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Naunym Schmiedeberg's Archives of Pharmacology, In press

Direct stimulation of adenylyl cyclase 9 by the fungicide imidazole miconazole

James Simpson[1], Adrienn Pálvölgyi[2] and Ferenc A. Antoni[1,2]
1 Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, Scotland, UK
2 Division of Preclinical Research, Egis Pharmaceuticals PLC, Budapest , Hungary

Correspondence:
F A Antoni MD DSc FRSE
Centre for Discovery Brain Sciences
University of Edinburgh
EDINBURGH EH8 9XD
Phone: +44 796 864 7338
Email : ferenc.antoni@ed.ac.uk

SUMMARY

Purpose: In mammals, nine genes encode trans-membrane adenylyl cyclase (tmAC) isoforms that synthesize the intracellular messenger compound cAMP from ATP. As cAMP is produced in virtually all types of cell, isoform-selective modulators of tmAC would have major research and therapeutic potential. This study investigated the effects of fungicide imidazoles previously shown to suppress cAMP production in various tissues on the activities of tmAC isoforms AC1, 2 and 9.

Methods: AC1, 2 or 9 stably expressed in human embryonic kidney 293 cells were investigated. Intact cells, as well as crude membranes were exposed to various imidazoles or known stimulators of tmAC and the ensuing changes in the production of cAMP analysed.

Results: In crude membranes, the activity of AC9 in the presence of GDP-β-S was enhanced by miconazole with an EC_{50} of ~8μM, while AC1 and AC2 were inhibited with an IC_{50} of ~20μM. Clotrimazole (10-100μM) was an inhibitor of all the ACs tested. Substrate saturation analysis indicated that miconazole increased the V_{max} of AC9 by 3-fold while having no effect on the K_m. In intact cells, the effect of miconazole on cAMP production through AC9 was additive with that of isoproterenol. The stimulation of cAMP production by miconazole was inhibited by Ca^{2+} and this could be prevented by the calcineurin blocker FK506.

Conclusion: Activation of AC9 by miconazole is through a mechanism distinct from that of forskolin, activated G proteins or the COOH-terminal mediated autoinhibition. However, it is subject to the AC9 isoform-specific inhibition by Ca^{2+}/calcineurin. Differential modulation of mammalian tmAC paralogues appears to be achievable by an imidazole with phenylated side-chains. Optimization of the lead-compound and exploration of the underlying mechanism(s) of action in more detail could exploit this further.

INTRODUCTION

Adenosine 3'5'monophosphate (cAMP) is formed from ATP by adenylyl cyclase (AC) and is an important second messenger molecule that is virtually ubiquitous in mammalian cells. Accordingly, cAMP is involved in several vital processes in the body ranging from spermatogenesis to cognition. Thus, drugs that target AC would have considerable therapeutic potential (Kerwin Jr, 1994, Serezani et al., 2008, Pierre et al., 2009, Seifert et al., 2012, Raker et al., 2016, Dessauer et al., 2017).

A family of nine different genes encodes trans-membrane tmAC (tmAC) in mammals. The tmAC protein paralogues encoded by these genes are structurally and functionally distinct (Taussig and Gilman, 1995, Krupinski and Cali, 1998, Antoni, 2000, Dessauer et al., 2017). Between species, the sequence homology of tmAC orthologues is high, typically 80-95%, whereas between tmAC isoforms of the same species it is only 20-40% (Krupinski and Cali, 1998). This points to specified physiologic functions for tmAC isoforms, which is corroborated by the differential tissue distributions of the respective mRNAs and proteins (Defer et al., 2000, Visel et al., 2006), as well as the selective deficits of biological functions caused by the targeted deletion of tmAC genes, reviewed in ref (Dessauer et al., 2017).
The catalytic core of tmACs is relatively well-preserved in all isoforms and requires the physical interaction of two distinct, yet structurally homologous cytoplasmic segments designated C1a and C2a (Fig. 1), respectively, reviewed in refs (Taussig and Gilman, 1995, Tesmer and Sprang, 1998). The C1a and C2a domains are several hundreds of amino acid residues apart and are flanked by non-conserved cytoplasmic sequences known as C1b and C2b, respectively, which are highly variable between isoforms (Taussig and Gilman, 1995, Krupinski and Cali, 1998). Moreover, significant sequence variation between tmAC isoforms can be observed in the transmembrane domains. Heterodimers formed from recombinantly expressed C1a and C2a domains (Tang and Gilman, 1995, Dessauer and Gilman, 1996) or the entire C1 and C2 domains (Scholich et al., 1997, Haunsø et al., 2003) retain catalytic activity and at least some of the regulatory properties of the ACs they have been derived from. It is currently hypothesized that the C1a-C2a complex is stable *in vivo* and stimulation of enzyme activity by $G_{s\alpha}$-GTP or the diterpene drug forskolin is by stabilisation of a catalytically active conformation of the heterodimer (Tesmer et al., 1999). Alternatively, it is possible that the C1a-C2a complex is not abundant *in vivo* and its formation is enhanced by $G_{s\alpha}$ or forskolin (Whisnant et al., 1996).

The requirement for heterodimerisation of C1a and C2a for catalysis in ACs raises the possibility that cAMP biosynthesis could be modulated in an isoform-specific manner by small ligands. Indeed, the details of several screens have been published, reviewed in refs. (Seifert et al., 2012, Dessauer et al., 2017). Overall, the compounds used target the active site (P-site inhibitors) or the forskolin binding-pocket formed at the interface of the C1a and C2b catalytic domains. More recently, a relatively selective inhibitor of AC1 that also stimulates the activities of AC2, 5, and 6 was reported (Brust et al., 2017). The mechanism of action of this compound awaits characterization.

The present study analysed the effects of variously substituted phenylated imidazoles (Fig. 2), currently in use as anti-fungal agents, on tmAC activity. Following on from an investigation into the mechanisms by which ketoconazole could suppress cortisol secretion without inducing the hypersecretion of corticotropin (Stalla et al., 1988), these drugs were

found to inhibit tmAC catalytic activity in anterior pituitary, as well as S49 lymphoid cells (Stalla et al., 1989, Watson, 1990). However, the compounds have not been tested on specified tmAC isoforms. Our focus was on AC9 (Antoni, 2016) as this isoform shows the lowest sequence homology with the rest of the tmACs, and thus appears to represent the best opportunity for selective targeting. In contrast to other tmACs, AC9 is insensitive to forskolin (Yan et al., 1998) and the currently known P-site inhibitors (Haunsø et al., 2003, Baldwin and Dessauer, 2018). In earlier work, we reported that miconazole had no discernible effect on cAMP production on an AC9 miniprotein (AC9_C1C2) constructed from the cytosolic C1 and C2 domains of the enzyme (Haunsø et al., 2003). Another imidazole, calmidazolium, markedly inhibited AC9_C1C2, but had a biphasic effect (stimulation at <5μM, inhibition at >5μM) on the holoenzyme in native membranes. As calmidazolium (Fig 2) is structurally related to both miconazole and clotrimazole, we investigated the possibility that these drugs have differential effects on AC9. Two other tmACs, AC1 and AC2, with different regulatory properties were used as comparators to AC9. We found that AC9 was stimulated 3-fold by miconazole, while AC1 as well as AC2 were markedly inhibited. Moreover, the stimulatory effect of miconazole was subject to the AC9-specific inhibition by Ca^{2+}/calcineurin.

MATERIALS AND METHODS

Reagents

If not otherwise indicated, all reagents were from Sigma-Aldrich, Poole, Dorset, U.K., and of the highest grade available. Further sources for reagents were: calyculin A, microcystin-LR (Alexis Corporation, Nottingham, U.K.), miconazole, clotrimazole, ketoconazole, (ICN Labs, Basingstoke, Hants, U.K.), creatine phosphokinase (Boehringer Mannheim, Lewes, East Sussex).

Assay of adenylyl cyclase

HEK293 cells stably overexpressing mouse (Antoni et al., 1998a) or human AC 9 (Paterson et al., 2000), human AC9 truncated at start of the C2b domain (AC9_Y1242, (Pálvölgyi et al., 2018)) rat AC2 (Feinstein et al., 1991), or bovine AC1 (Krupinski et al., 1989) were produced and propagated as described previously (Antoni et al., 1998a). The cells were used between 5 to 25 passages. Current evidence indicates, that species differences within mammalian tmAC isoforms are relatively minor. Thus, the salient regulatory properties of the respective isoforms are likely to be conserved across species (Taussig and Gilman, 1995, Krupinski and Cali, 1998, Dessauer et al., 2017). For the preparation of crude membranes, the cells were detached from the culture vessels in Mg^{2+} and Ca^{2+} free Hank's balanced salt solution containing 0.1% Na_2EDTA pH 7.4 and pelleted by centrifugation at 200 x g for 10 min at 20°C. The cell pellets were homogenized by 3 freeze-thaw cycles and trituration ($2x10^7$ cells/ml) in Tris-HCl 50mM, KCl 150mM, leupeptin 5μg/ml, pepstatin-A 1μg/ml, EGTA 1mM, Trasylol ™(Bayer) 15μl/ml, calyculin A 50nM, microcystin-LR 1μM, staurosporine 3μM, $MgCl_2$ 1mM, pH7.4 and crude membranes were prepared as previously described (Antoni et al., 1998a). The membrane pellet was washed twice with 4mM EGTA to deplete endogenous calmodulin and AC activity was determined as previously reported (Antoni et al., 1998a). The AC reaction mixture contained 100μM GDP-ß-S, which was included to block any effects of free G-protein α-subunits. Under these conditions the cyclase reaction was linear for at least 20 min. Protein content of the membrane samples was determined by the Coomassie blue method (Bradford, 1976).

cAMP accumulation in intact cells.

HEK293 cells that stably overexpress tmACs at levels ~20-fold above normal cellular concentration were maintained as previously described (Antoni et al., 1998a, Pálvölgyi et al., 2018). For the measurement of cAMP accumulation the cells were plated in growth medium in 24-well tissue culture trays. After 72-96h the medium was changed to Ham's F12 medium supplemented with Insulin-transferrin-sodium selenite media supplement (Sigma, I 1884) 1ml/L for 24h. Subsequently, the medium was exchanged to Ham's F12 containing 25mM HEPES pH7.4, 0.3 mM Na_2EGTA, 1μM thapsigargin, and 10μM ryanodine, in order to deplete intracellular pools of Ca^{2+}. Conditions of Ca^{2+} depletion were used because imidazole anti-fungal agents reportedly alter Ca^{2+}-handling in several cell types (Sargeant et al., 1994), which may have a secondary influence on the activity of ACs (see (Willoughby and Cooper, 2007) for review). Modulators of calcineurin were also introduced at this time and the cells were incubated for 20 min at 37°C. After 20 min, Ca^{2+} was applied in some cultures to reach 2.5mM extracellularly. After further 5 min of incubation inhibitors of cyclic nucleotide phosphodiesterase (PDE) were added : 1mM IBMX and 0.1 mM rolipram. The incubation was carried on for 10 min and stopped by the addition of HCl to 0.1 mM final concentration. In experiments with isoproterenol, the agonist was added after the 10 minutes of incubation with the PDE blockers, and the incubation was continued for a further 10min. The level of cAMP in the wells was determined as for membrane assays.

RESULTS

Expression of ACs in HEK293 cells

The AC activity in membranes prepared from AC1, AC2, or AC9 transfected cell lines was between 15-50 pmol cAMP /mg protein/min, whereas that of cells transfected with pcDNA3 skeleton vector alone was 1.3-2.9 pmol cAMP/mg protein/min. Thus, close to 90% of the tmAC activity in these crude membrane preparations is attributable to the transfected tmAC isoform, which correlated reasonably well with a previous estimate that AC9 protein was expressed at approximately 20x-fold higher than the physiologic level in stably transfected HEK293 cells (Antoni et al., 1998a).

Effects of imidazoles and other compounds on AC9 in crude membranes

Miconazole and econazole stimulated cAMP formation by AC9 with apparent EC_{50} values of 8 and 10 μM, respectively (Fig. 3). Ketoconazole caused no significant change of activity while clotrimazole was inhibitory, the maximal effect was 50% inhibition at 100μM. (Fig. 3). Imidazole (3-100μM) had no effect (not shown). Forskolin (10μM) produced an approximately 2-fold stimulation, and its effect was additive with that of a maximally effective concentration of miconazole (Fig 4A). Kinetic analysis of cAMP formation indicated that miconazole (30μM) increased the V_{max} of AC9 while the K_m for ATP remained unchanged (Fig 4B) (K_m [μM] *vehicle:* 176±33, *miconazole* 181±21; V_{max} [pmol/mg prot/min] *vehicle:* 55±11, *miconazole*: 162±43 means±S.D., n=3, $P<0.01$ for V_{max} by Student's t-test).

A blocker of calmodulin, J8 (MacNeil et al., 1988) had no significant effect on the activity of AC9 in membrane preparations or intact cells (Supplementary Fig 1). Moreover, the amphiphilic ligands trifluoperazine and 48/80, which influence the activity of numerous enzymes (Husebye and Flatmark, 1988), were without effect on AC9 in membranes at 100μM (Supplementary Fig 1).

Evidence for tmAC isoform selectivity of imidazoles in crude membranes

Miconazole inhibited cAMP synthesis by AC2 to 30% of control with an IC_{50} around 10μM (Fig 5A). Both clotrimazole and miconazole inhibited the activity of AC1 to 15 and 25% of the vehicle treated control, respectively (Fig 5B).

Effect of imidazoles in intact cells

Application of imidazoles to intact HEK293 cells expressing human AC9 gave results consonant with the studies in membranes: miconazole stimulated cAMP production, (EC_{50} was not determined as the plateau of the concentration-response curve for miconazole was not reached at 100μM) while clotrimazole was inhibitory (IC_{50} = 26μM) (Fig 6A). Fluconazole (100μM) was without effect (not shown).

A salient regulatory property of AC9 is its inhibition by Ca^{2+} involving a calcineurin-dependent process (Paterson et al., 1995, Paterson et al., 2000, Antoni, 2016). Miconazole-stimulated cAMP formation by AC9 was significantly reduced upon the introduction of 2.5 mM Ca^{2+} to the incubation medium (Fig 6B). The inhibitory effect was blocked by the calcineurin inhibitor FK506 (3 and10 μM) (Fig. 6B). L685,818 (50μM), an inactive FK506 analogue with respect to the inhibition of calcineurin, but a blocker of the prolylisomerase activity of FK-binding proteins (Dumont et al., 1992) had no effect .

We have previously shown that the C2b domain exerts a marked auto-inhibitory effect on the activation of AC9 by Gs-coupled receptors (Pálvölgyi et al., 2018), and therefore examined the possibility that miconazole stimulated AC9 by relieving this auto-inhibition. The effects of miconazole on full-length AC9 and AC9-Y1242, a COOH-terminally truncated human AC9 that lacks the C2b domain (Pálvölgyi et al., 2018), were identical. Thus, miconazole also activates AC9 in the absence of the auto-inhibitory domain (Fig 7A). As HEK293 cells express endogenous β2-adrenergic receptors (Rosethorne et al., 2010), the interaction between the beta agonist compound isoproterenol and miconazole was analysed. This was restricted to AC9_Y1242, because full-length AC9 is practically unresponsive to Gs-coupled receptors in HEK 293 cells (Pálvölgyi et al., 2018). As shown in Fig 7B, the effects of miconazole and a maximally effective concentration of isoproterenol (300 nM) on the production of cAMP in cells expressing AC9_Y1242 were additive (Two-way ANOVA, Interaction : $F(3, 24)$ 0.31, $P=0.82$, Miconazole concentration effect : $F(3,24)$ 206, $P<0.0001$, Isoproterenol effect : $F(1,24)$ 170, $P<0.001$).

DISCUSSION

The results presented here show that AC9 is directly stimulated by miconazole. Moreover, miconazole inhibited AC1 and AC2 thus indicating a potential for the differential modulation of tm-ACs by phenylated imidazoles.

It has been previously reported that anti-fungal imidazoles markedly inhibit cAMP formation in anterior pituitary cells (Stalla et al., 1989) as well as the S49 lymphoma cell line (Stalla et al., 1989, Watson, 1990). Both studies found that forskolin-stimulated cAMP production was drastically inhibited by miconazole. Importantly, AC9 is largely insensitive to forskolin (Yan et al., 1998). However, as both S49 and adenohypophysial cells contain multiple isoforms of tmAC, any selective actions of imidazoles may have been masked. The present study analysed stably transfected cell lines where ~90% of the production of cAMP is attributable to a specified tmAC, and hence the differences in the actions of imidazoles could be resolved.

Surprisingly, miconazole and its close structural analogue econazole stimulated the activity of AC9. In the case of the former, the V_{max} of the reaction was enhanced close to 3-fold, the K_m for Mg-ATP was unchanged. This observation indicates that the action of miconazole is distinct from that of forskolin, which generally increases the K_m for ATP (Dessauer and Gilman, 1996). Forskolin does not stimulate AC9 (Yan et al., 1998), furthermore, miconazole failed to stimulate cAMP production by the fused catalytic domains (AC9_C1C2) of AC9 (Haunsø et al., 2003), whereas it is well established that the target of forskolin is within these domains (Tang and Gilman, 1995, Tesmer and Sprang, 1998). Thus, it seems reasonable to conclude that miconazole stimulates AC9 *via* a mechanism distinct from that of forskolin on other tmACs (Tang and Gilman, 1995, Yan et al., 1998). With respect Gsα, which is a physiologic stimulator of tmACs, the studies in membranes were carried out in the presence of GDP-ß-S thus minimizing the contribution of G proteins. Furthermore, as AC9 is insensitive to inhibition by Gi (Baldwin and Dessauer, 2018), (Kleuss, Simpson and Antoni, unpublished data) an effect of miconazole on Gi also seems unlikely. Finally, in intact cells the effects of maximal beta-adrenergic receptor activation and miconazole on cAMP production were additive. Taken together, these findings also argue against a shared mechanism of action between G proteins and miconazole to stimulate AC9. Whether or not

the inhibition of AC9 by clotrimazole involves the same allosteric pathway as the effect of miconazole, remains to be explored.

We have not analysed the inhibition of AC1 and 2 or other AC paralogues by miconazole in further detail. As AC isoforms other than AC9 are all stimulated by forskolin, the results on AC1 and AC2 reported here are consonant with the previous findings of Stalla et al. and Watson (Stalla et al., 1989, Watson, 1990). It is of note, that inhibition of enzymatic activity by high concentrations (>10μM) of hydrophobic imidazoles in aqueous solution may be non-specific. Under these conditions, drugs may form colloidal aggregates that tend to cause protein unfolding, leading to a loss of biological function (Coan et al., 2009).

Taken together with previous work (Haunsø et al., 2003), the present findings indicate that miconazole targets the transmembrane domains to stimulate AC9. The transmembrane domains show considerable sequence variation between tmAC isoforms, and AC9 is the enzyme having the lowest level of sequence homology with the rest of the tmAC paralogues. Indeed, others have reported that the exchange of the trans-membrane domains of AC5 with that of AC7 led to a dramatic reduction of enzymatic activity (Seebacher et al., 2001). Moreover, chimeras between 6-transmembrane domain (6TM) receptors and tmAC catalytic domains show transduction of the activation of the 6TM by an extracellular ligand to tmAC (Beltz et al., 2016). Thus, the idea that the trans-membrane domains make a significant isoform specific contribution to tmAC catalytic activity is not unprecedented, but minimally explored experimentally (Bassler et al., 2018). Drugs that modulate the functional activity of other integral membrane proteins by interacting with their trans-membrane domains are well known (Kratochwil et al., 2011, Nury et al., 2011, Miller et al., 2017).

A further important feature of the miconazole stimulation of AC9 was that the inhibition by Ca^{2+}/calcineurin (Antoni et al., 1995, Antoni et al., 1998b) was preserved. This indicates that miconazole did not interfere with a salient feature of the isoform-specific physiological control of AC9. Tests of the specificity of the effects of imidazoles on ACs were carried out using other drugs that influence a wide range of enzymes (Husebye and Flatmark, 1988). Neither trifluoperazine nor compound 48/80 had sizeable effects on membrane AC9 activity under the conditions of the assay.

In summary, miconazole enhanced the activity of AC9 through a novel mechanism by apparently targeting the transmembrane domain(s) of the enzyme. The recent advances in structural analysis by cryo-electronmicroscopy technology (Renaud et al., 2018) should facilitate the understanding of the molecular mechanisms underlying the effects of imidazoles on tmACs and may lead to the development of novel pharmacons targeting this important family of proteins.

ACKNOWLEDGEMENTS

Funding

This work was supported by the Medical Research Council, U.K. and the Servier-Egis research co-operation agreement.

External input

We thank Dr Janice Paterson and Mrs Susan M. Smith for HEK293 cells expressing human AC9, Dr A. Friedrich, Fujisawa GmbH, Munich, Germany for FK506, Dr William Parsons, Merck and Co, Rahway N.J., for L685,818, and Dr H. Wachtel, Schering AG., Berlin, Germany for rolipram.

DISCLOSURES

Conflict of interest

AP and FAA were employees of Egis Pharmaceuticals PLC, Budapest, Hungary.

Contributions:

AP and JS, carried out experiments, collated and analysed data, FAA designed and carried out experiments, analysed and collated data, wrote the manuscript. All authors have agreed on the text of the submitted manuscript.

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LEGENDS TO THE FIGURES

Figure 1
Schematic representation of the structure of mammalian tmACs, following nomenclature suggested by Taussig and Gilman (Taussig and Gilman, 1995). Note the single polypeptide chain with six transmembrane segments resembling an ABC transporter protein. The C1a and C2b domains come in physical contact to form the catalytic core. The NH_2-terminal, the transmembrane, as well as the C1b and C2b domains are highly variable between tmAC paralogues.

Figure 2
Chemical structures of the imidazole compounds used in this study.

Figure 3
The effect of imidazoles on the activity of mouse AC9 in HEK293 cell membranes. Data are means ± S.D. n=4/group, expressed as percentage of cAMP levels in the presence of vehicle (dimethylsulfoxide at 0.7% v/v final concentration), representative of two identical experiments. * P<0.05, ** <0.01, *** <0.001 **** <0.0001 *vs.* the vehicle treated control group, one-way ANOVA followed by Dunnett's test for multiple comparisons.

Figure 4
Characterisation of the effect of miconazole on mouse AC9 in HEK293 cell membranes: (A) Additive effects of forskolin (10μM) and miconazole (30μM) on tmAC enzymatic activity. 2-ANOVA showed significant effects of both compounds and no interaction. Two-way ANOVA gave no significant interaction, effect of forskolin, $F(1, 12) = 34.13$, $P<0.0001$, miconazole $F(1, 12) = 258.1$ $P<0.0001$. Data are means ± S.D., n=4/group, representatives of 2 identical experiments. B) Miconazole (30μM) increased the V_{max} of cAMP formation by approximately 3-fold, while the K_m appeared unaltered. (K_m [μM] *vehicle:* 176±33 *miconazole* 181±21; V_{max} [pmol/mg prot/min] *vehicle:* 55±11 and *miconazole*: 162±43 means ± S.D., n=3, P<0.01 for V_{max} by Student's t-test).

Figure 5
Isoform selective action of imidazoles on tmAC activity in membranes prepared from cells overexpressing A) mouse AC9 and rat AC2. Note stimulation of AC9 by miconazole and inhibition of AC2. B) bovine AC1. Data are means ± S.D., n=4/group, representatives of four identical experiments. ** P<0.01, **** <0.0001 *vs.* the vehicle treated control group, one-way ANOVA followed by Dunnett's test for multiple comparisons.

Figure 6
The effects of imidazoles on cAMP accumulation by intact HEK293 cells overexpressing human AC9. (A) Concentration-dependent stimulation and inhibition by miconazole and clotrimazole, respectively. * P<0.05, ** <0.01, *** <0.001 **** <0.0001 *vs.* the vehicle treated control group, one-way ANOVA followed by Dunnett's test for multiple comparisons.
(B) Miconazole (30μM) induced cAMP accumulation was inhibited by Ca^{2+} (2.5mM) applied in the medium, and this effect was blocked by the calcineurin inhibitor compound FK506, but not by L685,818 an analogue of FK506 devoid of calcineurin inhibitory activity (Dumont et al., 1992). Two-way ANOVA showed significant interaction between the effects of Ca^{2+} and the calcineurin inhibitors $F(3, 24) = 5.911$, $P = 0.0036$. ** P<0.01 when compared with the

respective Ca^{2+}-free group, Sidak's *post-hoc* multiple comparisons test. All studies were carried out in the presence of 1 mM IBMX and 0.1 mM rolipram. Data are means±S.D., n=4/group, representatives of 4 (A) and 2 (B) identical experiments.

Figure 7

The effects of miconazole and isoproterenol to stimulate cAMP production by AC9 are additive. A) Comparison of the effect of miconazole on cAMP production by HEK 293 cells stably expressing human AC9 or AC9_Y1242. ** P<0.01 **** <0.0001 *vs.* the vehicle treated control group, one-way ANOVA followed by Dunnett's test for multiple comparisons. B) Effect of miconazole on cAMP production by HEK 293 cells expressing AC9_Y1242 in the presence of vehicle (triangles) or 300 nM isoproterenol (squares). AC9 was not tested as it barely responds to isoproterenol (Pálvölgyi et al., 2018). Two-way ANOVA gave no interaction, the effects of miconazole ($F(3, 24) = 205.6$, $P < 0.0001$) and isoproterenol ($F(1, 24) = 170.0$, $P < 0.0001$) were statistically significant. All studies were carried out in the presence of 1 mM IBMX and 0.1 mM rolipram. Data are means ± S.D., n=4/group. Representative of 2 independent experiments.

Figure 1

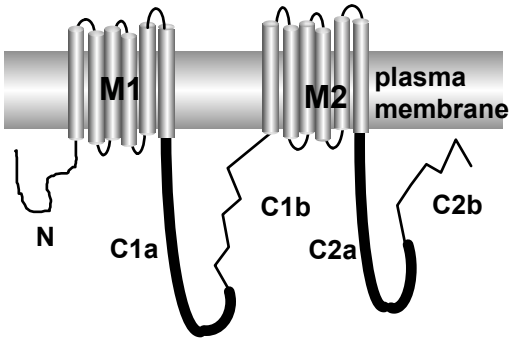
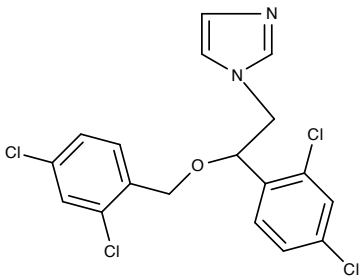


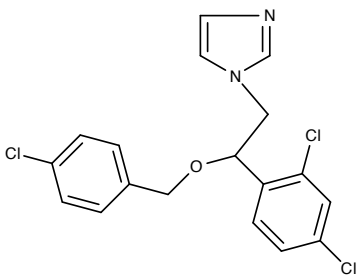
Figure 1

Figure 2

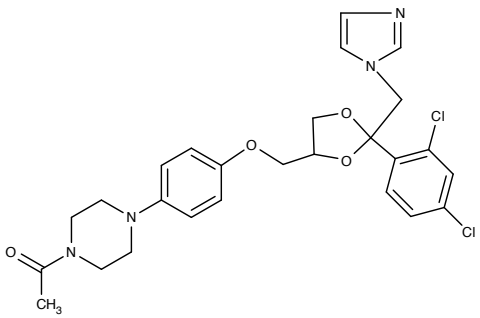
miconazole



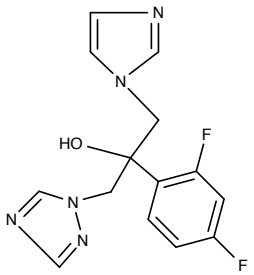
econazole



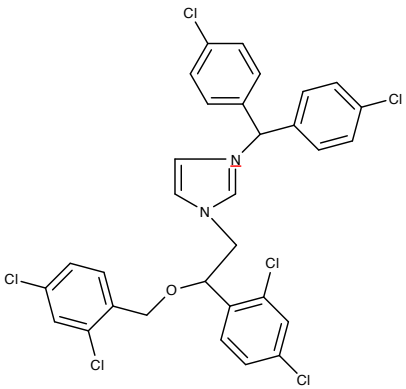
ketoconazole



fluconazole



calmidazolium



clotrimazole

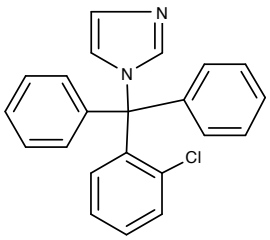


Figure 2

Figure 3

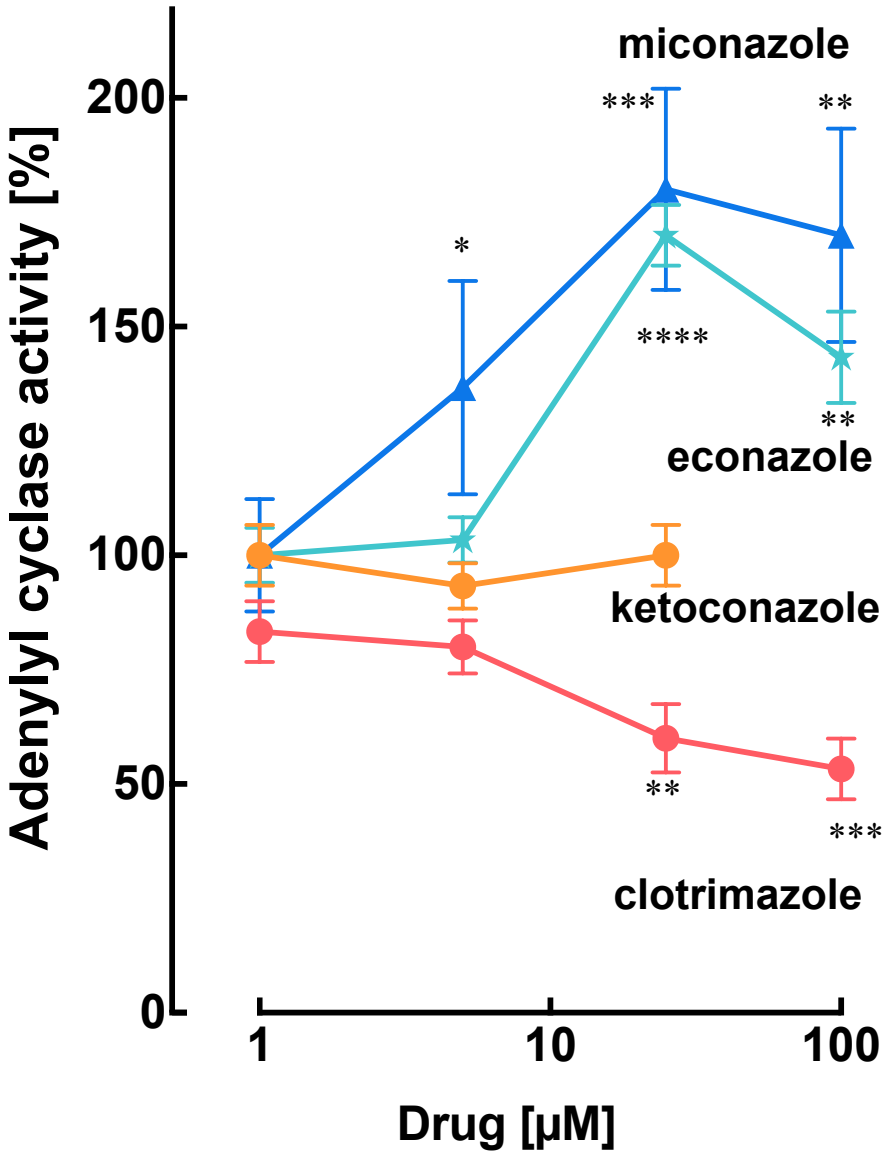


Figure 3

Figure 4

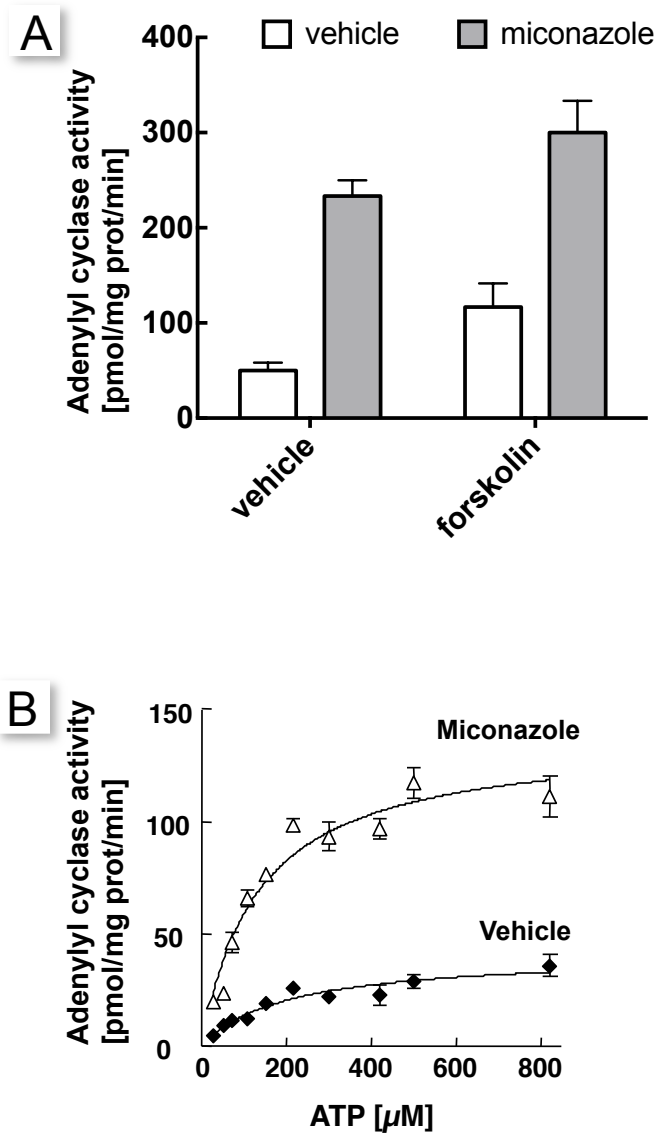

A
vehicle
miconazole
Adenylyl cyclase activity
[pmol/mg prot/min]
400
300
200
100
0
vehicle
forskolin
B
Adenylyl cyclase activity
[pmol/mg prot/min]
150
100
50
0
Miconazole
Vehicle
0
200
400
600
800
ATP [μM]

Figure 5

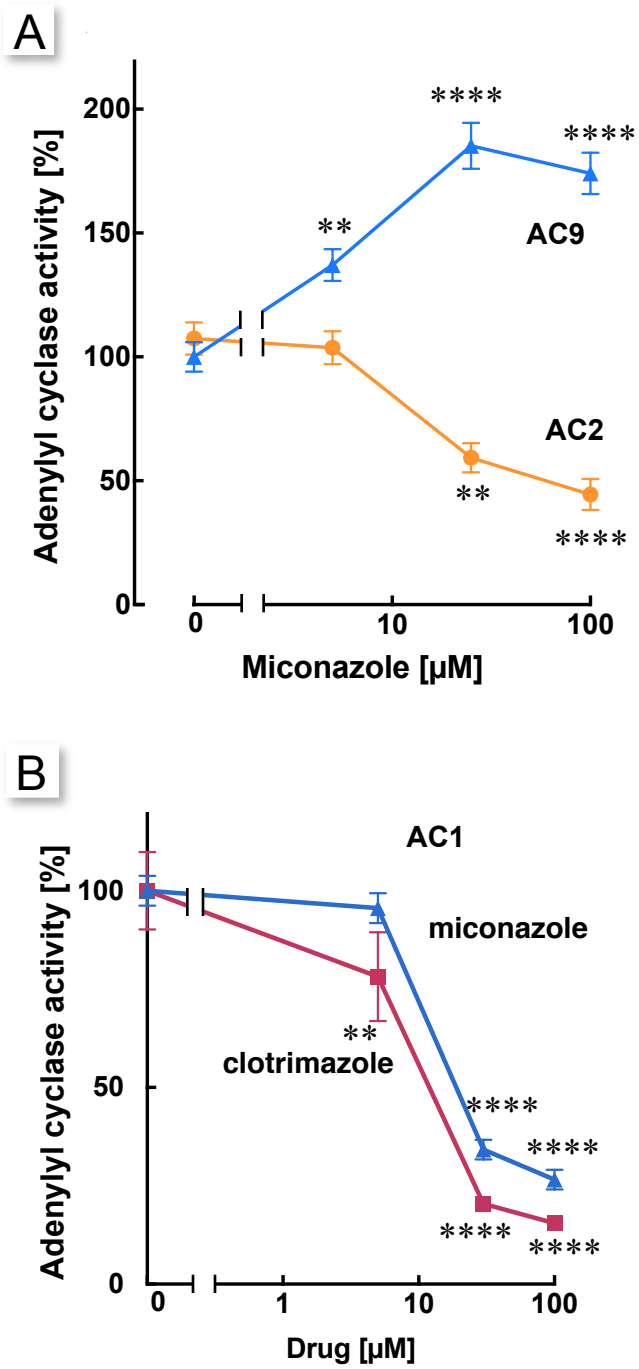


Figure 5

Figure 6

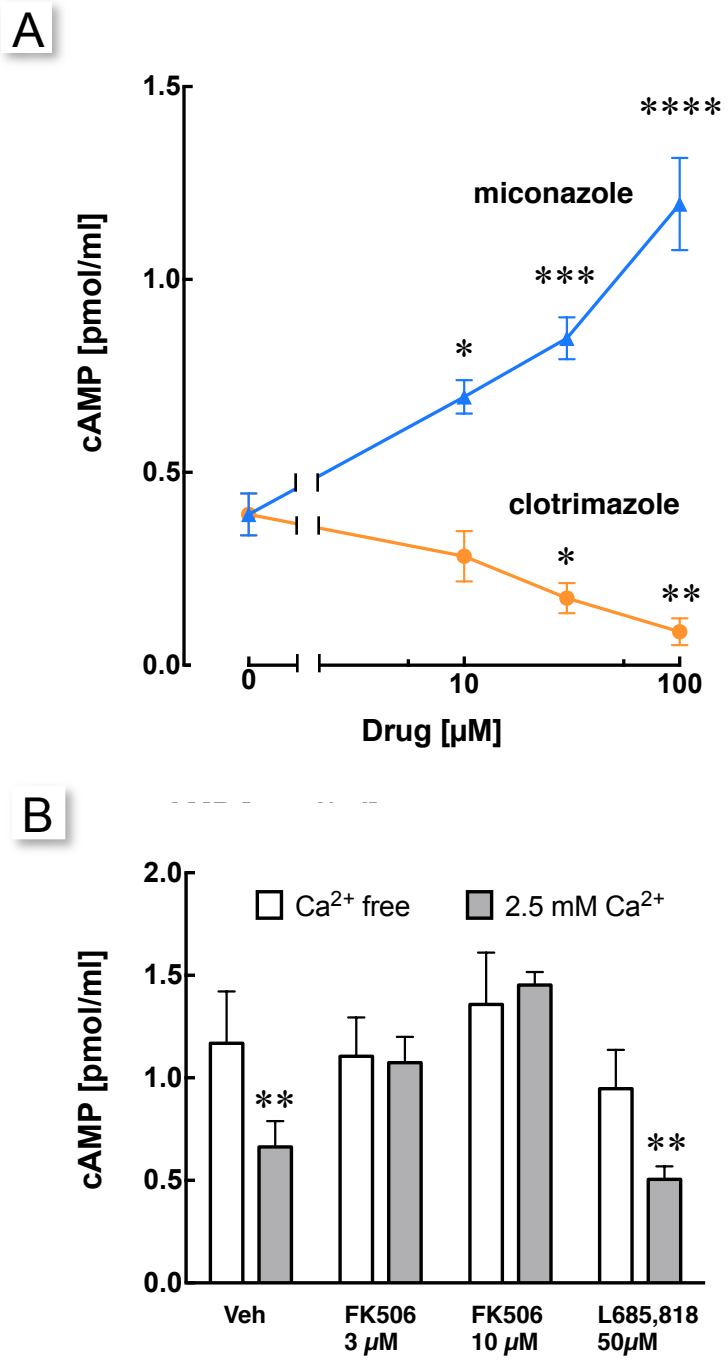
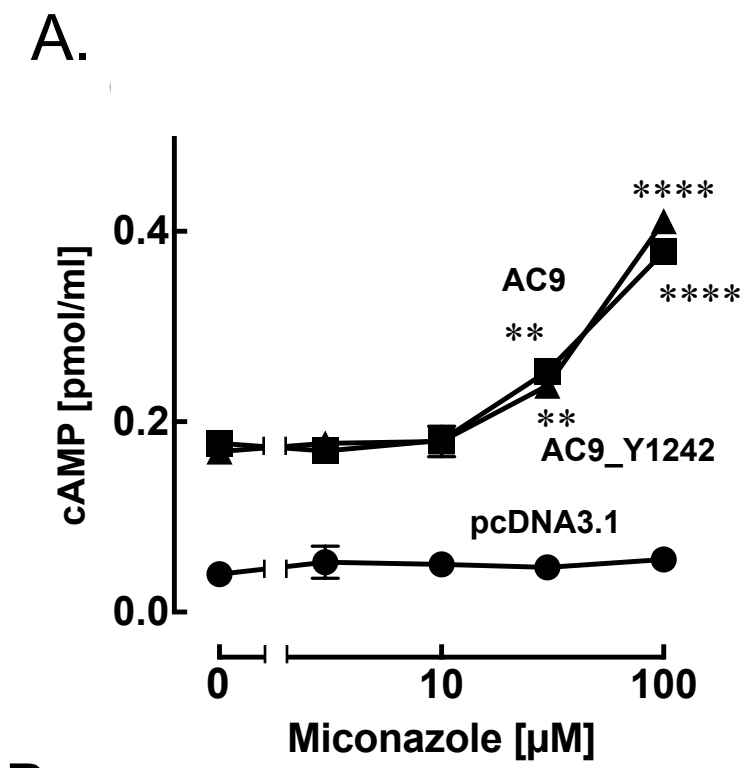
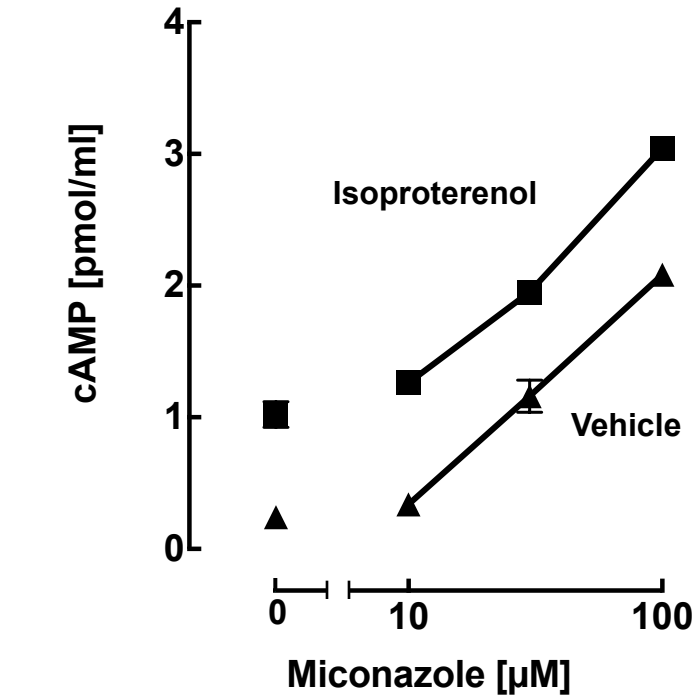

A
1.5
1.0
0.5
0.0
cAMP [pmol/ml]
miconazole
clotrimazole
****
***
*
*
**
0
10
100
Drug [μM]
B
2.0
1.5
1.0
0.5
0.0
cAMP [pmol/ml]
Ca2+ free
2.5 mM Ca2+
**
**
Veh
FK506 3 μM
FK506 10 μM
L685,818 50μM

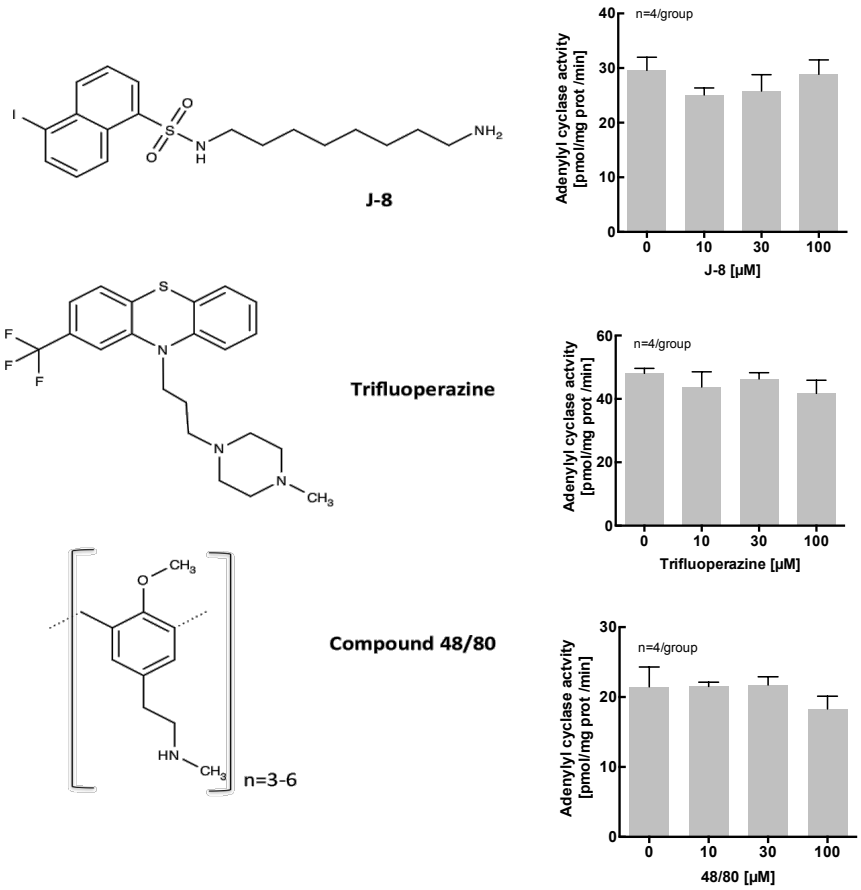
Figure 7


A.
cAMP [pmol/ml]
0.4
0.2
0.0
****
AC9
****
**
**
AC9_Y1242
pcDNA3.1
0
10
100
Miconazole [μM]


B.
cAMP [pmol/ml]
4
3
2
1
0
Isoproterenol
Vehicle
0
10
100
Miconazole [μM]

SUPPLEMENTARY DATA

Simpson et al., Direct stimulation of adenylyl cyclase 9 by the fungicide imidazole miconazole.



Suppl Fig 1. The effect of J8, trifluoperazine and 48/80 (a mixture of low-molecular weight polymers having a degree of polymerization between 3 to 6) on cAMP production in crude membranes prepared from HEK 293 cells stably overexpressing mouse AC9. Assays were carried out in the presence of 100 μM GDP-b-S to minimize the effects of G proteins. One-way ANOVA for J-8 F (3, 12) = 3.369, P = 0.0548 ; trifluoperazine: F (3, 12) = 2.660, P = 0.0956 ; Compound 48/80 F (3, 16) = 2.525, P = 0.0944