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Article

Nonsteroidal anti-inflammatory drugs as potential ecto-nucleotide phosphodiesterase inhibitors

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Phosphodiesterases (PDE) are group of enzymes which catalyze the hydrolysis of cAMP and cGMP. Since these cyclic phosphate moieties worked as intracellular second messengers in numerous physiological processes, their inhibition can affect normal physiology of living system. NSAIDs are among the frequently prescribed medications, because of their efficacy as analgesic, antipyretic and anti-inflammatory agents. They are known to block cyclooxygenase pathway. In limited data NSAIDs has been shown anti-tumor potential, and phosphodiesterase inhibition has assumed to be one of the mechanism. To date no further evaluation being done. Further, NSAIDs are classified as cyclooxygenase inhibitors and phosphodiesterase inhibition can imprint its side effects. This study first time investigates the effects of NSAIDs on phosphodiesterase 1 inhibition. The activity against snake venom phosphodiesterase 1 was assayed on a microtitre plate reader spectrophotometer. Selective COX-2 inhibitor, celecoxib, exhibited a potent PDE1 inhibitory activity, at therapeutic doses, with an IC_{50} value of 29.4 μM. The findings of our study are indicative of new pharmacological actions of cyclooxygenase inhibitors. This article presents the PDE inhibitory properties as a new effects of already existing drugs. These additional effects could be potentially helpful for researchers to assess other physiological and pathological states.

Keywords: Non-steroidal antiinflammatory drugs. Cyclooxygenase. Phosphodiesterase. Inflammation. cAMP.

ABBREVIATIONS

NSAIDs- Non-steroidal Antiinflammatory Drugs
cAMP- Cyclic Adenosine monophosphate
cGMP-Cyclic guanosine monophosphate
COX-Cyclooxygenase
PDE- Phosphodiesterase

INTRODUCTION

NSAIDs are among the most frequently prescribed medications because of their demonstrated efficacy as analgesic, antipyretic and antiinflammatory agents (Laine, 2001). Including all analgesics and antipyretics, these are the most widely used medications globally, i.e. 30% of all medicines used (Litalien, Jacqz-Aigrain, 2001). NSAIDs are used in management of fever, acute or chronic pain and inflammation in a wide spectrum of diseases. Their effectiveness has been proven in various clinical conditions, including osteoarthritis, rheumatoid arthritis, gout, dysmenorrhea, ankylosing spondylitis, headache and dental pain (Zochling *et al.*, 2006; Kean, Buchanan, 2005).

The basic mode of antiinflammatory actions of NSAIDs has been attributed to inhibition of the biosynthesis of prostaglandins, through the inhibition of key enzyme namely 'cyclooxygenase (COX)' (Vane, Botting, 1998; Vane, 1971). The inhibition of COX by NSAIDs yields clinical benefits and low toxicity (Fries *et al.*, 2004). Cyclooxygenases (COX) are involved in the formation of prostaglandins (PG), which further role in many physiological conditions, such as the

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dilation and constriction of blood vessels, contraction and relaxation of smooth muscles, control of blood pressure, and regulation of inflammation (Tasneem *et al*., 2019). It was previously believed that the effects of NSAIDs are due to the blockade of a single cyclooxygenase enzyme, later, the two distinct isoenzymes, namely COX-1 and COX-2 were discovered. Extensive studies now indicate that the clinical efficacy of NSAIDs are primarily due to the inhibition of COX-2, whereas the undesirable effects, e.g. gastric and intestinal mucosal damage and renal toxicity are due to the inhibition of COX-1 (Vane and Botting, 1998). Thus the specific COX-2 inhibitors lack the toxic effects of conventional NSAIDs. In brief, the NSAIDs include two groups, nonselective NSAIDs that block both cyclooxygenases-1 and -2, and COXIBs are selective COX-2 inhibitors. Previous studies with NSAIDs suggested that cGMP specific phosphodiesterase inhibition is an important COX-independent mechanism to suppress β-catenin signaling (Thompson *et al.,* 2000).

Phosphodiesterase

Ecto-nucleotide pyrophosphatases or Cyclic nucleotide pyrophosphatases or cyclic nucleotide phosphodiesterases are isoenzymes that catalyze the hydrolysis of the 3′, 5′-cyclic phosphate moiety of cAMP and/or cGMP to their 5′-nucleotide monophosphatase (Figure 1). Ligand binding to G-coupled protein receptor leads to activation of adenylate cyclase which is involved in conversion of ATP to cAMP. This action is terminated by PDE which hydrolyze the cAMP thus blocking the numerous physiological events (Figure 2). cAMP and cGMP are substrates for PDE1, and served as intracellular second messengers in the regulation of various physiological stimuli. This includes cardiac contractility, cardiac output, smooth muscle relaxation, neurodegeneration, vascular resistance, visceral motility, reduced immune and inflammatory activity of cells, neuroplasticity, reproduction and vision (Li, Yee, Beavo, *et al.,* 1999; Dousa, 1999).

Eleven members of phosphodiesterase (PDE) superfamily are identified uptil now. PDE4, PDE7 and PDE8 are specific for catalyzing the cleavage of cAMP, PDE5, PDE6 and PDE9 are specific for cGMP only. While PDE1, PDE2, PDE3, PDE10 and PDE11 can handle both the nucleotides as substrates (Dousa, 1999).

Phosphodiesterase 1

Nucleotide pyrophosphatases/phosphodiesterases (NPP1) or oligonucleate 5′- nucleotidohydrolase or PDE1, also called as Ca^{2+}/calmodulin – dependent cyclic

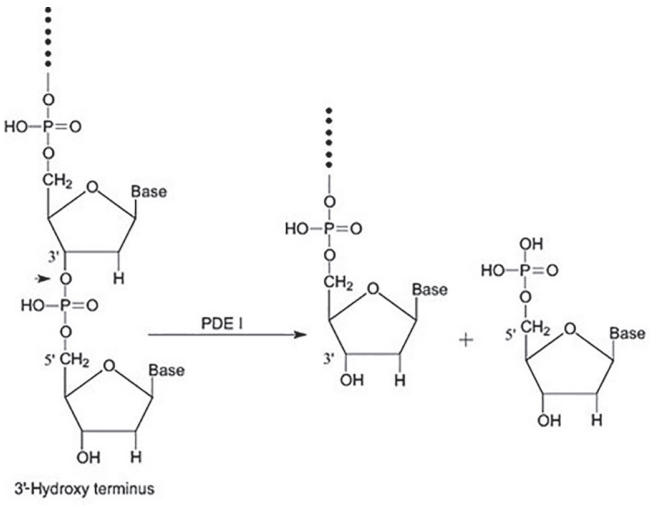

FIGURE 1 - PDE catalyzes the cleavage in the 3′- to 5′-direction to yield nucleoside 5′-phosphates.

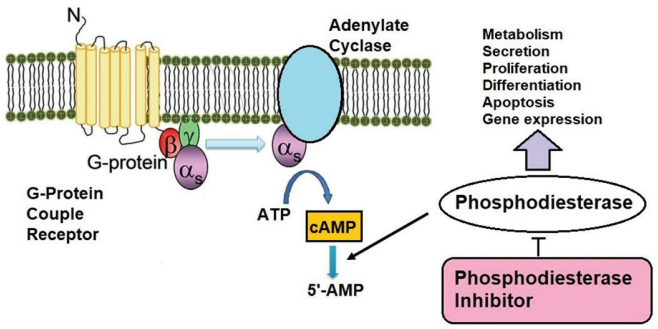

FIGURE 2 - G-Protein coupled receptor binding to ligand activates adenylate cyclase which form cAMP by ATP. Reaction is catalyzed by phosphodiesterase. Inhibition of phosphodiesterase leads to various physiological functions. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; 5'AMP, 5' Adenosine monophosphate.

nucleotide phosphodiesterase, was first reported by Kakiuchi and Yamazaki in 1970 in rat brain (Kakiuchi, Yamazaki, 1970). In early 1970's, the existence of protein activator "calmodulin" (CaM) was established which stimulates the PDE activity several folds. Three genes (PDE1A, PDE1B and PDE1C), with various splice variants, constitute PDE1 family. For the stimulation of PDE1 activity, the presence of both Ca^{2+} and calmodulin is required. Activity of PDE1 is triggered by binding of Ca^{2+} to free CaM to convert protein from its inactive state to activated conformation, which then associates with PDE1 to form active PDE. Activity of PDE1 is regulated by several different mechanisms, e.g. stimulate PDE1 phosphorylation, block phosphorylation and reverse phosphorylation.

Existence of PDE1 has been established in most mammalian tissues. Mammalian brain contains the highest

CaM-PDE activities. Additionally, PDE1 activity has been detected in other tissues, including heart, smooth muscles, lungs, and pancreas as well as in several other eukaryotes. This enzyme has also been presented in numerous immune cell types, such as lymphocytes and monocytes. The presence of PDE1 in many tissues and organisms highlights its importance and its pivotal role in signal transduction. PDE1 plays a significant role in a numerous physiological and pathological functions. It serves to regulate vascular smooth muscle contraction and participates in some interleukin regulation related allergic diseases (Sharma, Wang, 1986).

Inhibition of PDE1 may induce apoptosis in human leukemic cells (Zhang, Wang, Sharma, 1993). It is highly expressed in proliferating smooth muscle cells and is believed to be a major regulator of smooth muscle proliferation (Rotella, 2002). It also promotes arterial smooth muscle cell proliferation, hence could be beneficial in pathophysiology of atherosclerosis. According to some studies, it may also be involved in neuronal signaling (Sharma, Kalra, 1994). Clinically available PDE1 inhibitor, vinpocetine, is known to improve memory. Recently phosphodiesterases have attracted a major scientific attention as potential targets for PDE inhibitor-based antiparasitic drugs.

It is well known fact that numerous inflammatory mediators are produced during pro- and anti- inflammatory pathways, and among them TNFα and IL1β are well known to alter the cAMP levels and they are thought to do so primarily by way of an upregulation of cAMP-specific PDE expression and activity (Ghosh *et al.,* 2012). There are several lines of evidences suggest the role of COX independent targets for NSAIDs during cancer, apoptosis and neurodegenration (Grösch *et al.,* 2006; Elder *et al.,* 1997; Weggen *et al.,* 2001). One of the COX-independent mechanisms that has been proposed for the anticancer activity of NSAIDs involves inhibition of cGMP PDEs, resulting in increased intracellular cGMP levels and activation of cGMP signaling (Tinsley *et al.*, 2010). There are several studies that demonstrate NSAIDs attenuating their pleiotropic effects by altering the cyclic monophosphates. Tinsley and his co-workers have shown that the NSAID inhibits PDE5 activity in colon tumor cell lysates (Tinsley *et al.*, 2010). Another report suggest that NSAIDs inhibit the PDE and tumor cell growth (Piazza *et al.,* 2005; Thompson *et al.,* 2000). There are studies that show the role of COX inhibitors as PDE inhibitors but, to date, no data has been reported on extra-pharmacological effects of NSAIDs, as specific PDE1 inhibitors. During this study, we aim to find out the effects of COX inhibitors on phosphodiesterase1 enzyme.

The aim of the work has been to find out the pleiotropic effects of the drugs that could possibly be interfering with their known or intended therapeutic effects. We believe that these findings will contribute both in basic research, as well as in clinical development. Further clinical study for the PDE enzyme inhibition activity of celecoxib can provide beneficial effects in thrombosis and neurodegenerative conditions.

MATERIAL AND METHODS

Material

Bis(p-nitrophenyl) phosphate sodium salt and phosphodiesterase 1 enzyme were purchased from Sigma Chemical Co. (USA). EDTA was purchased from Merck (Germany). Aspirin, diclofenac, etodolac, flurbiprofen, meloxicam, naproxen, nimesulide, and piroxicam were purchased from Sigma chemical Co. (USA). Celecoxib was purchased from Bosch Pharmaceuticals (Pakistan). Ibuprofen was purchased from MP Biomedicals, Inc. (France). Indomethacin was purchased from TCI (Japan). NS-398 was purchased from Wako (Japan). The instrument used was microtitre plate reader spectrophotometer, Molecular Devices (USA). All the reagents used for buffer preparation were of analytical grade.

Method

Nucleotide pyrophosphatases / phosphodiesterases 1 inhibition assay

In this assay, the activity against the snake venom phosphodiesterase 1 (EC 3.1.4.1) was assayed by using the reported method (Razzell, Khorana, 1959) with the following modifications. *Tris*-HCl buffer 33 mM (pH 8.8), 30 mM Mg-acetate as a cofactor was added with 0.000742 U of enzyme phosphodiesterase 1 as a final concentrations using 96-well flat bottom plate and 0.33 mM *bis*(*p*-nitrophenyl) phosphate as a substrate. EDTA was used as positive controls having $IC_{50} = 277.69 \pm 2.52$ μM. After 30 min of incubation, the enzyme activity was monitored at 37 °C on a microtitre plate reader spectrophotometer (Molecular Devices, USA) by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 410 nm. All the reactions were performed in triplicate and the initial rates were measured as the rates of changes in the OD/min (optical density/min) and used in subsequent calculations.

The % inhibition was calculated by using the formula:

% Inhibition = 100-(OD of test/OD of control × 100).

IC_{50} Values were calculated by using the EZ-fit enzyme kinetics software from USA.

RESULTS AND DISCUSSION

Addition of celecoxib to PDE mixture at pharmacological concentrations (1 – 400 μM) inhibited the phosphodiesterase enzyme. The results show that celecoxib inhibits PDE with an IC_{50}= 29.43 ± 0.23 μM.

To examine whether the celecoxib mediated inhibition of snake venom PDE is a general property of COX inhibitors or is specific to celecoxib, we evaluated other inhibitors of COX-1 and COX-2 such as aspirin, diclofenac, etodolac, flurbiprofen, ibuprofen, indomethacin, meloxicam, naproxen, nimesulide, NS-398 and piroxicam. None of these compounds in a wide range of concentration showed any inhibitory effects on PDE. Instead, nimesulide, a selective COX-2 inhibitor, exhibited significant inhibitory activity against PDE1. The results show that nimesulide inhibits the PDE enzyme with an IC_{50} = 440.4 ± 5.92 μM (Table I).

TABLE I - Effects of COX inhibitors on phosphodiesterase 1 inhibition

	Drugs	IC_{50} (μM) ± SEM	% Inhibition
1.	Celecoxib	29.43 ± 0.23	84.8
2.	Nimesulide	440.40 ± 5.92	97.2
	EDTA Std.	277.69 ± 2.72	69.0

Progression of inflammation involve certain pro- and anti- inflammatory signaling pathways which results in the synthesis and activation of numerous inflammatory mediators (Serhan, Chiang, Van Dyke, 2008). Among those mediators, TNFα and IL1β are well known to alter the cAMP levels and they are thought to do so primarily by way of an upregulation of cAMP-specific PDE expression and activity (Ghosh *et al.,* 2012). To date, no data has been reported about NSAIDs extra-pharmacological effects including PDE1 inhibition. During our studies we observed that NSAIDs are inhibitors of PDE1 isoenzyme. PDE are isoenzymes which catalyze the hydrolysis of 3', 5'-cyclic phosphate moiety from cAMP and cGMP. PDE1 activity has been detected in many tissues which highlights its role in signal transduction in numerous physiological and pathological conditions (Dousa, 1999). Controlling or modulating the activity of PDE1 could be beneficial in many ways. For example, PDE1 serves to regulate the vascular smooth muscles contraction and proliferation and can be effective in atherosclerosis (Chan, Yan, 2011). Presence of PDE1 in mammalian brain makes it a target for the discovery of drugs for treatment or management of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Medina, 2011). PDE1 inhibition increases the levels of cAMP/cGMP in brain which leads to increase expression of neuronal plasticity-related genes and neuroprotective molecules. These make PDE1 inhibitory agents important drug candidates for the treatment of neurological conditions. On the other hand, inflammation also has a close relation with the onset and progression of various neurodegenerative diseases. For instance, activation of astrocytes, microglia, lymphocytes, and macrophages results in an increase release of cytokines, chemokines, neurotransmitters and ROS (Tansey, McCoy, Frank-Cannon, 2007). Uptil now the suggested possible mechanisms of action of NSAIDs in Alzheimer's disease are to alter the levels of $A\beta_{1\text{-}42}$ (Weggen *et al.,* 2001), activation of PPARγ (Jaradat *et al.,* 2001), inhibition of Ras and Ras-like GTP-binding proteins, and inhibiting multidrug resistance protein-4 (MRP-4). These evidences supported the role of inflammation in the pathogenesis of Alzheimer's disease. The classical inhibitor of PDE1, vinpocetine, has also been used in the treatment of cerebrovascular disorders and age-related memory impairments.

Celecoxib and nimesulide are specific COX-2 inhibitors, thus are more specific in reducing the inflammation. Celecoxib is a selective COX-2 inhibitor which was tested previously for its effects on phosphodiesterases types 4 and 5 (Soh *et al.,* 2008; Klein *et al.,* 2007). In particular, our results demonstrate the inhibitory effects of both COX-2 inhibitors on PDE1. Neuronal transmission through glutamate leads to an increase of intracellular Ca^{++}, and cAMP/cGMP levels, which further triggers the signaling pathways that will eventually lead to the activation of the transcription factors serum response factor and CREB (cAMP responsive element binding protein) (Vitolo *et al,* 2002). Since PDE I inhibition potentiates the glutamatergic transmission and improves the neuronal plasticity and memory functions and our study shows the celecoxib as strong inhibitor of PDE, lead to the conclusion that celecoxib may also be involved in potentiating glutamatergic transmissions. Similarly, Nimesulide is NSAID belong to the aryl group (sulfonanilide class) and it seems to exert its effects through several mechanisms. Our data suggest strong inhibitory activity of nimesulide on PDE1 in dose-response dependent manner. A better understanding and more studies on clinical patients might help to identify the role

of celecoxib and nimesulide in neurodegenerative diseases and atherosclerosis and to discover new indications of old drugs in the treatment of such diseases.

Our study clearly demonstrates the inhibitory effects of COX-2 inhibitors over phosphodiesterase enzyme. A better understanding and more studies on clinical patients might help to identify the role of celecoxib and nimesulide in Alzheimer's disease and atherosclerosis and to discover new indications of old drugs in the treatment of these diseases.

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