

Review

# Recent Advances in the Discovery of CK2 Allosteric Inhibitors: From Traditional Screening to Structure-Based Design

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**Abstract:** Protein kinase (CK2) has emerged as an attractive cancer therapeutic target and recent efforts have been made to develop its inhibitors. However, the development of selective inhibitors remains challenging because of the highly conserved ATP-binding pocket (orthosteric site) of kinase family. As an alternative strategy, allosteric inhibitors, by targeting the much more diversified allosteric site relative to the conserved ATP-binding site, achieve better pharmacological advantages than orthosteric inhibitors. Traditional serendipitous screening and structure-based design are robust tools for the discovery of CK2 allosteric inhibitors. In this review, we summarize the recent advances in the identification of CK2 allosteric inhibitors. Firstly, we briefly present the CK2 allosteric sites. Then, the allosteric inhibitors targeting the well-elucidated allosteric sites ( $\alpha/\beta$  interface,  $\alpha D$  pocket and interface between the Glycine-rich loop and  $\alpha C$ -helix) are highlighted in the discovery process and possible binding modes with the allosteric sites are described. This study is expected to provide valuable clues for the design of CK2 allosteric inhibitors.

**Keywords:** protein kinase (CK2); allosteric site; allosteric inhibitors; traditional screening

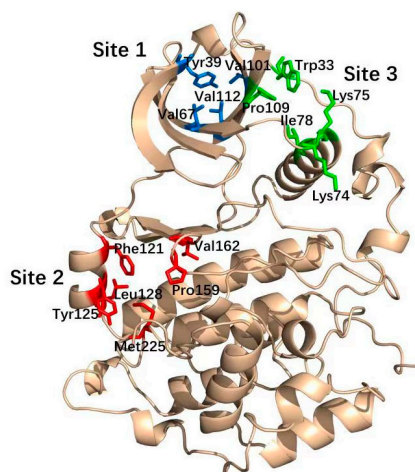
## 1. Introduction

Protein kinase (CK2) is a ubiquitous Ser/Thr kinase that phosphorylates more than 300 substrates and is also involved in several cellular processes as the cell survival promoter and apoptosis suppressor [1,2]. The CK2 holoenzyme is often described as a tetramer of two catalytic subunits (CK2 $\alpha$  or CK2 $\alpha'$ ) and two regulatory subunits (CK2 $\beta$ ). Interestingly, the CK2 holoenzyme complex is subject to disassembly and reassembly, and the irreversible nature of the CK2 holoenzyme makes the free population of each subunit, CK2 $\alpha$  (or CK2 $\alpha'$ ) and CK2 $\beta$ , co-exist in different cellular compartments [3]. Unlike other multi-subunit protein kinases, CK2 $\beta$  is not required for the activity of the catalytic subunits. Both the free catalytic subunit CK2 $\alpha$  (or CK2 $\alpha'$ ) and the CK2 holoenzyme complex are endowed with constitutive activity, while CK2 $\beta$  acts only as a targeting subunit and/or the central component of the stable tetrameric CK2 complex, affecting full enzymatic activity, stability, and CK2 $\beta$ -dependent substrate specificity. Hence, a limited number of protein substrates (eIF2 $\beta$ ) are phosphorylated by the CK2 holoenzyme complex, but not the noncomplexed CK2 $\alpha$  [4]. Meanwhile, CK2 is also involved in the regulation of Wnt signaling, NF- $\kappa$ B and PTEN/PI3K/Akt-PKB pathways by stabilization of the pro-oncogene and anti-apoptosis factors. Mounting evidence has demonstrated that over-expressed CK2 is correlated with many common solid tumor types, which include lung cancer, head and neck cancer, bladder cancer, or mesothelioma [5]. Consequently, this kinase has been regarded as a valuable

therapeutic target, and numerous efforts have been made towards the discovery of CK2 inhibitors for cancer therapy.

The growing amount of CK2 crystal structures in the Protein Data Bank provide valuable clues for the discovery of CK2 inhibitors. Diverse strategies to inhibit CK2 functions by small molecules have been explored in preclinical studies. By targeting the ATP-binding pocket, substrate-binding site, allosteric sites or CK2 $\alpha/\beta$  interface, a variety of CK2 inhibitors with the anti-cancer activity have been developed [6]. Like other kinases, the ATP-binding pocket of CK2 $\alpha$  has been considered as the orthosteric site to design ATP-competitive inhibitors, such as benzimidazole-derivative anthraquinone, tricyclic quinolone derivatives and natural products [7–9]. Unfortunately, although several ATP-competitive inhibitors of CK2, ranging from micromolar to nanomolar, have been discovered, most of them were impeded in their ability to become the promising drug candidates due to their lower specificity and diversity deficiencies [10]. Despite the fact that the potent compound CX-4945 ( $IC_{50} = 1.5$  nM against CK2 $\alpha$ ) has entered phase II clinical trials for the treatment of cholangiocarcinoma [11], this compound still suffered from low selectivity (dual-specificity tyrosine phosphorylation-regulated kinases (Dyrk) 1A and 1B with  $IC_{50}$  values of 6.8 and 6.4 nM, respectively) due to the highly conserved ATP-binding pocket of kinases [12]. Therefore, to overcome the general shortcomings, an alternative strategy strives to identify non-ATP-competitive inhibitors targeting sites outside of the conserved ATP site.

Allostery is the regulation of macromolecule function through the binding of a modulator to an allosteric site that is spatially and topographically distinct from its orthosteric site. Therefore, allosteric sites are regarded as attractive targets to develop non-ATP-competitive kinase inhibitors [13–15]. By targeting the much more diversified allosteric site relative to the highly conserved ATP-binding pocket, allosteric inhibitors might avoid the drawbacks of most conventional ATP-competitive inhibitors with their enhanced selectivity and reduced toxicity. For example, the subtype selective PDE4D allosteric inhibitor BPN14770 exhibited reduced vascular toxicity over earlier PDE4 inhibitors that lacked the subtype selectivity [16], and compound 8t, as the allosteric inhibitor of phosphoglycerate mutase 1, was demonstrated to delay tumor growth in the H1299 xenograft model without the obvious toxicity [17]. Notably, the identification of CK2 allosteric sites is a prerequisite to the discovery of allosteric inhibitors. To date, six allosteric sites of CK2 $\alpha$  have been identified using the combination of bioinformatics and biochemistry methods [18], and three well-elucidated sites among them have been targeted to discover CK2 allosteric inhibitors. As shown in Figure 1, Site 1,  $\alpha/\beta$  interface (Tyr 39, Val67, Val112 and Val101) has been confirmed to be occupied by the DRB, cyclic peptide (Pc) and W16 [19]. The  $\alpha$ D pocket named Site 2 was demonstrated as the allosteric pocket including Phe121, Pro125, Leu128, Pro159, Val162 and Met225. Recently, Bestgen et al. discovered aminothiazole derivatives as the allosteric modulators of CK2 by targeting the interface between the  $\alpha$ C helix and the glycine-rich loop (G-loop) [20].

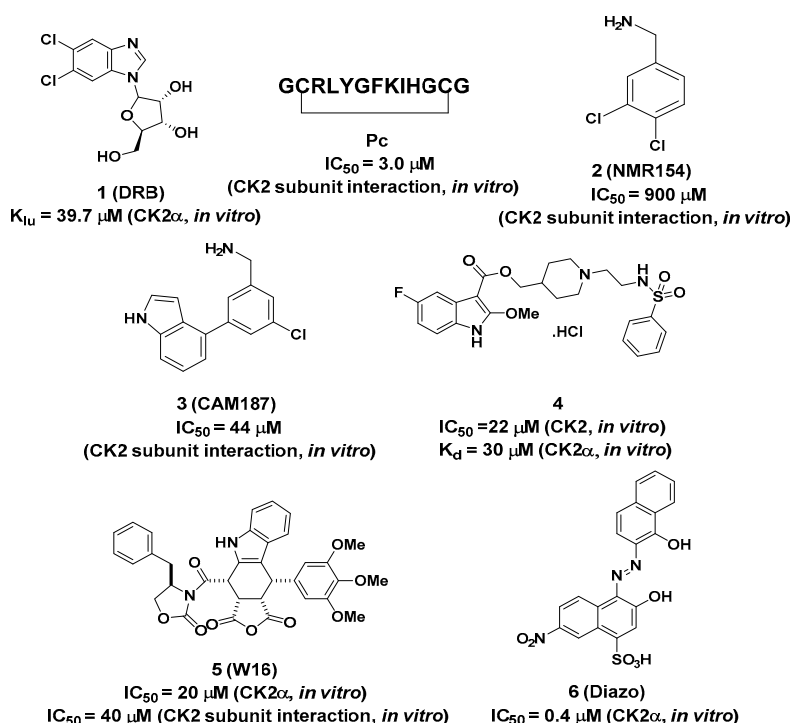


**Figure 1.** Three well-elucidated allosteric sites of CK2 $\alpha$  including Site 1 (blue), Site 2 (red) and Site 3 (green).

Most of the known CK2 allosteric hits were discovered by high-throughput screening (HTS) and virtual screening methods, which are the useful approaches to identify starting points against any target of interest for drug design [21,22]. This review highlights the recent progress in the development of CK2 allosteric inhibitors. We classified these inhibitors according to the allosteric sites where they bind, including Sites 1,2 and 3, and presented their discovery method and the key receptor–ligand interactions at the allosteric sites in detail.

## 2. Compounds Targeting Site 1

As indicated from the crystal structures of CK2 holoenzyme, (PDB ID: 1JWH) [23] and (PDB ID: 4IB5) [24], the subunit interface is located at a shallow groove consisting of hydrophobic residues of the G-, C-, and  $\beta$ 4/ $\beta$ 5 loops of CK2 $\alpha$  N-terminal domain. These observations provide a strategy to identify inhibitors that modulate interactions between the CK2 $\alpha$ /CK2 $\beta$  subunits as indicated in Figure 2. Although this interface was predicted as the druggable allosteric site, not all the molecules that bind here are the allosteric inhibitors.

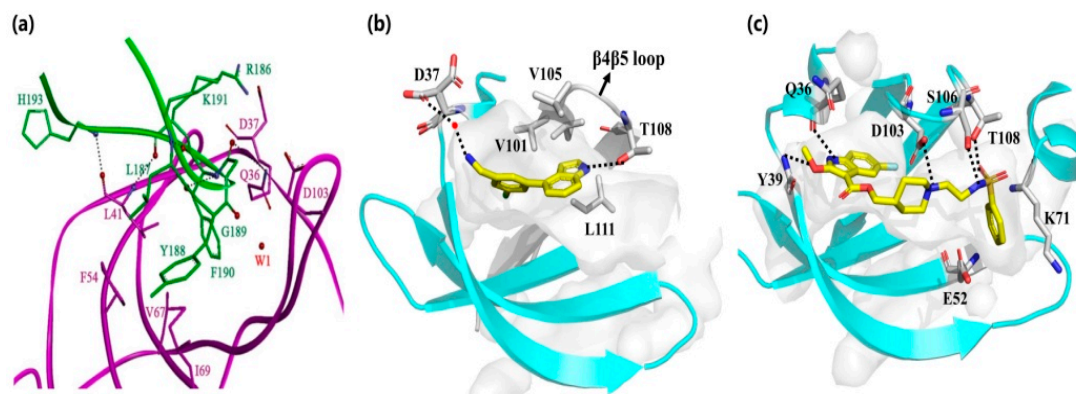


**Figure 2.** Structures of compounds (1, Pc, 2, 3 and 4) and allosteric inhibitors (5–6) binding to Site 1.

DRB was found to be the first small molecule with a dual binding mode, by targeting the ATP pocket and  $\alpha$ / $\beta$  interface simultaneously (PDB ID: 2RKP) [25]. This discovery presented a new druggable site out of the catalytic pocket on CK2 $\alpha$ , with the potential to induce the selective disruption of the CK2 $\alpha$ /CK2 $\beta$  subunit assembly. However, kinetic measurements indicated that the second DRB only disturbed the formation of CK2 $\alpha$ /CK2 $\beta$  holoenzyme, without any inhibitory effects on CK2 $\alpha$ .

Laudet et al. [26] designed a truncated CK2 $\beta$  mimic peptide (Pc) with the ability to antagonize the assembly of the CK2 holoenzyme through competition with CK2 $\beta$  binding to CK2 $\alpha$ . As shown in Figure 3, the hydrophobic interactions between the hotspot amino acids Y188 and residues F54 of the G-loop and I69 of the C-loop, as well as F190 with residues Q36 and L41 of the G-loop, V67 of the C-loop, and Val101 of the  $\beta$ 4/ $\beta$ 5 loop all contributed to the binding of Pc to CK2 $\alpha$ . Our group also revealed the dynamic structural features of CK2 $\alpha$ -Pc complex with the single mutation of Y188A and F190A [27]. By analyzing the contribution of key residues indicated from molecular dynamics simulations, a series of peptides with an improved binding affinity to CK2 $\alpha$  were rationally designed.

and synthesized, as well as anti-proliferative and pro-apoptotic effects against the HepG2 cell line [28]. Additionally, the introduction of halogens into the meta-position of F190 [29] or a novel covalent linker on Pc [30] have been explored as the possible improved versions. Unfortunately, none of the Pc derivatives are cell-permeable, and, thus, they all require a cell-penetrating peptide for delivery.



**Figure 3.** Interaction between (a) Pc, (b) compound 3 and (c) 4 with  $\alpha/\beta$  interface.

Fragment-based screening is also another powerful tool for the development of allosteric inhibitors. By screening the fragment library, an initial hit compound, 2 (NMR154), was found to bind in both the ATP pocket and the  $\alpha/\beta$  interface of CK2 with an  $IC_{50}$  of 900  $\mu\text{M}$  [31]. Through elaborate iterations, compound 3 (CAM187) was synthesized and exhibited potent inhibitory activity on CK2 ( $IC_{50} = 44 \mu\text{M}$ ) with the improving selectivity for  $\alpha/\beta$  interface binding only. As indicated from the co-crystal structure of CK2 $\alpha$ -CAM187, the biaryl part of this molecule fitted into the  $\alpha/\beta$  interface. The amine formed apolar interaction with D37 and the indole ring located under the  $\beta_4/\beta_5$  loop, alongside the interaction between the nitrogen atom and the OH group of T108. Due to the better drug-like properties of CAM187 relative to W16 and Diazo, the potent and selective fragment CAM187 could be regarded as a pharmacophoric group for the development of drug-like molecules.

In parallel with efforts to develop peptide-based inhibitors, compound 4 was optimized and validated to bind at the  $\alpha/\beta$  interface based on the lead compound discovered by virtual ligand docking and screening [32]. As shown in Figure 3, the fluorinated indole ring was buried in the hydrophobic pocket consisting of L41, F54, V67, V101, P104, V105, A110, and V112. Additionally, Q36 and Y39 formed polar interactions with the -NH- of the indole ring and the oxygen atom of ester, respectively. The left part, linked with ester, extended toward the top of the kinase P-loop with a salt bridge interaction between the nitrogen of the piperidine ring and the carbonyl of D103, as well as the hydrogen bond between sulfonamide and S106 and T108 of the  $\beta_4$ - $\beta_5$  loop (Figure 3b). Enzymatic assay confirmed that compound 4 blocked the CK2 $\alpha$ /CK2 $\beta$  interaction and inhibited phosphorylation of CK2 $\beta$ -dependent substrates. Furthermore, this compound exhibited better cell permeability than Pc with the impeded cell growth, migration and induced cell death effects on triple-negative breast cancer cells (MBA-MB-231). Therefore, this compound is the first example of a rationally designed small molecule that efficiently displaces CK2 $\beta$  from CK2 $\alpha$  in the cellular context.

DRB, Pc and compounds 3 and 4 only efficiently inhibited the CK2-holoenzyme mediated phosphorylation of CK2 $\beta$ -dependent substrates, but without inhibitory effects on the catalytic activity of CK2 $\alpha$ . Consequently, these mentioned compounds may not be considered the real allosteric inhibitors, but the allosteric site-binding molecule.

By a high-throughput screening of a collection of phyllotoxine indole-analogues, W16 was identified as the first chemical inhibitor that showed selective disruption of the CK2 $\alpha$ /CK2 $\beta$  assembly through binding to the CK2 $\alpha$ /CK2 $\beta$  interface [33]. Inhibition kinetic studies revealed that W16 induced a non-ATP-competitive inhibition of CK2 $\alpha$ . Unlike Pc, the binding of W16 into the subunit interface

may trigger a functional conformational change in CK2 $\alpha$  that exerted a negative influence on the ATP-binding site.

Azonaphthalene derivatives were also discovered as newly non-ATP-competitive CK2 inhibitors using an automated screening method, and compound **6** (Diazo) displayed an IC<sub>50</sub> of ~0.4  $\mu$ M on CK2 $\alpha$  [34]. Small-angle X-ray scattering experiments showed that this inhibition was due to the inactive conformation of CK2 $\alpha$  that blocked the binding of substrates upon the binding of these inhibitors. Diazo (at 5  $\mu$ M) selectively inhibited more than 95% of CK2 but had no effect on the other protein kinases tested, such as Pim-1, CDK2 and JAK2. However, as it was not possible to solve the crystal structure of CK2 $\alpha$ -Diazo, further structural analyses are still needed to reveal the inhibition mechanisms.

Overall, although all the mentioned compounds interfered with the specific interaction between CK2 $\alpha$  and CK2 $\beta$ , the inhibitory effects of them exerted on CK2 were different. Contrary to ATP-competitive inhibitors, DRB, Pc and compound **3** and **4** only inhibited the phosphorylation of CK2 $\beta$ -dependent substrates without affecting the catalytic activity of CK2 $\alpha$ . However, the binding of W16 and Diazo to the CK2 $\alpha/\beta$  interface may have triggered a negative effect on the active conformation of CK2 $\alpha$  that blocked the binding of ATP or substrates and, thus, inhibited the catalytic activity of CK2 $\alpha$ .

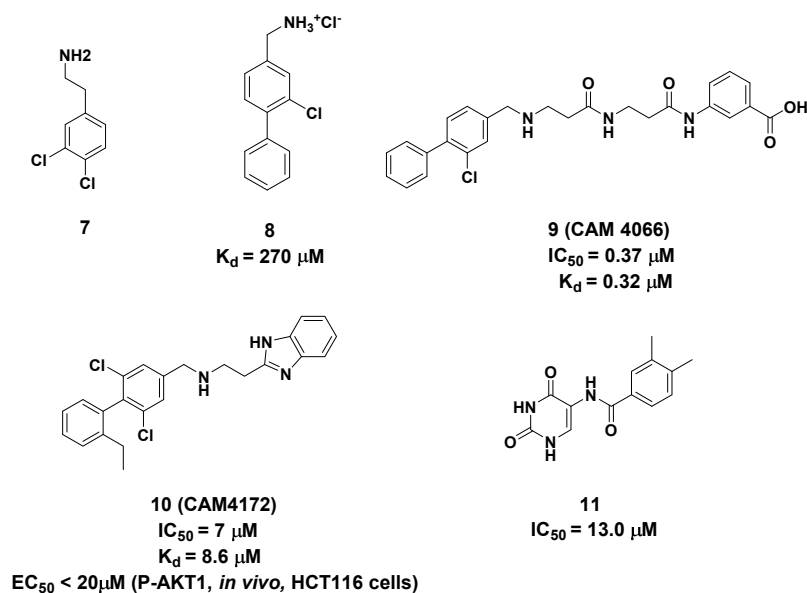
### 3. CAM4066 Derivatives and Uracil Analogues Targeting Site 2

Using a high concentration crystallographic screen method, compound **7** was identified as binding in the  $\alpha$ D site, but with lower K<sub>d</sub> values (Figure 4) [35]. The amine formed hydrogen bonds with the backbone carbonyls of V162 and P159, and the phenyl was accommodated into the hydrophobic pocket. Based on compound **7**, the replacement of the chlorine atom at Position 4 with a phenyl ring was optimized to make it bind more deeply into the  $\alpha$ D pocket. As a result, compound **8** showed the higher affinity of 270  $\mu$ M, although without any inhibition of CK2 kinase activity. With the introduction of the optimized flexible linker combining two pharmacophoric fragments, CAM4066 was synthesized and showed potent inhibitory effects and good selectivity on CK2 $\alpha$  (IC<sub>50</sub> = 0.37  $\mu$ M) [36]. As shown in Figure 5, the two pharmacophoric fragments were involved in forming hydrogen bonds with Pro159, Val162 and Lys68, respectively, while the linker formed the H-bonds with Asn118. Appropriate coupling between the linker and pharmacophore fragments was essential for the binding of CAM4066 with CK2 $\alpha$ . Our group explored the structural mechanism for the decreased inhibition of the rigid linker and non-ionizable substituted fragment (pre-CAM4066) against CK2 by molecular dynamics simulation (MD) studies [37].

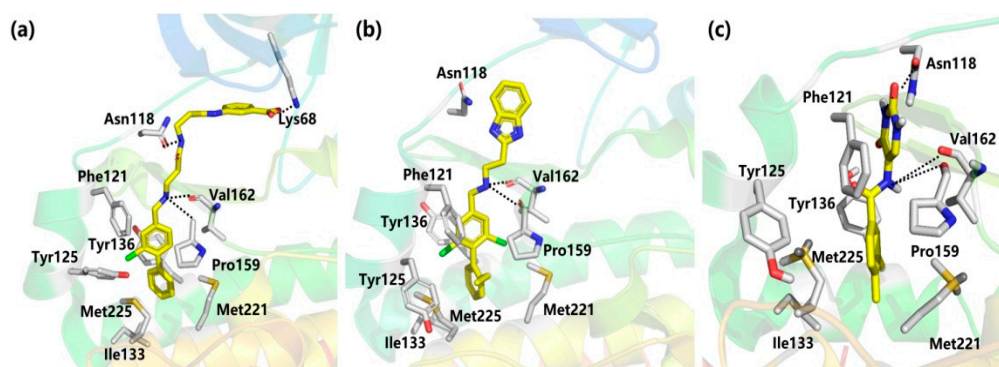
However, because CAM4066 interacted with the  $\alpha$ D pocket (Figure 5) and the positive area of the ATP-binding pocket simultaneously, despite its potent inhibition effect and high selectivity, this compound may not be considered as a classic allosteric inhibitor which binds completely outside the orthosteric pocket. With the aim of developing improved  $\alpha$ D-site binding inhibitors without reaching deep into the ATP-binding pocket, the second generation inhibitor CAM4712 (IC<sub>50</sub> = 7  $\mu$ M) [38] was designed by growing benzimidazole fragments near, but not inside, the mouth of ATP sites, which is a remarkable improvement relative to CAM4066. Similar to compound CAM4066, NH was entrapped in the hydrophobic site and formed an H-bond with Pro159 and Val162. Meanwhile, the benzimidazole fragment formed the  $\pi$ - $\pi$  interaction with His160. Competition experiments further confirmed that CAM4712 was the potent and selective allosteric inhibitor by binding in the  $\alpha$ D pocket without facilitating cellular uptake.

Besides the above mentioned  $\alpha$ D-site binding inhibitors CAM4066 and CAM4712, our group identified the novel allosteric inhibitors of CK2 $\alpha$  using the pharmacophore model and Alloscore-based virtual screening of ChemBridge fragment library [39]. By considering the filtration of fit values > 2.5 with the  $\alpha$ D pocket pharmacophore model, as well as the Alloscore value of 5.8, compound **11** combined with the uracil group was discovered to be an allosteric inhibitor of CK2 $\alpha$  (IC<sub>50</sub> = 13.0  $\mu$ M), which is similar to that of CAM4712. As indicated by the MD simulation result, the skeleton of this compound fitted well into the  $\alpha$ D pocket, including the polar interaction formed between the uracil group and the NH linker with Val162 and Asn118, as well as the hydrophobic packing of a phenyl ring with residues

Ile133, Tyr136, Met221 and Met225. Given the potent inhibitory effect of compound **11** against CK2 $\alpha$ , we made a further elaborate optimization of the linker and uracil substitution to discover the potent and selective CK2 allosteric inhibitors (unpublished manuscript).



**Figure 4.** Structure and biological activity of compounds targeting Site 2 ( $K_d$  and  $IC_{50}$  values tested *in vitro*).



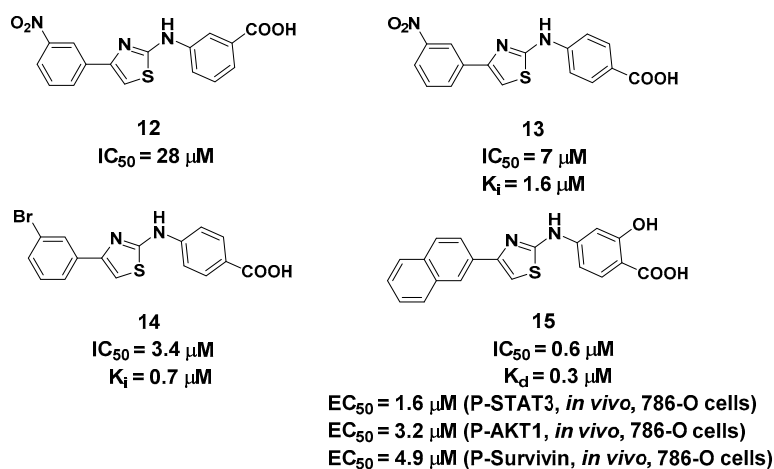
**Figure 5.** Interaction modes of compounds (a) **9** (CAM4066), (b) **10** (CAM4712) and (c) **11** with  $\alpha$ D pocket.

Despite the fact that CAM4712 could bind in the  $\alpha$ D pocket, and partially in the lip of the ATP site, this compound only showed good selectivity against the 20 closely related CMGC kinases in the panel at  $30 \mu\text{M}$ , with the exception of four kinases (CAMK1, SmMLCK, EF2K and SGK1). Given that Site 2 is near the mouth of the ATP-binding pocket, further optimization of allosteric inhibitors only targeting the unique  $\alpha$ D pocket is needed to improve selectivity towards CK2 $\alpha$ .

#### 4. 2-Aminothiazole Derivatives Targeting Site 3

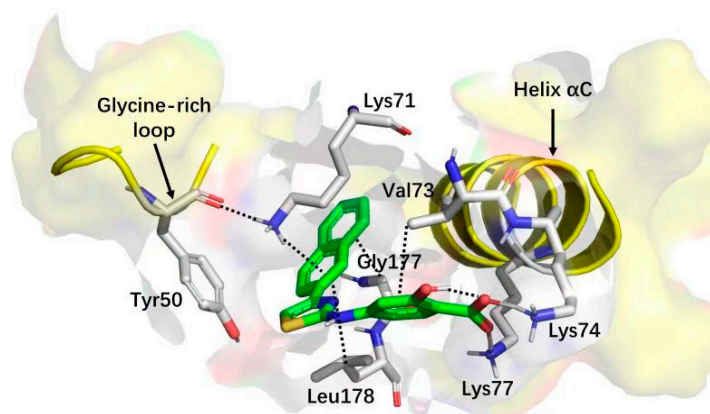
The serendipitous discovery of 2-aminothiazole derivatives (Figure 6) targeting the interface between the G-loop and  $\alpha$ C-helix is very intriguing [20,40]. Originally, Lomberget et al. aimed to identify hits targeting the CK2 $\alpha$ /CK2 $\beta$  interface using virtual ligand screening, and compound **12** was evaluated as the most active hit with an  $IC_{50}$  value of  $28 \mu\text{M}$  for CK2 $\alpha$ . However, elaborate biochemical experiments validated that compound **12** was not bound to the CK2 $\alpha$ /CK2 $\beta$  interface, and its inhibitory effects on CK2 were independent of the concentrations of both ATP, peptide substrate, and the presence

of CK2 $\beta$ . Further experiments were performed on compound **13**, which shared the same inhibition pattern as compound **12**, but with improved inhibitory effects ( $IC_{50} = 7.0 \mu M$ ) and higher selectivity with CK2 $\alpha$ .



**Figure 6.** Structure and biological activity of 2-aminothiazole derivatives ( $K_i$ ,  $K_d$  and  $IC_{50}$  values tested *in vitro*).

The structure–activity relationship indicated that the aminothiazole core and a carboxylic acid function were essential for inhibitory activity. By combining the saturation transfer difference NMR competition experiments, alanine scan mutational analysis, the native mass spectrometry-based detection, and molecular docking, the 2-aminothiazole derivatives were predicted to bind in the interface between the G-loop and  $\alpha C$ -helix by forming an H-bond, salt bridge (carboxylate with Lys74 and Lys77) or cation– $\pi$  interactions (naphthalene ring with Lys71) as shown in Figure 7. After the systematical optimization on the position 4 and amine of the aminothiazole core, compound **15** was identified as having submicromolar potency against CK2 $\alpha$  ( $IC_{50} = 0.6 \mu M$ ) with pro-apoptotic and anti-cell growth effects. As Site 3 is less conserved in the human kinome, the overall selectivity of **15**, as expressed by a Gini coefficient of 0.78, was higher than that of CX-4945 (Gini coefficient of 0.67), in particular, the main off-targets of CX-4945, Clk2 and Dyrk1A were not or were only weakly inhibited by **15**, respectively.



**Figure 7.** Cartoon/surface representation of the interaction between **15** with the G-loop and  $\alpha C$ -helix.

## 5. Conclusions

The currently available ATP-competitive CK2 inhibitors were impeded from being the drug candidates due to their off-targets and low selectivity. The structural diversity of the allosteric sites

endowed the allosteric inhibitors with improved pharmacological properties. This study outlined the recent process of allosteric inhibitors targeting three well-elucidated allosteric sites, including the  $\alpha/\beta$  interface,  $\alpha$ D pocket and the interface between the G-loop and  $\alpha$ C-helix. Most of the mentioned allosteric inhibitors shared the common planar aromatic scaffold with the non-rotatable bonds, which was complementary to the narrow hydrophobic allosteric sites. However, as most of the CK2 allosteric hits were discovered by high-throughput and virtual screening methods, it is still necessary to engage in further exploration of rational structural analysis and optimization, especially targeting the  $\alpha$ D pocket and the interface between the G-loop and  $\alpha$ C-helix.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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