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Der Pharma Chemica, 2010, 2(4): 397-403
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X

Synthesis and *invitro* spleen cell proliferative activity of novel oxime derivative of ibuprofenamide

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ABSTRACT

Many chronic inflammatory reactions are characterized by tissue infiltration of activated T-lymphocytes. The immunosuppressant activity of NSAIDs correlates with their ability to block transcription factors required for the expression of inducible response genes triggered by T-cell antigen receptor (TCR) following encounter with antigens including NF-KB, NF-AT and activated protein 1.The present work explores mechanism of NSAIDs involved in macrophage and T-cell mediated inflammatory response. Oxime derivative of ibuprofenamide was synthesized and evaluated for T-cell and B-cell proliferation activity (MTT assay) at concentrations ranging from 10-1000 µg/well. Results indicate suppression of the T-cell proliferation and stimulation of B-cell mitogen LPS.

Key words: Inflammation, MTT, NSAID, Oxime, TCR.

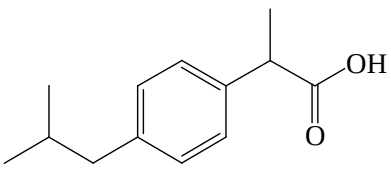
INTRODUCTION

Inflammation is a complex stereotypical reaction of the body expressing the response to damage of its cells and vascularised tissues. With inflammation, white blood cells are released to protect the body from injury [1]. These white blood cells have chemicals within them that, when leaked, induce swelling. The most common immune-derived cell type associated with tissue injury, however, is the macrophage which is responsible for the removal of dead tissue and debris by phagocytosis, lipid recycling and secretion of a wide range of cytokines. This release of cytokines and growth factors cause inflammation of the tissue [2].

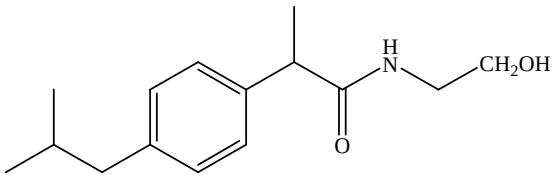
Some types of chronic inflammatory reactions are sustained by immune (hypersensitivity) reactions, and these are characterized by tissue infiltration by activated T-lymphocytes [3]. The cells of the immune system are widely distributed throughout the body, but when an infection or tissue damage occurs they concentrate at the site of damage.

The adaptive immunity mount more slow but focused response mechanism that require the recognition of specific molecular structures and generation of large number of antigen receptors, i.e. T-cell receptors and immunoglobulins, by somatic rearrangement processes in blast cells. When T-cells recognise a foreign antigen present in them, they attack the antigen bearing cell by cytotoxic T-cells, stimulating the antibody production by B-cells and induction of local inflammatory response [4]. B cells and T cells are the major types of lymphocytes and are derived from hematopoietic stem cells in the bone marrow. B cells are lymphocytes that play a large role in the humoral immune response (as opposed to the cell-mediated immune response, which is governed by T cells). The principal functions of B cells are to make antibodies against antigens, perform the role of Antigen Presenting Cells (APCs) and eventually develop into memory B cells after activation by antigen interaction. B cells are an essential component of the adaptive immune system.

Both B cells and T cells carry receptor molecules that recognize specific targets. T cells recognize a “non-self” target, such as a pathogen, only after antigens (small fragments of the pathogen) have been processed and presented in combination with a “self” receptor called a major histocompatibility complex (MHC) molecule. In contrast, the B cell antigen-specific receptor is an antibody molecule on the B cell surface, and recognizes whole pathogens without any need for antigen processing. Each lineage of B cell expresses a different antibody, so the complete set of B cell antigen receptors represent all the antibodies that the body can manufacture. The ultimate role of B-cell lymphocytes during inflammatory disorders is to produce antibodies, the effectors molecules of the humoral immune system [5]. The immunosuppressant activity of NSAIDs correlates with their ability to block transcription factors required for the expression of inducible response genes triggered by T-cell antigen receptor (TCR) following encounter with antigens, including NF-KB, NF-AT and activated protein 1 [6].
A common strategy in pharmaceutical research involves the use of well-established drugs as lead compounds to design new drug candidates with improved therapeutic properties. The prodrugs show comparable anti-inflammatory activity and lesser ulcerogenicity in comparison to the parent drug [7]. The free carboxylic acid group found in NSAIDs such as flurbiprofen and ibuprofen forms critical interactions with residues Arg-120, Glu-524, and Tyr-355 within the cyclooxygenase active site [8]. The masking of the ibuprofen-free carboxylic group seems to be principally the basis of this reduced topical irritant action [9]. Ibuprofen **(1)** has been modified into various heterocyclic amide derivatives having improved analgesic activity and lower ulcerogenic effects [10]. Aminoprofen[*N*-(β-hydroxy ethyl)-dl-2-(4'-isobutyl phenyl) propionamide] **(2)** an amide derivative of ibuprofen has been used for its topical anti-inflammatory activity which also metabolises into ibuprofen [11].



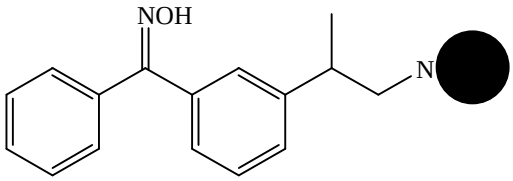
(1)



(2)

Schlegel and co-workers have reported the synthesis, and evaluation of anti-inflammatory and anti-ulcer activity of several bulky amine analogues of ketoprofen. It was found that activity was maintained on conversion of the keto group to oxime (**3)** [12]. Oxime derivatives of nabemutone and ketoprofen have been synthesized as cytochrome P450-selective prodrugs, and nabemutone

oxime has been found to possess anti-inflammatory activity. Also the oxime derivetives of various fenamates have shown to be inhibitors of prostaglandin biosynthesis. 2-[(2,6-dichloro-3-methylphenyl)amino] benzaldehyde-O-carboxymethyl oxime **(4)** inhibit the prostaglandin biosynthesis particularly the induced prostaglandin endoperoxide H synthase [13].



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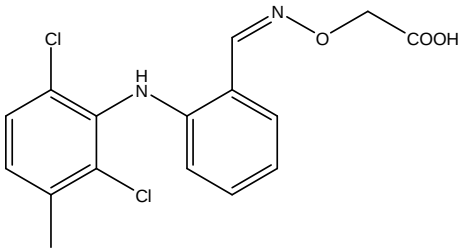


(4)

Thus, it was considered of interest to synthesise and study oxime derivatives of Ibuprofenamides. The study was planned to explore the mechanism of NSAIDs involved in macrophage and T-cell mediated inflammatory response (MTT assay).

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were obtained from S. D. Fine-Chem Ltd., E. Merck (India) limited, Sigma-Aldrich and Loba Chemie. Plates for thin layer chromatography (TLC) were prepared with silica gel G and activated at 110^{o}C for 30 min. Infrared (IR) spectra were obtained with Perkin Elmer 882 Spectrum and RXI, FT-IR model using a potassium bromide pellets. Elemental analyses were carried out on a Perkin–Elmer 2400 CHN elemental analyzer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with FT NMR Avance-II Brucker AC-400F (400 MHz) spectrometers at Regional Sophisticated Instrumentation Centre, Panjab University, Chandigarh using tetramethylsilane (TMS) as internal standard. The spin multiplicities are indicated by the symbols: *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *dd* (double doublet), *m* (multiplet) and *bs* (broad singlet).Melting points were determined on Veego-540 melting point apparatus and are uncorrected.

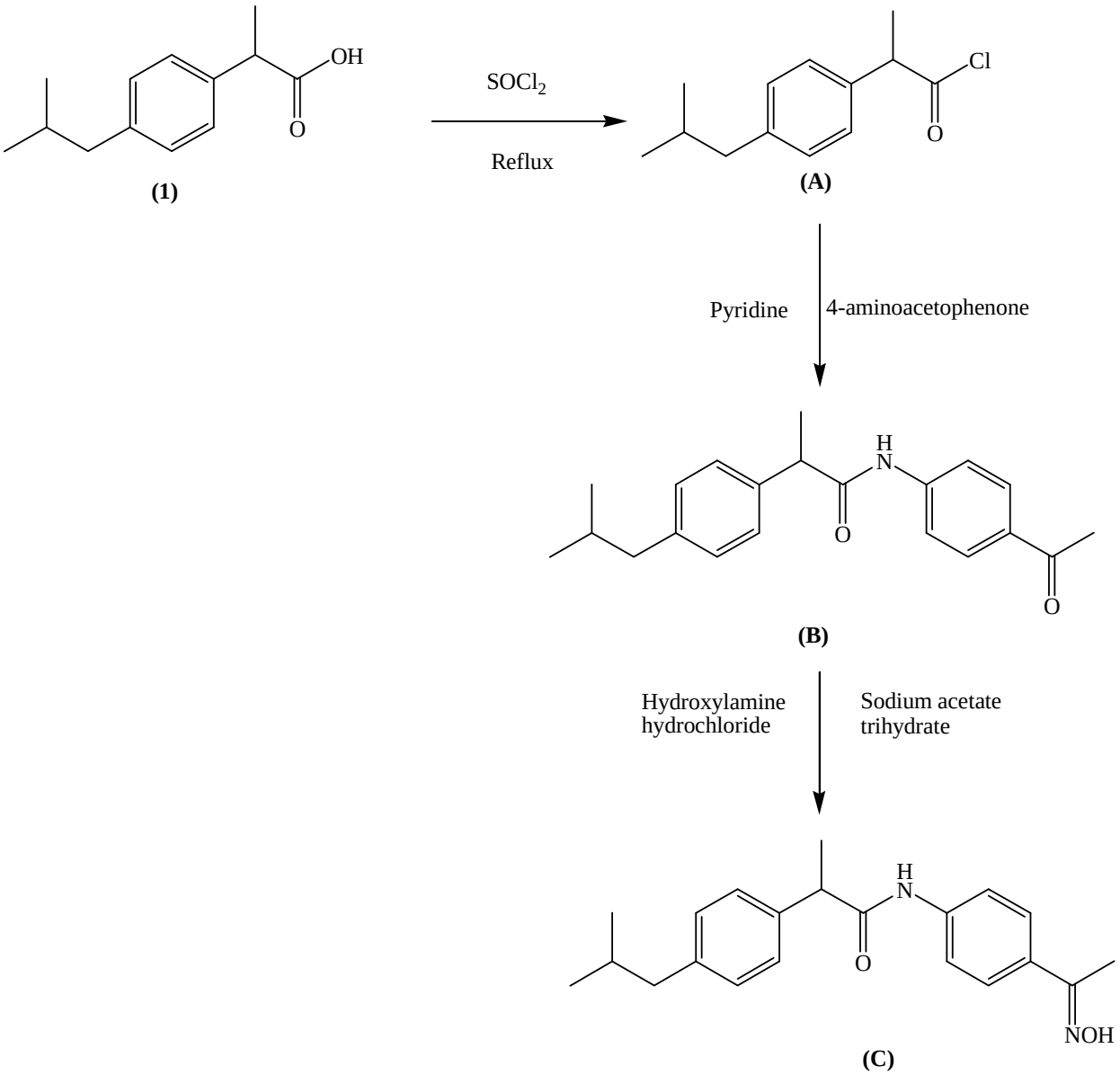
Chemistry

Ibuprofen obtained commercially was first converted into its acid chloride by conventional method using thionyl chloride to yield 2-(4-Isobutyl-phenyl)-propionyl chloride which was then converted to ibuprofenamide by treatment with 4-aminoacetophenone [14,15]. Ibuprofenamide was then treated with hydroxylamine hydrochloride and sodium acetate in ethanol to get the desired oxime derivative [16] of ibuprofenamide given in scheme 1. The structures of the synthesized compounds were confirmed by elemental analysis and spectral techniques such as FT-IR, ^{1}H-NMR and ^{13}C-NMR**.**

Experimental Procedures:

2-(4-Isobutyl-phenyl)-propionyl chloride (A)

Ibuprofen **(1)** (2.06 g, 0.01 mol) was stirred with freshly distilled thionyl chloride (5.95 mL,0.05mol) for 8 hr. Thionyl chloride was removed under reduced pressure to get 2-(4-isobutyl-phenyl)-propionyl chloride.



Scheme 1

2-(4-Isobutyl-phenyl)-N-(4-acetyl-phenyl)-propionamide (B)

To a mixture of 4-aminoacetophenone (1.35 g, 0.01 mol) and pyridine (2.0 mL) in acetone (25.0 mL) maintained at -10°C was added with stirring a solution of 2-(4-isobutyl-phenyl)-propionyl chloride **(A)** (2.25 g, 0.01 mol) in acetone (25.0 mL) over a period of 1 hr. The reaction mixture was stirred for 8 hr and poured into crushed ice. The residue obtained was filtered, dissolved in chloroform (100.0 mL), washed with 5% hydrochloric acid (3 × 50.0 mL), 5% sodium bicarbonate (3 × 50.0 mL) and finally with brine solution (2 × 25.0 mL). The organic layer was filtered, dried and crystallised from ethanol to get 2-(4-isobutyl-phenyl)-*N*-(4-acetyl-phenyl)-propionamide **(B)** (2.30 g, 71.21 % Yield); m.p. 81-83° C; IR (KBr): 3350, 3027, 2955, 1700, 1664, 1595, 1270 and 834 cm^{-1} ;^{1}H NMR ($CDCl_3$): δ 0.91 (*d*, 6H, *J* = 6.7 Hz, -CH(CH_3)$_2$), 1.59 (*d*, 3H, *J* = 7.1 Hz, -CHCH_3), 1.86 (*m*, 1H, -C*H*(CH_3)$_2$), 2.47 (*d*, 2H, *J* = 7.1 Hz, Ar-CH_2-), 2.54 (*s*, 3H, Ar-COCH_3), 3.67 (*q*, 1H, *J* = 7.1 Hz, -C*H*CH_3), 7.16 (*d*, 2H, Ar-*H*), 7.26 (*d*, 2H, *J* = 8.1 Hz, Ar-*H*), 7.35 (*bs*, 1H, -CON*H*-, Exchangeable with D_2O), 7.53 (*d*, 2H, Ar-*H*) and 7.89 (*d*, 2H, Ar-*H*) ;^{13}C NMR ($CDCl_3$): δ 18.42 (-CHCH_3), 22.41 (-CH(CH_3)$_2$), 26.44 (-*C*H(CH_3)$_2$), 30.20

(Ar-CO*C*H_3), 45.05 (-*C*HCH_3), 47.86 (Ar-*C*H_2-), 118.86 (2 × 2° Ar-*C*), 127.41 (2 × 2° Ar-*C*), 129.69 (2 × 2° Ar-*C*), 129.96 (2 × 2° Ar-*C*), 133.81 (1 × 3° Ar-*C*), 135.44 (1 × 3° Ar-*C*), 138.23 (1 × 3° Ar-*C*), 141.08 (1 × 3° Ar-*C*), 173.10 (-NH*C*O-) and 197.13 (Ar-*C*OCH_3) ;Calcd. for $C_{21}H_{25}NO_2$: N, 4.33. Found: N, 4.14

2-(4-Isobutyl-phenyl)-N-[4-(1-hydroxyimino-ethyl)-phenyl]-propionamide (C)

A solution of 2-(4-isobutyl-phenyl)-*N*-(4-acetyl-phenyl)-propionamide **(B)** (1.62 g, 0.005 mol), hydroxylamine hydrochloride (0.69 g, 0.01 mol) and sodium acetate trihydrate (1.36 g, 0.01 mol) in 25.0 mL ethanol was stirred and refluxed for 4 hr, cooled and added to the crushed ice. The precipitated oxime was filtered, dried and crystallised from ethanol to get 2-(4-Isobutyl-phenyl)-N-[4-(1-hydroxyimino-ethyl)-phenyl]-propionamide **(C)** (1.45 g, 86.83 % yield); m.p. 143-145° C;IR (KBr): 3345, 3035, 2954, 1661, 1592, 1521 and 827 cm^{-1} ;^{1}H NMR ($CDCl_3$): δ 0.89 (*d*, 6H, *J* = 6.7 Hz, -CH(C*H*$_3$)$_2$), 1.52 (*d*, 3H, *J* = 7.1 Hz, -CHC*H*$_3$), 1.86 (*m*, 1H, -C*H*(CH$_3$)$_2$), 2.20 (*s*, 3H, Ar-C(=NOH)C*H*$_3$), 2.44 (*d*, 2H, *J* = 7.2 Hz, Ar-C*H*$_2$-), 3.79 (*q*, 1H, *J* = 7.1 Hz, -C*H*CH$_3$), 7.10 (*d*, 2H, *J* = 8.0 Hz, Ar-*H*), 7.32 (*d*, 2H, , Ar-*H*), 7.54-7.61 (fused *dd*, 4H, Ar-*H*), 7.35 (*bs*, 1H, Ar-C(=NO*H*)CH$_3$, Exchangeable with D_2O), and 7.55 (*bs*, 1H, -CON*H*-, Exchangeable with D_2O) ; ^{13}C NMR ($CDCl_3$): δ 11.27 (Ar-C(=NOH)*C*H$_3$), 18.42 (-CH*C*H$_3$), 21.93 (-CH(*C*H$_3$)$_2$), 29.66 (-*C*H(CH$_3$)$_2$), 44.50 (-*C*HCH$_3$), 46.41 (Ar-*C*H$_2$-), 118.96 (2 × 2° Ar-*C*), 125.80 (2 × 2° Ar-*C*), 126.77 (2 × 2° Ar-*C*), 128.90 (2 × 2° Ar-*C*), 131.96 (1 × 3° Ar-*C*), 138.35 (1 × 3° Ar-*C*), 138.91 (1 × 3° Ar-*C*), 139.89(1 × 3° Ar-*C*), 153.41 (Ar-*C*(=NOH)CH$_3$) and 172.71 (-NH*C*O-) ; Calcd. for $C_{21}H_{26}N_2O_2$: C, 74.52; H, 7.74; N, 8.28. Found: C, 74.11; H, 7.57; N, 7.95.

Evaluation

In vitro Spleen Cell Proliferation Activity

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation [17].

MTT assay procedure

Balb/c Spleenocytes (single cell suspension) were prepared by disrupting the spleen between the glass slides in complete medium (RPMI 1640 with 10% Fetal bovine serum, 1% penicillin-streptomycin) [18]. After 10 min centrifugation at 1000 rpm to separate cells from debris, the cells were washed in RPMI medium, followed by lysis of erythrocytes using Tris ammonium chloride reagent. Spleenocytes 1 x 10^6 cells/ml were plated in 96 well plate in RPMI 1640 containing fetal bovine serum in presence or absence of T-cell stimulator Con-A (2.5µg/well) and in presence or absence of B-cell stimulator LPS (10µg/well). The assays were based on the cellular conversion of tetrazolium bromide also called MTT {3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium} bromide into a formazan product in the tissue culture medium that can be measured at 540nm [19]. Both the test compound and the control were used in three concentrations and placed in three wells assigned respectively to both test and control. The final data was calculated as mean of two experiments.

RESULTS AND DISCUSSION

Novel oxime derivative of ibuprofenamide was successfully synthesized using conventional method and characterised using various spectral techniques which was then further evaluated for their *in vitro* spleen cell proliferation activity using MTT assay. The whole assay was performed on three groups: control, standard (betamethasone) and test at three different concentrations ranging from 10-1000 µg/well. The results of MTT assay are given below in **Table 1**.

Treatment (µG/well)	Unstimulated cells	Stimulated cells Con-A	% Change	Stimulated cells LPS	% Change
Control	0.646 ± 0.03	0.890 ± 0.08	38	1.170 ± 0.18	81.2
Cells + BMS* (1000) Cells + BMS (100) Cells + BMS (10)	0.415 ± 0.046 0.297 ± 0.017 0.264 ± 0.066	0.280 ± 0.019 0.242 ± 0.02 0.213 ± 0.002	-68.5↓ -72.8↓ -76.06↓	0.645 ± 0.036 1.538 ± 0.290 1.319 ± 0.019	-44.87↓ 31.4↑ 12.73↑
Cells + Compound C (1000) Cells + Compound C (100) Cells + Compound C (10)	0.660 ± 0.04 0.211 ± 0.02 0.201 ± 0.02	0.3265 ± 0.016 0.213 ± 0.014 0.232 ± 0.02	-63.31↓ -76.06↓ -73.93↓	0.286 ± 0.052 1.463 ± 0.159 1.986 ± 0.186	-75.55↓ 25.04↑ 69.74↑

** Betamethasone was used as a positive control in concentration ranging from 1000 to 10µg/well. A minimum of 50% stimulation/inhibition is taken as the +ve and acceptable stimulation/inhibition*

[Table 1]

Result of spleen cell proliferation indicates that the oxime derivative showed promising immunomodulatory effects on T-cell and B-cell proliferation. From the table the absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes. As indicted in the table, oxime derivative of ibuprofenamide was more significant at 10µg (69.74↑) in responsiveness of murine splenocytes to B cell mitogen LPS, and 100 µg (-76.06↓) in suppressing the T cell activity in presence of Con-A. The above observations indicate the potential of oxime derivative of ibuprofenamide to suppress the T cell activity and stimulate B cell mitogen LPS and hence resulted in having immunomodulating potential. The results obtained above can be further explored for the design of compounds with immunomodulating behaviour for the treatment of inflammation.

Acknowledgement
Financial support from Council of Scientific and Industrial Research, New Delhi, (India) is highly acknowledged.

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