

The Protein Kinase Inhibitor Balanol: Structure–Activity Relationships and Structure-Based Computational Studies

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Abstract: Balanol, a fungal metabolite, is a potent ATP-competitive inhibitor of Protein Kinase C (PKC) and Protein Kinase A (PKA), important targets in oncology. Since its discovery in 1993, a number of studies have been performed in order to design selective and bioavailable balanol analogs. Several crystal structures of PKA in complex with balanol and a few analogs bound within the catalytic site have also been solved providing insight about the key interactions for binding. The PKA-balanol complex has also served as an interesting model system for structure-based ligand design and validation of a number of computational methodologies aimed at both understanding the physical basis for molecular recognition and addressing the important issue of protein flexibility in ligand binding. We provide an overview of the structure-activity relationships of balanol analogs and summarize the progress made in structural and computational studies involving balanol.

Key Words: Balanol, structure activity relationships, kinases, computational chemistry, x-ray crystal structure, protein flexibility, adenosine triphosphate.

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1. INTRODUCTION

Protein kinases belong to a diverse family of enzymes that have various regulatory roles, yet function similarly by catalyzing the phosphoryl transfer of the γ -phosphate of adenosine triphosphate (ATP) to an enzyme-specific protein substrate. Since many diseases including cancer, autoimmune disorders, cardiac disease and diabetes are associated with defects in protein phosphorylation and there are over 500 protein kinases in the human genome, this family (the “kinome”) has emerged as a major target in the design and development of small molecule inhibitors [1-3]. One such inhibitor is the natural product balanol (**1**), which has been consistently employed for the last 14 years, not only as a lead molecule, but also as a model compound by experimentalists as well as computational chemists. The purpose of this report is to present a comprehensive account of the structure-activity relationships (SARs) of balanol analogues and an overview of how X-ray crystallography and computational chemistry have contributed to our understanding of the issues of selectivity and protein flexibility in kinase inhibition using balanol as a model compound.

Balanol (Fig. 1), a fungal metabolite originally isolated and identified in 1993 from *Verticillium balanoides*, is regarded as one of the most potent naturally occurring inhibitors of Protein Kinase C (PKC) [4]. PKC encompasses a family of structurally related serine/threonine specific kinases widely distributed in tissues and cells [5]. PKC is activated by diacylglycerol (DAG) originating from receptor-mediated hydrolysis of membrane inositol phospholipids, a process that is responsible for initiating a cascade of cellular responses to extracellular stimuli such as hormones, neurotransmitters, and growth factors [6, 7]. Activated PKC catalyzes the transfer of the γ -phosphate of ATP to substrate proteins and, by doing so, relays transmembrane signals to biological systems involved in a number of cellular processes including gene expression, cell proliferation and differentiation [8]. Further, PKC has at least 10 different

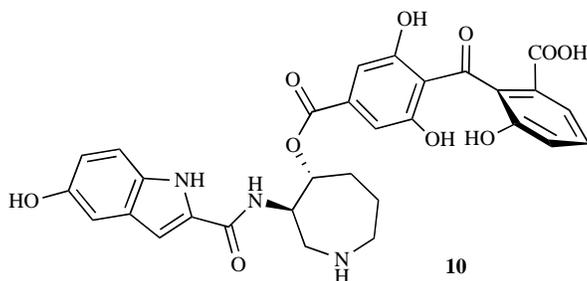
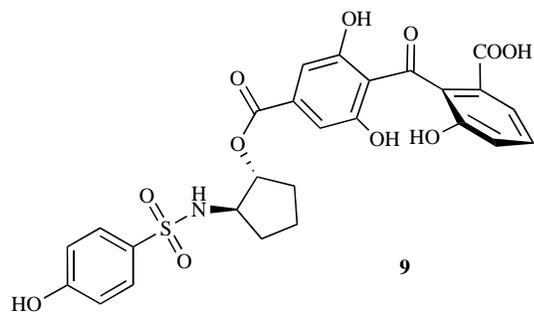
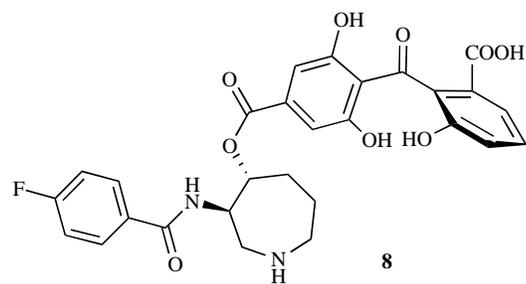
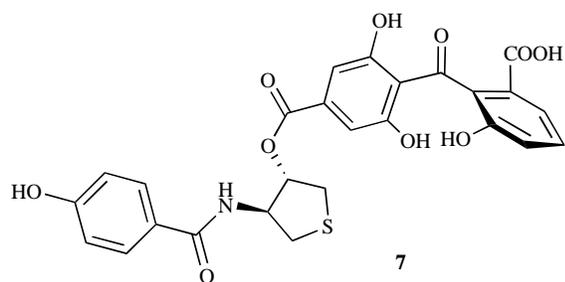
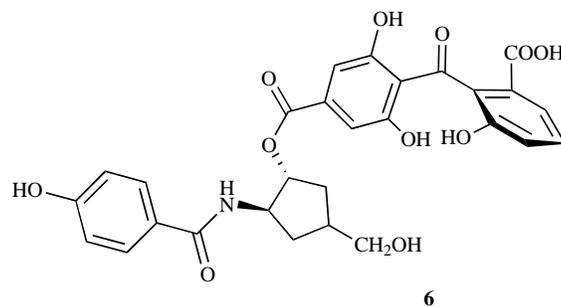
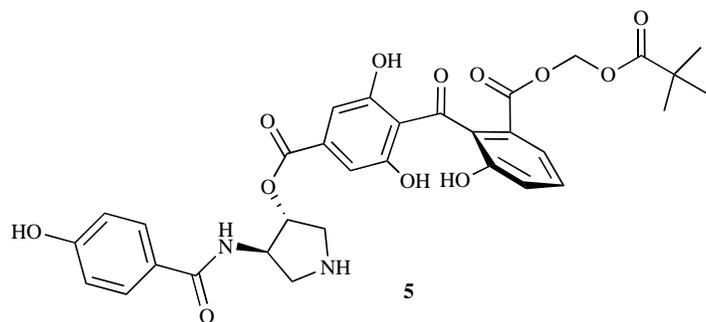
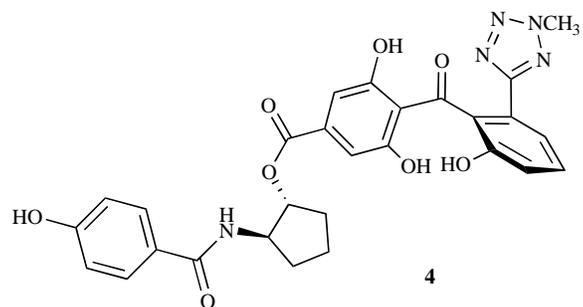
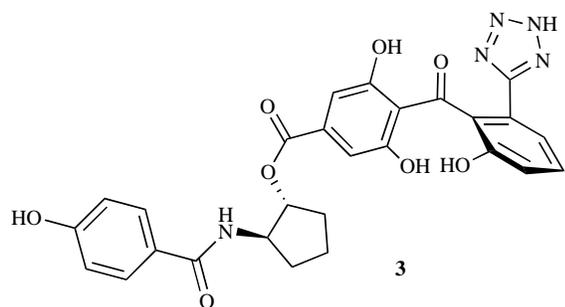
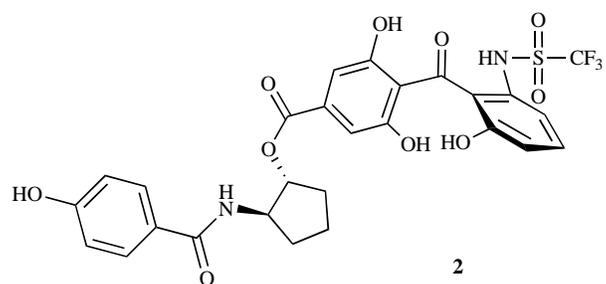
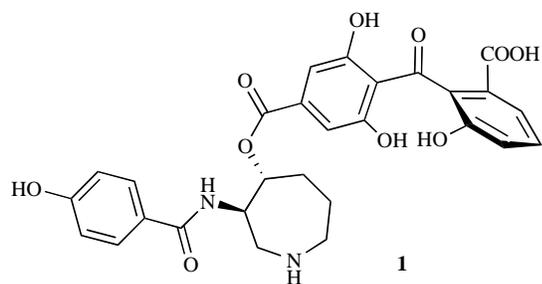
isoforms, which vary in both tissue expression and cellular compartmentalization [9]. PKC is a major receptor for tumor-promoting phorbol esters, which activate PKC in a way similar to DAG, and unregulated PKC activity has been related to a number of disease states including cancer, central nervous system disturbances, cardiovascular disorders, asthma, diabetes and HIV infections [10]. Apart from inhibiting PKC, balanol is a potent inhibitor of cyclic AMP-dependent protein kinase (PKA), another similar serine/threonine kinase important in physiology [11]. Balanol does not, however, inhibit the Src or epidermal growth factor receptor protein tyrosine kinases [11, 12].

Mechanistically, balanol inhibits PKC and PKA because it binds in the ATP binding pocket of these kinases with an affinity that is 3000 times greater than that of ATP (K_i value of balanol for both PKA and PKC is around 4 nM) [12]. We will discuss in detail this competitive inhibition with ATP in a later section on structural studies on PKA/PKC-balanol complexes. Fig. (2) shows the three-dimensional structures of balanol and ATP. Stereoelectronically, balanol is similar to ATP and also binds to an equivalent cleft in the kinase active site: the *p*-hydroxybenzamide group of balanol resembles the adenine base, the perhydroazepine ring resembles the ribose moiety, and the benzophenone resembles the triphosphate group. Balanol extracted from fungus is obtained in very low yield and, as early as 1994, two groups reported its total synthesis [13, 14]. Since then a number of studies have been devoted entirely to developing a more efficient synthesis of balanol and its enantiomers and analogues [15-38]. To review the synthetic chemistry of these molecules falls beyond the scope of this article, but we emphasize that balanol has been seen by organic chemists in the last decade as a challenging heterocyclic scaffold for synthetic purposes and also for structural modification. This endeavor, on the whole, has proved to be both successful and rewarding [39, 40].

2. STRUCTURE ACTIVITY RELATIONSHIPS

Balanol has been extensively modified, mainly in order to design not only PKC or PKA selective analogs (or even analogues selective for some PKC isoforms) but also inhibitors with improved

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(Fig. 1) contd....

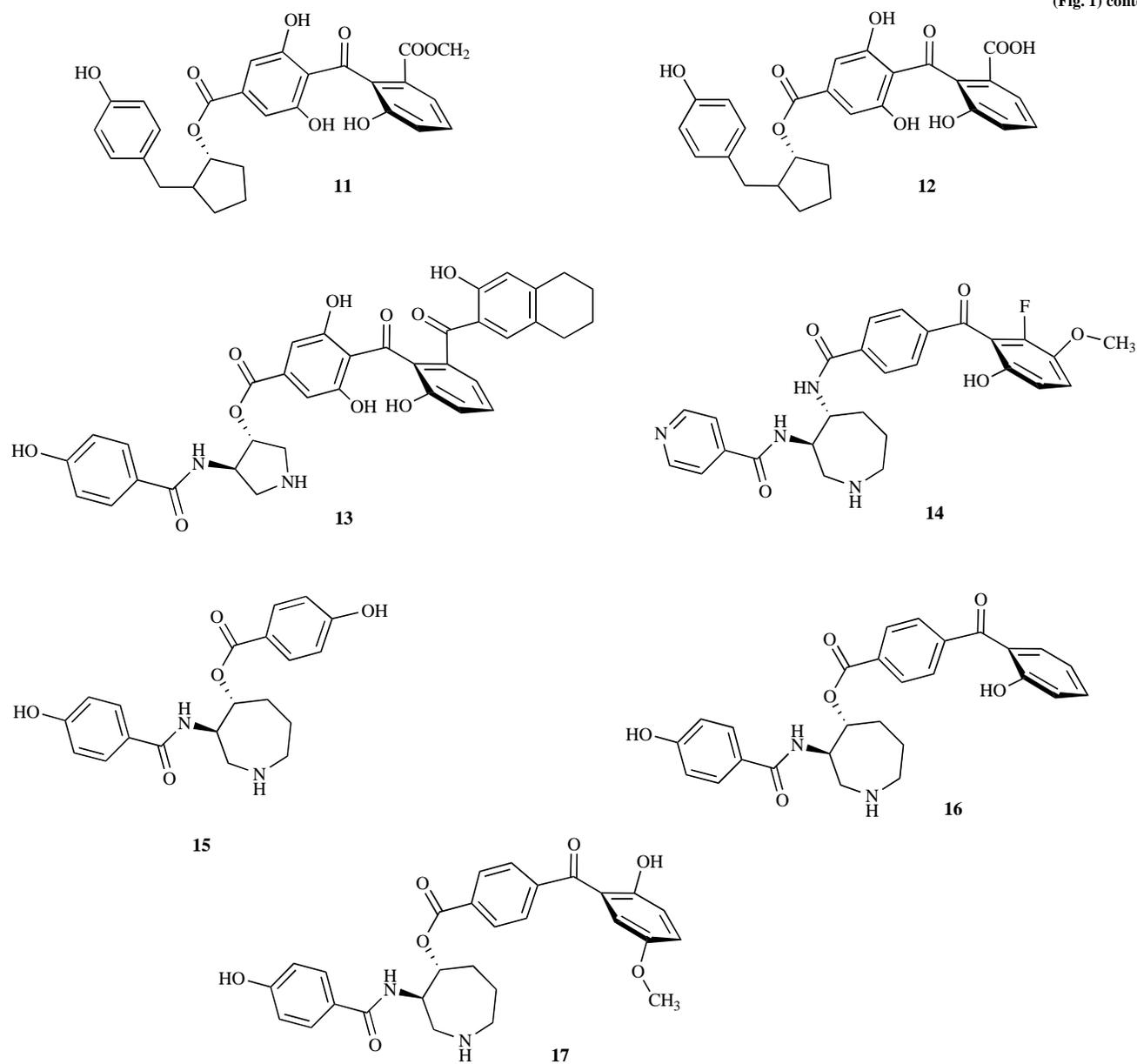


Fig. (1). Chemical structures of balanol (1) and some of its analogs.

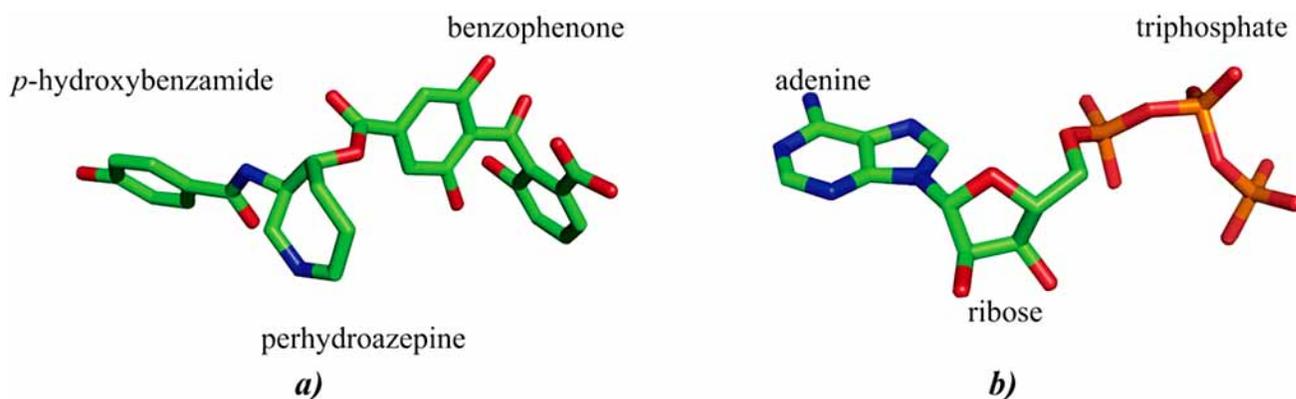


Fig. (2). Three-dimensional stick representations of (a) balanol and (b) adenosine triphosphate (ATP), as found in their respective complexes with PKA (PDB ids.: 1BX6 and 1ATP, respectively). Carbon atoms are colored in green, oxygens in red, nitrogens in blue and phosphorus atoms in yellow.

physical properties resulting in better cellular activity and bioavailability. Here, we discuss the SAR studies reported based on various substructure modifications in the balanol molecule. Just two years after its discovery, that is, in 1995 [41], a variety of related molecules bearing either a carboxylic acid (present in the benzophenone moiety) or several replacements (amides, sulfonamides, and tetrazoles) were synthesized and evaluated for PKC inhibitory activity. It was thought that an important feature of the benzophenone region of the molecule was the carboxylate moiety and, in fact, when the carboxylic acid was replaced by a simple proton or a hydroxyl group, the PKC inhibitory activity dramatically decreased. Nonetheless, while this acidic functionality appeared to be necessary for enzyme inhibition, its polar nature was thought to limit cell permeability. Other analogs possessing a carboxylic acid bioisosteric replacement that included an acidic proton, particularly trifluoromethylsulfonamide (**2**) and tetrazole (**3**), displayed excellent inhibition of PKC and showed good selectivity for PKC over PKA, with good cellular activity. Furthermore, the importance of the acidic hydrogen in this series was realized when it was shown that masking this proton (**4**) at the time of introducing the molecule into the assay was accompanied by reduction of its cellular activity.

This first SAR study was soon followed by work published in the same year (1995) that focused on the perhydroazepine moiety. Crane *et al.* [42], in an effort to enhance the cellular activity of balanol, prepared a series of benzophenone ester analogs of varying steric size and hydrolytic stability. These were evaluated for PKC inhibitory activity, with the results showing that small alkyl esters and the pivaloyloxymethyl ester analogs (e.g. **5**), displayed large increases in cellular PKC inhibition. No PKA inhibition data were reported in this study, so it was not apparent whether these modifications also resulted in any PKC selectivity. Some time later, Lai *et al.* [43] reported a series of seven balanol analogs in which only the perhydroazepine ring was replaced by a pyrrolidine or cyclopentane ring and small substitutions were introduced into the cyclopentane ring. Interestingly, these cyclopentane-based analogs were found to be, in general, more potent PKC inhibitors than balanol itself (e.g. **6**). Unfortunately, no PKA inhibitory or cellular activity data for these analogs were presented either. Subsequent work by Lai and Stamper [44] focused on several balanol analogs in which the perhydroazepine N atom was replaced by O, S, or C (e.g. **7**). Those molecules bearing O and S atoms were found to show enhanced PKC isozyme selectivity, despite the general trend in these analogs of being less potent relative to balanol. It became clear from these studies that the size and nature of the ring at the perhydroazepine position were crucial for potency. Thus it was shown [45] that a five-membered ring is favored over a seven-membered ring for the PKC inhibitory activity in the balanol series, and that a ring size of six atoms is unfavorable. In yet another study [46] it was shown that the azepine nitrogen is not essential as long as its surrogate (and also the replacement of the azepine ring itself) is able to hold the two aromatic side chains in a stereochemically correct manner. Additional modifications included replacing the perhydroazepine ring with an indane scaffold. Analogs containing either an indane or its racemic regioisomer were found to display highly potent PKC inhibitory activities and also showed excellent selectivity for PKC over PKA.

Other parts of the balanol molecule were also subjected to chemical variation, although not as exhaustively as was the perhydroazepine moiety, in order to design structurally diverse analogs. For example, Hu *et al.* [47] synthesized and evaluated a series of molecules incorporating modifications in the *p*-hydroxybenzamide moiety. In this study, the structural modification included substitution of (i) the 4-hydroxyphenyl group with variously substituted phenyl rings (e.g. **8**), (ii) the amide linkage with a sulfonamide or an ester group (e.g. **9**), and (iii) the 4-hydroxyphenyl substructure with a hydroxyl-substituted indole (**10**) or a hydroxybenzyl group (**11**). In general, these inhibitors were found to be less potent than

balanol, but a number of analogs were identified with improved isozyme selectivity. The SAR studies on these molecules also indicated that (i) for optimal general PKC inhibition a free 4-hydroxyl group in the benzamido portion of the molecule was required, (ii) the amide linkage of the benzamido moiety is important for PKC inhibition, and (iii) the conformation associated with the benzamido moiety appears to have a profound effect on PKC inhibition.

In another series [48], the carboxamide linkage between the *p*-hydroxybenzamide and perhydroazepine moieties was shortened to a single atom bridging unit. This study concluded that balanol analogs containing a cyclopentane skeleton and a methylene spacer in place of the carboxamide moiety were potent inhibitors of PKC isozymes and displayed excellent selectivity for PKC over PKA. Moreover, these analogs also displayed good cellular activity, which was further enhanced when the carboxylate moiety was masked as a methyl ester (as in **11**). Another interesting modification of balanol was reported by Defauw *et al.* [49] who combined the perhydroazepine ring and the *p*-hydroxybenzamide moiety into a single acyclic unit. Several low-micromolar to low-nanomolar inhibitors of PKC were reported in this study. In general, these acyclic analogs of balanol were found to be highly selective for PKC over PKA. Both the type and number of atoms linking the benzophenone ester to the *p*-hydroxyphenyl group necessary for optimal PKC inhibition were investigated. The most potent compounds contained a three-carbon linker in which the carboxamide moiety of balanol had been replaced by a methylene group. The effect of placing substituents on the three-carbon chain was also explored. The preferred compounds contained either a 2-phenylsulfonamido or a 1-methyl substituent and these compounds were found to be highly selective for PKC over PKA. One of the potent inhibitors reported in this study, the *anti* cyclopentane analog **12**, was found to have an IC₅₀ value of 0.003 μ M against PKC (β_1) and greater than 50 μ M against PKA. This inhibitor was also found to have an excellent cellular activity and to be easier to synthesize than balanol itself [49].

More recently, Lampe *et al.* [50] reported a series of balanol analogs with modified benzophenone substituents. These molecules were designed with the goal of uncovering features that could be used in the development of PKC inhibitors with a reduced polar character compared to balanol. The results of this study suggest that most of the benzophenone features are important for obtaining potent PKC inhibition. However, several modifications were found to lead to selective PKA inhibitors. In particular, and in agreement with earlier proposals, it was found that replacement of the benzophenone carboxylate with bioisosteric equivalents could lead to potent analogs (for instance, **13**). Furthermore, a tolerance for lipophilic substituents on the terminal benzophenone ring was uncovered. This study was published after the crystal structure of balanol in complex with PKA was reported in 1999 [51] and most of the SARs observed for PKA inhibition were justified on the basis of this three-dimensional structure. Despite this fact, however, none of the designed analogs was more potent than balanol, although several of them were, for the first time, selective over a number of PKC isoforms.

The interesting stereochemical role of the perhydroazepine moiety in balanol's kinase inhibition activity and selectivity was evident within a few years after its discovery. In addition, the crystal structure of the PKA-balanol complex triggered further interest in balanol from a rational design viewpoint. As we will discuss in the next sections, this crystal structure proved to be instrumental to both structural biologists and computational chemists to address a number of important issues in structure-based ligand design. It was in 2004 that Breitenlacher *et al.* [52] reported the structure-based optimization of azepane derivatives as PKA and PKB inhibitors. PKB, or protein kinase B, is another serine/threonine protein kinase that displays around 47% sequence identity with PKA in its catalytic domain (see below for a multiple sequence alignment of PKA,

PKB and PKC) and is also an important target in oncology [52]. In this series, five compounds were designed, synthesized and tested for activities against PKA and PKB, and also for plasma stability. Compound **14** was found to be plasma stable and highly active (IC_{50} for PKA = 2 nM, IC_{50} for PKB = 4 nM). Co-crystals with PKA were obtained for three compounds including **14** and all of them were found to bind in the active site of PKA in an equivalent way as balanol does (see next section). This work is very important for the design of balanol analogs, as **14** is not only more potent than balanol but also has better plasma stability and is suitable as a lead candidate for further optimization. The authors of this work were also interested in designing congeneric PKB selective inhibitors, which they were able to achieve recently [53] by using the PKA structure as a surrogate model for the PKB active site by carrying out two mutations in the catalytic domain of PKA (F188L and Q85E) and later solving the crystal structures of the mutant PKA catalytic domain in complex with the analogs (Protein Data Bank [PDB] identification codes 1XHA, 1XH4, 1XH5, 1XH6, 1XH7, 1XH8, and 1XH9).

3. STRUCTURAL STUDIES

In 1999, Narayana *et al.* [51] reported the crystal structure of balanol in complex with the catalytic domain of PKA at a resolution of 2.1 Å (PDB ID: 1BX6). This structure revealed the extensive complementary interactions between balanol and conserved PKA residues in the binding site. The model representation of this PKA-balanol complex depicted in Fig. (3) shows that balanol occupies the ATP binding pocket in a fashion similar to ATP itself, filling the adenine, ribose and triphosphate subsites (Fig. 2). These three binding regions can also be classified according to their respective functions of identification, accommodation and inhibition. In the apoenzyme, PKA is expected to adopt an open conformation,

thereby enabling the adenine-binding pocket to identify potential ligands. The adenine subsite can serve as an anchor point to orientate the ligand for binding to the other two regions. This hypothesized initial recognition event could trigger PKA's rearrangement from an open conformation to the intermediate or closed conformations that are observed in the crystal structures of complexes incorporating balanol and other active site binding ligands. This proposed mechanism is consistent with chemical footprinting studies involving the free catalytic subunit and also with the structure of a complex between the catalytic subunit and ATP plus two magnesium ions [54]. The ribose subsite must accommodate the ribose-like moiety linking the adenine-like portion of a potential ligand such as balanol to the ligand's phosphate-like groups. Polar and apolar interactions within the ribose-binding subsite involve PKA residues from both the large and the small lobe, thus further sequestering the ligand within the active-site and promoting loop closure. The role of the perhydroazepine ring in the interactions with the binding pocket is very interesting in terms of both its puckering and its protonation state. The azepine nitrogen is protonated and the logarithmic acid dissociation constant of this moiety is around 10. Further, it is reasonable to think that this nitrogen could be doubly protonated when balanol is bound to the active site, for example in PKA (Fig. 3), thus giving rise to the simultaneous formation of two hydrogen bonds, one with the backbone oxygen of Glu171 and a second one with the carboxylate of Asp184. However, free energy perturbation calculations from our lab (unpublished data) appear to support the single protonation state model for the perhydroazepine nitrogen and the engagement of the Asp184 carboxylate in a hydrogen bonding interaction with one of the hydroxyl groups attached to the central phenyl ring of balanol. Additionally, the puckering of this ring, as seen in the PKA-balanol crystal structure, as well as in other similar crystal structures, also suggests that the nitrogen's

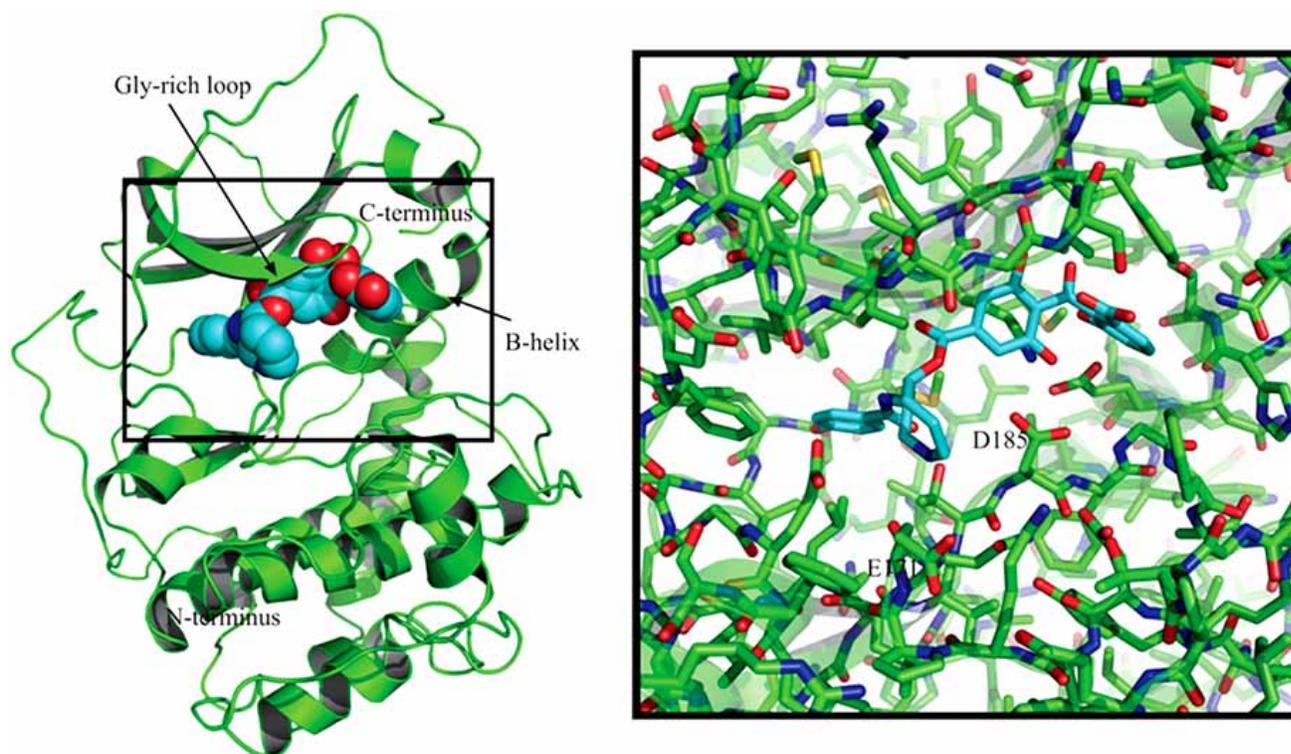


Fig. (3). Protein Kinase A (PKA) is shown complexed with balanol (PDB id.: 1BX6). On the left, balanol atoms within the ATP-binding site are shown as spheres whereas a ribbon representation is used for the protein. On the right, the active site is shown in atomic detail, and carbon atoms in balanol are colored in cyan.

position is better oriented for good hydrogen bonding to the backbone oxygen of Glu171. With nearly half of balanol's atoms being dragged into, accommodated, and oriented within the active site of PKA, the remaining half of the molecule is primed for high-affinity binding at the triphosphate subsite, which spans nearly the full length of the antiparallel strands that comprise the glycine-rich loop and where two-thirds of balanol's polar and non-polar interactions take place (Fig. 3).

The first crystal structure of the PKA-balanol complex was useful in the structure-based design of more potent and selective analogs, as discussed in the previous section [52, 53]. Additional crystal structures revealed that other balanol analogs position themselves in a similar fashion as balanol does, within the ATP-binding pocket. One recent, interesting structure-based design study was reported by Akamine *et al.* [55], in which three balanol analogs (**15**, **16** and **17**) were characterized as selective inhibitors of PKA over PKC. Compound **15** was reported to inhibit PKA with a K_i value of 5 nM and PKC with a K_i value of 2000 nM, compound **16** inhibited PKA with a K_i value of 0.3 nM and PKC with a K_i value of 670 nM, and compound **17** inhibited PKA with a K_i value of 200 nM and PKC with a K_i value of 3300 nM. The crystal structures of the PKA catalytic subunit in complex with each analog were solved (PDB identification codes 1REJ, 1REK and 1RE8) and a homology model of PKC was also built. This comparative study showed that the perhydroazepine region of balanol is PKA-sensitive whereas the terminal benzophenone part is PKC-sensitive, *i.e.* in PKA the benzophenone part is tolerant to modifications, but not in PKC, and *vice versa*. This proposal has been supported by SAR studies on several other balanol analogs. The observed selectivity is attributed to residue differences in the variable B helix and the C-terminal tail (Fig. 3). The sequence alignment of PKA, PKB and PKC depicted in Fig. (4) shows that, despite the high sequence conservation of residues in close proximity to the inhibitor in the PKA-balanol complex, there are also some structural differences in these three kinases that can be exploited to provide selectivity to some of the balanol analogs, as exemplified above.

4. COMPUTATIONAL STUDIES

The first PKA-balanol structure attracted the attention of computational chemists [56] as soon as it was made available and still continues to be exploited [57-60]. Since the deposition of the crystal structure with the PDB, the PKA-balanol complex has become a good test case to study the physical determinants of molecular recognition and selectivity, as well as issues related to the importance of considering protein flexibility in ligand binding. Hunenberger *et al.* [56] reported on the determinants of ligand binding by applying a theoretical model based on continuum electrostatics and a surface area-dependent nonpolar term to the calculation of binding affinities towards the catalytic subunit of PKA of several balanol derivatives and also other ATP analogs. These calculations reproduced the experimental trends and provided insights into the driving forces responsible for binding. Protein-ligand affinity was proposed to be governed by nonpolar interactions as hydrogen bonds were seen to represent a negligible contribution because their formation in the complex entails the desolvation of the interacting partners. Nonetheless, since the binding affinity decreases when hydrogen-bonding groups of the ligand remain unsatisfied in the complex, the disposition of these groups are deemed to be crucial for binding specificity. Following on this work, Wong *et al.* [61] illustrated a computational approach that extended the usefulness of a single protein-inhibitor complex structure (PKA-balanol) as an aid for the general design of protein kinase inhibitors. Using the PKA-balanol complex as a guide, the authors of this study analyzed and compared the distribution of amino acid types near the protein-ligand interface for nearly 400 kinases. This comparative sequence/structure analysis identified a number of sites that are more variable in amino acid types amongst the kinases analyzed, and these were proposed as useful sites to consider in the design of specific protein

kinase inhibitors [61, 62]. This study took advantage of the PKA-balanol complex from the standpoint of methodology development, as it led to further improvements in a semiempirical approach that is used to predict binding affinities employing an implicit-solvent binding model. The PKA-balanol complex still continues to be used as a model system for computational studies. Thus, very recently Wang and Wong [57] proposed a novel QM/MM/PBSA (quantum mechanics/molecular mechanics/Poisson-Boltzmann-surface area) method to rank-order protein-ligand binding affinities and a series of balanol derivatives was successfully used as a test case to validate the new approach.

Besides being used in the theoretical prediction of binding affinities, the PKA-balanol complex has also served as a good example to study the role of protein flexibility in molecular docking. In yet another computational study [63], molecular docking of balanol to PKA snapshots generated from a molecular dynamics simulation was performed using the automated docking program AutoDock [64]. When these snapshots were taken from the simulation of the protein-ligand complex, the correct docking structure could be recovered easily by the docking algorithm in all cases. However, when the snapshots were taken from the simulation of the ligand-free protein (which is likely to represent a more general situation in practice), several clusters of structures were found. Of the 10 docking runs for each snapshot, at least one structure was found to be close to the correctly docked structure. Consequently, it was proposed that a useful way to identify the correctly docked structure was to locate that which appeared most frequently as the lowest energy structure in the docking experiments using different snapshots as the target protein. In a similar study, Kovacs *et al.* [65, 66] studied conformational sampling of protein flexibility and used the structure of the PKA-balanol complex as a test case. Normal modes in internal generalized coordinates furnish an alternative way to represent receptor flexibility. Perturbation along these modes preserves covalent geometry and is especially well suited for representing mid- and large-scale movements. Working on a reduced spring model of PKA, the authors of this study showed that only four modes have significant relevance on the glycine-rich loop. Alternative protein backbone conformations were generated by perturbing the structure along these relevant modes, and those conformations were complexed with balanol and then subjected to global energy minimization to optimize side-chain positioning. The starting crystal structure and the one thus generated were used to perform a small-scale receptor-ensemble docking. It was shown that docking to the resulting ensemble decreases both the root-mean-square deviation values with respect to the experimental structure and enhances the enrichment factors. In another recent study, Zhao *et al.* [60] reported the development of a new docking software called FLIPDock (Flexible LIgand-Protein Docking) and successfully used balanol as a test case.

5. CONCLUSIONS

In this review, we have provided an outline of the development of the natural product protein kinase inhibitor balanol over the last 14 years not only as a lead compound but also as a challenging model system for synthetic chemists, structural biologists and computational scientists. Balanol has been extensively modified in order to design selective as well as bioavailable analogs and success has been achieved in this direction. The crystal structure of the PKA-balanol complex showed that balanol, an ATP mimic, indeed binds in a fashion similar to ATP itself, in the active site of the kinase. This structure triggered a handful of studies to rationally design selective balanol analogs. Also, a structural basis for the selectivity of balanol analogs over PKA/PKC was proposed. Computational work has also exploited the PKA-balanol complex structure to gain insight into the physical basis for the potent activity and selective molecular recognition exerted by balanol. Some methodological developments in computational chemistry found the PKA-balanol complex to be a good test case, especially to account for the role of

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PKA -----
PKB -----
PKC MADPAAGPPPSEGEESTVRFARKGALRQKNVHEVKNHKFTARFFKQPTFCSHCTDFIWGF 60

PKA -----
PKB -----
PKC GKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPASDDPRSKHKFKIHTYSSPTFCDHCGS 120

PKA -----MGNAAAAKKGSEQES-----V 16
PKB -----MNEVSVIKEGWLHHRG-----EYIKTWRP 24
PKC LLYGLIHQGMKCDTCMMNVHKRCVMNVPSLCGTDHTERRGRIYIQAHDREVLIIVVVRDA 180
      * . : . . .

PKA KEFLAKAKEDFLKKWEDP----- 34
PKB RYFLLKSDGSFIGYKERPEAPDQTLPLNNSVAECQLMKTERPRPNTFVIRCLQWTTVI 84
PKC KNLVMPDPNGLSDPYVKLKLIPDPKSESKQKTKTIKCSLNPEWNETFRFQLKESDKDRRL 240
      : : : . :

PKA -----
PKB ERTFHVDSPDEREEWIRAIQMVANS LKQRGPG----- 116
PKC SVEIWDWDLTSRNDFMGSLSFGIS ELQKAGVDGWFKLLSQEEGEYFNVPVPEGSEGNEE 300

PKA -----SQNTAQLDHFDRIKTLGTGSFGRVMLVK 62
PKB -----EDAMDYKCGSPSDSSTSEMMEVAVSKARAKVTMNDFDYLKLLGKGTFGKVLVR 170
PKC LRQKFERAKIGQGTKAPEEKTANTISKFDNNGNRDRMKLTDNFNLMVLGKGSFGKVMLSE 360
      : : : . * : : * * * : * * * : * .

PKA HKESGNHYAMKILDKQKVVVKLQIEHTLNEKRILQAVN-FPFLVKLEFSFKDNSNLYMVM 121
PKB EKATGRYYAMKILRKEVIAKDEVAHTVTESRVLQNTN-HPFLTALKYAFQTHDRLCFVM 229
PKC RKGTDELYAVKILKQVVIQDDDVECTMVEKRVLALPGKPPFLTQLHSCFQTMDRLYFVM 420
      . * . . . * * : * * : : : * : * . * * * * * . * . * : . * : * *

PKA EYVPGGEMFSLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQGYI 181
PKB EYANGGDLFFHLSRERVFTEDRARFYGAEIVSALEYLHSTDVVYRDIKLENLMLDKDGH 289
PKC EYVNGGDLMYHIQQVGRFKEPHAVFYAAEIAIGLFFLQSKGIYRDLKLDNVMLDSEGH 480
      * * . * : : * : : * . * : * * * * * . : : * * . : * * * * : * : : * * : *

PKA QVTDGFAKR--VKGRTW-TLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPP 238
PKB KITDFGLSKEGISDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLP 349
PKC KIADFGMCKENIWDGVTTKTCGTPDYIAPEI IAYQPYGKSVDDWWAFVLLYEMLAGQAP 540
      : : * * : . * . * * * * * : * * * * : : * : : * * * : * * * * *

PKA FFADQPIQIYEKIVSGKVRFPSPHFSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWF 298
PKB FYNQDHERLFELILMEEIRFPRTLGPPEAKSLLAGLLKDPKQRLGGGSPSDAKEVMEHRFF 409
PKC FEGEDEDELQFSIMEHNVAYPKSMSKEAVAICKGLMTKHPGKRLGCGPEGERDIKEHAFF 600
      * : : . : : * : : : * : : : : . * : . : * * . . : : * : *

PKA ATTDWIAIYQRKVEAPFIPKFKGPGDTSNEDD-YEEEEIRVSINEKCG-----KE 347
PKB LSINWQDVVQKLLPPFKPQVTSEVDTRYFDDEFTAQSITITPPDRYDSLGSLELDQORTH 469
PKC RYIDWEKLERKEIQPPYKPKARDKRDTSNFDKEFTROPVELTPTDKLFIMN----LDQNE 656
      : * : : : : . * : * . * * * * . : : : : : : :

PKA FTEF----- 351
PKB FPQFSYSASIRE--- 481
PKC FAGFSYTNPEFVINV 671
      * . *

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Fig. (4). ClustalW [67] sequence alignment of the catalytic subunits of protein kinases PKA (NCBI: P27791), PKB (NCBI: P47197) and PKC (NCBI: P68403). Residues making up the balanol binding site in PKA are highlighted in gray (all residues within a distance of 5 Å around balanol in the PKA-balanol crystal structure). “*”, “:”, and “.” stand for identical, conserved and semi-conserved amino acids, respectively.

protein flexibility in molecular docking. We hope this progressive journey of balanol from a fungus to the computer will continue, and

will eventually result in the design and development of promising clinical candidates.

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