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Partial structures of ketoconazole as modulators of the large conductance calcium-activated potassium channel (BK_{Ca})

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Abstract—A series of partial structures of ketoconazole has been synthesized and tested for activity on the large conductance calcium-activated potassium channel (BK) in bovine smooth muscle cells. This has provided openers and blockers of the channel. The results suggest that the phenyl and phenoxy moieties are important for interaction with BK, whereas the imidazole group is unimportant. The properties of the phenoxy moiety seem to determine whether the compounds act to open or block the channel.

The large conductance calcium-activated potassium channel (BK) is a ubiquitously expressed potassium channel that has been implicated in a variety of physiological processes.[1] The channels are activated both by depolarization and by elevated calcium levels. In smooth muscle, they have been shown to maintain myogenic tone by shortening the action potential.[2] Furthermore, BK channels have been implicated in the regulation of neurotransmitter release. Given its importance in maintaining myogenic tone,[3] activation of the BK channel presents a possible mechanism for the treatment of asthma or hypertension. In addition to this, a potassium channel opener has been shown to confer neuroprotection in a rat model of permanent stroke.[4]

BK channels are membrane-bound channels that contain α- and β-subunits, examples of which have been cloned from a variety of tissues.[5–7] Several types of β-subunits exist and this, along with splice variation of the α-subunit, contributes to the heterogeneity of native BK channels.[8] This may prove to be important in the design of therapeutic agents that act by activation of the BK channel.

Several agents have been reported to activate the BK channel. Natural products such as soyasaponins[9] and CAF603[10] have been shown to open the channel. In addition to these, synthetic biaryls such as NS-004,[11] NS1608[12] and 3-aryloxindoles[4] activate BK. The antifungal drug ketoconazole (Chart 1) has been reported to activate BK channels in ferret portal vein[13] and subsequently shown to inhibit the BK channel in GH3 pyramidal neurons in rat.[14] Given its structural divergence from the other known BK channel openers, it was seen as an interesting target for investigation.

Taking ketoconazole as the lead, various partial structures (Fig. 1) were synthesized and tested to discover which parts of the ketoconazole molecule may be important for activity.

Compound **1** was synthesized according to the published route.[15] Hydrolysis of the benzoyl ester yielded the alcohol **2** that was reacted with *p*-toluene sulfonyl chloride to yield **3**. Reaction of **3** with the appropriate phenoxide in DMF or DMSO yielded compounds **4**, **5**, **6**, **7** and **8** (Scheme 1, Table 1). To obtain **6**, the aniline was protected as an imine. The fully protected product after reaction with **3** was not isolated. After flash chromatography, a mixture of aniline **6** and imine was obtained. This was treated with hydrazine to convert any remaining imine to the desired product **6**. Compound **9** was obtained by treating **8** with KOH in *i*-PrOH according to a literature procedure.[18] It was also considered worthwhile to test the benzoyl **1** and tosyl **3** derivatives, which were synthetic intermediates.

Keywords: Potassium channels; Opener; Blocker; Large conductance; BK_{Ca}.
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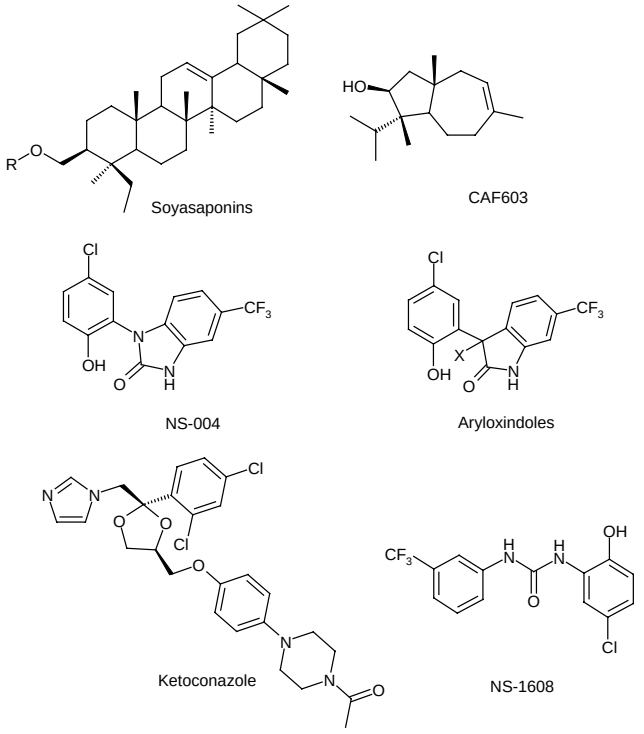


Chart 1. Known BK channel activators.

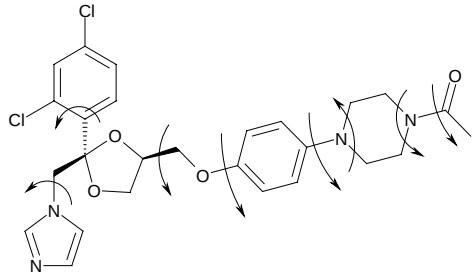
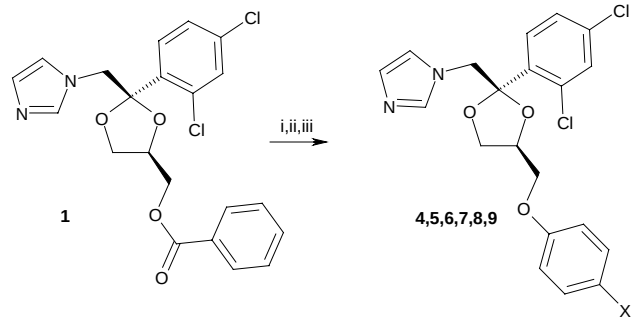


Figure 1. Structure of ketoconazole showing the notional removal of subunits.

The amine group in **6** was introduced via benzyliminophenol **10**[16] which was formed in good yield by stirring 4-aminophenol and benzaldehyde in methanol at room temperature. To synthesize the piperidino-compound **7**, 4-bromoanisole was aminated according to a Buchwald–Hartwig procedure[17] and demethylated using BBr_3 in dichloromethane to yield the phenol **11**. 1-Acetyl-4-(4-methoxyphenyl)piperazine **12** and acetyl-4-(4-hydroxyphenyl)piperazine **13** were prepared according to literature procedures[19] (Scheme 2). Compound **13** was used to prepare compounds **8** (Table 1), **16** and **17** (Scheme 3, Table 2).

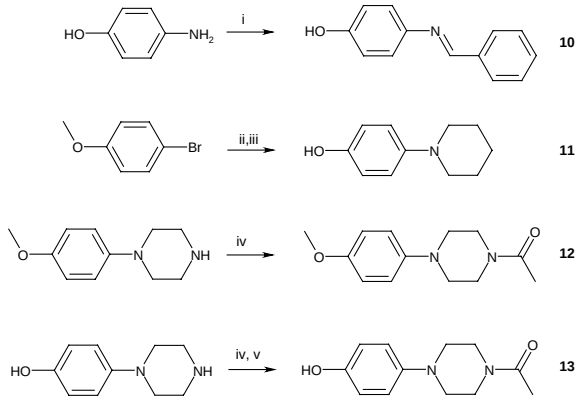
The compounds **14** and **15** were synthesized by formation of the acetal followed by tosylation (Scheme 3, Table 2). Substitution of the tosylate **14** with phenoxide of **13** in DMSO yielded **16**. Similarly **17** was synthesized from **15**. For these initial investigations **16** was tested as the racemate and **17** as a mixture of diastereomers.



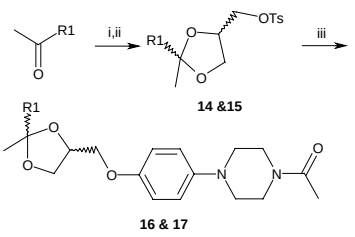
Scheme 1. Synthesis of ketoconazole analogues. Reagents and conditions: (i) **1**, NaOH, dioxane reflux; (ii) **2**, tosyl chloride, pyridine; (iii) **3**, phenol, NaH, DMSO. 0–80 °C.

Table 1. Structures of compounds **1**–**9**

	Substituent	Activity BK
1	COPh	Blocker
2	H	Opener
3	Tosyl	None
4	Phenyl	Blocker
5	4-Bromophenyl	Blocker
6	4-Aminophenyl	Opener
7	4-(*N*-Piperidino)phenyl	Blocker
8	4-(4-Acetyl-*N*-piperazino)phenyl (ketoconazole)	Opener
9	4-(*N*-Piperazino)phenyl	Opener



Scheme 2. Synthesis of 4-substituted phenols. Reagents and conditions: (i) Benzaldehyde MeOH, rt; (ii) $Pd_2(dba)_3$, P(o-Tol)$_3$, piperidine, LHMDS, toluene 80 °C; (iii) BBr_3, DCM, −78 °C–rt; (iv) Ac_2O, pyridine, DCM; (v) K_2CO_3, MeOH, rt.



Scheme 3. Synthesis of tosylates **14** and **15** and replacement by substitution to give **16** and **17**. Reagents and conditions: (i) Glycerol, PTSA, benzene/butanol, reflux Dean–Stark; (ii) tosyl chloride, pyridine; (iii) **13**, NaH, DMSO. 0–80 °C.

Table 2. Structures of compounds **14**–**17**

	R^1	Stereochemistry
14	Methyl	Racemic
15	2,4-Dichlorophenyl	Racemic & 1:1 cis and trans
16	Methyl	Racemic
17	2,4-Dichlorophenyl	Racemic & 1:1 cis and trans

A culture of bovine pulmonary artery (BPASM) derived by explant culture was kindly supplied by Dr R. Corder, William Harvey Research Institute, London, UK. The cells used in the study were from passages 12 to 19. Cultures were maintained in DMEM (Invitrogen) supplemented with 10% foetal calf serum, 2 mM L-glutamine and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For electrophysiology, the cells were plated onto 35 mm tissue culture dishes and used within 1–3 days.

Whole cell BK currents were recorded using an EPC7 patch-clamp amplifier essentially as described.[20] Briefly, cells were superfused with a solution containing (in mM): NaCl 136, KCl 6, $MgCl_2$, 1, $CaCl_2$ 2, glucose 10 and HEPES 10. pH was adjusted to 7.4 with 1 M NaOH. The pipette filling solution contained (in mM): KCl 136, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10 and EGTA 2. The pH was adjusted to 7.2 with 1 M KOH. Free Ca^{2+} was calculated to be 150 nM. Patch pipettes were fabricated from 1.5-mm borosilicate capillaries (Clarke ElectroMedical), fire polished and coated with Sylgard Resin and had resistances of 3–5 MΩ when filled with internal solution. All experiments were conducted at room temperature (23–25 °C).

Routinely cells were clamped at a holding potential of −20 mV. Voltage dependent activation of BK currents could be elicited by applying 100 mS voltage jumps to more positive potentials. The effect of test compounds was assessed by measuring the current activated on stepping from the holding potential to +80 mV and expressed as the difference between the current in the presence and absence of test compounds as a percentage of the current in its absence. Each concentration of tested compound was applied to at least three different cells and the effect is reported in Table 3 as the mean change in current at +80 mV ± SEM. As an internal control the effectiveness of ketoconazole (30 μM) was tested on every cell included in the study. Since ketoconazole has already been shown to act on BK, it was used as the reference agent in this study.[13]

Table 3. Quantitative results for the compounds tested

	Activity BK	*n*	% Inhibition (concn)	% Opening (concn)
1	Blocker	3	40 ± 8 (30 μM)	
2	Opener	3		47 ± 3 (100 μM)
3	None		2 ± 30 (30 μM)	
4	Blocker	3	73 ± 7 (30 μM)	
5	Blocker	4	95 ± 35 (30 μM)	
6	Opener	3		566 ± 200 (30 μM)
7	Blocker	3	80 ± 6 (10 μM)	
8	Opener	5		74 ± 14 (10 μM)
		31		218 ± 38 (30 μM)
		9		348 ± 32 (100 μM)
9	Opener	3		310 ± 83 (30 μM)
12	None	3	9 ± 17 (100 μM)	
16	None	3	17 ± 6 (100 μM)	
17	Opener	4		235 ± 57 (30 μM)

Activity is shown as the % decrease (blockade) or increase (opening) of baseline current at the given concentration and *n* = number of observations.

Stock solutions of test compounds were prepared in DMSO at a concentration of 2×10^{-2} M and diluted in bath solution to give the desired final concentration. The concentration of DMSO did not exceed 0.5% v/v and at this concentration had no visible effect on the current.

Activity of partial structures (Table 3): Major dissection of the ketoconazole molecule gave weakly active or inactive fragments; thus, **2** and **12** were notionally obtained by splitting of the ether linkage. Compound **2**, derived from the removal of the phenylpiperazine moiety, showed a little activity. Compound **12**, having the phenylpiperazine moiety found in ketoconazole **8**, was inactive (no effect at 100 μM) and was still not rendered active when combined with the dioxalane ring as in **16** (no effect at 100 μM). Thus, it appears that either the 2,4-dichlorophenyl group and/or the imidazolyl ring of ketoconazole are very important for activity.

Maintaining the imidazole and dichlorophenyl moieties, while varying the acetylpiperazine group, yielded some interesting results. Removing the acetyl group gave the piperazine **9**, which was a more effective channel opener than ketoconazole. Splitting of the acetylpiperazine ring to leave the structure having a *p*-aminophenoxy group gave compound **6**, which was also more potent than ketoconazole.

On the other hand, removing the acetylpiperazine ring entirely gave the phenoxy compound **4**, which blocked the channel. Likewise, replacing the acetylpiperazine ring by other groups such as piperidine (**7**) or bromine (**5**) gave channel blockers. Replacing the phenoxy group by benzoate gave a weak blocker (**1**) or by *p*-toluenesulfonate gave compound (**3**), which had no effect at 30 μM. Removal of the imidazole ring gave compound **17**, which had potency similar to that of ketoconazole as a channel opener. This finding is similar to the finding that clotrimazole can block the BK channel even after removal of the imidazole moiety.[13] Given that this moiety is also crucial for the cytochrome P450 activity of ketoconazole, the authors would suggest that the action on BK is not due to a potentiating effect via cytochrome. Also it has been shown that ketoconazole acts on excised patches of smooth muscle cells which suggests a direct action or an action close to the channel.[13]

Given that the piperazines **8** and **9**, and the aniline **6**, act as channel openers, it would appear that an anilino nitrogen atom is the common feature conferring this mode of action. Such a group might act as a hydrogen-bond acceptor or increase the electron density in the phenoxy ring. It is therefore surprising to find that piperidine **7** (which also possesses an anilino *N*) is a

blocker. Perhaps, the differentiation between these compounds is that **6**, **8** and **9** have hydrophilic substituents, whereas for **7**, the piperidino group is lipophilic.

Overall these results show that the phenyl groups seem to be important for binding to the BK channel, whereas the imidazole group does not. They also point to the properties of the phenoxy moiety as being crucial for determining the type of activity.

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