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Recent Advances in the Discovery of Small Molecules Targeting Exchange Proteins Directly Activated by cAMP (EPAC)

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Abstract

cAMP is a pivotal second messenger that regulates numerous biological processes under physiological and pathological conditions, including cancer, diabetes, heart failure, inflammation and neurological disorders. In the past, all effects of cAMP were initially believed to be mediated by PKA and cyclic nucleotide-regulated ion channels. Since the discovery of EPAC proteins in 1998, accumulating evidence has demonstrated that the net cellular effects of cAMP are also regulated by EPAC. The pursuit of the biological functions of EPAC has benefited from the development and applications of a growing number of pharmacological probes targeting EPAC proteins. In this Perspective, we seek to provide a concise update on recent advances in the development of chemical entities including various membrane-permeable analogues of cAMP and newly discovered EPAC-specific ligands from high throughput assays and hit-to-lead optimizations.

1. Introduction

Cyclic adenosine monophosphate (cAMP, cyclic AMP or 3′–5′-cyclic adenosine monophosphate) is a pivotal second messenger derived from its precursor adenosine triphosphate (ATP). A wide variety of extracellular ligands bind to G-protein coupled receptors (GPCRs), activate adenylate cyclases (ACs) to catalyze the conversion of intracellular ATP to pyrophosphate and cAMP.[1,2] cAMP regulates a number of key biological processes under physiological and pathological conditions, including neuronal signaling, gluconeogenesis, glycogenolysis, lipogenesis, cardiac and smooth muscle contraction, secretory processes, ion channel conductance, learning and memory.[1]

The local concentration and distribution of intracellular cAMP is regulated by ACs and the cyclic nucleotide phosphodiesterases (PDEs). Generally, numerous extracellular signals trigger a series of the conformational changes of GPCRs on the cell surface. Typically, Gs protein stimulates ACs to increase cAMP production inside the cell, whereas Gi protein

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CONFLICT OF INTERESTS
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inhibits ACs and lowers the level of cAMP.[2–6] Meanwhile, the intercellular level of cAMP is degraded by PDEs which catalyze conversion of cAMP to 5′-AMP.[7]

In the past, all effects of cAMP were initially believed to be mediated by protein kinase A (PKA) and cyclic nucleotide-regulated ion channels.[8–11] In 1998, two independent groups reported their findings that PKA-independent mechanism of cAMP action was regulated by a family of guanine nucleotide exchange factors (GEFs) called cAMP-GEFs which are also named as exchange protein directly activated by cAMP (EPAC).[12,13] Since then, remarkable progress has been made on elucidating the molecular mechanism of EPAC proteins over the last fifteen years. Meanwhile, probing the biological functions of EPAC has been significantly facilitated by the development and applications of small-molecule EPAC ligands including various membrane-permeable analogues of cAMP and newly discovered EPAC-specific antagonists. Consequently, numerous additional biological functions of EPAC have been uncovered. This review briefly summarizes the structures of EPAC family members, EPAC signaling pathway and biological functions, and also provides a perspective on recent advances in the discovery of new chemical entities targeting EPAC proteins. In addition, these valuable pharmacological tools including cAMP analogues and EPAC antagonists have led to a greater understanding of the important role of EPAC proteins in different diseases, establishing EPAC proteins as novel molecular targets for new therapeutic strategies against various human diseases including cancer, diabetes, heart failure, inflammation and neurological disorders.

2. EPAC Family and EPAC2 Protein Structures

To date, two isoforms of EPAC have been identified, EPAC1 and EPAC2, which are also known as RAPGEF3 (cAMP-GEF-I) and RAPGEF4 (cAMP-GEF-II), respectively.[12–14] As depicted in Figure 1, each EPAC family member composes an auto-inhibitory amino-terminal regulatory region and a carboxyl-terminal catalytic region for activation of Rap GTPase.[14–18] The regulatory region contains a Dishevelled Egl-10 Pleckstrin (DEP) domain and at least one functional cyclic nucleotide binding domain (CNBD, one for EPAC1 and two for EPAC2). The carboxyl-terminal catalytic region consists of a Ras exchange motif (REM) domain and a Ras association (RA) domain as well as the CDC25-homology domain (CDC25-HD). The CDC25-homology domain is responsible for guanine nucleotide exchange activity and catalyzes the exchange of G-protein-bound GDP for GTP on the Ras-like small GTPases Rap1 and Rap2 isoforms.[19,20]

The two EPAC isoforms EPAC1 and EPAC2 are mostly expressed in both mature and developing tissues with different expression levels. EPAC1 is highly expressed in central nervous system, adipose tissue, blood vessels, kidney, ovary, and uterus, while EPAC2 is most detectable in the central nervous system, adrenal gland, and pancreas.[21] EPAC2 has been found to exist as three splice variants. EPAC2A is expressed in cerebral cortex and pancreatic islets and has two cAMP-binding domains, whereas expression of EPAC2B is restricted to adrenal glands without cAMP-binding domain A. EPAC2C is found in the liver and lack of cAMP-binding domain A and DEP domain.[22]

The X-ray crystal structure determinations and analysis of two different conformations of EPAC2 protein have revealed in atomic detail that cAMP binding causes conformation change allowing the catalytic region to be available for the binding of Rap (Figure 2).[15,16] NMR spectroscopy studies, peptide amide hydrogen/deuterium exchange mass spectrometry (DXMS) approach, small angle X-ray scattering, fluorescence resonance energy transfer (FRET) studies and molecular dynamics (MD) simulations further demonstrated the mechanism of autoinhibition and activation of EPAC2.[23–32] In the absence of cAMP, the apo-EPAC2 protein exists in an autoinhibited conformation (inactive state), in which the cyclic nucleotide–binding domains sterically hinder the access of down-regulating effectors to the catalytic domain, effectively inhibiting the guanine nucleotide exchange activity. Upon binding of cAMP, a sequence of structural reorganizations within the cyclic nucleotide–binding domains allows the regulatory domain to move to the back side of the catalytic region, exposing the Rap-binding site of the catalytic region. It is worth noting that while X-ray structural analysis reveals no significant conformational changes between the CDC25-HD in inactive and active stages,[15,16] DXMS studies indicate that in addition to relieving the steric hindrance imposed upon the catalytic lobe by the regulatory lobe, cAMP may also act as an allosteric modulator affecting the interaction between EPAC2 and Rap1 by inducing conformational changes at the ionic latch/hairpin structure, which is directly involved in Rap1 binding.[32]

3. EPAC Signaling Pathway and Biological Functions

Cellular cAMP levels are modulated by hormones and neuromediators with specific effects on the homeostasis of multicellular organisms. The elevation in intracellular concentration of cAMP after Gs protein-coupled receptors activation is sufficient to activate EPAC. As a part of the downstream targets of cAMP, EPAC proteins as guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP on the small GTPases Rap1 and Rap2.[14] Rap1 and Rap2 as members of the Ras family are involved in the network of multiple proteins to regulate cell adhesion and cell junction. EPAC proteins act as the critical upstream effectors, among many other Rap GEFs, controlling the Rap-mediated biological processes of cAMP.[19,33] The signaling pathway of EPAC proteins is depicted in Figure 3. Accumulating evidence has demonstrated that EPAC signaling has a significant contribution in various biological processes, including cardiac function, insulin secretion, neuronal function, vascular function, inflammation, renal function, and airway function.[34,35]

3.1. EPAC and Cardiac Function

In a collection of specialized muscle cells called cardiac myocytes, cAMP modulates a series of cardiac functions such as heart rate, cardiac contractility and relaxation.[36,37] Sustained elevation of cAMP induces hypertrophy, cardiac dysfunction and eventually to the development of heart failure.[38–43] Given the critical role of cAMP in cardiac function, more and more studies focused on the cardiac function of EPAC proteins, providing plenty of evidence that EPAC is a new regulator in cardiac physiology and pathophysiology.[37,38,44–49] Overexpression of EPAC1 in left ventricular hypertrophy of animal models and patients demonstrated that EPAC1 contributes to the progression of heart failure.[47,48] Very recent work using knockout mice for EPAC1, EPAC2, or both has

revealed that β_1-adrenergic receptor (β_1-AR) activation causes EPAC2-mediated sarcoplasmic reticulum (SR) Ca^{2+} leak and arrhythmias via Ca^{2+}/calmodulin-dependent protein kinase IIδ (CaMKIIδ)-dependent phosphorylation of ryanodine receptor 2 (RyR2)-S2814, indicating that this pathway contributes to β-AR-induced arrhythmias and reduced cardiac function.[50] Thus, EPAC proteins represent novel therapeutic targets for the treatment of cardiac disorders.

3.2. EPAC in Insulin Secretion

EPAC2 is highly expressed in the insulin-producing pancreatic β-cells.[51] A growing body of biological studies have demonstrated that EPAC2/Rap1 signaling pathway is essential in the regulation of the dynamics of insulin secretion.[52] The initial studies including *in vivo* experiments in EPAC2 knockout mice indicated that the traditional antidiabetic drugs sulfonylureas might interact with EPAC2 to activate Rap1.[53,54] Despite the controversial debate, this finding implicates that EPAC2 contributes to glucose-induced insulin secretion.[55–57] Additional studies suggest that several intracellular signaling pathways with relation to insulin secretion are linked with EPAC2 such as recent discovered pathway EPAC2-Rim2-Rab3.[58] The relevant genetic and pharmacological studies concerning EPACs' involvement in glucose homeostasis and energy balance, through regulation of leptin and insulin signaling pathways, were discussed in a very recent review.[59] The accumulating evidence supports that EPAC may represent a novel target to provide therapeutic potential for the treatment of diabetes and obesity.

3.3. Neuronal Function of EPAC

Both EPAC1 and EPAC2 are predominant in the brain including the hippocampus, striatum, and prefrontal cortex, cerebral cortex, cerebellum, olfactory bulb, thalamus, habenula, and pituitary.[13,60] Compelling evidence has demonstrated that EPAC proteins have a significant contribution in the development and function of the nervous system.[40,61] In addition, a recent study using EPAC knockout mice provided the direct *in vivo* evidence that EPAC1 and EPAC2 proteins synergistically regulated neurological functions in the brain for processing spatial learning and social interactions.[62] Additional studies also showed that EPAC2-deficient mice exhibited robust deficits in social and communication behaviors, indicating potential contributions in brain disorders.[63] Thus, targeting EPAC signaling pathway can be considered as a promising strategy for the treatment of various neurological disorders such as autism, anxiety and depression, schizophrenia and Alzheimer's disease.[63–73]

3.4. EPAC and Vascular Function

The vascular endothelium regulates the extravasation of solutes, macromolecules, and cells between the circulating blood and the adjacent tissues. Accumulating evidence suggests that the EPAC signaling pathway controls cAMP-mediated hormones on endothelial function including cell adhesion, endothelial barrier function and gap junction formation in vascular endothelial cells.[74–86] In addition, EPAC1-Rap signaling has been reported to regulate vascular permeability, intracellular responses, extracellular matrix adhesion and migration and inflammatory processes.[87–89]

3.5. EPAC in Inflammation

As mentioned above, EPAC1 modulates inflammatory processes by regulating the immune responses. During inflammation, leukocytes extravasate from the blood into the extravascular space to regulate leukocyte and transendothelial migration and integrin-mediated adhesion. Several studies have shown that EPAC1 activation modulates numerous inflammatory mediators in various leukocytes.[33,79] In addition, EPAC1 enhances leukocyte adhesion and migration as a proinflammatory mediator.[34,71,83,90–94] Intriguingly, recent studies using two mouse models of hyperalgesic priming provide new insights into the role of EPAC in chronic inflammatory pain. It has demonstrated that increasing the expression of GPCR kinase 2 (GRK2) in nociceptors or decreasing EPAC1 inhibited chronic hyperalgesia, suggesting that therapies targeted at tuning the balance between GRK2 and EPAC1 levels may have promise to prevent and treat chronic pain.[95]

3.6. Renal Function of EPAC

EPAC proteins are also involved in many physiological processes in the kidney including ion transport and cellular proliferation. EPAC1 and EPAC2 are highly expressed by all three segments of the proximal tubules and are enriched at the brush border membrane.[96] Recent studies indicate that EPAC1 and/or EPAC2 represent a novel therapeutic target for diabetes-related diseases, particularly kidney failure. Although the exact mechanism of EPAC proteins -especially the contribution of EPAC1 and EPAC2 - to these diseases remains to be elucidated, the development of EPAC-specific activators or inhibitors as pharmacological probes will expedite the discovery process and biological characterization.[97–101]

3.7. Airway Function of EPAC

Because of the critical role of cAMP in modulating airway functions, it was anticipated that cAMP-induced regulation of these processes including airway smooth muscle contraction and proliferation could be mediated by EPAC. Recent studies in human airway smooth muscle cells showed that cigarette smoke extract reduced EPAC1 expression.[71] Interestingly, the reduction of EPAC1 expression was also found in lung tissue from patients with chronic obstructive pulmonary disease (COPD).[71] More and more findings have demonstrated that EPAC-mediated signaling represents a novel key pathway in airway fibroblasts and airway smooth muscle cells.[102–104]

4. Small Molecules Targeting EPAC

Since the discovery of the EPAC proteins in 1998, to better understand their functions and elucidate the EPAC-mediated signaling pathways under both physiological and pathological conditions, there is an increasing need to develop EPAC-specific ligands including agonists and antagonists as pharmacological probes and potential molecular therapeutics for human diseases. Several EPAC-selective and PKA-selective cAMP analogues have been identified as powerful pharmacological tools to distinguish the signaling pathways of EPAC and PKA in numerous biological processes.[105,106] However, cAMP analogues could not discriminate the functions of EPAC1 and EPAC2 as two major isoforms share extensive sequence homology. Although some non-nucleotide small molecules such as brefeldin A were shown to inhibit EPAC function, there was lack of evidence to suggest that these compounds

directly interact with EPAC.[65,107–109] Early in 2012, Cheng's research group reported a robust high throughput screening (HTS) assay by using a fluorescent cAMP derivative to identify EPAC-specific antagonists from compound libraries[110–112]. After that, Lezoualc'h and colleagues reported their validated fluorescence-based HTS assay on the basis of the ability of EPAC to regulate the nucleotide exchange activity of Rap1 for screening EPAC ligands[113]. Recently, by collaborating with Cheng, our team made substantial medicinal chemistry effort on hit-to-lead optimizations.[114–116]. The structure-activity relationships of cAMP analogues and newly discovered EPAC-specific antagonists, as well as the potential applications of these chemical probes for new therapeutic strategies will be discussed herein.

4.1. cAMP analogues as EPAC ligands

Early systematic studies focusing on structure-activity relationships of cAMP derivatives suggest that modification on 3′- and 5′-positions of the cyclophosphate ring of cAMP (**1**) with nitrogen or sulfur diminishes the affinity of cAMP analogues towards PKA (Figure 4). Further introduction of various substituents at these positions was found to be inappropriate.[117,118] The cAMP binding to PKA is sensitive to most modifications of the cyclic phosphate ring, while modifications on the exocyclic phosphate-oxygen result in several more interesting findings. For example, replacement of the exocyclic phosphate-oxygen by sulfur generated two diastereoisomers, Sp-cAMPS (**2**) and Rp-cAMPS (**3**), respectively (Figure 4).[119–121] Compound **2** is an activator of both PKA and EPAC, but has an affinity about 10-fold lower than cAMP. Compound **3** is a weak partial activator of PKA that acts as a competitive inhibitor at physiological PKA concentrations. It binds weakly to EPAC, where it acts as an inhibitor.[122] Taken together, these results indicate that modification on the cyclic nucleotide is very sensitive for the activation properties.[122–126] Further modification on **2** and **3** at the position 2 or 8 of the adenine base with different spacers has provided a set of valuable tools for chemical proteomics approaches.[123]

More recently, there has been remarkable progress in understanding the molecular mechanism of PKA and EPAC activation. In particular, X-ray crystal structure determinations of PKA and EPAC2 have revealed the structural information of the amino acid residues around the cAMP binding site. From comparison of the primary amino acid sequences of the different cAMP-binding domains between PKA and EPAC, it was noticed that there is a highly conserved glutamate residue only present in the cAMP binding domains of the PKA.[71,127,128] Insight into the structural basis indicated that 2′-OH on the ribose ring is essential for the binding to the conserved glutamate residue in the cAMP binding domain of PKA.

Several systematic structure–activity relationship investigations were carried out on 2′-OH on the ribose ring, the N^6-position and the 8-position of the adenine moiety of cAMP. A variety of new cAMP analogues displayed the ability to selectively activate EPAC or PKA. 2′-deoxy-cAMP (**4**) with the 2′-OH moiety replaced by hydrogen selectively activated EPAC without any effect towards PKA (Figure 5).[122,129] Despite compound **4** exhibiting some selectivity towards EPAC versus PKA, it is not an ideal EPAC activator due to a binding affinity >1000-fold less than cAMP itself.[71] Further detailed evaluation showed that 2′-*O*-alkyl-modified cAMP analogues such as 2′-*O*-Me-cAMP (**5**) displayed more potent

EPAC1 activation than cAMP and were unable to activate PKA, and these compounds displayed about 10 to 100-fold improved EPAC/PKA binding selectivity (Figure 5).[64] These findings further suggest that the 2′-OH group is important for binding affinity and selectivity.[64,106,127,128,130]

In contrast, modifications on the N^6-position of cAMP abolished the ability of cAMP analogues to activate EPAC but not PKA. For instance, 6-Bnz-cAMP (**6**) and N^6-phenyl-cAMP (**7**) are full PKA activators and inefficient EPAC activators (Figure 5).[64,122] This implies that suitably 6-substituted cAMP analogues may differentiate their selectivity between PKA and EPAC. It is worth mentioning that the generally low efficiency of the 6-modified analogues is likely not ascribed to the steric hindrance resulting from the introduction of bulky substituents. Some analogues with small groups, such as an oxygen atom at 6-position (like that in cGMP), are even less bulkier than that of cAMP and still have a lower affinity to EPAC.[61]

Systematical modifications on the 8-position of cAMP revealed that 8-substituted cAMP analogues possess differences in charge accommodation, hydrogen bonding and adaptability between cAMP homologous binding sites.[129,131,132] In addition, the lipophilicity of cAMP analogues with the hydrophobic substitutions at 8-position has a major impact on their cell membrane permeability.[106] As shown in Figure 6, several 2′-*O*-Me-cAMP analogues with modifications on the 8-position have been synthesized and their abilities to activate EPAC and PKA were compared. 8-Br-2′-*O*-Me-cAMP (**8**) and 8-pCPT-2′-*O*-Me-cAMP (**9**, a.k.a. 007) have been found not to activate PKA with an enhanced EPAC/PKA binding selectivity about three orders of magnitude. Detailed SAR studies have revealed that the *S*-phenyl ring on 8-position with *para*-substituted chloro (**9**), methoxy (**10**), or hydroxyl (**11**) can improve the binding affinity selectively towards EPAC (Figure 6).

EPAC-selective cAMP analog **9** was administered in several animal models, demonstrating its biological applications under physiological circumstances. For instance, EPAC activation by intraplantar injection of **9** was found to induce hyperalgesia via a PKCε-dependent route.[95,133] Intrarenal administration of **9** reduced renal failure in a mouse model for ischemia-reperfusion injury.[134] Intrahippocampal injection of **9** improved fear memory retrieval in contextual fear conditioning through regulating hippocampal EPAC2 signaling.[135] Although **9** was used as a handy pharmacological tool to elucidate the critical role of EPAC proteins in cAMP signaling pathway, the relatively low membrane permeability limited its further biological applications due to its negatively charged phosphate group. Introduction of an acetoxymethyl ester to **9** to mask the negatively charged, singly bonded oxygen on the phosphate group generated the labile ester **12** which consists of the equatorial and the axial isomers (Figure 7).[136] This prodrug-like molecule is capable of penetrating cell membranes more efficiently and can be intracellularly hydrolyzed directly by cellular esterases to release the biologically active parent compound **9**. 8-pCPT-2′-*O*-Me-cAMP-AM (**12**) has thus become a powerful tool in EPAC and PKA related research. Compound Sp-8-pCPT-2′-*O*-Me-cAMPS (**13**) has been further developed as a highly specific EPAC activator with high lipophilicity and membrane permeability, while it is not metabolized by phosphodiesterases (Figure 7).[68,136,137] Despite significant progress on development of cAMP analogues as EPAC ligands, most of the selectivity

studies with these cAMP analogues were based on EPAC1, none of which has been known to distinguish between the isoforms EPAC1 and EPAC2. In addition, the off-target effects of cAMP analogues including **9** to other targets such as PDEs are also an issue that needs to be addressed.[4,7,137]

4.2. Non-nucleotide Small Molecules as EPAC Antagonists

As discussed above, although the EPAC-selective cAMP analog **9** as an EPAC activator has proven very useful for the elucidation of EPAC-mediated signaling pathways, there remains an urgent need to develop non-nucleotide small molecules as additional EPAC-selective and isoform-specific ligands (e.g. antagonists) for expediting the discovery of new biological roles and functions of EPAC in human diseases as well as potential therapeutic strategies.

Cheng and colleagues first reported their sensitive and robust HTS assay for identifying antagonists that directly compete with a fluorescent cAMP derivative 8-NBD-cAMP (**14**, Figure 7) in binding to purified full-length EPAC2.[110] This assay was based on the finding that the fluorescent signal changed in a dose-dependent manner after binding of 8-NBD-cAMP to EPAC2. In addition, more than 100-fold increase of the fluorescent signal was altered under near saturating EPAC2 concentration. Furthermore, the addition of excess unlabelled cAMP decreased the fluorescent signal in a dose-dependent manner. Consistent with previous findings,[138] alteration of the magnitude of fluorescence signal was only modest with a limited increase upon 8-NBD-cAMP binding to EPAC1 protein. Nevertheless, the significant reversible fluorescence change made it a remarkable readout for developing a sensitive and robust HTS assay for screening EPAC antagonists with high reproducibility.[110]

The pilot screening was preformed simply and effectively in 96-well format by using the NCI DTP (Developmental Therapeutics Program) diversity set library. Three compounds, NSC45576 (**15**), NSC119911 (**16**) and NSC686365 (**17**) (Figure 8) have been identified to significantly decrease the fluorescence signal with apparent IC_{50} values of 1.7 μM, 3.8 μM and 7.9 μM, respectively. Further validation demonstrated that these compounds not only inhibited EPAC2 GEF activity but also inhibited EPAC1-mediated Rap1 nucleotide exchange at 25 μM.[110] The counter-screening assays measuring type I and II PKA holoenzyme activities were performed to test the specificity of these compounds. The results revealed that **15** and **17** do not block cAMP mediated PKA activation suggesting that these two compounds are EPAC specific antagonists that selectively inhibit cAMP-induced EPAC activation.

Further studies through a more extensive screen of the Maybridge Hitfinder compound library (14,400 compounds) under a 384-well format led to the discovery of seven HTS hits **18–24** (ESI-04 to ESI-10) that are able to inhibit EPAC2 activity completely at 25 μM in the presence of the equal concentration of cAMP.[111]

As ESI-06 (**20**) and ESI-08 (**22**) shared the same fragment of 5-cyano-6-oxo-1,6-dihydro-pyrimidine (Figure 9), further exploration of the structural determinants on this scaffold have been investigated.[114] Extensive structure–activity relationship (SAR) analysis has resulted in the identification of two more potent EPAC antagonists HJC0197 (**25**) and

HJC0198 (**26**) (Figure 10). Compound **26** with a cyclopropyl moiety and **25** with the 6-cyclopentyl moiety instead of the 6-cyclohexyl group displayed enhanced activity with the IC_{50} values of 4.0 μM and 5.9 μM, respectively. Further evaluation revealed that **25** also inhibits EPAC1-mediated Rap1-GDP exchange activity, while **26** is a little more specific for EPAC2.[114]

Molecular docking studies using the AutoDock Vina algorithm[114,139,140] revealed that the hydrophobic groups of C-6 position and 2-position are critical for the binding affinity towards EPAC2. The SAR results and the representative docking data also indicated that optimization on C-6 position may lead to identification of more EPAC2-specific antagonists. As depicted in Figure 11A, the cyclopentyl group at the C-6 position interacts with the hydrophobic residues of Phe367, Ala415 and Ala416, while the hydrophobic *S*-benzyl moiety at 2-position forms interactions with Leu406 and Leu449. Further evaluation of **25** and **26** showed that both compounds did not alter cAMP-induced type I and II PKA holoenzymes activation at 25 μM, suggesting that both compounds are EPAC-specific inhibitors. The results in HEK293/EPAC1 and HEK293/EPAC2 cells have demonstrated that **25** and **26** can suppress EPAC1 and EPAC2 function *in vitro*.[114]

Further evaluation of cAMP-mediated EPAC1 GEF activity of seven hits (Figure 9) has revealed that compounds ESI-05 (**19**) and ESI-07 (**21**) are unable to inhibit EPAC1 GEF activity. Additional investigation on the specificity of **19** and **21** showed that neither of them displayed significant effects towards PKA, indicating that they are specific targeting EPAC2 protein. It is important to note that ESI-05 and ESI-07 inhibited EPAC2 activity with IC_{50} values of 0.4 ± 0.1 μM and 0.6 ± 0.1 μM, respectively, whereas cAMP showed an IC_{50} value of 40 ± 1 μM. Further studies demonstrated that **19** and **21** selectively modulated EPAC activation in living cells. Moreover, EPAC- and PKA-based fluorescence resonance energy transfer (FRET) sensor in living cells and Rap1-GTP pull-down assays further confirmed these results.[111] Recent detailed biophysical study further confirmed **19** as a direct and selective inhibitor of EPAC2 with a binding affinity about 20-fold higher than cAMP.[141]

As **19** and **21** are exclusively EPAC2-specific antagonists with no effect toward EPAC1 protein, the potential mechanism of these small chemical probes was further investigated to analyze the effect of **21** binding to EPAC2 protein using deuterium exchange mass spectrometry (DXMS) approach. The resulting data indicated that EPAC2-specific antagonists may bind to the interface of CNBD-A and CNBD-B and lock the EPAC2 protein in its autoinhibitory conformation.[111] Although other possible binding modes might not be excluded, the identification and characterization of these pharmacological probes capable of selectively targeting the EPAC2 protein represent a major milestone in the exploration of the complex cAMP signaling pathway. These EPAC2-specific antagonists have provided a new platform for further development.

Previous SAR studies and the molecular docking results demonstrated that appropriate hydrophobic moieties of the molecules play an essential role for the specificity and potency towards EPAC2 protein. Based on the scaffold of **19**, the 2,4,6-trimethylphenyl moiety was retained as a privileged fragment in the preliminary medicinal chemistry effort for hit-to-lead optimization. Consistent with the previous SAR studies, the 2,4,6-trimethylphenyl ring

as an appropriate hydrophobic pharmacophore is important for the potency. For instance, compound **27** with such a privileged fragment and another appropriate hydrophobic group was also found to be a potent antagonist with an IC_{50} of 0.7 μM, while the methoxy compound **28** exhibited only moderate potency (Figure 12).[115]

Based on the above SAR findings and the hit ESI-10 (**24**), a series of *N*,*N*-diarylamine derivatives have been designed, synthesized and characterized.[115] Compound **29** showed an enhanced IC_{50} of 3.8 μM in comparison with **24**. It is worth highlighting that **30** and HJC0338 (**31**) with the 3,5-dichloro or 2,5-dichloro displayed high potency with IC_{50} values of 0.9 and 0.4 μM, respectively (Figure 12). However, compound **32** containing a thiazole ring was found inactive with a dramatic loss of activity. SAR analysis of these *N*,*N*-diarylamine analogues further demonstrated that an appropriate hydrophobic phenyl moiety is preferable for competing with 8-NBD-cAMP binding to EPAC2.[115]

On the basis of the structural features of the preliminary SAR studies and the hit ESI-05, a series of arylsulfonamide derivatives as a novel chemical entity of EPAC2 antagonists have been designed and characterized (Figure 12).[115] Compound **33** with an indole ring displayed a moderate potency with an IC_{50} of 1.2 μM. Compound **34** with the 2-ethylpyrrole moiety as a hydrophobic fragment showed higher potency with an IC_{50} of 0.5 μM, while 2,4-dimethylpyrrole derivative HJC0350 (**35**) displayed a significantly enhanced activity with an IC_{50} of 0.3 μM. The systematic and extensive SAR results have also revealed that appropriate hydrophobic pharmacophores are important for the potency of these EPAC ligands. Further validation studies have demonstrated that these three series including diarylsulfones, *N*,*N*-diarylamines, and arylsulfonamides (Figure 12) are EPAC2-specific antagonists. In addition, live cell imaging studies using EPAC1, EPAC2, or PKA FRET reporters also confirmed that compound **35** is an effective EPAC2-specific antagonist.[115]

3-(5-*tert*-Butyl-isoxazol-3-yl)-2-[(3-chlorophenyl)-hydrazono]-3-oxo-propionitrile (ESI-09, **23**) has also been indentified and characterized as a novel non-cyclic nucleotide EPAC2 antagonist with an IC_{50} of 7.0 μM (Figure 9).[112] Meanwhile, **23** inhibited cAMP-mediated EPAC1 GEF activity with an IC_{50} of 3.2 μM but no effect towards PKA, indicating that it is an EPAC-specific antagonist. Molecular docking studies based on the scaffold of **23** provided useful insight to design more potent antagonists. As shown in Figure 11B, the *tert*-butylisoxazolyl moiety forms a hydrogen bond with the residue Gly404 and interacts with the hydrophobic residues of Phe367, Leu406, Ala407, and Ala415. Meanwhile, 3-chlorophenyl fragment forms hydrophobic interactions with residues Val386, Val394 and Leu397. Further biological functional investigation showed that **23** specifically suppressed Akt phosphorylation, EPAC-mediated Rap1 activation and insulin secretion in pancreatic β cells. Using hit **23** as a pharmacological probe, the functional roles of EPAC1 in pancreatic cancer migration and invasion have been discovered, indicating that EPAC1 may represent an attractive therapeutic target for pancreatic cancer.[112]

Previous studies have revealed that activation of cAMP-EPAC pathway in hypothalamus induced multiple indices of leptin resistance, suggesting that EPAC may represent a novel pharmacological target for obesity.[142] A more recent study demonstrated that resistance to high fat diet in EPAC1 knockout mice improved glucose tolerance, heightened leptin

signaling and induced obesity. Compound **23** enhanced leptin signaling in organotypic hypothalamic slice culture system. Furthermore, administration of **23** in wild-type mice significantly reduced plasma leptin. These findings support the notion that EPAC1 may represent a novel therapeutic target for diabetes or obesity.[143]

Very recent studies showed that genetic EPAC1 knockout mice protected them from fatal rickettsiosis, nearly completely blocked rickettsial attachment and/or invasion into the endothelial cells.[144] Importantly, pharmacological inhibition of EPAC1 *in vivo* using **23** completely recapitulates the EPAC1 knockout phenotype. Treatment with **23** in wild-type mice significantly protected them against rickettsial infection with much milder disease manifestations and dramatically improved survival, indicating that EPAC1 is a potential target for the prevention and treatment of fatal rickettsioses. **23** as the selective pharmacological probe has proven successful in unraveling the *in vivo* functions of EPAC1 to overcome potential limitations of knockout mouse models, and may provide potential novel therapeutics.[144] Meanwhile, **23** showed excellent tolerability during the *in vivo* studies, indicating its low toxicity to animals.[144] In addition, **23** displays no significant inhibitory effects on PDEs, as well as very weak inhibitory activities towards hERG and CYP450 enzymes (unpublished data). All these combined observations support that such non-nucleotide small molecules may have more advantages in terms of off-target effects and toxicities than cAMP analogs, although more extensive preclinical ADMET assessments remain to be explored for the clinical development.

Since compound **23** was identified as a novel EPAC-specific pharmacological probe to discern the physiologic functions of EPAC *in vitro* and *in vivo*, a practical and efficient method to readily prepare **23** at large scale was developed including a one-pot synthesis of isoxazole synthon, modified protocol for cyanomethyl ketone and a coupling step.[116] The development of this efficient synthetic route for **23** will greatly facilitate the ongoing medicinal chemistry efforts of lead optimization and SAR studies in searching for new molecules with improved DMPK profiles. This is particularly important in light of a recent report by Rehmann suggesting that compounds **23** and **25** exhibit non-specific effects on protein stability when used at high concentrations above 50 μM.[141] While more detailed characterizations are required to sort out the actual causes and mechanisms of such effects, the ability of **23** to recapitulate the genetic EPAC knockout phenotypes in multiple biological systems both *in vitro* and *in vivo* suggests that this class of compounds is specific to EPAC at lower concentrations or appropriate doses.[112,143,144] Therefore, it is imperative to further optimize these lead compounds through rational drug design approaches to develop advanced candidates for IND-enabling studies.

Lezoualc'h and colleagues reported another functional fluorescence-based high throughput screening assay to identify EPAC inhibitor compounds from chemical libraries.[113] This variant of the assay is on the basis of the ability of EPAC to catalyze the nucleotide exchange activity of Rap1.[145] Tetrahydroquinoline CE3F4 (**36**) has been identified as EPAC1 inhibitor that blocked EPAC1-induced Rap1 activation both in cell-free systems and in intact cells and did not influence protein kinase A holoenzyme activity (Figure 13).[113] Preliminary structure-activity study revealed that the formyl moiety on position 1 (**37**) and the bromo group on position 5 (**38**) of this class of analogues were essential for their activity

targeting the EPAC1 (Figure 13). Although the additional data revealed that **36** and its analogues could also inhibit EPAC2, **36** and related compounds may serve as new chemical leads for further structural optimizations and useful pharmacological tools for determining the biological functions of EPAC1 in a wide range of biological processes.[113,146] Further investigation on the activity of the (*R*)- and (*S*)-enantiomers of **36** revealed that (*R*)-CE3F4 (**39**) is a more potent cAMP antagonist than the racemic **36** and (*S*)-enantiomer (Figure 13). (*R*)-enantiomer **39** displays 10-fold selectivity for EPAC1 over EPAC2. The biochemical evaluation of tetrahydroquinoline analogues is very helpful in elucidating the structural features that are essential for high EPAC1 inhibitory activity.[146]

5. Conclusions and Future Directions

Since the discovery of EPAC proteins as the novel cAMP mediators in 1998, significant progress has been made by many pioneers in discriminating between PKA- and EPAC-mediated effects under physiological and pathophysiological circumstances.[147] X-ray crystal structure determinations and NMR spectroscopy techniques have provided novel insights into the mechanism of cAMP activation of cyclic nucleotide binding domains. In particular, the development of the EPAC-selective cAMP analog **9** (8-pCPT-2′-*O*-Me-cAMP, 007) through rational design based on structure/sequence alignment analysis contributed to identification of new biological functions. Moreover, its prodrug **12** (8-pCPT-2′-*O*-Me-cAMP-AM, 007-AM) with improved cell permeability further delivered the reliable insights into the multiplicity of the biologic properties of EPAC. However, the potency, selectivity and specificity, as well as the off-target effects of cAMP analogues remain essential for further improvement.

Recent studies on development of the sensitive and robust HTS assays provided highly valuable non-nucleotide small molecules as EPAC-specific ligands (e.g. antagonists) for elucidating the signaling pathways and dissecting the physiological functions. Meanwhile, it is important to screen the specificity of new EPAC modulators using cells with deletion of EPAC1, EPAC2 or both, as well as their off-target effects to other proteins such as PDEs. Thus, developing new methodologies of chemical biology in this regard is also urgently needed. It is worthy to mention that the combination of genetic knockout animal models and small molecular EPAC-specific modulators has proven very useful to reveal the *in vivo* physiological functions of EPAC. Treatment with the EPAC-specific ligands with favorable *in vivo* bioavailability in wild-type animal models may overcome the potential shortcomings of the genetic knockout manipulations such as the secondary effects due to the complex signaling pathways and the possible physiological compensation.

It is the opinion of these authors that further HTS efforts to discover new chemical entities as EPAC-specific activators, and further medicinal chemistry efforts to optimize the currently available antagonists with enhanced potency, specificity to each of the isoforms, and drug-like properties, as well as the *in vivo* pharmacological evaluation in relevant disease models of lead candidates are highly anticipated to pave the way for the ultimate development of clinical applications. Given that EPAC proteins have been implicated to play important roles in major human pathological conditions such as cancer, neurological disorders, infectious disease, heart disease and diabetes, we believe that it is of great value in

the development of pharmacological tools to probe the functions of EPAC in disease processes, and it is the hope that such efforts will eventually lead us to the development of advanced compounds into a clinical setting as potential mechanism-based therapies for those diseases.

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ABBREVIATIONS USED

cAMP	3′–5′-cyclic adenosine monophosphate or cyclic AMP
ATP	adenosine triphosphate
GPCRs	G-protein coupled receptors
ACs	adenylate cyclases
PDEs	phosphodiesterases
PKA	protein kinase A
GEFs	guanine nucleotide exchange factors
EPAC	exchange protein directly activated by cyclic AMP
CNBD	cyclic nucleotide binding domain
DEP	Dishevelled Egl-10 Pleckstrin
REM	Ras exchange motif
RA	Ras association
CDC25-HD	CDC25-homology domain
Rap	Ras related protein
DXMS	deuterium exchange mass spectrometry
FRET	fluorescence resonance energy transfer
MD	molecular dynamics
β_1-AR	β_1-adrenergic receptor
SR	sarcoplasmic reticulum
CaMKIIδ	Ca^{2+}/calmodulin-dependent protein kinase IIδ
RyR2	ryanodine receptor 2
GRK2	GPCR kinase 2
COPD	chronic obstructive pulmonary disease

HTS	high throughput screening
SAR	structure-activity relationship
PKCε	Protein kinase Cε
CYP450	cytochrome P450 enzymes
hERG	the human ether-à-go-go-related gene
8-NBD-cAMP	8-(2-[7-Nitro-4-benzofurazanyl]aminoethyl-thio) adenosine-3′, 5′-cyclic monophosphate
DTP	Developmental Therapeutics Program
ADMET	absorption, distribution, metabolism, excretion and toxicity
DMPK	drug metabolism and pharmacokinetics
IND	Investigational New Drug

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Biographies

Haijun Chen received a dual degree (B.S. in chemistry and B.E. in bioengineering) from Fuzhou University, China in 2003. He earned his Ph.D. in medicinal chemistry from Shanghai Institute of Materia Medica, under the supervision of Dr. Fajun Nan in 2008. After graduation, he worked at ShangPharm for two years and then did his postdoctoral training at University of Texas Medical Branch (UTMB) under the direction of Dr. Jia Zhou. Currently, Dr. Chen is an Associate Professor at Fuzhou University. His research focuses on the rational drug design and synthesis of novel agents for the diagnosis and treatment of cancer and CNS disorders.

Christopher Wild studied biology and chemistry at the California State University, Northridge, where he received his Bachelor and Master of Science degrees under the direction of Dr. Gagik Melikyan. Subsequently, he worked as a program chemist for ChemicoMays, CA and a research chemist at Celenese, TX where he was a member of the acetyl catalyst development team under the tutelage of Dr. Michael Nutt. Currently, Christopher is a member of the chemistry faculty at San Jacinto College, TX, and is pursuing a Ph.D. as a Keck Research Fellow at UTMB under the supervision of Dr. Jia Zhou. His research focus is on the design and synthesis of small molecule inhibitors of EPAC and allosteric modulators of 5-HT_{2C} receptor.

Xiaobin Zhou received his B.S. in pharmacy from South-Central University for Nationalities, China in 2012. He is currently pursuing his Ph.D. degree in medicinal chemistry at the College of Chemistry and Chemical Engineering, Fuzhou University. His main interest focuses on the design and synthesis of small molecules targeting EPAC proteins.

Na Ye earned her Ph.D. in medicinal chemistry from Shanghai Institute of Materia Medica, Chinese Academy of Sciences, under the supervision of Dr. Ao Zhang in 2013. She is currently pursuing her postdoctoral training under the direction of Dr. Jia Zhou at UTMB. Her research topics focus on the target-based drug design and chemical synthesis of

bioactive small molecules including EPAC-specific inhibitors as pharmacological probes and drug candidates for the treatment of CNS disorders, cancer, and other human diseases.

Xiaodong Cheng received a PhD in biochemistry and molecular biology from UTMB in 1994. After completing his postdoctoral fellowship at the University of California, San Diego in 1999, he returned to UTMB as a faculty member in the Department of Pharmacology and Toxicology. Currently, he is a Professor in the Department of Integrative Biology and Pharmacology, the University of Texas Health Science Center at Houston. His research has focused on applying multidisciplinary approaches to understand the structure and function of exchange protein directly activated by cAMP (EPAC) and oncogene KRAS. Recently, his research group has discovered novel small molecule inhibitors for EPAC proteins, established animal disease models, and is in the process of evaluating the therapeutic potential of targeting EPAC proteins.

Jia Zhou received his Ph.D. in organic chemistry in 1997 from Nankai University, China. Then he joined the chemistry faculty in the same university and was promoted to Associate Professor there. In 1999, he started his postdoctoral training in organic chemistry with Dr. Sidney M. Hecht at the University of Virginia. After further postdoctoral training in medicinal chemistry with Dr. Alan P. Kozikowski at Georgetown University, he has conducted research at Acenta Discovery, and PsychoGenics, Inc. as a Senior Principal Scientist for 7 years. Dr. Zhou is currently an Associate Professor (tenured) at the Chemical Biology Program, Department of Pharmacology and Toxicology at UTMB, leading a drug discovery research group. He is an author of more than 70 papers and an inventor of 9 patents.

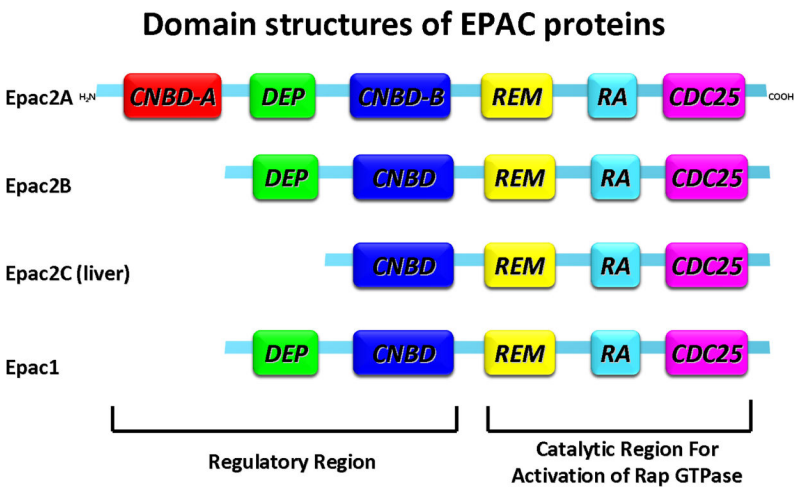


Figure 1.

Domain structures of EPAC proteins. Each EPAC family member composes an auto-inhibitory amino-terminal regulatory region and a carboxyl-terminal catalytic region for activation of Rap GTPase. The regulatory region contains a Dishevelled Egl-10 Pleckstrin (DEP) domain and at least one functional cyclic nucleotide–binding domain (CNBD). The carboxyl-terminal catalytic region consists of a Ras exchange motif (REM) domain and a Ras association (RA) domain as well as the CDC25-homology domain (CDC25-HD). The CDC25-homology domain is responsible for guanine nucleotide exchange activity and catalyzes the exchange of G-protein-bound GDP for GTP on the Ras-like small GTPases Rap1 and Rap2 isoforms.

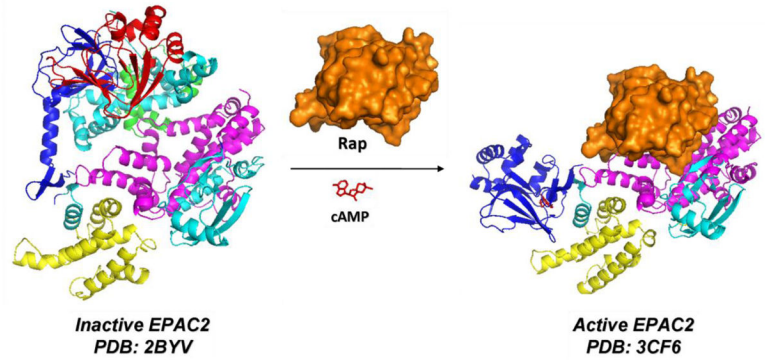


Figure 2.

The X-ray crystal structures of inactive EPAC2 (the full-length apo-EPAC2, PDB: 2BYV) and active EPAC2 (EPAC2Δ305:Sp-cAMPS:Rap1B complex, PDB: 3CF6). CNBD-A, DEP domain, CNBD-B, REM domain, RA domain, and CDC25-HD are colored in red, green, blue, yellow, cyan, and magentas, respectively. Upon binding of cAMP, a sequence of structural reorganizations within the cyclic nucleotide–binding domains allows the regulatory domain to open and move to the back side of the catalytic region. This conformational change leads to the exposure of the catalytic region for binding of Rap GTPase to catalyze the exchange of GDP for GTP.

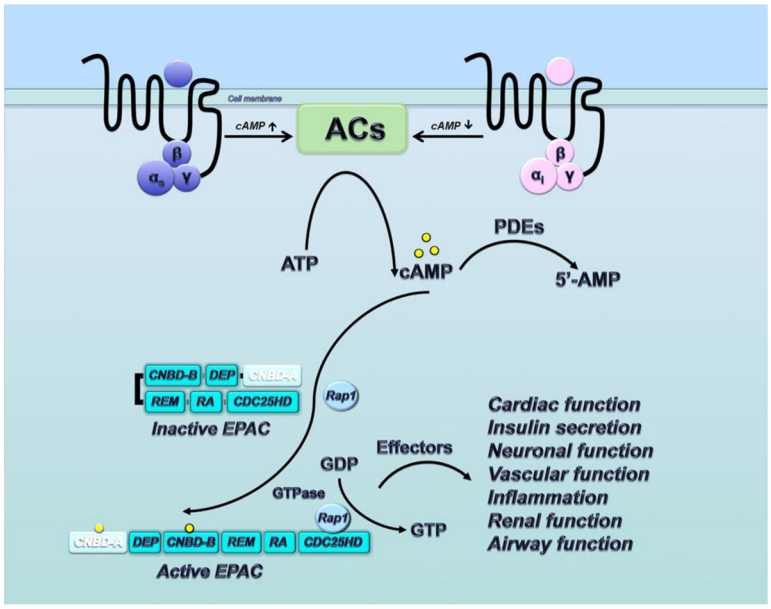


Figure 3.

Signaling pathway of EPAC proteins. EPAC1 only contains one functional cyclic nucleotide binding domain (CNBD-B), while EPAC2 contains two CNBDs (CNBD-A and CNBD-B).

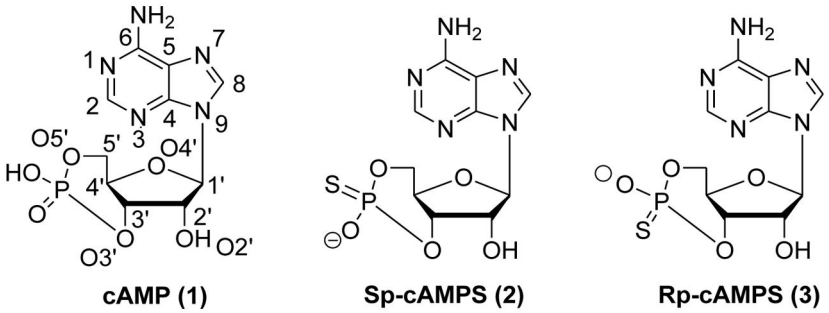


Figure 4.

Chemical structures of cAMP molecule (**1**) and cAMP analogues with modified exocyclic oxygen atom (**2** and **3**).

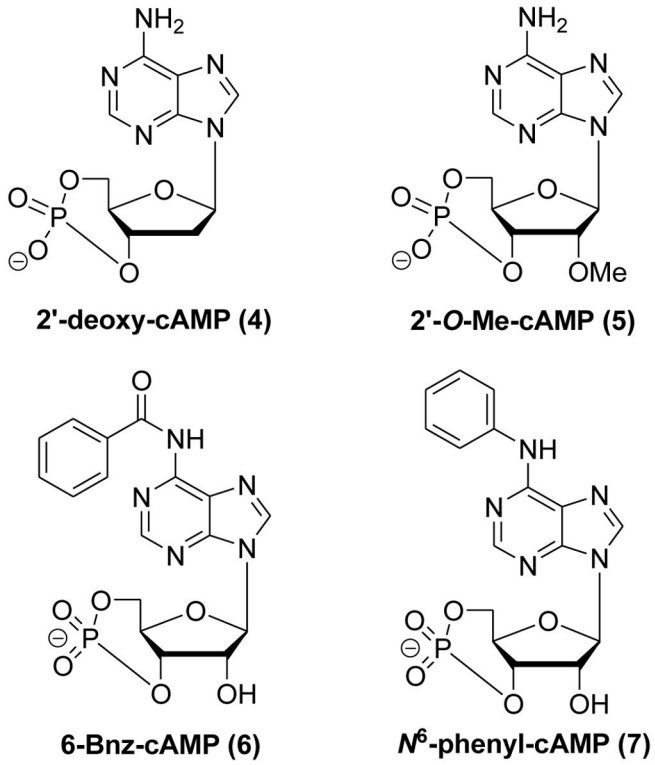


Figure 5.
Chemical structures of 2′-deoxy-cAMP (**4**), 2′-*O*-Me-cAMP (**5**), 6-Bnz-cAMP (**6**) and N^6-phenyl-cAMP (**7**).

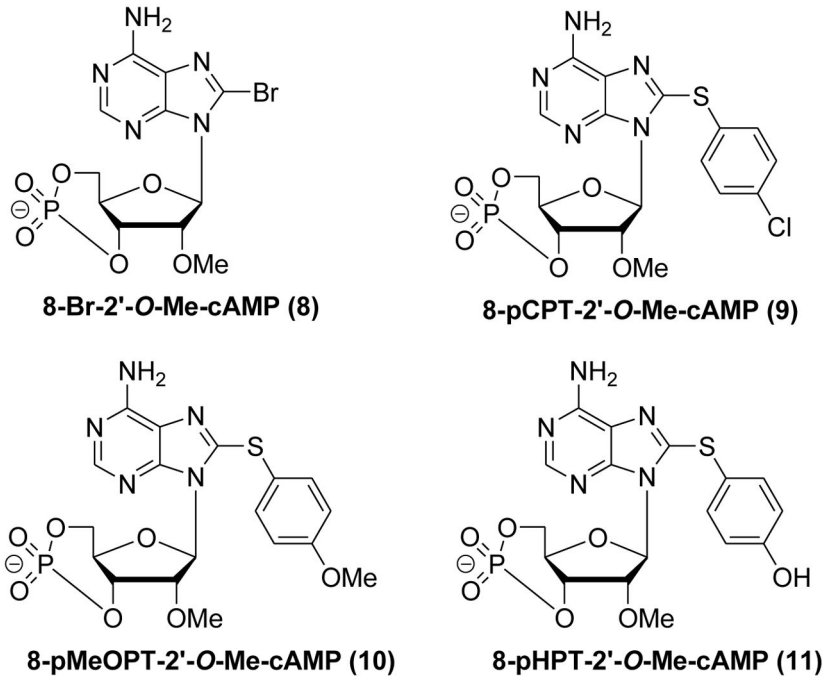


Figure 6.

Chemical structures of 8-Br-2′-*O*-Me-cAMP (**8**), 8-pCPT-2′-*O*-Me-cAMP (**9**), 8-pMeOPT-2′-*O*-Me-cAMP (**10**) and 8-pHPT-2′-*O*-Me-cAMP (**11**).

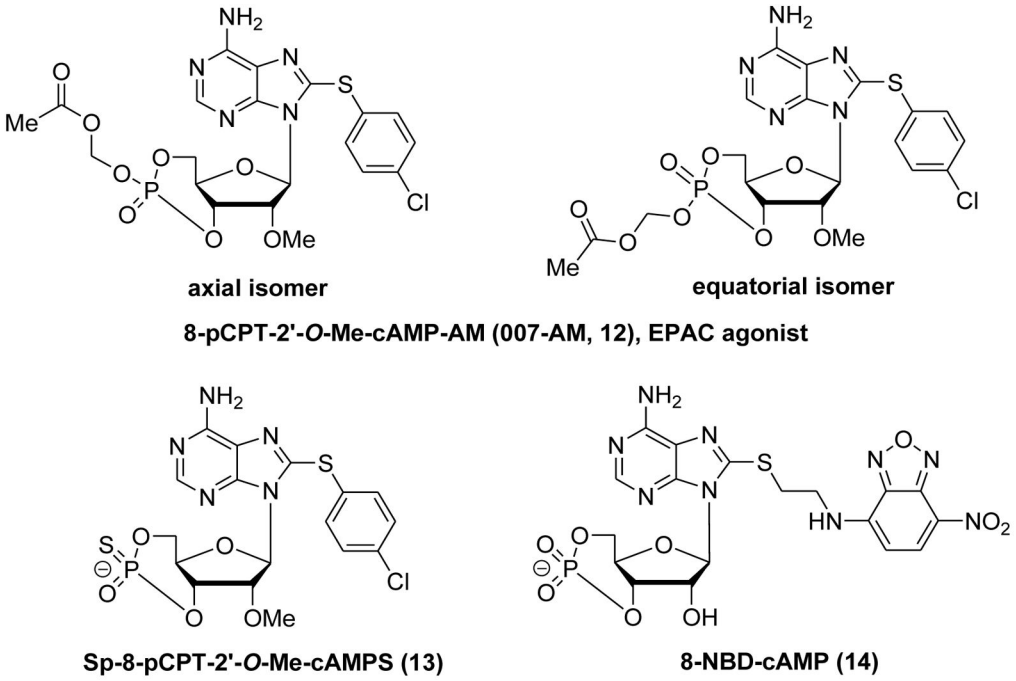


Figure 7.

Chemical structures of 8-pCPT-2′-*O*-Me-cAMP-AM (**12**), Sp-8-pCPT-2′-*O*-Me-cAMPS (**13**) and 8-(2-[7-nitro-4-benzofurazanyl]aminoethylthio)adenosine-3′, 5′-cyclic monophosphate (**14**, 8-NBD-cAMP).

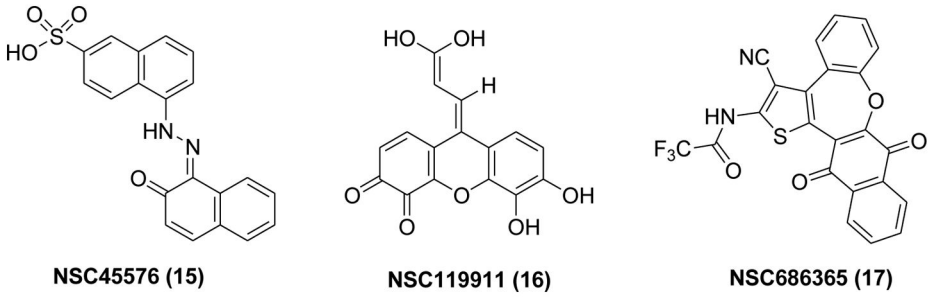


Figure 8.
Chemical structures of identified EPAC inhibitors **15–17**.

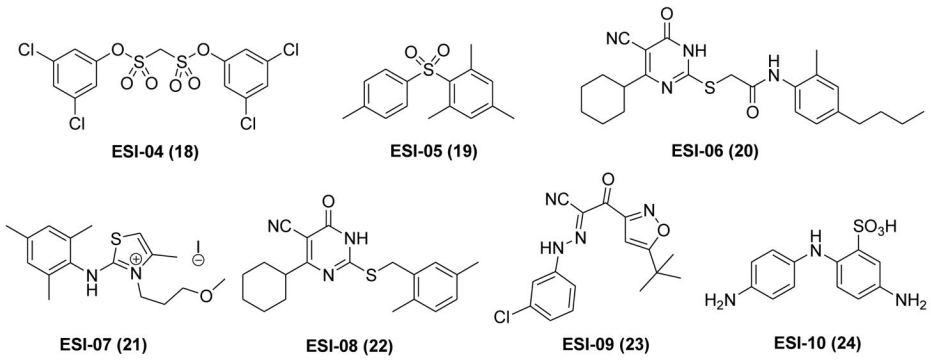
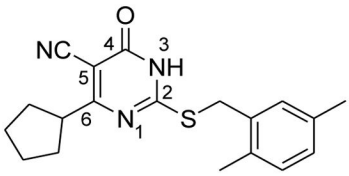
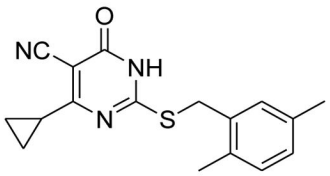


Figure 9.
Chemical structures of HTS hits **18–24**.



HJC0197 (25)



HJC0198 (26)

Figure 10.
Chemical structures of **25** and **26**.

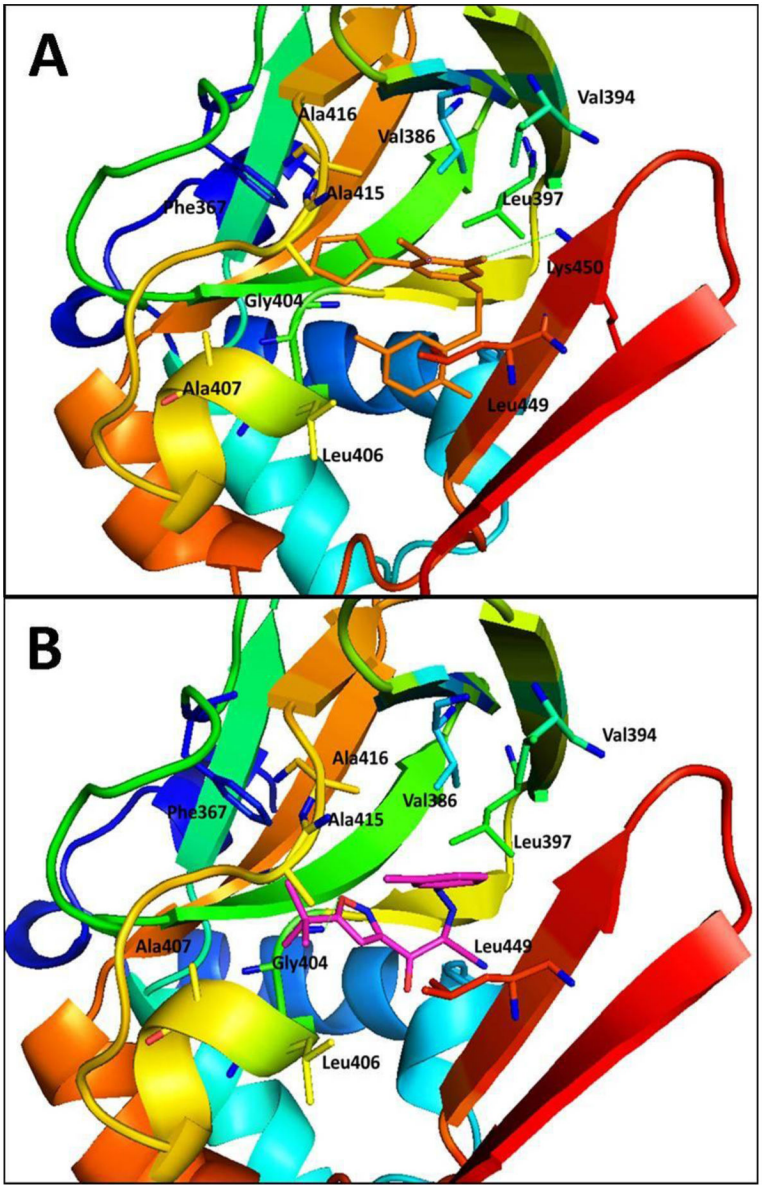


Figure 11.

Predicted binding mode and molecular docking of **25**, and **23** into the cAMP binding domain B (CNBD-B) of EPAC2 protein. Important residues are drawn in sticks. Hydrogen bonds are shown as dashed green lines. (A) Binding mode of **25** (orange). The cyclopentyl group at the C-6 position interacts with the hydrophobic residues of Phe367, Ala415 and Ala416, while the hydrophobic *S*-benzyl moiety of 2-position forms interactions with Leu406 and Leu449. (B) Binding mode of **23** (pink). The *tert*-butylisoxazolyl moiety forms a hydrogen bond with the residue Gly404 and interacts with the hydrophobic residues of Phe367, Leu406, Ala407, and Ala415. Meanwhile, 3-chlorophenyl fragment forms hydrophobic interactions with residues Val386, Val394 and Leu397.

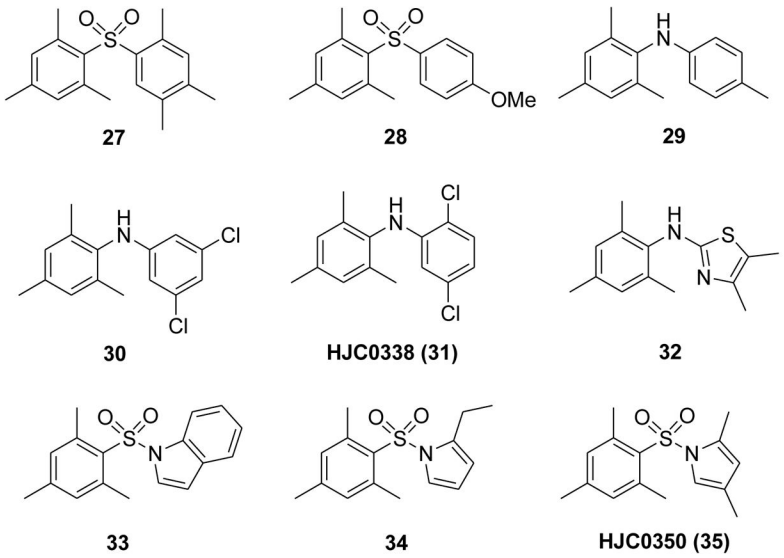


Figure 12.

Chemical structures of representative diarylsulfones **27**, **28** and *N*,*N*-diarylamines **29–32**, and arylsulfonamides **33–35**.

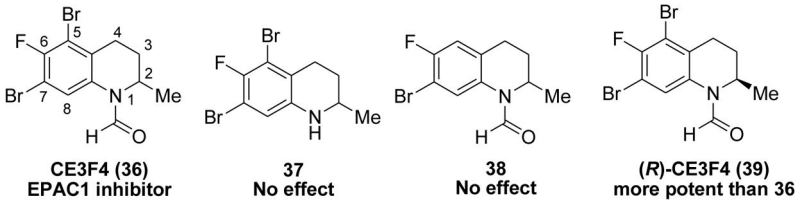


Figure 13.
Chemical structures of tetrahydroquinoline analogues **36–39**.