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Synthesized oxime and ketone derivatives of ibuprofen, have higher hepatic safety profile and hepatoprotective potential against acute CCl_4 - 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 CCl_4-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 CCl_4 to induce hepatotoxicity. Groups 6,7,8: treated with IBP, OI or KI respectively 30 minutes before CCl_4 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 CCl_4, 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 CCl_4-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 (CCl_4), 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 CCl_4 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 cytokine 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) sulphonanilides 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 besidesvarious 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].

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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 CCl_4-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 CCl_4 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

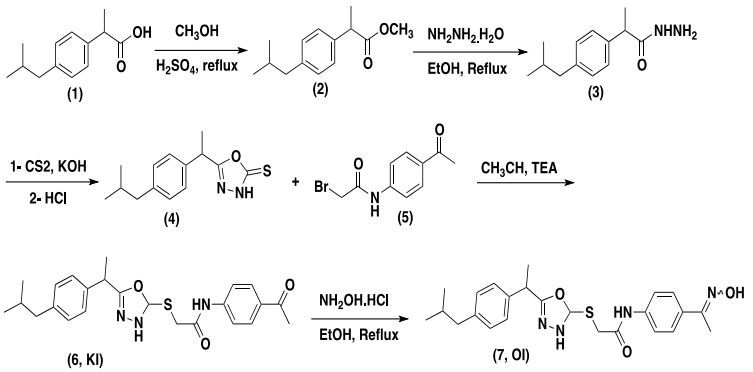
The designed IBP derivatives were synthesized as reported [15]. Ibuprofen/oxadiazole hybrid was synthesized through the cyclization of the ibuprofen hydrazide that was obtained through hydrazinolysis of methyl ester of ibuprofen (Scheme 1). Reaction of oxadiazole with *N*-(4-acetylphenyl)-2-bromoacetamide 2 gave the corresponding ketone **(6, KI)**, which is further reacted with $NH_2OH.HCl$ 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 ^{13}C 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: CCl_4 treated group (received single i.p injection of CCl_4 in a dose 1 mg/kg) dissolved in olive oil solution in 1:1 ratio [18]**.**
- Group 6: IBP+CCl_4 treated rats (received single i.p. injection of IBP, 30 min before CCl_4 injection).
- Group 7: OI+CCl_4 treated rats (received single i.p. injection of OI, 30 min before CCl_4 injection).
- Group 8: KI+CCl_4 treated rats (received single i.p. injection of KI, 30 min before CCl_4 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 CCl_4 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.



Scheme 1: Synthesis of KI and OI derivatives

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). Oneway 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 CCl_4-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, CCl_4 treated rats have livers with slightly distorted hepatocytes with some scattered inflammatory cells and congested areas in between. IBP+CCl_4 treated rats had hepatocytes with marked inflammation and congestion in portal tract and in between hepatocytes. In OI+CCl_4 rats the hepatocytes are with focus of inflammatory cells and congestion. In KI+CCl_4 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 CCl_4-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 CCl_4 treated groups compared to normal control group (a: p< 0.05). Activities of ALT, AST were significantly lower in IBP+CCl_4, OI+CCl_4 and KI+CCl_4 compared to CCl_4-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+CCl_4, OI+CCl_4 and KI+CCl_4 compared to CCl_4-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)**.

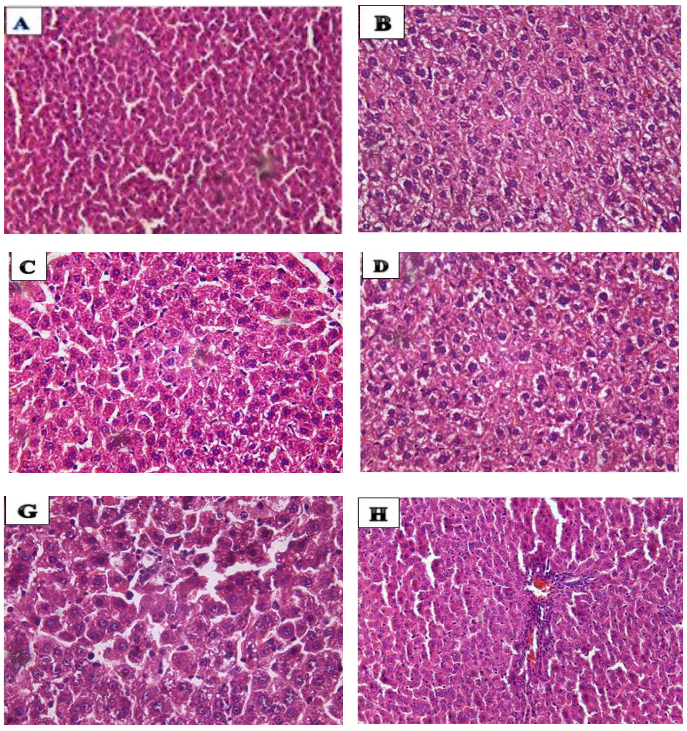


Figure 1: Histopathologicl Examination. A: normal control group with normal liver; **B:** OI treated group with normal hepatocytes with little intervening inflammatory cells and small scattered areas of congestion; **C:** KI treated group with normal hepatocytes, little intervening inflammatory cells and small scattered areas of congestion; **D:** IBP treated group with mild fatty change and small areas of congestion; **E:** CCl_4 treated group with slightly distorted hepatocytes with some scattered inflammatory cells and congested areas in between; **F:** OI+CCl_4 group focus of inflammatory cells and congestion; **G:** KI+CCl_4 group with distorted cords and sinusoids of hepatocytes with some scattered inflammatory cells and areas of congestion; **H:** IBP+CCl_4 group with moderate fatty change and marked inflammation and congestion in portal tract and in between hepatocytes.

3.3. Effect of IBP, OI and KI on TNF-α, VEGF and NO levels in normal and CCl_4-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+CCl_4, OI+CCl_4 and KI+CCl_4 compared to CCl_4-control group (b: p< 0.05) **(Table 1)**.

Table 1: Biochemical assessment

	Control N=5	IBP N=5	OI N=5	KI N=5	CCl4 N=5	IBP + CCl4 N=5	OI + CCl4 N=5	KI + CCl4 N=5	P value
Rat weight	186±6.5	186.4±16.5	177±17.2	182±10.4	168.2±17.3	169.4±13.9	164.8±10.8	169.2±22.7	0.169
Absolute Liver weight	4.2±0.3	4.5±0.6	4.4±0.4	4.9±0.6	5.1±0.3	5 ±0.9	4.6±0.4	4.6±0.4	0.098
Relative Liver Weight	2.25	2.42	2.48	2.69	3.03	2.96	2.79	2.72	0.064
ALT	41.8±16.7	a 127.4±13.7	a 114.6±10	a 120.8±13.3	a 317.1±22.8	b 234.7±84.7	b,c 124.5±18.6	b,c 122.1±29.8	<0.001*
AST	24.9±5.2	a 206.2±71	a 137.7±15.4	a 142±32.9	a 470.1±60.2	b 5096.9±2	b,c 38162.9±	b,c 177.3±80.1	<0.001*
T. bilirubin	1.9±0.3	a ±1.034.4	1.9±0.7	2.9±0.8	a 10.6±1.1	b 4.7±1.1	b,c 2.5±0.2	b,c 2.6±0.4	<0.001*
D. bilirubin	0.9±0.3	a 2.4±0.9	1.2±0.4	1.5±0.6	a 4±0.5	b 2.5±1.1	b,c 1.1±0.13	b,c 1.3±0.31	<0.001*
TNF	11.9±2.9	a 38.6±3.6	a 31.2±5.6	a 33.4±2.3	a 67.6±6.8	b 47.2±1.9	b,c 35.4±6.3	b,c 34±4.4	<0.001*
VEGF	307.4±46.3	a 453.4±17.6	a 435.4±8.2	a 511.4±22.9	a 597±27.5	541±26	562.4±20.4	559±38.4	<0.001*
NO	14.9±6.1	a 15.4±3.5	a 23.1±2.7	a 15.2±2.9	a 41.6±2.3	b,d 20.6±2.3	b 29.5±1.7	b,d 21.7±2.1	<0.001*

Data expressed as range / mean ± SD; *: Significant difference between the eight groups using One-way ANOVA test with post hoc Tukey analysis between each two groups; a: Significant difference with control group ($p<0.05$); b: Significant difference with CCl_4 group ($p<0.05$); c: Significant difference with IBP+CCl_4 group ($p<0.05$); d: Significant difference with OI+CCl_4 group ($p<0.05$)

4. DISCUSSION

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 CCl_4- 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 CCl_4-induced hepatotoxicity.

In the current study, CCl_4 caused expected significant histopathological and biochemical abnormalities (increase in all liver function tests and serum levels of TNF-α, VEGF and NO). CCl_4 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 CCl_4 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, CCl_3* and CCl_3OO□ radicals are produced, then oxidative stress follows producing lipid peroxidation and inflammatory response. CCl_4 also causes NFκB activation and transforming growth factor (TGF-β) production and eventually liver fibrosis [4]. In addition, CCl_4 increases hepatic Inducible nitric oxide synthase (iNOS) mRNA and nitric oxide (NO) levels. This leads to iNOS-derived NO production, activates down-stream of inflammatory cytokines as NF-*κ*B followed by reactive oxygen species generation [23].

In the current study, when IBP, OI and KI were given alone, they all caused hepatocellular damage (higher ALT, AST serum activities), but only IBP, showed cholestatic damage (higher total and direct bilirubin serum levels). Histopathological manifestations were less evident in OI and KI compared to IBP. Abnormal hepatic architecture was previously reported in rat liver sections treated with ibuprofen; the hepatocytes showed prominent vesicular nuclei, granular cytoplasm and dilated

sinusoids [24]**.** Although serious liver toxicity with ibuprofen are rarely reported, three hepatitis C patients case series, who developed more than five-fold increase in liver enzyme transaminases after ibuprofen ingestion, even in therapeutic doses was reported [17].

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 sulhydryl 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 CCl_4-intoxicated rats, they ameliorated most of CCl_4–induced hepatotoxic effects. They improved liver histopathological manifestations and liver function tests. IBP has shown clear antioxidant properties may be through inhibition of NFκB activation [7]. IBP can prevent CCl_4- induced lipid peroxidation via increasing glutathione, catalase and super oxide dismutase levels. IBP attenuated macrophages recruitment/activation in liver after Fas stimulation, which is critical for glutathione depletion in CCl_4-induced liver injury, thus antioxidant activity of IBP may explain, in part, their anti-fibrotic potential [36]. IBP was also reported to have anti-fibrotic effects which may be due to the inhibition of COX. IBP prevented the expression of TGF-β induced by CCl_4 administration. TGF-β is one of the most effective profibrogenic mediators because it stimulates the hepatic stellate cell (HSC) phenotypic change from quiescent state to the proliferating phenotype capable of increasing production and deposition of extracellular matrix (ECM). Treatment with IBP avoided the deposition of collagen fibers according to the degree of the effect on the TGF-β expression [2].

In the current study, IBP, OI and KI caused significant lower levels of serum TNF-α and NO, but did not had any significant effect on elevated VEGF levels, compared to their corresponding drug controls. OI and KI caused more significant decrease in CCL_4-intoxicated groups compared to IBP. It was reported that IBP down-regulated iNOS mRNA in Lipopolysaccharides and interferon gamma treated rats. Also, Liu et al reported that chronic treatment with IBP prevented the increase in renal iNOS expression of diabetic nephropathy rats [37]. IBP also inhibited the inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β) activity, thereby preventing 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 CCl_4-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 CCl_4. When administered to CCl_4 intoxicated rats, they did not exaggerate CCl_4 hepatotoxicity; on the contrary, they reversed most of CCl_4-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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