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Short communication

The inhibitory profile of Ibudilast against the human phosphodiesterase enzyme family

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Abstract

Ibudilast is widely used in Japan to treat ischemic stroke and bronchial asthma. Its mode of action is through the inhibition of cyclic nucleotide phosphodiesterases (PDEs). Growing evidence suggests this compound has utility in a range of neurological conditions linked to its ability to elevate cellular cyclic nucleotide concentrations, however limited data exists on Ibudilast's action on individual PDE families. We therefore used an extensive panel of human PDE enzymes to define the PDE inhibitory profile of this compound. Ibudilast preferentially inhibits PDE3A, PDE4, PDE10 and PDE11 with lesser inhibition of a number of other families. The significance of these findings is discussed in relation to Ibudilast's observed effects on certain disease states.

Keywords: Phosphodiesterase; Ibudilast; Neurological disease; Respiratory disease

1. Introduction

Cyclic nucleotides are key second messengers within cells exerting powerful effects in many signalling pathways, thus controlling their concentrations can have potent effects in many disease states. Phosphodiesterases (PDEs) provide the only means of degrading cyclic nucleotides, therefore these enzymes play an essential role in cyclic nucleotide signalling. There are 11 known human PDE families, able to hydrolyse cAMP, cGMP or, in some cases, both cAMP and cGMP (Lugnier, in press) (see Table 1). Some families are generated from multiple genes, and most genes can generate multiple isoforms, thus there are over 50 known PDE isoforms. These enzymes vary by their substrate specificity and affinity, tissue distribution and sub-cellular localisation. This large number of diverse family members, possessing such potent effects on many cellular control pathways makes these enzymes attractive targets for specific drug development (Dyke and Montana, 2002; Lugnier, in press).

Ibudilast is known as a non-selective inhibitor of cyclic nucleotide phosphodiesterases (Souness et al., 1994) and is widely used in Japan to treat both ischemic stroke and bronchial asthma. The clinical uses are based on Ibudilast's ability to inhibit the aggregation of platelets (Ohashi et al., 1986; Kishi et al., 2000), improve cerebral blood flow (Fukuyama et al., 1993) and attenuate allergic reactions (Nagai et al., 1983; Nishino et al., 1984; Ohashi et al., 1993) through the inhibition of PDEs.

In addition to its effects in stroke and asthma, Ibudilast has also been shown to exhibit a number of beneficial effects in the brain (Tominaga et al., 1996; Yoshioka et al., 1997, 2002). By elevating cGMP, it attenuates H_2O_2 induced apoptosis of astrocytes (Takuma et al., 2001a,b). It also selectively vasodilates cerebral blood vessels without reducing systemic blood pressure. In addition, Ibudilast has been shown to inhibit TNFα (tumour necrosis factor-α) release from astrocytes and microglial cells (Suzumura et al., 1999), reducing neuronal degeneration. Recent studies have shown that Ibudilast may be

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Table 1
Human PDE panel

Enzyme	Substrate	Standard inhibitor	Standard inhibitor IC_{50} (μM)		Ibudilast K_i (μM)
			Published	Measured	
PDE1A3	cAMP	Vinpocetine	20	24±2.7 (n=3)	39.2±4.6
PDE1A3	cGMP	n.d.	n.d.	n.d.	93.5±5.3
PDE2A3	cAMP	EHNA	3	3±0.3 (n=3)	92.8±5.4
PDE3A	cAMP	Cilostazol	0.12	0.18±0.009 (n=10)	9.5±0.63
PDE3B	cAMP	Cilostazol	n.d.	3.2±0.2	77.6±9.9
PDE4A4	cAMP	Rolipram	1.6	1.5±0.11 (n=7)	4.1±0.19
PDE4B2	cAMP	Rolipram	1.6	1.2±0.08 (n=6)	3.3±0.44
PDE4C2	cAMP	Rolipram	6.6	6.5±1.03 (n=5)	6.3±0.94
PDE4D3	cAMP	Rolipram	0.68	0.66±0.15 (n=6)	3.7±0.27
PDE5A2	cGMP	Sildenafil	0.004	0.004±0.0005 (n=4)	51.6±1.8
PDE7A2	cAMP	Dipyridamole	42	47 (n=2)	57.8±11
PDE8A1	cAMP	Dipyridamole	9	8.5±0.49 (n=3)	49.7±5.8
PDE9A2	cGMP	Zaprinast	35	33 (n=2)	>400
PDE10A1	cAMP	Dipyridamole	1.2	1.3±0.1 (n=4)	2.22±0.28
PDE10A1	cGMP	Dipyridamole	0.45	1.1	1.27±0.2
PDE11A1	cAMP	Dipyridamole	0.82−1.8	1.36±0.07 (n=8)	8.9±1.56
PDE11A1	cGMP	Dipyridamole	0.37	1.87	43.1±6.2

Shows the full-length PDE isoforms which were cloned, expressed in insect cells and partially purified for this study. Cyclic nucleotide substrates used are shown. Standard inhibitors for each isoform are shown with published IC_{50} values for these inhibitors. IC_{50} values for these inhibitors against our PDE enzymes are shown. IC_{50} measurements were carried out in duplicate. Where no n value is given, this is the averaged data from one experiment. Where n=2, the mean value from two experiments is given. Where n>2, the mean±S.E.M. is given. n.d.=no data. K_i values for Ibudilast against our PDE enzymes are presented as mean±S.E.M.; n=4 for these measurements.

useful as a neuroprotective and anti-dementia agent (Mizuno et al., 2004) and as a potential therapy for multiple sclerosis (Feng et al., 2004) and Krabbe's disease (Kagitani-Shimono et al., 2005).

Although Ibudilast is known to act by elevating cyclic nucleotides, there is some confusion over its true PDE inhibitory profile. Previous studies have found that it inhibits platelet derived PDE3 and PDE5 at low micromolar concentrations (Kishi et al., 2000). Inhibition studies using PDE enzymes obtained from other tissue sources have alternatively identified Ibudilast as a potent inhibitor of PDE4 (Souness et al., 1994; Murashima et al., 1998).

This study was performed to determine the inhibitory profile of Ibudilast against an extensive panel of human PDEs. We cloned human genes representing at least one member of all known PDE families except PDE6. Human PDE enzymes were produced in insect cells using a baculovirus expression system. We found that rather than being a broad range PDE inhibitor Ibudilast inhibited few PDEs to any significant degree with the most striking inhibition being against PDE3A, PDE4, PDE10 and PDE11.

2. Materials and methods

2.1. Cloning and expression of PDE enzymes

PDEs (see Table 1) were cloned by reverse transcription-polymerase chain reaction (RT-PCR) of human heart and brain total RNA (Clontech). Primer sequences used for cloning were based on sequence information deposited in Genbank. In all cases the full length gene was cloned and expressed using the Gateway (Invitrogen) baculovirus expression system according to the manufacturer's protocols. After infection of Sf9 cells for 90 h, enzymes were partially purified from the cells by ion exchange chromatography except PDE3B which is a membrane protein. We therefore used this enzyme as a crude membrane preparation in the PDE assay. In all cases endogenous PDEs contributed less than 1% of the total PDE activity.

2.2. PDE assay

PDE enzyme activity was determined using a modification of the method of Thompson and Appleman (1971). Briefly, PDEs were incubated for 20 min at 30 °C in 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.1 μCi tritiated cyclic nucleotide, 0.1 or 1 μM unlabelled cyclic nucleotide and Ibudilast or standard PDE inhibitor ranging from 0.3 nM to 400 μM. After incubation at 70 °C for 2 min, assays were cooled on ice for 10 min. Then 25 μl of *Crotalus atrox* venom (1 mg/ml) was added to each assay and incubated at 30 °C for 10 min. Then 200 μl of a 1:1:1 (v/v/v) slurry of Dowex 1X8 200–400 MESH CI resin, ethanol and water were added, assays shaken at 4 °C for 20 min and centrifuged at 3000×g for 5 min. 50 μl of supernatant was mixed with 200 μl Microscint 20 (Perkin Elmer) and counted in a TopCount NXT scintillation counter (Perkin Elmer). The cyclic nucleotide concentration in the assays was at or below the K_m of the substrate for the enzyme. In all cases PDE concentration was adjusted so that the maximum substrate hydrolysis was less than 10% of the total. IC_{50}'s were calculated using GraphPad Prism. K_i's were calculated from the IC_{50}'s using the Cheng–Prussoff equation.

Ibudilast (3-isobutyryl-2-isopropylpyrazolo[1,5-*a*]pyridine) was supplied by Kyorin Pharmaceutical Company Ltd.

3. Results

3.1. Expression of full length human PDEs

A comprehensive panel of human PDE enzymes (Table 1) was established by expressing the relevant genes in insect cells. Soluble lysates of these cells contained high levels of PDE activity except in the case of PDE3B where PDE activity was associated with the membranes. Enzymes were prepared as described in Materials and methods and used for inhibition studies. All enzymes were validated using previously characterised standard inhibitors for each family. These results (Table 1) show that our enzymes gave IC_{50} values very close, if not identical, to previously published values. The IC_{50} values we obtained for Rolipram against the PDE4s were consistent with our PDE4s being in a low affinity binding state.

3.2. The PDE inhibitory profile of Ibudilast

We measured the inhibitory effect of Ibudilast against our human PDE panel. PDE3A, PDE4, PDE10 and PDE11 were most sensitive to inhibition by Ibudilast (Table 1). Ibudilast did not exhibit PDE4 subfamily selectivity with K_i values ranging from 3.3 μM to 6.3 μM. Inhibition of PDE10 by Ibudilast was essentially the same with cAMP and cGMP as substrates yielding K_i values of 2.2 μM and 1.3 μM, respectively. PDE11 was tested for inhibition using both cAMP and cGMP substrates and cAMP hydrolysis was sensitive to inhibition with a K_i value of 8.9 μM. PDE3A was inhibited by Ibudilast with a K_i of 9.5 μM.

4. Discussion

Ibudilast is widely regarded to be a broad range PDE inhibitor. In this study we have shown that Ibudilast has marked selectivity for PDE3A, PDE4, PDE10 and PDE11. The inhibitory activity was measured against an extensive panel of recombinant full length human PDEs derived from the same cellular background. We believe this is the first time the PDE inhibitory profile of Ibudilast has been systematically assessed against such a large number of PDE families at the same time.

Ibudilast had a potency against our PDE4 preparations of a similar order to that of the classical PDE4 inhibitor Rolipram. Our results showing Ibudilast to be a potent inhibitor of PDE4 are consistent with previous reports using eosinophil and HUVEC derived PDE enzymes (Souness et al., 1994; Murashima et al., 1998).

Our finding that Ibudilast did not significantly inhibit PDE5 (K_i of 51.6 μM) conflicts with an earlier report of an IC_{50} for PDE5 of 2.2 μM (Kishi et al., 2000). The reason for this discrepancy is not clear, however these authors used a PDE5 enzyme preparation from platelets and it is possible that other cGMP PDEs may have been present. We used recombinant human enzymes produced in an insect cell system and further enriched by ion exchange chromatography. Endogenous PDE activity was negligible and we were certain that we measured inhibitor effects on individual PDEs. Our PDE5A2 was inhibited by the standard inhibitor Sildenafil with an IC_{50} value identical to the published values. We also showed that PDE5A1 and PDE5A3 were not significantly inhibited by Ibudilast ($K_i > 50$ μM in both cases, data not shown).

Ibudilast is currently used in the treatment of both asthma and as a vascular improver after stroke. A number of studies exist detailing the effects of this compound upon neurological disease conditions (Tominaga et al., 1996; Yoshioka et al., 1997). Whilst these effects link increases in cellular concentration of cAMP (Yoshioka et al., 1998) or cGMP (Yoshioka et al., 2000) with measured positive outcomes, such as reduction in TNFα release (Suzumura et al., 1999) or attenuation of H_2O_2 induced apoptosis (Takuma et al., 2001a,b), little work has been done to investigate the PDE families involved.

The ability of Ibudilast to inhibit both PDE3A and PDE4 may explain the effects this compound exert in respiratory conditions. This combination has been shown to reduce symptoms of asthma (Underwood et al., 1994).

The combination of PDE4 and PDE10 inhibition may explain the positive effect of Ibudilast on neurological conditions. PDE4 is highly expressed in many regions of the brain, and PDE4 inhibitors are also known to act within the brain, serving as anti-depressants (Takahashi et al., 1999; Zhang et al., 2002a,b) and memory enhancing agents (Bourtchouladze et al., 2003). The PDE4 inhibitor Rolipram has been shown to inhibit TNFα, O_2^- and NO (nitrous oxide) release from microglial cells (Zhang et al., 2002a,b), accounting for this compound's good neuroregenerative properties. Elevating cAMP levels through PDE4 inhibition and thus increasing the activity of the protein kinase, PKA, has also been shown to reverse amyloid β-peptide induced inhibition of long-term potentiation in Alzheimer's disease (Vitolo et al., 2002). Although the clinical relevance of PDE10 is not yet fully documented it is also known to be abundant in the brain (Fujishige et al., 1999). This enzyme is particularly rich in the putamen and caudate nucleus, areas both linked with Parkinson's disease (Fujishige et al., 1999; Soderling et al., 1999). Rodefer et al. (2005) have shown that PDE10 inhibition may be an effective therapy for the treatment of schizophrenia. Thus, PDE10 is a potential target for compounds useful in treating neurological disorders.

We also noted significant inhibition of PDE11 by Ibudilast. The physiological role of PDE11 is not known and PDE11 knockout mice do not show any obvious phenotype (Wayman et al., 2005). Therefore, it is not possible to say whether PDE11 inhibition contributes to the positive effects of Ibudilast.

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