovalent, according to their ligand–host-protein interactions. Covalent drugs are compounds bearing a reactive, electrophilic warhead, following binding at the active site of a target and then reacting with a specific nucleophilic residue (usually cysteine) at the target site by formation of a stable covalent adduct [1]. Noncovalent drugs refer to compounds that do not form a covalent bond with a host protein. Compared with noncovalent drugs, covalent drugs have potential pharmacological advantages, including enhanced potency, selectivity and prolonged duration of drug action [2–6]. These effects favor administration of lower doses of the covalent drugs to the patient. Owing to continuous target suppression, covalent drugs are resistant to the emergence of drug-resistance mutations [7]. For instance, AZD9292 and CO-1686 enable the effective inhibition of the erlotinib/gefitinib-resistant epidermal growth factor receptor (EGFR) T790M mutant by formation of a covalent bond with Cys797 [7]. In addition, 'undruggable' oncogenic proteins with shallow binding sites are often amenable to covalent inhibition such as the K-Ras4B G12C mutant [8,9]. Despite successful therapies for a myriad of indications such as cancer and hepatitis C by covalent drugs [10], they frequently suffer disadvantages from safety concerns such as off-target effects [11–13]. By contrast, the metabolism of covalent drugs generates chemically reactive drug metabolites that indiscriminately react with other cellular macromolecules, DNA bases and/or the endogenous antioxidant glutathione and eventually lead to immune-mediated toxicity. These rare adverse events are referred to as idiosyncratic adverse drug reactions (IADRs) [14].

A global and in-depth analysis of proteins targeted by covalent kinase inhibitors in human cancer cells using activity-based protein profiling in tandem with quantitative mass spectrometry (MS) have recently pinpointed that the off-target effects of covalent kinase inhibitors tend to be confined to proteins that harbor conserved functional cysteines at the active site [15]. To surmount such major obstacles, huge enthusiasm has been directed at the design of covalent ligands bound to the nonconserved allosteric sites – distinct sites that are topographically and spatially distinct from the highly conserved active (orthosteric) site [16–18]. Allosteric regulation, the 'second secret of life' [19], fine-tunes virtually most biological processes, including signal transduction, enzyme activity, metabolism and transport [20–22]. The propagation of allosteric signals from allosteric sites induced by effectors binding to different, often distant, orthosteric sites enables exquisite control of protein activity [23–26]. Protein kinases and G-protein-coupled receptors (GPCRs) have highly conserved orthosteric sites in protein families. An orthosteric ligand binds to the orthosteric site of one protein, which can also bind to the same sites of

Corresponding author: Zhang, J. (jian.zhang@sjtu.edu.cn)

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homologous family members. However, an allosteric modulator binds to the nonconserved allosteric site of one protein, which is more specific and selective compared with an orthosteric ligand [27]. In addition, orthosteric ligands need to compete with the endogenous ligands bound to the orthosteric sites, thereby shutting off the normal protein functions. By contrast, allosteric modulators do not compete with the endogenous ligands. Indeed, allosteric modulators function when the endogenous ligands are bound to the orthosteric sites, which allow processing of the natural cellular functions in the presence of allosteric modulators and endogenous ligands [22,28]. This effect endows allosteric modulators with fewer side-effects and lower toxicity [17,18,21]. Despite the distinctive advantages conferred by allosteric modulators, they tend to bind to their targets with a slightly lower potency compared with orthosteric ligands [29], as demonstrated by a comprehensive analysis of a large set of allosteric and non-allosteric ligands from the ChEMBL database. An alternative approach to improve binding potency of allosteric modulators is the design of covalent allosteric modulators that target the specific nucleophilic residues in the vicinity of allosteric modulators within allosteric sites. As such, covalent allosteric modulators not only possess the pharmacological merits of covalent ligands but also have the additional benefit of the higher specificity and lower toxicity of allosteric modulators. Thus, covalent allosteric modulators have emerged as a novel direction to follow in therapeutic drug development [30]. In this review, we survey the recent advances in the design of covalent allosteric modulators with a focus on successful examples. First, a widespread interest in the development of covalent allosteric modulators is exemplified across a broad range of bona fide targets, including GTPases, protein kinases, GPCRs, molecular chaperones and ribonucleases. Finally, current challenges facing the design of covalent allosteric modulators and future perspectives are discussed.

Design of covalent allosteric modulators: some typical examples

K-Ras4B GTPase

The quintessential example for design of covalent allosteric modulators as therapeutic agents is the small GTPase K-Ras4B, the most frequently mutated oncogene in human cancer [31]. Owing to the extremely high affinity of K-Ras4B protein for its GDP and GTP substrates and the lack of any evident suitable pockets on the K-Ras4B surface where a molecule might bind tightly, pharmacological inhibition of K-Ras4B has thus remained intractable for the treatment of K-Ras4B-driven cancers, rendering Ras oncoprotein still undruggable for over three decades [32–35]. The G12C mutation, one of the three most-common Ras mutants in cancer, introduces a non-native cysteine residue in the P-loop adjacent to the switch I and switch II regions wrapped in the nucleotide-binding site, which can be utilized to design covalent allosteric inhibitors between a thiol warhead and the target cysteine through disulfide trapping (or tethering) [36].

On the basis of an extensive battery of biochemical, SAR and crystallographic studies, Ostrem *et al.* [8] recently identified a disulfide-containing compound **1** (Fig. 1), which is capable of significantly modifying K-Ras4B G12C but is resistant to label wild-type K-Ras4B. The determination of co-crystal structure of K-Ras4B^{G12C}–GDP in complex with **1** (PDB ID 4LUC) (Fig. 1) displayed that this compound covalently modifies Cys12 and binds in a pocket beneath the switch II region, which does not overlap with the nucleotide-binding site. The allosteric binding site of **1** is named the switch II pocket (S-IIP). To replace the reversible thiol with irreversible warheads to improve potency the authors further identified a vinylsulfonamide-containing compound **2** and acrylamide-containing compounds **3** and **4** that covalently modify Cys12 of K-Ras4B^{G12C}. The determination of co-crystal structures of K-Ras4B^{G12C}–GDP–**2** (PDB ID 4LYF) (Fig. 1) and K-Ras4B^{G12C}–GDP–**3** (PDB ID 4M21) (Fig. 1) complexes showed that each compound can bind to the allosteric S-IIP and forms a disulfide bond with Cys12. Very recently, Patricelli *et al.* [9] performed structural modification based on the scaffold of **4** and identified the most potent compound: ARS-853 (Fig. 1). The determination of the co-crystal structure of K-Ras4B^{G12C}–GDP in complex with ARS-853 (PDB ID 5F2E) (Fig. 1) ascertained that it concurrently binds to the previously characterized S-IIP through covalent modification of Cys12. Furthermore, it showed improved activity against cancer cell lines containing K-Ras4B^{G12C} with IC_{50} values in the low micromolar range. Cellular effects of ARS-853 on the Ras-mediated signaling in cells confirmed that ARS-853 can inhibit downstream signaling of K-Ras4B^{G12C}, including c-Raf, ERK and Akt. Taken together, these results suggest that targeting the allosteric S-IIP of K-Ras4B^{G12C} provides a promising new paradigm for generating anti-Ras therapeutics, supporting the druggability of K-Ras4B^{G12C} by attaching to S-IIP.

Antiapoptotic MCL-1

Myeloid cell leukemia 1 (MCL-1) is an antiapoptotic member of the BCL-2 family of proteins and has emerged as a ubiquitous resistance factor in human cancers [37]. Like BCL-2, the antiapoptotic mechanism of MCL-1 in cancer cells is rooted in the burial of the BH3 killer domains of proapoptotic BCL-2 family members by attaching to a surface groove of MCL-1, referred to as the BH3-binding groove [38]. Thus, pharmacological inhibition of MCL-1 via disengagement of BCL-2 BH3 domains with the BH3-binding groove of MCL-1 is of fundamental importance for cancer drug development [39], because antiapoptotic MCL-1 has currently been recognized as a 'top ten' pathologic factor in a myriad of human cancers.

Recently, Lee *et al.* [40] attempted an alternative mechanism for MCL-1 inhibition by design of small-molecule covalent compounds bound to a new allosteric site distant from the active site – the BH3-binding groove. On the basis of a series of dilution and fluorescence polarization binding assays as well as MS analyses, the authors identified a small-molecule compound named MCL-1 allosteric inhibitor molecule 1 (MAIM1) (Fig. 2), which covalently modifies Cys286 at a helix α6 regulatory site located on the opposite face of the protein from the canonical BH3-binding groove. The covalent adduct was formed between the cyclic enone in the naphthoquinone moiety of MAIM1 and Cys286. C286S mutagenesis abrogated the effect of MAIM1 on the inhibitory activity of MCL-1ΔNΔC in the presence of the proapoptotic BID BH3 domain, highlighting the necessity of Cys286 for MAIM1 activity. However, an atomic resolution view of the association between MCL-1ΔNΔC and MAIM1 is unavailable. Further biochemical and cellular data, together with molecular dynamics (MD) simulations, uncovered that the allosteric mechanism for

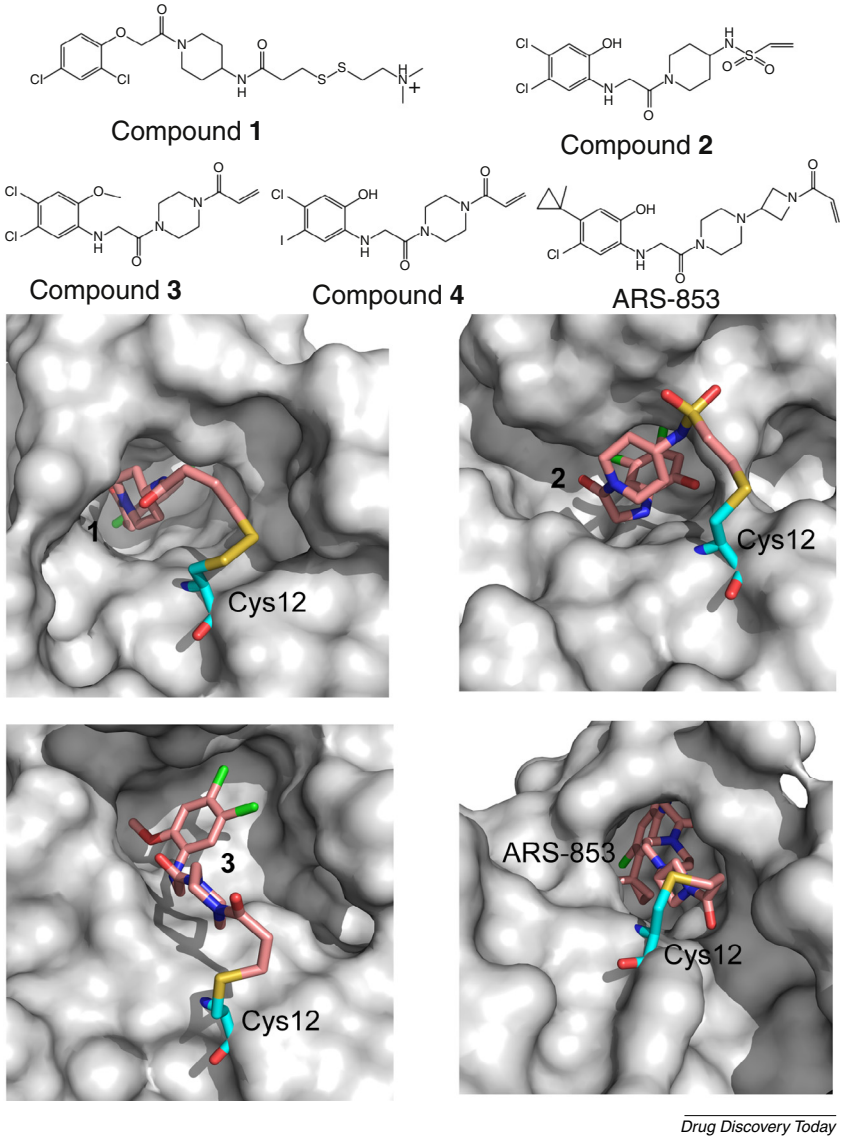

FIGURE 1

Chemical structures of compounds **1**, **2**, **3**, **4** and ARS-853. Co-crystal structures of **1** (PDB ID 4LUC), **2** (PDB ID 4LYF), **3** (PDB ID 4M21) and ARS-853 (PDB ID 5F2E) bound to an allosteric switch II pocket (S-IIP) through covalent modification of a mutated cysteine (G12C) of K-Ras4B.

MAIM1 inhibition of MCL-1ΔNΔC is involved in the impairment of the ability of the canonical BH3 groove to interact with BH3 domains induced by covalent modification of Cys286 by MAIM1, thereby leading to suppress the antiapoptotic activity of MCL-1 implicated in oncogenesis and chemoresistance. Collectively, this result suggests that targeting of MCL-1 through covalent modulation of Cys286 by small-molecule compounds offers a potentially novel avenue for the allosteric inhibition of antiapoptotic activity of MCL-1.

Akt1 kinase

As a serine/threonine kinase, Akt [also called protein kinase B (PKB)] plays a key part in a broad spectrum of cellular functions, including cellular growth and proliferation, metabolism, gene expression and differentiation [41]. Akt is a key regulator involved in the phosphoinositide 3-kinase (PI3K) signaling pathway – one of the most frequently activated proliferation pathways in cancer [42]. The architecture of Akt1 includes three domains; the N-terminal pleckstrin homology (PH) domain acts as a phosphoinositide-binding module and the hydrophobic motif is located at the C terminus adjacent to the catalytic kinase domain in the central region of the protein. In an autoinhibited 'PH-in' state, the intramolecular interaction between PH and kinase domains maintains Akt1 in a closed, inactive conformation. By contrast, in a 'PH-out' state, the disassociation of PH domain with the kinase domain exposes T308 in the activation loop capable of being phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) [42], resulting in the activation of Akt1. Akt1 dysregulation is frequently associated with cancer in humans, such as breast, colorectal and ovarian cancers [41]. Because of the extremely conserved ATP-binding site in kinases throughout the human kinome, targeting allosteric sites of kinases, as a novel tactic, is the preferred option to achieve enhanced selectivity or reduced toxicity.

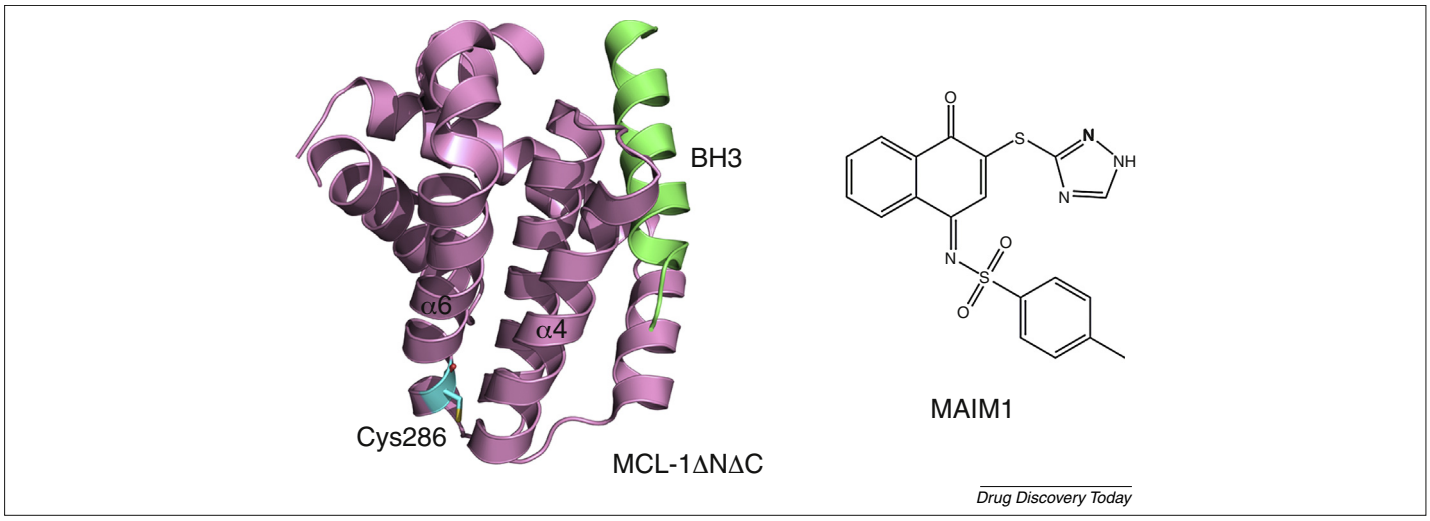

FIGURE 2

MCL-1 allosteric inhibitor molecule 1 (MAIM1) covalently modifies Cys286 (cyan) at a helix α6 regulatory site of MCL-1ΔNΔC (pink) (PDB ID 3MK8). The location of the BH3-binding groove is colored in lime.

The co-crystal structure of Akt1 complexed with an allosteric inhibitor VIII (Fig. 3) reveals that the inhibitor VIII is located in an allosteric binding site formed at the interface of the PH domain and the N- and C-lobes of the kinase domain [43]. This resulting VIII binding results in Akt1 in its closed, inactive PH-in conformation. Structural analysis of the Akt1–VIII complex unveils that two nonreactive cysteines: Cys296 and Cys310 at the activation loop, are located proximal to the inhibitor VIII and could be covalently bound to VIII through attachment of suitable warheads. On the basis of this notion, Weisner *et al.* [44] recently designed the most potent compound **5** bearing a Michael acceptor to covalently modify Cys296 or Cys310 (Fig. 3), with a significantly lower binding affinity of **5** (IC_{50} 0.2 ± 0.1 nM) to Akt1 compared with previously reported allosteric and orthosteric Akt1 inhibitors, such as MK-2206 (IC_{50} 6.5 ± 0.8 nM) and GSK690693 (IC_{50} 2.3 ± 0.3 nM). Replacement of the Michael acceptor on **5** with a nonreactive structurally similar moiety resulted in loss of ~40-fold binding affinity, bolstering the covalent modification of Akt1 by **5** for its higher potency. ESI-MS/MS analyses corroborated that **5** covalently binds to Akt1 at Cys296 and Cys310. Biochemical assays of **5** to Akt1 against a panel of 100 different protein kinases with similar features revealed a

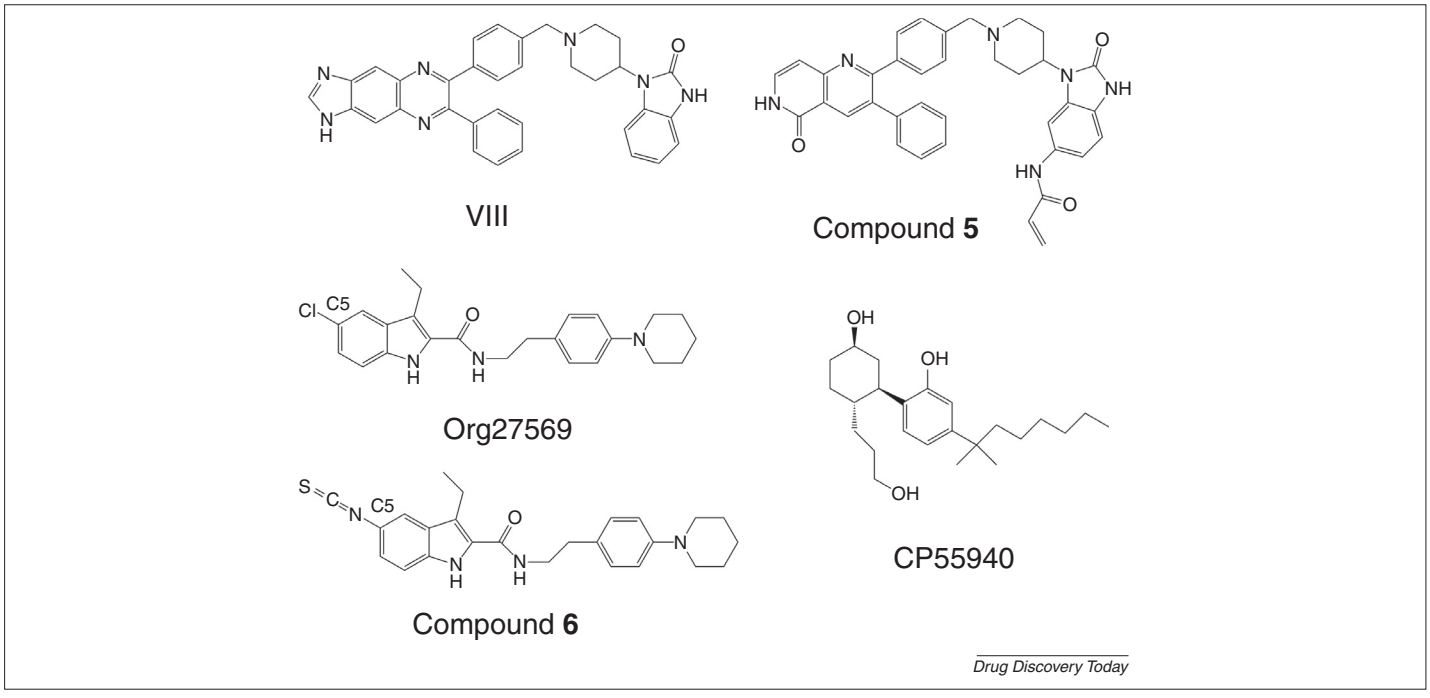

FIGURE 3

Chemical structures of compounds VIII, **5**, Org27569, CP55940 and **6**.

prominent selectivity profile of **5** to Akt1. Furthermore, cellular studies exhibited that **5** impairs Akt1 phosphorylation in cancer cells. Cumulatively, the allosteric covalent inhibitor **5** bearing high potency and selectivity represents a novel opportunity in taming Akt1 inhibition for therapeutic intervention.

CB1R GPCR

Cannabinoid 1 receptor (CB1R), as a class-A GPCR, is expressed predominantly in the brain and is involved in a variety of physiological processes, encompassing learning, memory, mood and cardiovascular regulation [45]. Dysregulated CB1R activity has been implicated in the etiology of many human diseases, such as neurodegenerative diseases, multiple sclerosis and colorectal tumors. As a result, design of small-molecule modulators as therapeutic potential is an appealing strategy to realize improved CB1R-mediated signaling. Accumulating evidence indicates that orthosteric antagonists of CB1R are germane in many side-effects such as depression and anxiety owing to the additional inverse agonist activity of these compounds [46], thwarting their utility in the clinic. As an alternative approach, design of CB1R allosteric modulators offers therapeutic advantages compared with orthosteric ligands. Org27569 (Fig. 3) acts as a positive allosteric modulator of the orthosteric ligand CP55940 binding affinity and a negative allosteric modulator of CP55940 signaling efficacy and potency [47]. Kulkarni *et al.* [48] recently replaced the chlorine atom at the C5 position of Org27569 with an isothiocyanate reactive group, yielding compound **6** (Fig. 3) which has the potential to engage in covalent interaction with CB1R; this experimental paradigm is also called ligand-assisted protein structure. Functional assays revealed that **6** was more potent and efficacious than the parent Org27569 in CB1R-dependent β-arrestin recruitment and cAMP accumulation and had the highest functional selectivity (83-fold) for β-arrestin against cAMP. More remarkably, compared with the parent Org27569, **6** did not exhibit inverse agonism, which can rule out psychotropic side-effects caused by CB1R orthosteric antagonists/inverse agonists. Despite the functional importance of the allosteric covalent compound **6** in CB1R-mediated signaling, there is a pressing need for determination of the co-crystal structure between human CB1R and **6** to characterize the CB1R allosteric site and their detailed atomic-level interaction.

Hsp70 chaperone

Eukaryotic heat shock protein 70 (Hsp70), an ATP-dependent molecular chaperone, is essential for the proper folding of a large body of client proteins. Members of the Hsp70 family have been associated with cell proliferation, cell survival and cell apoptosis. Dysregulation of Hsp70 has thus been implicated in multiple diseases such as cancer, neurodegenerative disease, autoimmunity and infection [49]. The chaperone activity of Hsp70 is allosterically modulated by intramolecular interactions between its two domains: the substrate-binding domain (SBD) and nucleotide-binding domain (NBD) [50]. In the ATP-bound state, the two domains are tightly coupled to each other, which led to the α-helical 'lid' domain of SBD (αSBD) to adopt an open conformation and subsequently fast release substrates. By contrast, in the ADP-bound state, the allosteric communication between the two domains is disrupted, leading to the αSBD in the closed conformation and enhancing affinity for substrates. From a therapeutic perspective, design of potent inhibitors that compete with ATP or bind to Hsp70 allosteric sites provides an avenue to decouple allosteric communication between the two domains [51], thereby leading to the inhibition of Hsp70 activity which contributes to an effective cancer therapy. Recently, based on genome-wide yeast assays, Hassan *et al.* [52] identified the Hsp70 protein family as a potential molecular target for a fungal metabolite, novolactone (Fig. 4). Biochemical characterization of novolactone and LC-MS analysis of Hsp70 in the presence or absence of novolactone elucidated that novolactone forms a covalent complex with Glu444 of Hsp70. The solved co-crystal structure (PDB ID 4WV7) between the Hsp70 SBD and novolactone unequivocally validated this prediction and further pinpointed that novolactone binds to an allosteric site at the interface of the SBD and the NBD (Fig. 4). Structural comparison between unbound and novolactone-bound Hsp70 crystal structures suggests that the allosteric communication between the SBD and the NBD is disrupted in response to novolactone binding, thereby resulting in Hsp70 in a catalytically incompetent conformation that impairs ATP-induced substrate release and inhibits refolding activities. This result indicates that allosteric capture of an inhibited Hsp70 conformation through covalent modification of Glu444 by novolactone opens a new horizon for the future of Hsp70 drug discovery.

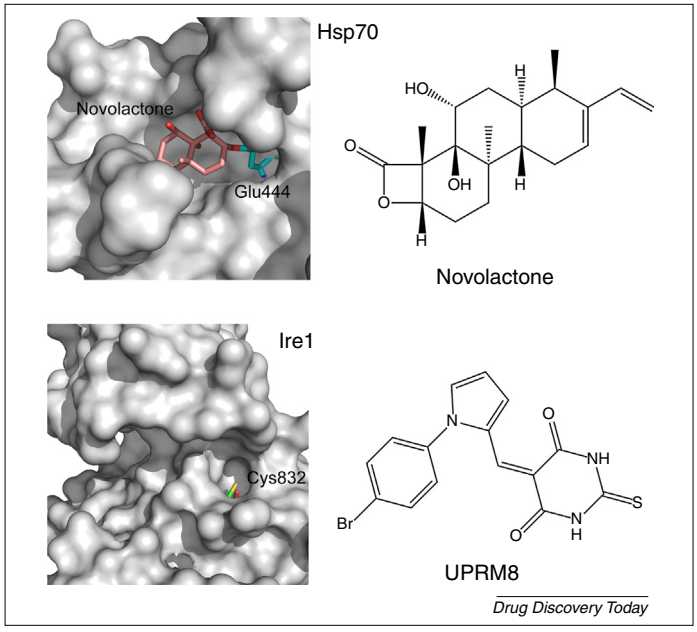

FIGURE 4

Co-crystal structure of novolactone bound to an allosteric site through covalent modification of Glu444 of Hsp70 (PDB ID 4LUC). Chemical structure of covalent allosteric modulator, unfolded protein response modulator 8 (UPRM8), bound to yeast Ire1 through covalent modification of Cys832.

Ire1 ribonuclease

Ire1, a transmembrane kinase/ribonuclease, is responsible for the unfolded protein response (UPR) – a cellular stress response related to the endoplasmic reticulum [53]. Overactivation of UPR is involved in some cancers and inhibition of Ire1 has been proposed as a promising strategy for the development of high-fidelity Ire1 inhibitors. On the basis of a specific Cys832 at the DFG + 2 position in the activation loop of yeast Ire1, Waller *et al.* [54] performed a

high-throughput screen of a small-molecule combinatorial library containing electrophilic compounds to covalently modify Cys832. Three UPR modulators (UPRMs) – pyrimidinediones – were identified to prevent Ire1-dependent HAC1 mRNA splicing. Among them, UPRM8 (Fig. 4) was selected for further mechanistic investigation. Biochemical characterization of UPRM8 revealed that it inhibits yeast Ire1 ribonuclease activity and human Ire1 catalytic activity through an allosteric mechanism. MS analysis and mutagenesis unraveled that inhibition of yeast Ire1 activity by UPRM8 occurs by formation of a covalent complex between Cys832 in the Ire1 activation loop and UPRM8. In fact, human Ire1 includes α and β isoforms; human Ire1α and Ire1β contain the conserved cysteine at the DFG + 2 position. On the basis of this observation, UPRM8 could covalently modify human Ire1α and Ire1β, leading to off-target polypharmacology. However, this hypothesis still needs to be tested by *in vitro* kinase and ribonuclease activities of human Ire1. Although URPM8 represents a potential covalent allosteric modulator of Ire1, the exact binding paradigm for URPM8 within the Ire1 allosteric site is still required to be unearthed by X-ray crystallography for the development of more-potent and -selective inhibitors of Ire1.

Concluding remarks: current challenges and future directions

Despite the numerous examples of successful covalent ligands advanced into the market, the repertoire of covalent allosteric modulators is only emerging. Covalent allosteric modulators bear dual pharmacological merits of covalent ligands and allosteric modulators, including the increased biochemical efficiency and higher specificity, the prolonged duration of action and the lower toxicity, the mitigation of the emergence of therapy-induced drug resistance, and the increased probability of targeting 'shallow' binding sites of intractable targets, but the design of covalent allosteric modulators is highly challenging. First, a major stumbling block in designing covalent allosteric modulators is the identification of allosteric sites in proteins, which is the first step in covalent allosteric drug discovery. However, it is not trivial to find allosteric sites via current experimental approaches; in fact, the vast majority of known allosteric sites have fortuitously been discovered through biochemical experiments. To our delight, a host of computational approaches based on sequence, structure and dynamics has recently been developed aiming to analyze and single out potential allosteric sites in proteins [55–58]. Nonconserved nucleophilic residues such as cysteines in the vicinity of the lead compounds within potential allosteric sites can be cherry-picked via a complete bioinformatics analysis of a protein family and the reactive, electrophilic warheads should be attached in the parent small-molecule compounds wherein the two reactive centers (nucleophile and electrophile), coupled with structure-based drug design, must be occupied in close contact and in the correct geometry for the chemical transformation to occur. Second, hyperactive warheads might lead to drug-induced toxicity such as hepatoxicity, mutagenicity or carcinogenicity. To prevent unwanted off-target covalent modifications and to attenuate the risks of IADRs, less reactive and safer functional groups called soft electrophilic warheads such as an acrylamide group can be utilized to specifically and moderately react with nucleophilic residues in the topology of an enzyme or receptor binding site. Furthermore, advanced analytical techniques such as LC-MS/MS methodology for proteomic applications and high-field NMR spectroscopy analysis of purified adducts should be adopted to minimize the potential risks of reactive drug metabolites at the initial discovery or lead optimization phase [59,60]. In summary, we expect that comprehensive mechanistic understanding of protein allosteric modulation in concert with biochemical and clinical data from covalent drugs will provide valuable avenues toward the design of covalent allosteric modulators, which is an up-and-coming trend in drug discovery.

Conflicts of interest

The authors declare that they do not have any conflict of interest related to this manuscript.

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