Adenylyl cyclases as innovative therapeutic goals

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Pharmacological modulation of intracellular cyclic AMP (cAMP) signalling could provide new therapeutic and experimental tools. Although drugs interfering with this pathway have traditionally targeted membrane receptors, the effector enzyme adenylyl cyclase (AC), which functions as a signalling catalyst, also presents an interesting target. Thus, development of isoform-selective stimulator and/or inhibitor compounds for AC could lead to organ-specific pharmacotherapeutics for treating heart failure, cancer and neurodegenerative diseases. In this review, the potential of AC as the object of drug therapy is discussed.

Introduction

The enzyme adenylyl cyclase (AC) catalyses the conversion of ATP into the universal second messenger cyclic adenosine-3′,5′-monophosphate (cAMP), which regulates several physiological functions in mammals including memory, learning, heart contractility, respiration and lipolysis [1–3]. An altered cAMP system is therefore implicated in numerous human diseases [1–3].

cAMP is generated by two families of enzymes from the class III AC superfamily (AC; E.C. 4.6.1.1) [4]: one family comprises of transmembrane ACs, which are encoded by nine distinct genes (tmACs AC1 to AC9), and which play key roles in cellular responses to extracellular signals. They are modulated through heterotrimeric G-proteins in response to stimulation of G-protein coupled receptors (GPCRs), and other encompassing cytoplasmic enzymes referred to as 'soluble' ACs (sAC, AC10), which are directly activated by calcium and the cellular metabolites bicarbonate and ATP. Therefore, sAC has been postulated to act as an intracellular metabolic sensor [5].

cAMP activates protein kinase A (PKA), leading to a phosphorylation cascade with multiple cellular targets, and is ultimately broken down to 5′-AMP by cyclic nucleotide phosphodiesterases (PDEs). The balance between the formation of cAMP by ACs and degradation by PDEs determines cellular cAMP levels.

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To date, the AC signal transduction cascade has been pharmacologically targeted through GPCR agonists or antagonists and PDEs inhibitors. However, increasing therapeutic interest currently surrounds the development of new approaches for the manipulation of GPCR signalling at steps distal to receptors. AC is an attractive pharmacological target and has been reported to be a central relay station which receives and amplifies many primary signals such as changes in extracellular ionic composition and hormone and neurotransmitter binding to GPCRs [1]. On the basis of the efficacy (maximal response) and potency (affinity, sensitivity, EC_{50}, Ka) of all the GPCR/Gs/AC signalling pathway components, Ostrom *et al.* [6] showed AC as the most crucial. Interestingly, AC has been referred to as a downstream 'bottleneck' for the GPCR/Gs signal, limiting the maximum efficacy of the system in terms of maximal cAMP generation. This central role indicates the 'signal strength', that is the stoichiometric relationship between the components of this amplification cascade [7]. Furthermore, AC is not subject to the desensitisation occurring during long-term receptor agonist stimulation with consequent loss of drug efficacy [8]. Moreover, mammalian AC isoforms show distinct regulatory properties and tissue distribution, suggesting the feasibility of developing tissue-targeted isoform-specific AC activators and inhibitors. Indeed, the generation of mice with targeted disruption of genes for the various AC isoforms has greatly enhanced the understanding of their *in vivo* relevance [1].

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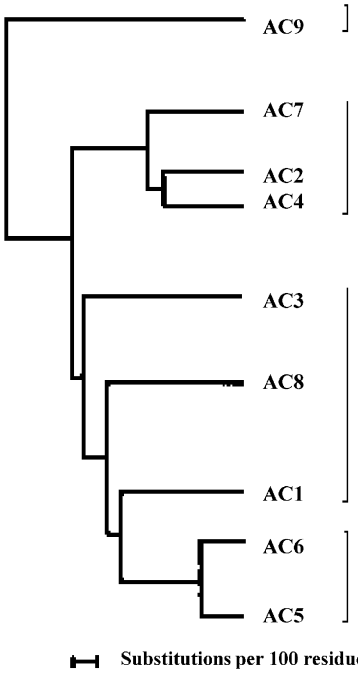
TABLE 1

Classification of mammalian ACs following to their phylogenetic tree and their regulation by endogenous modulators and tissue expression

Phylogenetic tree	Isoforms	Group	Activators	Inhibitors	Tissue distribution
	AC9	Group 4	Gsα	Calcineurin, P-site analogs	Scheletal muscle, brain, lung, liver
	AC7	Group 2	Gsα, FSK, Gβγ, PKC	P-site analogs	Lung, heart, spleen, Kidney
	AC2		Gsα, FSK, Gβγ, PKC	P-site analogs	Brain, olphactory bulb
	AC4		Gsα, FSK, Gβγ	P-site analogs	Kidney, brain, liver
	AC3	Group 1	Gsα, FSK,Ca^{2+}/CaM	CaM Kinase II, P-site analogs	Olfactory neurones, brain
	AC8		Gsα, FSK, Ca^{2+}/CaM	P-site analogs	Brain
	AC1		Gsα, FSK, Ca2+/CaM	Giα, G βγ, CaM Kinase IV, P-site analogs	Brain, retina
	AC6	Group 3	Gsα, FSK	Giα, PKA, PKC, Ca^{2+}, P-site analogs	Heart> brain>kidney, testis, liver
	AC5		Gsα, FSK, PKC and ζ	Giα, PKA, Gβγ, Ca^{2+}, P-site analogs	Brain>Heart
	Substitutions per 100 residues				

Gsα: G-protein stimulatory α subunit; FSK: forskolin; Gβγ: G-protein βγ subunits; PKC: protein kinase C; CaM: calmodulin; Giα: G-protein inhibitory subunit α; PKA: protein kinase A; PKζ: protein kinase ζ.

Classification, physiological regulation and tissue distribution of ACs

The nine membrane-bound AC subtypes are described in Table 1. As each AC isoform is characterised by distinct biochemical properties and tissue distribution, they might represent the biochemical signature of tissue cAMP generation in various tissues [9,10]. For example, the calcium-inhibitable AC type 5 is the major isoform expressed in the adult heart; PKC-sensitive type II is expressed in the lung; calmodulin-sensitive types 1 and 8 are expressed mainly in neuronal tissues; type 3 is expressed abundantly not only in olfactory tissues, but also in other tissues including the lungs, atria and adipose tissue; and types 4 and 7 are widely expressed [10]. The different ACs act as an integrative core in response to many signals, such as Gsα, Giα (inhibitory) or Gβγ (stimulatory or inhibitory, depending on the enzyme), protein kinases such as PKA, PKC and Ca^{2+}/calmodulin kinase or Ca^{2+} alone [11]. The effects of these activators are often highly synergistic or conditional, highlighting the function of ACs as common integrative target for separate inputs [11]. Variance in the cAMP accumulation properties of the various AC isoforms has been reported [12]; in particular, the sustained cAMP accumulation property of AC6 [12] could make it an alternative to PDE inhibitors.

Structure of ACs

TmACs contain 12 transmembrane helices arranged in two sets of six separated by a large hydrophilic domain formed by two intracellular lobes generally referred to as C1 and C2 (Fig. 1) [13]. The nucleotide-binding site of the tmACs, which has been designated 'P'-site, consists of two homologous cytoplasmic domains, C1a and C2a, within the intracellular lobes. Its occupancy traditionally requires molecules characterised by the presence of an intact adenine ring [14–16]. These domains comprise approximately 230 amino acids that share at least 50% similarity across the AC family. Mutagenesis analysis has revealed that residues from both C1a and C2a domains contribute to binding and catalysis of ATP. X-ray crystal structures of the catalytic site depict AC as a receptor characterised by three main features: (i) a phosphate binding region containing two metal ions (normally Mg^{2+} or Mn^{2+}), (ii) a purine ring binding site possessing H-bond donating and accepting side-chains suitably oriented for affixing adenosine and (iii) a hydrophobic pocket [13].

Main approaches to pharmacological control of ACs

The catalytic site of ACs can be pharmacologically targeted by specific activators or nucleotides, which inhibit AC either competitively or non-competitively [1,17–20]. Information on structure/activity relationships (SARs) and structure/selectivity relationships (SSRs) for the development of drugs to regulate cAMP signals in various tissues gets increasing, although the design of AC isoform-selective drugs is still in its infancy. Indeed, to produce therapeutic effects, an AC inhibitor or stimulator should have a K_D value in the low nanomolar range and a selectivity index greater than 100. Currently, valuable design projects to obtain selective AC ligands are in progress. This review will provide an overview of the known

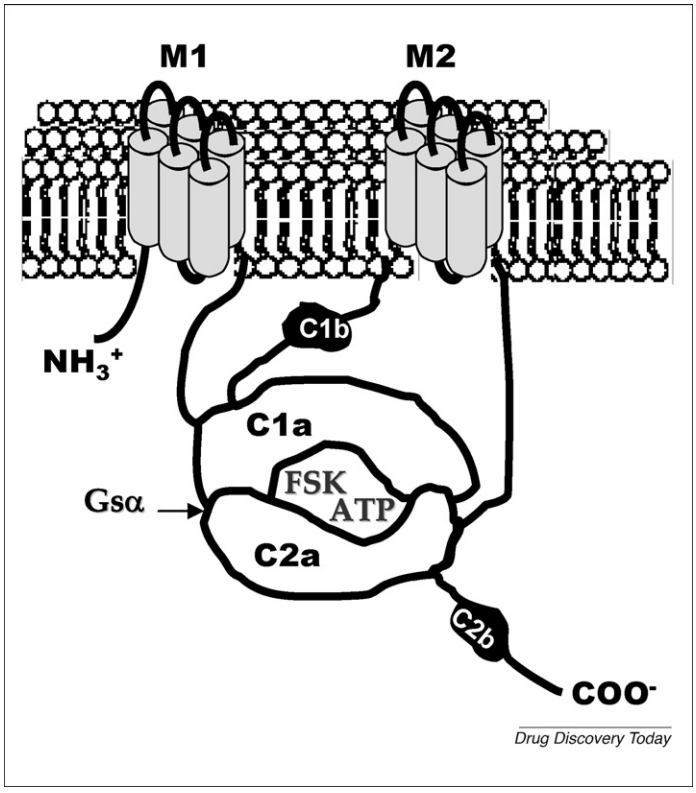


FIGURE 1

Schematic diagram of the proposed structure and membrane topology of adenylyl cyclase. M1 and M2 denote the two regions of the six transmembrane-spanning domains in the AC molecule. C1a and C2a are the first and the second cytoplasmic catalytic domains, respectively, which, facing each other, form the catalytic core inside a crevice. FSK and ATP bind the catalytic core at opposite sites. C1b is the first cytoplasmic-linker domain and C2b is the second non-catalytic domain. The C2b domain is present only in AC1, AC2, AC3 and AC8 isoforms.

SARs for the main AC activators and inhibitors, followed by a summary of the physiological and therapeutical relevance of the specific activation or inhibition of that isoforms of each AC subgroup most expressed in a tissue-specific manner.

SARs of AC stimulators

The best known direct activator of AC is the diterpene forskolin (FSK) (Fig. 2), a hydrophobic activator of all mammalian membrane-bound ACs, excluding type 9. Its intravenous administration is difficult owing to its low water-solubility [20].

Crystallographic studies have revealed that FSK interacts with AC at a hydrophobic site created by the C1 and C2 catalytic subunits. Specifically, FSK binds to the opposite end of the site where ATP binds within the catalytic core, and anneals the two cytoplasmatic domains by a combination of hydrophobic and hydrogen bonding interactions, thereby enhancing enzymatic activity [21–23]. Hydrogen bonds are formed between amino acids highly conserved among AC types 1–8 and the C_1-OH group, C_7-acetyl group or C_{11}-OH group of diterpenes [24]. In accordance with these data, initial experimental studies indicate that modification at the C_1, C_9 and C_{11} positions of FSK results in loss of AC stimulatory activity [10]. Crystallographic studies have also permitted the identification of a relatively large open space near the C_6 and C_7 FSK positions [21], suggesting that the presence of new substituents at positions 6 and 7 of FSK might allow to obtain novel selective activators for the different AC isoforms [10,17,25,26]. Indeed, it was shown in insect cells overexpressing one of the 2, 3 or 5 AC isoforms, that the 6-[*N*-(2-isothiocyanoethyl)aminocarbonyl] FSK derivative (FD1, Fig. 2), which is an irreversible AC1 inhibitor [27], was also a more potent activator of AC2, compared to AC3 and AC5. Similar behaviour was found for the 6-(4-acrylbutyryl) FSK derivative (FD2, Fig. 2) [10]. It has therefore been hypothesised that selective stimulators of AC2 might be obtained using FSK derivatives with functional groups at position 6 able to interact with Lys^{896}, unique to this AC isoform.

The FSK derivatives 7-deacetyl-7-hydroxamino FSK and 5,6-dehydroxy-7-deacetyl-7-nicotinoyl FSK (FD3 or FD4, respectively, Fig. 2) are able to selectively enhance the stimulatory activity of AC3, suggesting that this effect can be obtained by polar substitution at position 7, as well as by the presence of a double bond between the C_5 and C_6 atoms of the diterpene ring [10].

NKH477 6-[3-(dimethylamino)propionyl]FSK (FD5, Fig. 2) is currently employed to stimulate cardiac ACs in patients with congestive heart failure [28,29]. It selectively enhances AC5 with respect to AC2 and AC3 [10,17,25,30]. Interestingly, AC5 selectivity is further augmented with the compound 6-[3-(dimethylamino)propionyl]-14-15-dihydroforskolin (FD6, Fig. 2), characterised by the absence of the double bond in position 13 of the diterpene ring.

Very recently, the effect of several FSK derivatives on AC1, AC2 and AC5 expressed in insect cell membranes has been analysed to find their EC_{50} and efficacy values [26]. As displayed in Table 2, the derivatives 1,9-dideoxyFSK (1,9dd-FSK) and 7-deacetyl, 1-deoxyFSK (7DA1d-FSK) appeared to be totally ineffective towards the three isoforms, whereas 7-deacetylFSK (7DA-FSK), 6-acetyl,7-deacetylFSK (6A7DA-FSK), 7-deacetyl-7-(*N*-methylpiperazino-γ-butyryloxy)FSK (DMB-FSK, Fig. 2) and 9-deoxyforskolin (9d-FSK) acted as partial agonists showing AC1 selectivity. Notably, the derivatives 1-deoxyFSK (1d-FSK) and 7-deacetyl-1,9-dideoxyFSK (7DA1,9dd-FSK) showed only inverse agonist activity on AC2, with EC_{50} values in the micromolar range. Finally, the derivative obtained by the substitution of the small C_7-acetyl group with the large BODIPY group (BODIPY–FSK, Fig. 2) yielded potencies higher than that of FSK, acting as a partial agonist for isoforms 1 and 5 but as an inverse agonist for isoform 2. Molecular modelling studies suggest that the BODIPY group is located outside the FSK binding site, and its interactions with surrounding amino acids induce the diverse effects on the different AC isoforms [26]. Table 2 summarises the effects of the AC stimulators described above.

The ineffective derivative 1,9dd-FSK was identified also as mixed competive/non-competive inhibitor versus the stimulatory activity of the AC1, AC2 and AC5 isoforms [26]. Very recently this behaviour has been attributed to the absence of the C_1-OH substituent in the diterpene ring. In particular, it has been suggested that diterpenes are all able to promote the C1/C2 assembly, but those without the C_1-OH substituent cannot form, in a second step, an hydrogen bond able to promote the conformational switch necessary for the catalysis activation. The strong hydrophobic interactions of 1,9dd-FSK with C1/C2 domains should impair ligand exchange, inducing an apparent non-competitive inhibition [31].

Recently, benzyloxybenzylaldehyde derivatives have been proposed as AC activators, although their chemical structure is totally

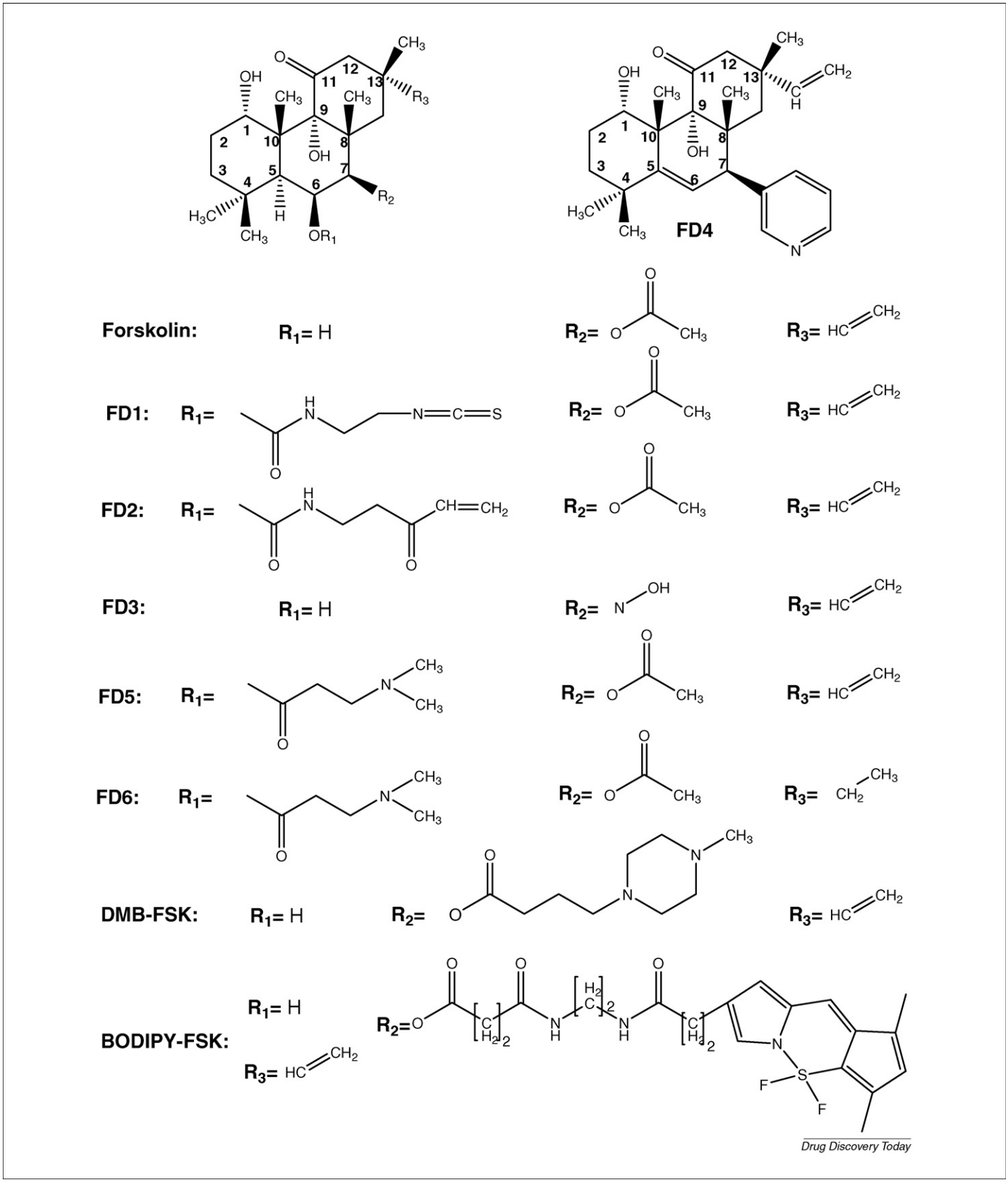


FIGURE 2

Chemical formulae of forskolin; 6-[*N*-(2-isothiocyanoethyl)aminocarbonyl]forskolin (FD1), 6-(4-acrylbutyryl)forskolin (FD2); 7-deacetyl-7-hydroxaminoforskolin (FD3); 5,6-dehydroxy-7-deacetyl-7-nicotinoylforskolin (FD4); 6-[3-(dimethylamino)propionyl]forskolin (FD5; 6-[3-(dimethylamino)propionyl]-14-15-dihydroforskolin (FD6); 7-deacetyl-7-(*N*-methylpiperazino-γ-butyryloxy)forskolin, (DMB-FSK) and BODIPY–forskolin (BODIPY–FSK).

TABLE 2

Rank order of catalytic activity stimulation, potency (EC50) and efficacy showed by FSK and FSK-analogues toward various AC isoforms

Abbreviation	Stimulatory effect[a] or potency[b]	Efficacy
FD1	Type 2 ≫ type 3 > type 5[a]	–
FD2	Type 2 > type 3 ≈ type 5[a]	–
FD3	Type 3 > type 2 ≈ type 5[a]	–
FD4	Type 3 > type 2 ≈ type 5[a]	–
FD5	(NKH477) type 5 > type 2 ≈ type 3[a]	–
FD6	(DMAP) type 5 ≫ type 2 > type 3[a]	–
FSK	Type 1 > type 5 > type 2[b]	100% efficacy
DMB-FSK	Type 1 > type 2 > type 5[b]	Type 5 > type 1 > type 2
7DA-FSK	Type 1 > type 5 > type 2[b]	Type 1 ≈ type 2 > type 5
6A7DA-FSK	Type 1 > type 5 > type 2[b]	Type 5 > type 1 > type 2
9d-FSK	Type 1 > type 5 > type 2[b]	Type 1 ≈ type 2 > type 5
1d-FSK	Type 2; type 1 and type 5 ineffective[b]	Type 2 inverse agonist
1,9dd-FSK	Ineffective	Ineffective
7DA1,9dd-FSK	Type 2; type 1 and type 5 ineffective[b]	Type 2 inverse agonist
7DA1d-FSK	Ineffective	Ineffective
BODIPY-FSK	Type 1 ≈ type 2 > type 5[b]	Type 1 > type 5; type 2 inverse agonist

FD1: 6-[*N*-(2-isothiocyanoethyl)aminocarbonyl]forskolin; FD2: 6-(4-acrylbutyryl)forskolin; FD3: 7-deacetyl-7-hydroxaminoforskolin; FD4: 5,6-dehydroxy-7-deacetyl-7-nicotinoylforskolin; FD5: (NKH477); 6-[3-(dimethylamino)propionyl]forskolin; FD6 (DMAP): 6-[3-(dimethylamino)propionyl]-14-15-dihydroforskolin; FSK: forskolin; DMB-FSK: 7-deacetyl-7-(*N*-methylpiperazino-γ-butyryloxy)forskolin; 7DA-FSK: 7-deacetylforskolin; 6A7DA-FSK: 6-acetyl,7-deacetylforskolin; 9d-FSK: 9-deoxyforskolin; 1d-FSK: 1-deoxyforskolin; 1,9dd-FSK: 1,9-dideoxyforskolin; 7DA1,9dd-FSK: 7-deacetyl-1,9-dideoxyforskolin; 7DA1d-FSK: 7-deacetyl-1-deoxyforskolin; BODIPY–FSK: BODIPY–forskolin.

[a] Rank order of stimulatory effect on the catalytic activity of various AC isoforms produced by FSK-analogues (from Refs. [15,17,19]). The stimulatory effects were obtained by employing 50 μM FSK derivatives in the presence of 5 mM $MgCl_2$.

[b] Rank order of potency (EC_{50}) and efficacy of FSK and FSK-analogues toward various AC isoforms (from Ref. [20]). The values range in the micromolar order of magnitude and were obtained by employing increasing concentrations of the drugs in the presence of 10 mM $MgCl_2$ and 3% (v/v) DMSO.

different from that of forskolin [32]. They might thus constitute a new scaffold for the development of AC-activating drugs.

SARs of AC inhibitors

The early known class of AC inhibitors is that of P-site inhibitors. Although they have an adenine moiety, their inhibition is non-competitive with respect to the substrate ATP [33]. P-site inhibitors, indeed, bind AC in the presence of pyrosphosphate and, as demonstrated by crystallographic studies, they form a dead-end complex preventing the interaction of other substrates with P-sites [33,34].

There are several adenosine derivatives among P-site inhibitors, which lack certain ribose ring hydroxide groups that can be substituted by mono or polyphosphate groups (Fig. 3). In particular, pyrophosphate groups at position 3′ of the ribose ring have been demonstrated to provide potent inhibitors of rat brain AC. The order of inhibitory potency is adenosine < 2′-deoxyadenosine < 3′-AMP < 2′-deoxy-3′-AMP < 2′,5′-dideoxy-3′-AMP < 2′,5′-dideoxy-3′-ADP < 2′,5′-dideoxy-3′-ATP < 2′,5′-dideoxyadenosine-3′-tetraphosphate [35–38]. The IC_{50} value of the latter was found to be 7.4 nM for rat brain AC [37], indicating that it is a very potent AC1 inhibitor.

Studies into AC1, 2 and 6 indicate that 2′-deoxy-3′-AMP is slightly selective for AC1, over type 2 and 6 ACs. Moreover, ribose substituted P-ligands such as 9-(cyclopentyl)-adenine (CPA) and 9-(tetrahydrofuryl)-adenine (THFA, Fig. 3) appeared to selectively inhibit AC1 rather than types 2 and 6 [39]. These compounds all showed IC_{50} values in the micromolar range. It was also demonstrated that CPA and THFA are highly selective for AC5 over the 2 and 3 isoforms, whereas 2′-deoxy-3′-AMP and 3′-AMP inhibit AC3 and AC5 potently and AC2 weakly [10,25,40].

Considering that P-site inhibitors with an intact adenine ring can induce side effects such as the inhibition of DNA synthesis [41], some researchers have sought new AC inhibitors characterised by the absence of the adenine moiety [10]. On the basis of the crystallographic studies, the sequence $=C^2H–N^1=C^6(NH_2)$ was identified as an essential pharmacophore for AC5 inhibitors, and the compound 2-amino-7-(2-furanyl)-7,8-dihydro-5(6H)-quinazolinone (NKY80, Fig. 3) was recognised as a less potent AC inhibitor than THFA but showed similar selectivity for AC5 [10]. It was confirmed that the IC_{50} values of NKY80 were 14.8 μM for AC5, but 226 and 2630 μM for AC3 and AC2, respectively. Modifications of the furan moiety of NKY80 induced a drastic decrease in potency towards AC [40].

A new class of P-site inhibitors has been obtained by joining hydroxamic acid, which is characterised by chelating properties, to the adenine ring. This strategy was devised taking into account that ATP requires the presence of the divalent cation Mg^{2+} to bind the P-site and induce the enzymatic activity of AC [42]. Hydroxamic acid probably mimics the ATP phosphate groups by forming a substrate complex with Mg^{2+}. Several P-site inhibitors with metal chelating properties (PMC) have also been synthesised [43–45] by joining hydroxamic acid to the adenine ring via various flexible acyclic linkers. Only the derivative 1R,4R-3-(6-aminopurin-9-yl)-cyclopentane-carboxylic acid hydroxamide (PMC-6, Fig. 3) has been identified as a selective, potent AC5 inhibitor (IC_{50} = 0.32 μM, 11.1 μM and 65.3 μM for AC5, AC3 and AC2, respectively).

Recently, 2′(3′)-*O*-(*N*-methylanthraniloyl) (MANT) nucleotides have been identified as a new class of potent AC inhibitors. Indeed, 2′(3′)-*O*-(*N*-methylanthraniloyl)-5′-[γ-thio]triphosphate] (MANT-GTPγS) and 2′(3′)-*O*-(*N*-methylanthraniloyl)-guanosine5′-[β,γ-imido]-triphosphate (MANT-GppNHp, Fig. 3) have been found to inhibit FSK-stimulated AC in S49 lymphoma and Sf9 insect cells with IC_{50} values in the high affinity nanomolar range. This inhibition appeared competitive with respect to the substrate ATP [46]. Moreover, MANT-nucleotides were discovered to inhibit type 5 and 6 ACs more potently than the others [18]. Currently, crystallographic and biochemical studies are being performed to analyse the molecular interactions of MANT-nucleotides with AC [19,47], which might provide information for the design of new AC inhibitors. It is known that MANT-nucleotides bind the C1 and C2 catalytic subunits in a reverse orientation with respect to the ATP analogues without bulky substituents at the 2′(3′) position. The reverse orientation induces the guanine moiety to form with the C1 and C2 domains hydrogen bonds analogues to those formed by adenine.

Studies performed on murine cardiac myocytes of wild-type and AC type 5-deficient mice demonstrated that MANT-GTPγS is able to attenuate the L-type calcium current stimulated by isoproterenol, acting predominantly via AC5 inhibition [48].

One of the problems related to MANT-inhibitors is their low membrane permeability. This problem is currently being addressed by designing prodrugs that can be deprotected and phosphorylated after permeation into cells [49]. In fact, it has been demonstrated that MANT-nucleoside-5′-disphosphated are phosphorylated to the corresponding trisphosphates by cellular kinases [18].

An important aspect of the MANT-GTPγS derivative is its selectivity for tmACs, with respect to soluble ACs [19], unlike the other inhibitors described above. By contrast, two new sAC inhibitors with poor or no inhibitory effect on tmACs have been developed and characterised *in vitro* [50]. These inhibitors were created by a docking study based on the crystal structure of the catalytic core of a cyanobacterial homologue of human sAC bound with 2-hydroxy-17β-estradiol, an AC inhibitor able to interact in a newly identified pocket for catechol estrogens [51]. The selected compounds were 3,20-dioxopregn-4-en-21-yl-4-bromobenzenesulfonate and 1,2,3,4,5,6,7,8,13,13,14,14-dodecachloro-1,4,4a,4b,5,8,8a,12b-octahydro-11-sulfo-1,4:5,8-dimethanotriphenylene-10-carboxylic acid; the latter is depicted in Fig. 3. These inhibitors showed IC_{50} values in the low micromolar range on purified recombinant mammalian sAC, but a poor or insignificant inhibition of tmACs. These data suggest that investigations into the catechol estrogen binding site of AC might permit the identification of potent and selective sAC inhibitors.

Physiological and therapeutical relevance of AC isoforms

Currently, several AC isoforms are known potentially involved in therapeutics against brain and heart diseases. In particular, AC1 and AC2 appear targets for treating Alzheimer's and other neurodegenerative diseases; AC5 seems implied in human motor dysfunctions. Moreover, AC5 and AC6 play important roles in acute or chronic heart failure and pressure overload. These aspects are described as following.

AC1, AC2 and AC8 isoforms

Pharmacological implications of brain targeting

Ca^{2+}/calmodulin-activated AC1 and AC8 are expressed at high levels in neuronal tissues such as the hippocampus and several cortical regions. Conversely, AC8 can be regarded merely as a Ca^{2+} detector, being approximately five times less sensitive to Ca^{2+} than AC1 and not synergistically stimulated by Gs-coupled receptors and Ca^{2+} [52]. Additionally, as AC8 is located in extra-cerebral tissues, AC1 is a more attractive drug target owing to its neurospecificity and expression pattern restricted to areas of the brain involved in learning and memory. Ca^{2+} enhances cAMP signals triggering AC1, which in turn allows synergism between Ca^{2+} and cAMP-activated kinases, and positive feedback regulation of Ca^{2+} channels by cAMP-dependent protein kinase. Indeed, drugs that increase cAMP in specific brain areas in response to a synaptic signal might enhance synaptic plasticity and memory formation [52].

It could be argued that compounds stimulating AC1 in the presence of Ca^{2+}/CaM may provide the greatest level of specificity and therapeutic value as memory-enhancing drugs. Wang and Storm [53] have proposed an interesting pharmacological viewpoint in which drugs that specifically enhance Ca^{2+}-stimulated AC1 activity may be preferable to those sustaining an increased cAMP level, such as PDE inhibitors. This means that AC1 could supply a 'pharmacological window of opportunity' to augment cAMP in specific areas of brain without the side affects associated with cAMP increases in other tissues [53]. Thus, it is plausible that selective AC1 activators like BODIPY–FSK could be of clinical relevance for treating Alzheimer's disease [26], where a decreased expression of AC1 has been demonstrated [54]. Furthermore, types of AC other than Ca^{2+}/calmodulin-stimulated ACs might affect the learning processes [55]. In particular, reduction in Ca^{2+}-insensitive AC activity and in AC2 mRNA expression levels was associated with hippocampus-dependent memory formation in adult mice [55,56], but not in aged mice [57]. It was hence suggested that downregulation of AC2 is permissive for acquisition of hippocampus-dependent memory formation, whereas an upregulation of AC1 is crucial to accurately encode, store or use information. Effects of hippocampal injection of forskolin on memory formation are also consistent with this interpretation [58]. These data emphasise the importance for selectively targeting AC isoforms and further, they reinforce the potential therapeutic action of BODYPY–FSK, which effectively stimulates AC1 and also inhibits AC2 as inverse agonist.

The usefulness of AC1-selective inhibitors in treating neurodegenerative conditions or stroke-related injury has also been hypothesised. Indeed, Wang *et al.* [59] demonstrated that cortical neurons from mice deficient in AC1 are resistant to glutamate-induced neuronal toxicity. Neuronal excitotoxicity has been linked to the activation of *N*-methyl-D-aspartate (NMDA) receptors (NMDARs), and appears to play a role in neuronal death associated with stroke, Huntington's disease and other degenerative disorders. AC1 has also been proposed as the major Ca^{2+} sensor for NMDAR activation and excitotoxicity, with respect to AC8. These findings indicate that AC1 plays an isoform-specific role in modulation of NMDA receptor-dependent neuronal excitotoxicity, and might therefore become a therapeutic target [60].

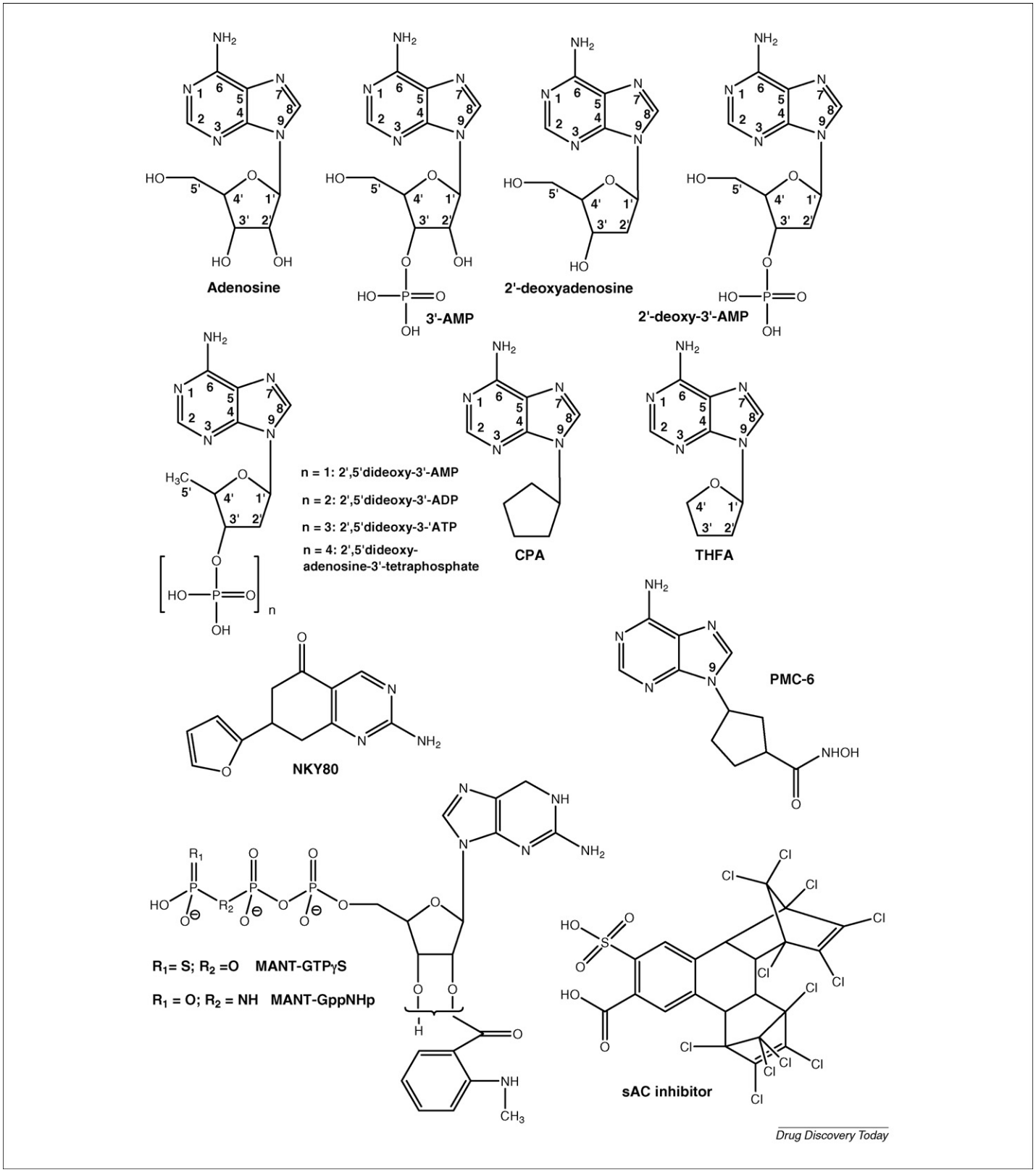


FIGURE 3

Chemical formulae of P-site inhibitors; 9-(cyclopentyl)-adenine (CPA); 9-(tetrahydrofuryl)-adenine (THFA); 2-amino-7-(2-furanyl)-7,8-dihydro-5(6H)-quinazolinone (NKY80); 1R,4R-3-(6-aminopurin-9-yl)-cyclopentane-carboxylic acid hydroxamide (PMC-6); 2′(3′)-*O*-(*N*-methylanthraniloyl)-5′-[γ-thio]triphosphate] (MANT-GTPγS) and 2′(3′)-*O*-(*N*-methylanthraniloyl)-guanosine5′-[β,γ-imido]-triphosphate (MANT-GppNHp) – these two compounds show a spontaneous isomerisation between the 2′- and 3′-MANT-substituted structure; 1,2,3,4,5,6,7,8,13,13,14,14-dodecachloro-1,4,4a,4b,5,8,8a,12b-octahydro-11-sulfo-1,4:5,8-dimethanotriphenylene-10-carboxylic acid (sAC inhibitor).

To target the AC1, AC2 and AC8 neurospecific isoforms, compounds must be engineered taking into account not only their molecular framework, but also their ability to cross the blood–brain barrier (BBB) depending, for example, on their lipophilic properties. Perhaps for this reason, development of AC1-targeted drugs is still in its infancy compared to, for example, the heart.

AC5 and AC6 isoforms

Although millimolar (i.e. non-physiological) concentrations of Ca^{2+} inhibit all AC isoforms, AC5 and AC6 are inhibited by concentrations of Ca^{2+} at micromolar concentrations, well within the dynamic range of intracellular levels [61].

Pharmacological implications of striatum targeting

AC5 is the dominant isoform in the adult striatum, where it has been revealed as the major AC subtype in transducing dopamine 1 and 2 receptor signalling [62]. Therefore, genetic disruption of AC5 leads to a major loss of AC activity in the striatum and a small increase in the expression of a few other AC isoforms. These findings implicate that targeting an AC isoform, such as AC5, in future pharmacotherapy might be an effective way of treating human motor dysfunction [62].

The drugs targeted to striatum-expressed AC5 can be also manipulated to reduce delivery to the brain and increase distribution to the heart by altering their passage through the blood–brain barrier. Thus, AC5-targeted drugs could generate neuronal modulation of heart activity without inducing unwanted brain effects.

Pharmacological implications of heart targeting

AC5 and AC6 are the major isoforms in the heart; AC5 is dominantly expressed in adult cardiomyocytes, but poorly expressed or absent in other peripheral tissues such as the lungs [40]. Conversely, AC6 is expressed primarily in foetal cardiomyocytes, whereas adult cardiac fibroblasts express abundant AC6 [17].

Direct activation of AC5, not its receptor, might serve as an alternative to the common β-adrenoceptor (β-AR) blockade therapy in the treatment of cardiovascular diseases, as the enzyme integrates the input from the multiple receptor system [40]. It has recently been demonstrated that AC5 deletion plays a protective role for the heart against chronic β-AR stimulation and chronic pressure overload by attenuating the decline in cardiac function and defending against increased apoptosis. These findings make AC5 potentially important for future β-AR blockade therapy, in which suppression of AC5 activity might be advantageous for treating heart failure [63]. Indeed, absence of AC5 results in more effective desensitisation after long-term catecholamine stress, and protects against the development of myocyte apoptosis and deterioration in cardiac function, potentially elucidating a novel treatment approach [63].

Furthermore, potential new insights into controlling longevity have been revealed by a study of the AC5 gene [64]. This study indicates that genetic disruption of AC5 increases mouse lifespan and confers resistance to ageing-related conditions, including bone loss and cardiomyopathies. Consequently, AC5 inhibitors might be valuable drugs in the prevention of age-related heart disease to prolong the human lifespan [64]. By contrast, the cardioprotective and lifespan extending effects of heart-specific AC6 overexpression in transgenic mice are not negligible [65,66]. To date, a correlation between AC6 upregulation and AC5 deletion is unknown.

Poor water solubility and a broad spectrum of non-specific pharmacological activities have hampered FSK clinical application. The water-soluble FSK derivative NKH477 (FD5) was introduced as an inotropic agent in acute heart failure, as colforsin daropate hydrochloride (CDH), in Japan by the Nippon Kayaku Company [67]. NKH477 has significantly reduced bio-distribution in the brain and fewer neuronal effects than FSK, presumably because of poor permeation through the blood–brain barrier [30]. NKH477 has been demonstrated to stimulate all tissue ACs, in particular cardiac AC, more potently than FSK. By contrast, FD6 stimulates AC more potently than FSK only in the heart. Thus, both NKH477 and FD6 stimulate cardiac AC more potently than FSK, but FD6 seems to be more selective than NKH477 [30]. Therefore, agents that directly and selectively activate AC5 might mimic superselective β-agonist in terms of cardiac specificity, and could be useful in the treatment of acute heart failure, being AC5 the major AC isoform mediating acute β-adrenergic stimulation in mouse heart [30,67].

In this regard, FD6 and NKH477 have been reported to serve as the prototype of such superselective compounds [30].

Among the P-site inhibitors, PMC-6 is the most potent and selective inhibitor of AC5, also in comparison to RS-2 (NKY80), the most selective, but not cell membrane-permeable, known inhibitor of AC5 so far [40].

Concluding remarks

Development of AC isoform-selective stimulators or inhibitors offers the advantage of a tissue-specific manipulation of cAMP. Indeed, ACs show distinctive features, such as the absence of desensitisation, maximal signalling capacity along their pathway, and variance in catalytic activity between the various isoforms, in terms of cAMP accumulation over degradation. Actually, owing to its sustained cAMP accumulation property, AC6 could be suggested as an alternative target to the employ of PDE inhibitors.

The heart-specific AC5 and AC6 offer the best results in pharmacotherapeutics. Interestingly, the MANT-nucleotides appear selective inhibitors for the tmACs, in particular AC5 and AC6, over the sACs, showing potencies in the nanomolar range. Prodrugs able to increase their membrane permeability have been identified. This class of drugs might constitute an alternative to the classical β-adrenergic-antagonist therapy against chronic heart failure.

Among stimulators, the AC5-selective FSK derivative NKH477 is clinically employed against acute heart failure. BODIPY–FSK was recognised not only as a relatively potent AC1 stimulator but also as an AC2 inverse agonist. This behaviour makes it an interesting candidate drug against brain diseases.

Currently, the design of AC ligands is still hindered by some drawbacks such as the absence of ideal compounds having K_D values in the low nanomolar range together with a selectivity index higher than 100. Moreover, the referenced compounds cannot been systematically examined across all the ACs, since some of them, such as types 4 and 7, do not have so high catalytic activity that is readily measurable, even when they are overexpressed in insect cells. Again, a reduced therapeutic usefulness of AC-selective drugs might be suggested for that tissues expressing several different AC isoforms [68]. However, it must be considered

that the AC isoforms expression is known at mRNA but not at tissue protein levels, since antibodies with requisite sensitivity are lacking [69]. In particular, knowledge that usually only the catalytic activity of an isoform predominates in a specific tissue supports the AC-selective ligands relevance [40]. Moreover, when catalytic activities of several isoforms match in a same tissue, their interaction with a same drug might even constitute a therapeutic advantage, as above described for BODIPY–FSK capability to improve memory and learning. On these grounds, it is reasonable to spend more efforts in developing such drugs.

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