Synthesized oxime and ketone derivatives of ibuprofen, have higher hepatic safety profile and hepatoprotective potential against acute CCl₄ - induced hepatotoxicity in rats

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Abstract
Despite previously reported high hepatic safety profile of ibuprofen (IBP), but other reports oppose its use in hepatic patients. The aim of this study is to evaluate the possible effect of IBP besides its oxime (OI) and ketone (KI) derivatives in both normal liver and in acute CCl₄-induced hepatotoxicity. Sixty adult male Wistar rats were used, divided into 8 groups. Group 1: received saline water as normal control. Groups 2,3,4: treated with IBP, OI or KI respectively. Group 5: treated with CCl₄ to induce hepatotoxicity. Groups 6,7,8: treated with IBP, OI or KI respectively 30 minutes before CCl₄ administration. Current results showed that despite the apparent hepatotoxic effects of IBP, which were less evident in OI and KI, on normal liver that may be explained by possible immunological idiosyncrasy, they ameliorated both hepatocellular and cholestatic damage induced by CCl₄, which may be attributed to their anti-inflammatory and anti-oxidant potential. OI and KI derivatives, rather than IBP, showed higher hepatic safety profile and stronger hepatoprotective potential against acute CCl₄-induced hepatotoxicity, which favor their use, instead of IBP, in concurrent hepatic diseases.

Key words
Ibuprofen, Oxime, Ketone, Carbon tetrachloride, Hepatotoxicity

1. Introduction
The liver is an important target for the detoxification and deposition of exogenous and endogenous substances. Carbon tetrachloride (CCl₄), a common hepatotoxin, is the most widely used model for liver injury in laboratory animals, triggering liver injury and causing hepatocyte degeneration and cellular death [1-2]. It acts by initiating lipid peroxidation, thereby causing injuries to various organs as kidney, heart, brain, testis and liver [3]. Liver, in particular, is susceptible to oxidative stress due to production of CCl₄ metabolites besides cytokines, which propagate inflammatory response [4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for treating inflammation, pain, and fever not only by inhibition of cyclooxygenases (COXs) [5], but also by their antioxidant potential and their ability to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation [6]. IBP can inhibit neutrophil aggregation besides degranulation and proinflammatory cytokite release by immune cells in vivo and in vitro [7].

The NSAIDs chemical classification identifies four major groups of molecules: (1) carboxylic acids; (2) oxicams carboxamides; (3) pyrazole/furanones; and (4) sulfonanilides diaryl-substituted [8]. Ibuprofen (2-(4-isobutylphenyl) propanoic acid) the first propanoic acid NSAIDs member which marketed successfully. Due to the presence of a single asymmetric carbon atom, the molecule can exist as the (S)-(+) (dextro) or (R)-(−) (levo) isomers. While conventional ibuprofen can occur as a racemic mixture of both isomers, but nearly all of the prostaglandin synthetase inhibitory activity resides with the (S)-(+) isomer and the (S)-(+) single isomer is available in some territories [9]. IBP exerts its anti-inflammatory and analgesic effects mainly by inhibition of the formation of prostanoids. Prostaglandin E₂, a primary mediator of pyresis, is triggered in the hypothalamus by certain pyrogens such as cytokines, endotoxin besides various products from activated leukocytes [10].

Various systemic and local side effect, especially gastrointestinal (GIT) side effects, were reported in long-term use of NSAIDs frequently [11]. Systemic side effects may be attributed to non-selective inhibition of COXs, while the free carboxylic functional group may be associated with the local GI tract irritation. Many strategies were suggested to achieve an anti-inflammatory agent avoiding such side effects. Replacement of the free carboxylic functional group with other heterocyclic bio-isosters such as 1,3,4 oxadiazole [12], 1,2,4-triazole [13], and 1,3,4 thiadiazole [14] caused decrease in gastric upset and enhancement of anti-inflammatory activity [15].
Regarding hepatotoxicity, a few number of hepatotoxicity reports about IBP are associated to both hepatocellular and cholestatic liver damage [16]. Up till now, the use of IBP in hepatic patients is still controversial [2, 17]. The aim of this study was to evaluate the possible effects of IBP and its OI and KI derivatives in both normal liver and in acute CCl₄-induced hepatotoxicity to evaluate their possible impact when used in concurrent liver diseases.

2. MATERIALS AND METHODS

2.1. Animals

The present study was conducted on 60 adult male Wistar rats (14-15) week old, weighing (150-200 g) purchased from El-Neil pharmaceutical company, Egypt. Rats were housed in a standard experimental condition with temperature rang 20-22°C, humidity, 50±5% and night/day cycle, 12 hours). Rats had free access to commercial laboratory chow and tap water all over the time of the experiment. Animals were left for 2 weeks to acclimatize before the start of the experiment. Animal experiments were performed according to the Institutional Animal Care and Use Committee of Faculty of Pharmacy, Minia University, Egypt.

2.2. Chemicals, kits and drugs

OI and KI derivatives were synthesized in Medicinal Chemistry Department, Faculty of Pharmacy, Minia University according to their reported procedure [15]. IBP and CCl₄ were obtained from Sigma Aldrich Corporation, USA. Alanine aminotransferase (ALT) kit, aspartate aminotransferase (AST) kits were obtained from (Reactivos GPL, Barcelona, and España), total and direct bilirubin assay kits, total Nitrite colorimetric kit were obtained from (Biodiagnostics, Egypt). Tumor necrosis factor- α (TNF-α) kit and vascular endothelial growth factor (VEGF) kit were obtained from (Abcam, USA).

Chemistry

The designed IBP derivatives were synthesized as reported [15]. Ibuprofen/oxadiazole hybrid was synthesized through the cyclization of the ibuprofen hydrazide that was obtained through hydrazinolysis of methyl ester of ibuprofen (Scheme 1). Reaction of oxadiazole with N-(4-acetylphenyl)-2-bromoacetamide 2 gave the corresponding ketone (6, KI), which is further reacted with NH₃⋅HCl to yield the oxime (7, OI). Formation of the oxime was confirmed through the appearance of a singlet signal of OH group in the offset region (11.09 ppm) in ¹H NMR and also through shift of the ketonic C=O from (196.95) to C=N at(152.47) ppm in ¹³C NMR as previously reported [15].

2.3. Grouping

Rats were divided into 8 groups, n= (5-10):

- Group 1: received saline water and acted as normal control.
- Group 2: IBP drug control group (received single i.p injection of 2mg IBP dissolved in 0.5 mL 0.1% dimethyl sulfoxide (DMSO) [15].
- Group 3: OI drug control group (received single i.p. injection of 4.3mg of OI dissolved in 0.5 mL 0.1% DMSO [15].
- Group 4: KI drug control group (received single i.p injection of 4.2mg of KI dissolved in 0.5 mL 0.1% DMSO [15].
- Group 5: CCl₄ treated group (received single i.p injection of CCl₄ in a dose 1 mg/kg) dissolved in olive oil solution in 1:1 ratio [18].
- Group 6: IBP+CCl₄ treated rats (received single i.p. injection of IBP, 30 min before CCl₄ injection).
- Group 7: OI+CCl₄ treated rats (received single i.p. injection of OI, 30 min before CCl₄ injection).
- Group 8: KI+CCl₄ treated rats (received single i.p. injection of KI, 30 min before CCl₄ injection).

IBP, OI and KI doses were calculated in equimolar doses to (0.05 mol, 17 mg/kg) of the standard drug (indomethacin) [18]. After 24 hours from CCl₄ administration, all animals in all groups were sacrificed under ether anesthesia then blood samples were collected from jugular veins and were left to clot for a period of 30 minutes at room temperature for serum isolation. Then the liver was dissected washed by normal saline and weighed then put in 10% formalin for histopathological evaluation.

2.4. Biochemical Assessment

2.4.1. Determination of liver function tests

Determination of ALT, AST and total and direct bilirubin were done by colorimetric commercial kits according to the manufacturer instructions.

2.4.2. Determination of TNF-α, VEGF and NO serum levels

Quantitative determination was performed using a sandwich enzyme linked immune sorbent assay (ELISA) kit (Abcam,
USA). While Bio-diagnostic nitrite assay kit was used as an accurate and convenient colorimetric method for measurement of endogenous nitrite concentration and as indicator of nitric oxide production in biological fluids [19].

2.5. Histopathological assessment

Livers were identified and observed for any gross appearance and color change. Liver samples were fixed in 10% formalin, specimens, cleared in xylene and embedded in paraffin blocks then 4 microns sections were collected on glass slides, deparaffinized and stained by Hematoxylin and eosin (H&E) stain and examined through an electric light microscope.

2.6. Statistical analysis

Results were expressed as range and means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by the Tukey post analysis test was used to analyze the results for statistically significant difference. p values less than 0.05 were considered significant. Graph Pad Prism was used for statistical calculations (version 5.01 for Windows, Graphpad Software, and San Diego California USA.

3. RESULTS

3.1. Effect of IBP, OI and KI on liver histopathology in normal and CCl4-intoxicated rats.

In the present study, the histopathological examination showed that normal control group showed normal hepatocytes separated by normal sinusoids; the hepatocytes are rounded and showing central nucleus and eosinophilic cytoplasm. IBP treated group showed hepatocytes with mild fatty change and small areas of congestion and inflammation is detected. OI and KI treated group showed slightly normal hepatocytes with little intervening inflammatory cells and small scattered areas of congestion. On the other hand, CCl4 treated rats have livers with slightly distorted hepatocytes with some scattered inflammatory cells and congested areas in between. IBP+CCl4 treated rats had hepatocytes with marked inflammation and congestion in portal tract and in between hepatocytes. In OI+CCl4 treated rats the hepatocytes are with focus of inflammatory cells and congestion. In KI+CCl4 sections examined from the liver revealed distorted cords and sinusoids of hepatocytes with some scattered inflammatory cells and areas of congestion (Figure 1).

3.2. Effect of IBP, OI and KI on relative liver weight and liver function tests in normal and CCl4-intoxicated rats.

No significant difference was observed in relative liver weight, calculated as absolute liver weight/whole body weight ratio (p >0.05). Activities of ALT, AST were significantly higher in IB, OI, KI, and CCl4 treated groups compared to normal control group (a: p< 0.05). Activities of ALT, AST were significantly lower in IBP+CCl4, OI+CCl4 and KI+CCl4 compared to CCl4-control group (b: p< 0.05). OI and KI groups showed significantly lower ALT, AST activities compared to IBP (c: p< 0.05) (Table 1).

IBP only, but not OI or KI, showed significantly higher levels in total and direct bilirubin compared to normal control (a: p< 0.05). Levels of total and direct bilirubin were significantly lower in IBP+CCl4, OI+CCl4 and KI+CCl4 compared to CCl4-control group (b: p< 0.05). Levels of total and direct bilirubin were also significantly lower in OI and KI groups compared to IBP (c: p< 0.05) (Table 1).

3.3. Effect of IBP, OI and KI on TNF-α, VEGF and NO levels in normal and CCl4-intoxicated rats.

Levels of TNF-α, VEGF and NO levels were significantly higher in OI, KI, IBP and CCl4 treated groups compared to normal control group (a: p< 0.05). Levels of TNF-α, and NO, but not VEGF, were significantly lower in IBP+CCl4, OI+CCl4 and KI+CCl4 compared to CCl4-control group (b: p< 0.05) (Table 1).
Drug induced-hepatotoxicity is a major concern in clinical practice. IBP is one of the most commonly used over the counter drugs all over the world [5]. The most reported side effects are related to GIT. Oxime and ketone derivatives was reported to have less GIT side effects [13, 15]. But various concerns regarding possible hepatotoxicity have been raised. Despite having relatively safe hepatic profile [16], their use in hepatic patients is still under question. While it may have beneficial effects in CCl₄-induced liver fibrosis [2]. However, other reports oppose their use in hepatic disease like chronic hepatitis C [17]. The current study evaluated the impact of IBP, OI and KI in both normal liver and acute CCl₄-induced hepatotoxicity. In the current study, CCl₄ caused expected significant histopathological and biochemical abnormalities (increase in all liver function tests and serum levels of TNF-α, VEGF and NO). CCl₄ was reported to cause marked hepatic damage manifested as morphological non-transparent white punctate foci which are indicative of focal damage along with histopathological abnormalities in the form of inflammatory infiltrate, congestion and distorted hepatocytes [3-20]. Such changes may be attributed to CCl₄ metabolic products, as trichloromethyl and proxy chloromethyl free radicals, produced by the oxygenase system of cytochrome P450 in endoplasmic reticulum. The trichloromethyl radical reacts with different biological substances such as proteins, nucleic acids, amino acids fatty acids and lipids [21, 22]. CCl₄ is initially activated by cytochrome 2E1, 2B1 and 2B2, CCl₄ radical reacts with different biological substances such as proteins, nucleic acids, amino acids fatty acids and lipids [21, 22]. 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The mechanism of IBP-induced hepatotoxicity is not fully clear, but definitely thought to be multi-factorial. It may be largely immunological idiosyncrasy proved by rapid onset which suggests a toxic metabolic byproduct, while accompanied hypersensitivity response points to an immuno-allergic reaction [25]. IBP-induced liver injury severity ranges from asymptomatic increase in serum aminotransferase levels to acute cholestatic hepatitis or acute liver failure which may require transplantation. However, in most instances, complete recovery is expected after several months if drug is stopped. Recurrence of hepatic injury is related to IBP re-exposure which should be avoided in such cases [26].

Other possible mechanisms of NSAIDs-related hepatotoxicity were investigated by in vitro animal models using rat liver mitochondria, freshly isolated rat hepatocytes showed that diphenylamine, a common component in NSAIDs structure, can uncouple oxidative phosphorylation leading to decrease in hepatic ATP content and eventually hepatocyte injury [27]. In the current study, when IBP, OI and KI given alone, they caused significant higher TNF-α, VEGF and NO levels compared to normal control. NO is a short-lived pluripotent free radical molecule which influences various physiological functions as neurotransmission, blood flow regulation and immune response [28]. Induction of iNOS lead to induction of tissue damage through peroxynitrite formation [29], which can react with sulphydryl residues in DNA and cell membranes, causing lipid peroxidation and finally cytotoxicity [30]. Similarly, IBP-induced gastric mucosal injury may be mediated by increased NO level, which may be reduced by inhibition of nitric oxide synthase [31]. VEGF is a common angiogenic growth factor promoting angiogenesis and tissue regeneration [32]. Elevated circulating VEGF levels may be related to various models of liver impairment. It may contribute to liver fibrosis development [33]. It was reported that IBP intensified secretion of LPS-induced VEGF in human micro-vascular endothelial cells culture [34,35].

In the current study, when IBP, OI and KI were given to CCl₄-intoxicated rats, they ameliorated most of CCl₄-induced hepatotoxic effects. They improved liver histopathological manifestations and liver function tests. IBP has shown clear antioxidant properties may be through inhibition of NFκB translocation of NFκB, thus affecting TNF-α expression [7].

Conclusion

In summary, the current work highlighted the possible beneficial potential of IBP, OI and KI derivatives in experimental model of acute CCl₄-induced hepatotoxicity. Despite the apparent hepatotoxic effects of IBP, which was less evident in OI and KI, on normal liver that may be explained by immunological idiosyncrasy, interestingly, they ameliorated partially or totally hepatocellular and cholestatic damage caused by CCl₄. When administered to CCl₄ intoxicated rats, they did not exaggerate CCl₄ hepatotoxicity; on the contrary, they reversed most of CCl₄-induced hepatotoxic effects, which may be attributed to their anti-inflammatory and anti-oxidant potential. This effect was more pronounced in OI and KI derivatives rather than IBP itself. Higher hepatic safety profile and stronger hepatoprotective effect of OI and KI, favor their use instead of IBP in concurrent hepatic diseases.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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