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Structure–activity relationship exploration of Kv1.3 blockers based on diphenoxylate

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ABSTRACT

Diphenoxylate, a well-known opioid agonist and anti-diarrhoeal agent, was recently found to block Kv1.3 potassium channels, which have been proposed as potential therapeutic targets for a range of autoimmune diseases. The molecular basis for this Kv1.3 blockade was assessed by the selective removal of functional groups from the structure of diphenoxylate as well as a number of other structural variations. Removal of the nitrile functional group and replacement of the C-4 piperidinyl substituents resulted in several compounds with submicromolar IC_{50} values.

Autoimmune disorders are characterised by an organism mistakenly mounting an immune response against itself. Examples of these disorders include multiple sclerosis (MS) psoriasis, type-1 diabetes, Crohn's Disease, and rheumatoid arthritis.[1] The aberrant immune response is mediated by autoantibodies and self-reactive lymphocytes. The lymphocytes involved are T cells and once activated they proliferate and cause tissue damage. One particular type of T cells known as effector memory (T_{EM}) cells have been linked to autoimmune disorders.[2–4]

Human T cells express two types of K+ channels, the voltage-gated Kv1.3 and the calcium-activated KCa3.1 channel.[5,6] Both Kv1.3 and KCa3.1 play a role in regulating membrane potential and calcium signalling during the activation of T cells.[5] Calcium influx, which is crucial to the process, is only possible if the T cells are able to maintain a negative membrane potential through a counterbalancing potassium efflux via Kv1.3 and/or KCa3.1 channels.[5,7,8]

Blockade of Kv1.3 or KCa3.1 channels is a possible method for treating autoimmune and inflammatory diseases by suppressing T cell proliferation and modulating their activities.[9] Importantly, naïve and central memory T cells (T_{CM}) upregulate KCa3.1 channels upon activation leaving the number of Kv1.3 channels largely unchanged. The opposite occurs in T_{EM} cells which upregulate Kv1.3 channels when activated.[5] Blockade of the Kv1.3 channel therefore provides an opportunity for intervention by therapeutic agents, leaving naïve and T_{CM} cells free to address other immunogenic threats (e.g., infections).

A number of studies have shown that blockade of Kv1.3 potassium channels results in functional inhibition of T cell activation/proliferation and cytokine secretion.[5,8,10] In one study, the potent Kv1.3 blocking peptide ShK and a series of related analogues were able to treat both adoptive transfer and chronic relapsing experimental autoimmune encephalomyelitis (EAE) in rats.[11] Small molecule blockers of Kv1.3 channels have also been investigated.[12] The most potent of these compounds is PAP-1 (IC_{50} 2 nM) which has been shown to suppress delayed type hypersensitivity (DTH) and allergic contact dermatitis (ACD) in Lewis rats when dosed orally, by ip injection or topically.[13,14]

In searching for new compounds that might have clinical potential as Kv1.3 channel blockers we noticed that diphenoxylate (**1**) had been shown, in a small clinical trial, to successfully treat psoriasis and other inflammatory skin conditions.[15,16] Diphenoxylate also shows structural similarity to a number of Kv1.3 blockers (Fig. 1) and when assessed it was found to block Kv1.3 channels with an IC_{50} of 5 μM.[12] With a view to optimising the Kv1.3 blockade shown by diphenoxylate, we examined which elements of the chemical structure are required for biological activity.

As our principle objective was to delineate the pharmacophoric elements of diphenoxylate with respect to Kv1.3 blockade, analogues were prepared where one or more of the functional elements of **1** were removed or altered. The first reports of

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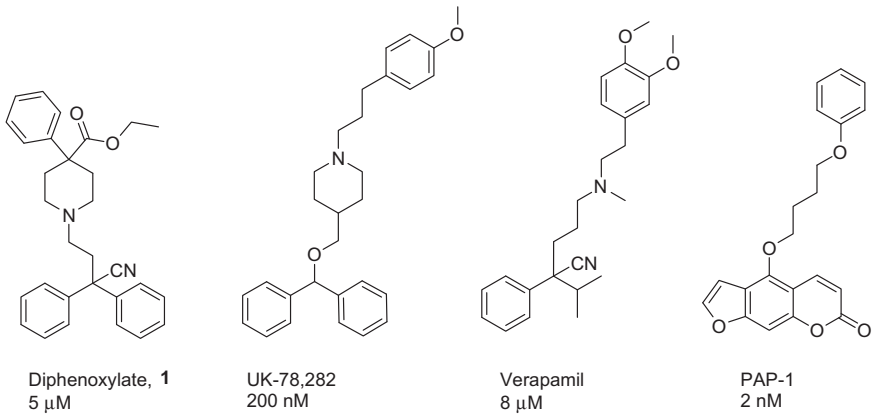


Figure 1. Structures and Kv1.3 blocking activity of diphenoxylate (**1**), UK-78,282, verapamil and PAP-1.

diphenoxylate synthesis date back to Janssen et al. in 1959[17] and that synthesis provided the basis for the work described here. As exemplified in the synthesis of the methyl ester **2**, it was found that the alkylation of the key piperidine precursor (**3**) with diphenylbromopropionitrile (**4**) could be accelerated and the yield improved by microwave heating in acetonitrile (ACN) in the presence of *N,N*-diisopropylethylamine (DIPEA). This synthetic step (Scheme 1) formed the basis for all analogues described here, with variations derived from commercial or synthesized building blocks. Full synthesis details are provided as Supplementary data.

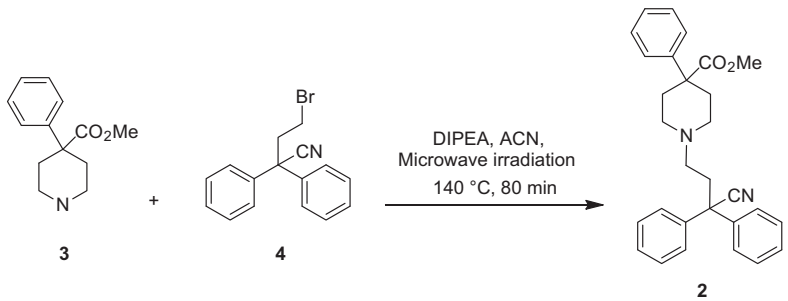
Other compounds prepared in this way included **5**, **6** and **7** in which the ester, piperidine 4-phenyl substituent and the nitrile were replaced by a hydrogen atom, respectively or **8** where both piperidine 4-substituents were removed. In compound **9** the 2-biphenylbutyronitrile portion is replaced by a simple phenylpropionyl group. Other analogues that were prepared included replacements of the piperidine ring such as piperazine (**10**, **11**) and tetrahydropyridine (**12**). Replacement of the ester with a hydroxyl group (**13**) was undertaken as the 4-phenylpiperidin-4-ol group is a well-known fragment in established drugs. The activity of **13** discussed later, prompted the removal of the 4-phenyl ring to produce **14** and the related piperidine-3-ol analogue (**15**). To complement our SAR exploration, the truncated analogues **16** and **17** were also produced. A further series of analogues based on compound **14** were synthesized to produce **18**, **19**, **20** and **21**.

Compounds were assessed for their ability to block Kv1.3 channels using manual whole-cell patch-clamp as previously described.[14,18] Briefly, L929 cells stably expressing Kv1.3 channels were subjected to depolarizing step pulses from −80 mV to +40 mV to elicit Kv1.3 currents. Compounds were manually perfused and in most cases 3–5 different concentrations were tested at least 2–3 times. All compounds were washed out again to differentiate true pharmacological effects from unspecific current 'run-down'. IC_{50} values were determined by fitting the reduction of area under the current curve after reaching equilibrium block to the Hill equation. Full details are provided as Supplementary data.

The compounds tested demonstrated a range of potencies, clearly showing the specific contributions of the diphenoxylate substructures. In the simplest analogue, replacement of the ethyl ester with a methyl ester (**2**) resulted in a marginal improvement in activity (Table 1). Removing the ethyl ester from diphenoxylate altogether in **5**, also slightly improved blocking potency. In contrast, removal of the phenyl ring from the piperidine (**6**) resulted in a substantial decrease in activity (~50-fold). Compound **7** which lacked the cyano group was sixfold more active than diphenoxylate itself.

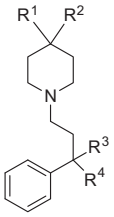
Of the more extensively pruned compounds, compound **8** was fourfold less potent than diphenoxylate demonstrating that while the ester is not needed, further removal of the phenyl ring was not well tolerated suggesting that this aromatic group is required for activity (Table 1). Pruning of the diphenylmethane/cyano moiety of diphenoxylate had a significant effect on activity. Removal of both the cyano group and one phenyl ring from this group (**9**) resulted in a similar improvement in potency relative to compound **7**. Compound **9** has been flagged as an interesting substance for future optimization.

Having systematically removed groups from the structure of diphenoxylate, we investigated the substitution of other functional groups and isosteres at the 4-position of the piperidine ring. More radical changes were also made to help clarify this SAR and confirm whether the ester functionality is necessary for Kv1.3 channel blockade. Isosteric replacement of the piperidine ring with a piperazine ring (**10**) resulted in a twofold improvement in activity. Extension of the aromatic ring from the piperazine nitrogen with the insertion of a methylene carbon (**11**) was also tolerated and



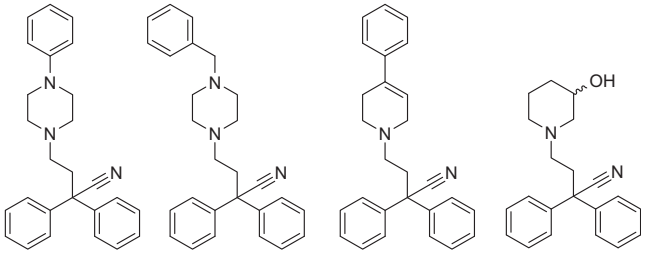
Scheme 1. Synthesis of diphenoxylate analogue **2**.

Table 1
Kv1.3 blockade



Compound	R^1	R^2	R^3	R^4	Kv1.3 (IC_{50} μM)	lipE
Diphenoxylate, **1**	$-C_6H_5$	$-C{=}OOC_2H_5$	–CN	$-C_6H_5$	5.0[12]	1.03
2	$-C_6H_5$	$-C{=}OOCH_3$	–CN	$-C_6H_5$	2.52 ± 0.15	1.81
5	$-C_6H_5$	–H	–CN	$-C_6H_5$	2.61 ± 0.11	1.77
6	–H	$-C{=}OOC_2H_5$	–CN	$-C_6H_5$	230 ± 7.5	1.05
7	$-C_6H_5$	$-C{=}OOC_2H_5$	–H	$-C_6H_5$	0.90 ± 0.10	1.35
8	–H	–H	–CN	$-C_6H_5$	20.5 ± 1.6	2.42
9	$-C_6H_5$	$-C{=}OOC_2H_5$	–H	–H	0.80 ± 0.10	2.63
10[a]					1.86 ± 0.03	2.21
11[a]					1.27 ± 0.10	2.45
12[a]					4.20 ± 0.80	1.02
13	$-C_6H_5$	–OH	–CN	$-C_6H_5$	1.70 ± 0.20	2.89
14	–H	–OH	–CN	$-C_6H_5$	0.75 ± 0.05	4.66
15[a]					>100	–
16	–H	–OH	–H	$-C_6H_5$	~75.0	–
17	–H	–OH	–H	–H	>100	–
18	–H	$-CH_3$	–CN	$-C_6H_5$	5.80 ± 0.3	2.47
19	–H	$-OCH_3$	–CN	$-C_6H_5$	>100	–
20	–H	$-NH_2$	–CN	$-C_6H_5$	>100	–
21	–H	–NHBoc	–CN	$-C_6H_5$	~25.0	–

a.



led to a similar IC_{50} value. Compound **12** has reduced flexibility relative to **5** yet the difference in their potencies is only slight (Table 1). Again this demonstrated that removal of the ester was not detrimental to Kv1.3 blockade.

Replacing the ethyl ester on diphenoxylate with a hydroxyl group (**13**) resulted in a threefold improvement in activity. Interestingly **14**, which introduces a hydroxyl group in the 4 position of the piperidine ring thus replacing the phenyl ring and ester, displayed a sevenfold increase in activity over diphenoxylate. As a result we prepared a range of analogues based on this compound. The first of these, compound **15**, showed no blocking activity below a concentration of 100 μM highlighting the importance of the hydroxyl group position (Table 1).

Removing the cyano group from **14** affords **16** which showed a 100-fold decrease in Kv1.3 blocking activity. This SAR is in direct contrast to that seen between diphenoxylate and **7** where the removal of the cyano group led to improved blocking ability. Removal of both the cyano group and one phenyl ring (**17**) completely abolished activity. The addition of a methyl group at the 4-position of the piperidine ring (**18**) restored activity and is comparable with diphenoxylate (Table 1). Compound **19** also showed no activity and this was somewhat surprising given the minor change to a methoxy group and presumably there is a need for a hydrogen bond donor. This is somewhat contradicted with the structure of **20** which showed no activity with a primary amine substituent. While the primary amine can function as a hydrogen bond donor, its basic character may explain its poor blocking ability. The precursor to **20** (**21**) was also tested and showed diminished activity (IC_{50} = ~25 μM). For the majority of the analogues of compound **14**, alteration of the 4-hydroxy substituent as well as the cyano group and diphenylmethane moiety abolished activity.

Diphenoxylate has already been shown to be able to treat inflammatory skin conditions including the autoimmune disorder psoriasis[15,16] and this clinical observation, though unproven in a large clinical trial, is consistent with modest Kv1.3 channel blockade.[12] Efforts to produce a suitable topical formulation of diphenoxylate would seem unlikely due to the side-effect potential of this narcotic agent.

Exploitation of diphenoxylate as a lead compound for developing Kv1.3 channel blockers presents a number of challenges.[12] Diphenoxylate has moderate potency and a relatively high molecular weight and lipophilicity. Compounds with high log*P* values are often found to be able to block potassium channels and the need to demonstrate a clear SAR for diphenoxylate was imperative.

From a structural perspective we saw partial similarities to the known Kv1.3 blockers UK 78,282, verapamil and PAP-1 (Fig. 1). These included the basic aliphatic nitrogen, diphenylmethyl moiety, cyano group and the location of aromatic rings at both ends of the molecules. We began by pruning these functional groups on diphenoxylate to explore their effect on Kv1.3 blockade. In some cases these groups were replaced by other functional groups of varying sizes and properties.

The principle outcomes were the demonstration that Kv1.3 blockade could be maintained and even enhanced by the removal of one or more of diphenoxylate's key functional groups. For example, removal of the R^4-phenyl ring and the nitrile was tolerated such that compound **9** (IC_{50} 0.8 μM) had improved potency. The replacement of the phenyl and carboxylic ester substituents by a single hydroxyl group in compound **14** also showed improved activity (IC_{50} 0.75 μM). The combination of these modifications however, yielded a poorly active compound **17** (IC_{50} > 100 μM). This contradicted the overall pharmacophore that we had tentatively proposed[12] for Kv1.3 inhibitors. The inner cavity, which constitutes the binding site for most small molecule Kv1.3 blockers is somewhat large[19] and it seems probable that the compounds bind in various ways such that a single pharmacophore will not describe the SAR.

From a drug discovery perspective, the retention of blockade with both reduced molecular size and lipophilicity is encouraging. While PAP-1 shows good potency it has no ionizable functional groups and a log*P* value of 4.03[14] resulting in a relatively low oral availability of only 25% and solubility issues for formulation.[20] Both compounds **14** and **9** have reduced *C*log$D_{7.4}$ values relative to diphenoxylate as well as reduced molecular weights (320.4 and 351.5, respectively). Diphenoxylate is a poorly soluble substance due to its relatively high lipophilicity (*C*log$D_{7.4}$ 4.27). The lipophilicity of compound **9** was reduced (*C*log$D_{7.4}$ 3.47) however, the removal of groups to generate compound **14** resulted in a substantially lower *C*log$D_{7.4}$ value of 1.46.

The concept of lipophilic ligand efficiency (LipE) has recently emerged as an influential descriptor to assist lead optimization[21] and is calculated by subtracting the pIC_{50} value from *C*log$D_{7.4}$. Values of LipE above 5.5 have been proposed as a target for developing orally dosed compounds.[21] PAP-1 has a LipE value of 4.67 for Kv1.3 blockade, which is in stark contrast to diphenoxylate (LipE 1.03 for Kv1.3). Compound **9** shows an improved LipE value of 2.63 relative to diphenoxylate while compound **14** has a LipE value (LipE 4.66) that matches that of PAP-1. Clearly these are steps in the right direction showing that these compounds have improved properties and are worthy of further optimization.

Our work also highlighted a lack of clear SAR when compound **14** was investigated further. No improvement in activity could be gained from the direct modification of **14** which suggests that more radical changes may need to be explored. Unlike **7**, removal of the cyano group in **16** led to a 100-fold decrease in its IC_{50}. Also in contrast to **9**, the removal of a cyano group plus a phenyl ring (**17**) also resulted in no activity. The difference in SAR between diphenoxylate and **14** suggests that these two compounds are likely to have two distinct binding modes and/or locations within the Kv1.3 channel. It is possible that these compounds may also be binding to a different state of the channel (i.e., open or closed) which may account for their dissimilar SAR.

Another important aspect of investigating the SAR of diphenoxylate is to place focus on the ester group which is linked to its activity at opioid receptors. The ester on diphenoxylate is metabolised in vivo to the carboxylic acid (diphenoxin) which is the active opioid agent.[22] Any future work would need to monitor mu opioid activity and avoiding an ester would need to be considered. Compound **14** circumvents this problem, however any optimization of **9** would need to bear this in mind.

This study has identified two new series of Kv1.3 blockers derived from the anti-diarrhoeal compound diphenoxylate. Successive deletion of functional groups was able to improve activity although the SAR was not consistent between the compound classes. Removal of the ester, cyano and an aromatic ring were tolerated and in many cases improved activity. These deletions also reduced both MW and lipophilicity presenting compounds worthy of further investigation. There is a need for Kv1.3 blockers with improved selectivity and biopharmaceutical properties, and this study provides a starting point for further investigations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.080.

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