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New Inhibitors of the FVB2 Subunit from Mammalian Kv1 Potassium Channels

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Short communication

New inhibitors of the Kv β_2 subunit from mammalian Kv1 potassium channelsKumari Alka^a, J. Oliver Dolly^b, Barry J. Ryan^a, Gary T.M. Henehan^{a,*}^a School of Food Science and Environmental Health, Dublin Institute of Technology (DIT), Cathal Brugha Street, Dublin 1, Ireland^b International Centre for Neurotherapeutics, Dublin City University, Dublin 9, Ireland

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ABSTRACT

The role of the redox state of Kv β subunits in the modulation of Kv1 potassium channels has been well documented over the past few years. It has been suggested that a molecule that binds to or inhibits the aldo-keto reductase activity of Kv β might affect the modulation of channel properties. Previous studies of possible modulators of channel activity have shown that cortisone and some related compounds are able to physically dissociate the channel components by binding to a site at the interface between α and β subunits. Herein, we describe some new inhibitors of rat brain Kv β_2 , identified using an assay based on multiple substrate turnover. This approach allows one to focus on molecules that specifically block NADPH oxidation. These studies showed that, at 0.5 mM, 3,4-dihydroxyphenylacetic acid (DOPAC) was an inhibitor of Kv β_2 turnover yielding a ~40–50% reduction in the aldehyde reductase activity of this subunit. Other significant inhibitors include the bioflavonoid, rutin and the polyphenol resveratrol; some of the known cardioprotective effects of these molecules may be attributable to Kv1 channel modulation. Cortisone or catechol caused moderate inhibition of Kv β_2 turnover, and the aldo-keto reductases inhibitor valproate had an even smaller effect.

Despite the importance of the Kv1 channels in a number of disease states, there have been few Kv β_2 inhibitors reported. While the ones identified in this study are only effective at high concentrations, they could serve as tools to decipher the role of Kv β_2 *in vivo* and, eventually, inform the development of novel therapeutics.

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1. Introduction

The Shaker voltage-gated K⁺ channels (Kv1 superfamily) control the efflux of K⁺ through cell membranes and, thereby, dampen membrane excitability. An auxiliary β -subunit (Kv β), associated with the cytoplasmic face of Kv proteins (Dolly et al., 1994; Parcej et al., 1992; Scott et al., 1994) has sequence homology with aldo-keto reductase (AKR) enzymes (Gulbis et al., 1999; Long et al., 2005). The catalytic C-terminal of Kv β_2 has a tightly, but non-covalently, bound nicotinamide (NADPH) cofactor. Most significantly, Kv β is known to modulate the channel's activity in a manner that depends on the redox state of the bound cofactor. This mechanism is thought to proceed *via* redox dependent conformational changes (Weng et al., 2006; Pan et al., 2008b, 2011). The Kv β subunit is catalytically active as an aldo-keto reductase being able to reversibly oxidise a range of alcohols to their corresponding aldehydes (Alka et al., 2010; Tipparaju et al., 2008).

Allelic deletion of Kv β is directly linked to severe epilepsy including infantile spasm (Heilstedt et al., 2001) while loss-of-function mutations in pore forming α -subunits are associated with convulsive disorders (Adelman et al., 1995; Guan et al., 2006; Imbrici et al., 2006, 2007). It has been suggested that inhibitors of the action of Kv β might prove useful as therapeutics (Pan et al., 2008a, 2012). Despite this, there have been few reports of compounds that might inhibit Kv β .

In 2008, Pan et al., screened a number of potential Kv β inhibitors and showed that cortisone can bind to Kv β proteins at two different sites: one close to the enzyme bound NADPH and another at the interface between Kv β and Kv1 α subunits (Pan et al., 2008a). They further showed that cortisone promotes dissociation of Kv β from Kv1 subunits, a finding that focused attention on inhibitors that might target the interface between subunits in Kv1 channels. Subsequently, these authors tested 25 cortisone analogues for their ability to potentiate Kv1 channels by binding to Kv β and promoting its dissociation (Pan et al., 2012). Their work identified fluticasone as a potential lead for designing more efficient small molecules that might influence channel function by dissociating the Kv1–Kv β assembly. Nevertheless, there is little or no

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information on molecules, other than substrates and corticosteroids, that can bind to the active site of the Kv β subunit. Predicting inhibitors for this protein is difficult since even studies on substrate specificity have proven somewhat confusing (Alka et al., 2010; Tipparaju et al., 2008).

Herein, the interactions of a variety of small molecules with the active site of Kv β 2 were probed. The tightly bound NADPH on Kv β 2 may dissociate and allow measurement of multiple turnovers (Weng et al., 2006). We exploit this activity to screen for Kv β 2 inhibitors that have a direct influence on substrate turnover. This approach utilised a simple HPLC-based assay to monitor the Kv β 2-catalysed reduction of 4-nitrobenzaldehyde (see Alka et al., 2010). This assay was used for screening of small molecule inhibitors that might bind to Kv β 2 and inhibit the redox reaction. In this way, inhibitors were sought for NADPH oxidation rather than modulators of channel function, since this oxidation step is known to influence channel activity.

2. Materials and methods

2.1. Overexpression and purification of Kv β 2

The rat brain Kv β 2 protein was overexpressed in, and purified from, *Escherichia coli* BL21 (DE3, plysS) cells transformed with the pET15b-Kv β 2 construct containing an N-terminal His-tag, described in Alka et al., 2010. The purified protein was dialysed against 2 L of prechilled 0.2 M potassium phosphate, pH 7.5 for 36 h with three changes.

2.2. HPLC assay for measuring the inhibition of Kv β 2-mediated 4-nitrobenzaldehyde reduction

Two different HPLC separation methods (Isocratic and Gradient) were used to carry out the inhibition studies for the Kv β 2 mediated-reduction of 4-nitrobenzaldehyde. The one chosen depended on the effective separation of the inhibitor from components of the reaction mixture. All inhibition studies were carried out in duplicate in 0.2 M potassium phosphate buffer, pH 7.5 containing 0.2 mM NADPH at 37 °C in the presence of ~0.5 mg of Kv β 2, an appropriate concentration of each test inhibitor and 500 μ M of 4-nitrobenzaldehyde as substrate (final volume of 0.25 ml). A 100 mM stock solution of all inhibitors was prepared in DMSO. The concentration of DMSO in the assay mixture was less than or equal to 1% (v/v). DMSO at this concentration was found to have no effect on the 4-nitrobenzaldehyde reductase activity of Kv β 2. The inhibitor was incubated with the enzyme for 20 min before the addition of substrate, which was added last, and the reaction further incubated for 30 min at 22 °C before quenching by adding an equal volume of the HPLC mobile phase consisting of methanol/trifluoroacetic acid/water (60: 0.1: 39.9, v/v/v). Aliquots, (10 μ l), of the resultant mixture were analysed on a Nucleosil C18 (3.9 \times 150 mm) HPLC column, using Waters 2695 family of Separations modules (Waters Corporation, Milford, USA) with monitoring by a Waters 2998 photodiode array detector at 274 nm. The column temperature was 37 °C. Controls without enzyme were used to monitor any background reaction between the inhibitor and the substrate 4-nitrobenzaldehyde.

2.2.1. Gradient HPLC assay

Solvent A was a mixture of methanol/trifluoroacetic acid/water (60: 0.1: 39.9) while solvent B comprised methanol/trifluoroacetic acid/water (20: 0.1: 79.9). The following linear gradient was used for the separation: 20–100% B (15 min), 100% B (5 min) and 100–20% B (0.1 min) at 0.9 mL/min. The run time was 20 min. Standard curves constructed using each method separately for

4-nitrobenzaldehyde and 4-nitrobenzyl alcohol were used to determine concentrations of product in each of the reaction mixtures.

2.2.2. Isocratic elution method

Solvent A (100%) was used as mobile phase with a total acquisition time of 10 min for the isocratic elution method while the procedure for determination of product concentration was the same as described above (Section 2.2.1)

2.3. Fluorescence measurement of inhibitor binding to Kv β 2

Fluorescence scans of Kv β 2 with bound NADPH were recorded on a Perkin Elmer fluorescence spectrometer LS50B at 22 °C. Binding studies were carried out in a quartz cuvette containing ~2.0 μ M Kv β 2-bound NADPH in 0.2 M potassium phosphate buffer, pH 7.5 at 22 °C. The reaction volume was 2 ml. All inhibitor solutions (Stock concentration 100 mM) were prepared in DMSO and then diluted into 0.2 M potassium phosphate buffer, pH 7.5 to the required concentration, keeping the content of DMSO in the reaction mixture equal to or below 1% (v/v). DMSO at this concentration had no effect on enzyme-bound NADPH. The fluorimeter excitation wavelength used was 360 nm with a slit size of 15 nm and emission spectra were acquired from 300 to 600 nm. After a spectrum was recorded, 10 μ l of inhibitor stock solution was added into the Kv β 2 solution and mixed. A spectrum was recorded immediately after mixing (1 min) and at different time points afterwards.

3. Results and discussion

3.1. Inhibitors of the β -subunit from voltage-sensitive potassium channels

Table 1 shows the compounds tested for their ability to inhibit Kv β 2-mediated reduction of 4-nitrobenzaldehyde. Although it has previously proven somewhat difficult to find molecules that bind to Kv β 2 as inhibitors in this study several molecules were found that were capable of binding to, and inhibiting, Kv β 2.

3.1.1. DOPAC and catechol

3,4-Dihydroxyphenylacetic acid (DOPAC), the acid metabolite of dopamine inhibited Kv β 2 in a concentration-dependent and saturable manner (Fig. 1A). Under the conditions used in this study, DOPAC was found to be the most effective inhibitor of Kv β 2 of all the molecules tested, showing ~40% reduction in 4-nitrobenzaldehyde reductase activity (Table 1). The inhibition was found to be concentration dependent (Fig. 1A). Interestingly, catechol, which has a structure like DOPAC but without the acetate group, was found to be far less effective (16% Inhibition, see Table 1). While the observed DOPAC inhibition is only 40%, it is of interest to find that this neurotransmitter metabolite is capable of binding to Kv β 2, particularly considering findings regarding the role of Kv1 channels in regulating dopamine release (see Martel et al., 2011).

3.1.2. Cortisone

Structural studies have shown cortisone to bind at two different sites on Kv β 2: one close to the NADPH cofactor binding site and another at an interface site between the α and β subunits (Pan et al., 2008a). It was, therefore, expected that cortisone might inhibit Kv β 2. Indeed, this study shows that cortisone does inhibit Kv β 2 activity but only by ~18% (Table 1). This inhibition was further shown to be concentration dependent (Fig. 1B). Such a relatively modest inhibition by cortisone supports the idea that its main effect on channel activity is likely *via* dissociation of Kv β 2 from Kv1 (Pan et al., 2008a).

Table 1Inhibitors of Kv β 2-mediated aldehyde reduction. All data are expressed as mean of triplicate measurements.

Inhibitor (0.5 mM)	Description of inhibitor	Percentage inhibition ^a
Sodium valproate	Classical AKR inhibitor/anticonvulsant	10 \pm 0.8
4-Nitrobenzoic acid	Kv β 2 catalysed reaction product	0
4-Chlorobenzaldehyde	Substrate analogue	6 \pm 0.5
4-Fluorobenzaldehyde	Substrate analogue	3 \pm 0.8
Anisaldehyde	Substrate analogue	0
Tolualdehyde	Substrate analogue	0
Chloral hydrate	Sedative and hypnotic drug	0
4-nitroacetophenone	Aromatic ketone	6 \pm 0.6
Rutin hydrate	Plant flavanoid	38 \pm 2
Resveratrol	Chemopreventive/cardioprotective/antiaging	38 \pm 1
Cortisone	Steroid hormone	18 \pm 2
Pyrazole	Analgesic/anti-inflammatory	0
Cyproheptadine	Antidepressant	0
Amitriptyline hydrochloride	Antidepressant	0
D-Sorbitol	Sugar alcohol	0
Chlorpromazine hydrochloride	Antipsychotic drug	0
DOPAC	Dopamine metabolite	40 \pm 4
Catechol	Structural variant of DOPAC	16 \pm 0.7
5-Hydroxyindole-3-acetic acid	Serotonin metabolite	0

^a The amount of 4-nitrobenzyl alcohol produced as a result of Kv β 2-mediated multiple turnover of 4-nitrobenzaldehyde with no inhibitor was taken as 100%. Activities in the presence of inhibitor were expressed as a percentage of control 4-nitrobenzaldehyde reductase activity. Enzyme activity was measured as described in Section 2.

3.1.3. Sodium valproate

This anti-convulsant drug, which is a classical inhibitor of many aldo-keto reductases, (Hinshelwood et al., 2002; Todaka et al., 2000) proved to be a rather poor inhibitor showing a 20% reduction in 4-nitrobenzaldehyde activity of Kv β 2 at 0.5 mM (Table 1).

3.1.4. Rutin

Rutin, a naturally-occurring plant flavanoid known to inhibit aldose reductase from rat brain (Wermuth et al., 1982), reduced Kv β 2 aldehyde reductase activity by \sim 38% at 0.5 mM (Table 1). A concentration-dependent increase in inhibition was observed

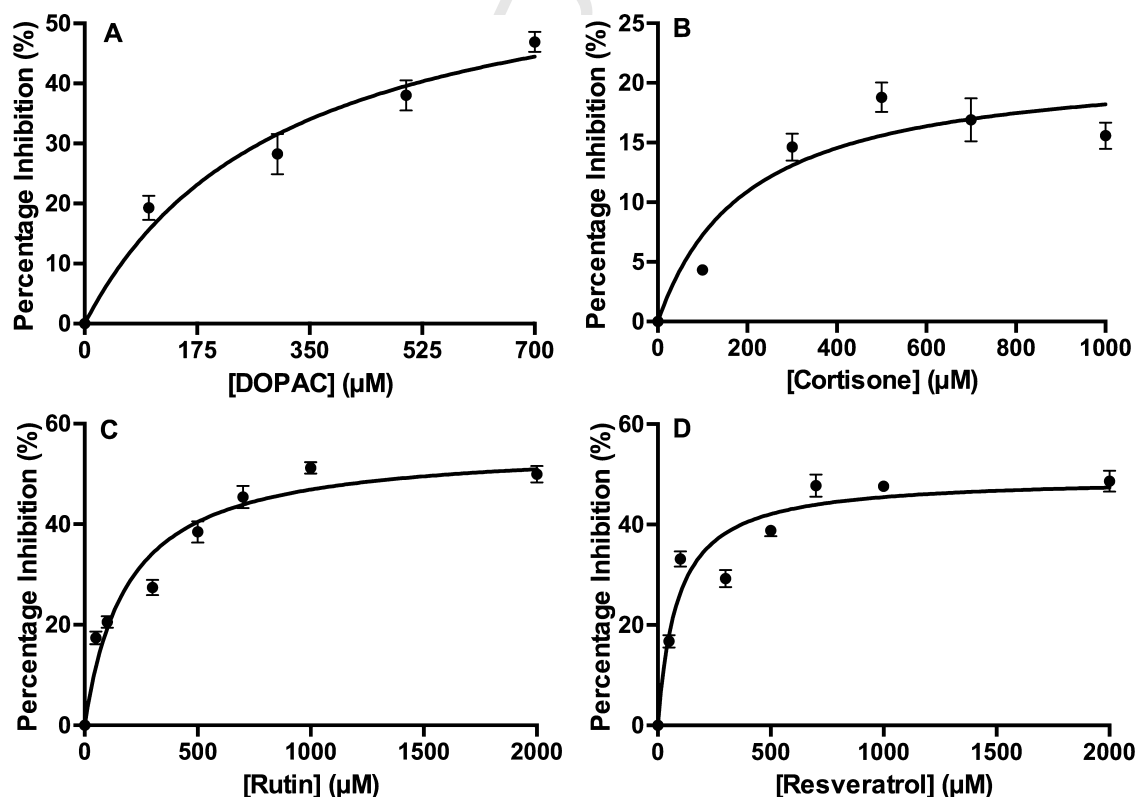


Fig. 1. Concentration dependent inhibition of Kv β 2 by various inhibitors. The data show the percentage inhibition as a function of increasing concentration of (A) DOPAC (0–700 μ M), (B) cortisone (0–1000 μ M), (C) rutin (0–2000 μ M), (D) Resveratrol (0–2000 μ M). The assay mixture contained \sim 0.5 mg Kv β 2 in 0.2 M potassium phosphate buffer (pH 7.5), along with 500 μ M 4-nitrobenzaldehyde as substrate, 200 μ M NADPH and various concentrations of inhibitor in a final reaction volume of 250 μ l at 37 $^{\circ}$ C. After the addition of inhibitor to Kv β 2 containing NADPH, the reaction was initiated by the addition of the substrate (4-nitrobenzaldehyde) and incubated for 30 min. The amounts of the reduced product (4-nitrobenzylalcohol) formed were analysed by HPLC, as described in Section 2. Percentage inhibition of the 4-nitrobenzaldehyde reductase activity of Kv β 2 at different starting concentrations of inhibitor was plotted against inhibitor concentration. Data are expressed as the mean of triplicate measurements. The points were fitted to a rectangular hyperbolic curve using prism 6.0.

(Fig. 1C), giving a maximal level of inhibition only slightly less than that observed with DOPAC.

3.1.5. Resveratrol

Kv β 2 inhibition by resveratrol was examined since it has been shown to have chemopreventive, cardioprotective, and anti-ageing properties which may be mediated by inhibition of a reductase (Buryanovskyy et al., 2004). Resveratrol (0.5 mM) inhibited Kv β 2 activity in a concentration-dependent manner but to a maximal level of \sim 40% (Fig. 1D). It is interesting to speculate that some of the cardioprotective effects attributed to resveratrol might be mediated by Kv β 2 inhibition.

3.1.6. Other compounds tested

4-Nitrobenzoic acid showed no inhibition showing that Kv β 2 was not liable to product inhibition under these conditions. anisaldehyde, cyproheptadine, chloral hydrate, D-sorbitol, chlorpromazine and 5-hydroxyindole-3-acetic acid all failed to inhibit Kv β 2. The antidepressants amitriptyline and cyproheptadine (a known potassium channel blocker, Wooltorton and Mathie, 1993) were, likewise, devoid of effect on Kv β 2. The antipsychotic drug, chlorpromazine and the serotonin metabolite 5-hydroxyindole-3-acetic acid showed no measurable inhibition of Kv β 2.

3.1.7. Summary of inhibition findings

As with the substrate specificity studies mentioned above it is difficult to deduce common features that will define a Kv β 2 inhibitor. A variety of aldehydes might be expected to be inhibitors or substrates. However, the fully hydrated aldehyde, chloral hydrate, did not inhibit neither did a several aromatic aldehydes although 6% inhibition was observed with 4-chlorobenzaldehyde. Sodium valproate did show some inhibition (10%) indicating that a carboxylate might be necessary for inhibition, however, 4-nitrobenzoate did not inhibit. On the other hand, 5-nitroacetophenone did inhibit albeit by only 6%. Catechol inhibited Kv β 2 by 16% but DOPAC which consists of catechol bearing an acetate group inhibited by 40%. This again suggests that a carboxylate is desirable for inhibition. Large multi ring structures such as amitriptyline, chlorpromazine and cyproheptadine were not inhibitory presumably due to their bulk. On the other hand the multi ring structure of rutin was inhibitory. These findings probably reflect an active site with a high degree of specificity. The most significant inhibition was observed with DOPAC, rutin hydrate and resveratrol.

3.2. Inhibitor binding studies

It was possible that some of the compounds tested might be binding to sites other than the active site of Kv β 2. In 2008, Pan et al., identified an interface site that promotes dissociation of Kv β 2 from Kv1. It is not known if this site will bind cortisone in the absence of Kv1 α subunits. Moreover, because some of the compounds tested might be substrates for Kv β 2, the binding of the inhibitors to the Kv β 2-active site was examined by monitoring the quenching of the cofactor fluorescence peak at 450 nm. This effect on fluorescence intensity is different from that induced by a substrate, which oxidises the Kv β 2-bound NADPH and, thereby, eliminates the fluorescence peak (Alka et al., 2010; Pan et al., 2008a). Binding studies carried out for cortisone, DOPAC, catechol, rutin and resveratrol yielded reductions in the fluorescence intensity at 450 nm of $16 \pm 0.7\%$, $7 \pm 0.3\%$, $12 \pm 0.8\%$ and $16 \pm 1.1\%$, respectively immediately after their addition; the decreased signals remained almost constant for 20 min. The data for DOPAC and cortisone are illustrated in Fig. 2A and B; findings with the latter agree well with a report from Pan et al., 2009. In the same year, Tipparaju et al., reported cortisone as a poor substrate for Kv β 2; this was not

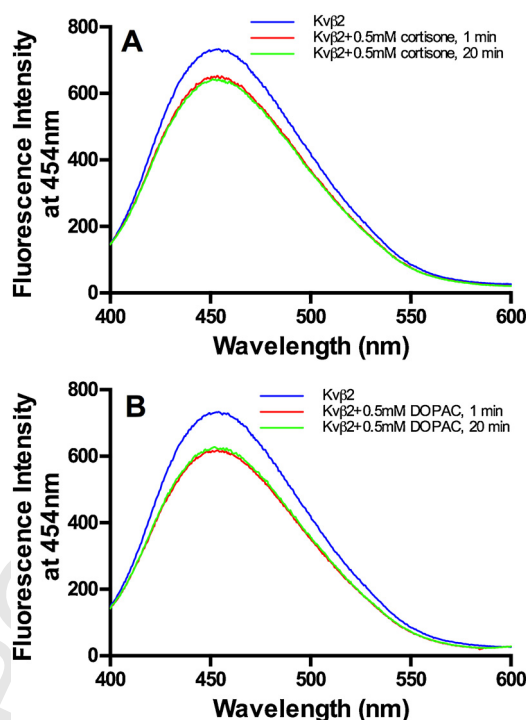


Fig. 2. Fluorescence quenching studies of Kv β 2. Fluorescence spectra of Kv β 2 before (blue) and 20 min after mixing (shown in red and green) with 0.5 mM cortisone/DOPAC (A and B respectively) showing 16 and 7% reduction in fluorescence at 450 nm. The change in fluorescence of the Kv β 2 bound cofactor occurred immediately after the addition of the inhibitor and remained stable for 20 min. All assays were carried out as detailed in Section 2. The addition of 10 μ l DMSO showed no significant reduction in the fluorescence spectrum of Kv β 2 under the conditions used (data not shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

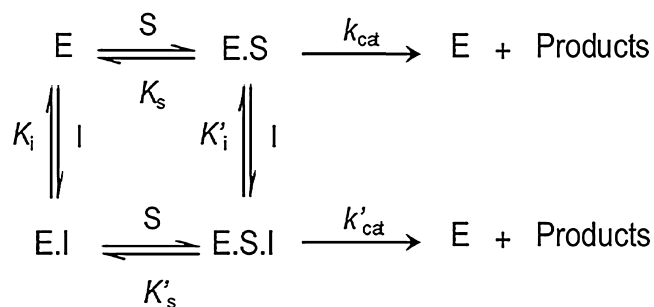
observed in the present study. Indeed, none of the inhibitors tested identified in this study were Kv β 2 substrates.

4. Conclusion

Previous studies of modulators of Kv1 channel activity have focused on cortisone and its analogues (Pan et al., 2008a, 2012). The present study advances the search for inhibitors of Kv β 2 by identifying several new compounds that bind to this subunit that specifically inhibit substrate turnover and, therefore, are likely to have a quite different mechanism of action from cortisone which appears to exert its influence in large part by dissociating Kv β from Kv1.

It is surprising that, given the sequence homology between Kv β proteins and AKR enzymes, there is very little inhibition of this subunit activity by classical AKR inhibitors; in fact, there are few compounds that appear to bind tightly to this protein and this includes substrates (see Pan et al., 2012; Alka et al., 2010).

Interestingly, DOPAC (the acid degradation product of dopamine) was found to be an effective inhibitor of Kv β 2. Dopamine is released by several areas of the brain and these findings make DOPAC a possible *in vivo* effector of brain Kv β 2. However, the concentration at which inhibition was observed is quite high. DOPAC is only the second physiological compound shown to inhibit Kv β 2 activity. Physiologically, Dopamine undergoes catabolism by monoamine oxidase to 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further oxidised to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase. It is unlikely that DOPAC levels *in vivo* will approach those used in this study (Jing et al., 2007) unless in localised microenvironments.



Scheme 1. Scheme showing partial inhibition of an enzyme catalysed reaction where E, S and I represent enzyme, substrate and inhibitor, respectively. Terms k_{cat} and k'_{cat} represent the catalytic rate constants for the breakdown of the ES and ESI complexes, respectively (McDonald and Tipton, 2002).

Cortisone was found to influence aldehyde turnover as predicted, but rutin and resveratrol were at least as effective in this regard. Resveratrol, a polyphenolic compound found in red wine, is well known for its cardioprotective (Markus and Morris, 2008; Nicholson et al., 2008) and anti-cancer effects (Chen et al., 2004; He et al., 2011). Its observed inhibition of the catalytic activity of Kv β 2 may relate to some of the therapeutic properties exhibited by resveratrol.

It is acknowledged that the concentrations used to achieve Kv β 2 inhibition are high and the degree of inhibition does not exceed 40% in any cases. The lack of inhibition studies in the literature for this subunit may be due to difficulties in identifying any molecules that can bind to Kv β 2. It is important to note that cortisone does not greatly inhibit aldehyde turnover by this subunit but it has nonetheless been shown to have a significant effect on channel activity (see Pan et al., 2008a).

It is important to note that while the inhibitors examined here showed concentration dependence they did not give rise to complete inhibition in any case. This precluded the measurement of IC₅₀ values. Moreover, a plot of enzyme activity versus inhibitor concentration was saturable. Inhibition of this kind is normally regarded as partial and can be explained by assuming that inhibitor binding does not fully exclude substrate turnover (Scheme 1).

An alternative explanation, where inhibition is caused by binding at a site other than the active site in a manner that constrains substrate binding, is also possible but was discounted on the basis of the fluorescent quenching studies. Moreover, direct binding of cortisone to the active site was observed previously (Pan et al., 2008a). Of course, such an explanation might be considered where such binding gave rise to perturbation of the active site in a way that caused fluorescence quenching. Further kinetic studies are needed to distinguish these possibilities.

The compounds described here may help to provide a starting point to stimulate further exploration of Kv β 2 inhibitors. In summary, a number of molecules were identified that bind to and inhibit Kv β 2-mediated p-nitrobenzaldehyde reduction, including the steroid cortisone.

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