

POSSIBLE IMMUNOMODULATING EFFECT KETOPROFEN AND KETOPROFEN LYSINE SALT ON THE BRAIN

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ABSTRACT

Ketoprofen lysine salt (KL) is a new non steroidal antiinflammatory drug (NSAID) competing with ketoprofen (K) on the market. The former is believed to have gastroprotective properties, the latter to kill acute pain. Many people drink ethanol and use NSAIDs on the days after. The aim of the study was to find out if K and KL have any immunomodulating effect on the brain after ethyl alcohol intoxication in a rat model.

The experiment was carried out on 36 female Wistar rats. Animals were bred at the Experimental Medicine Centre at the Medical University of Lublin. The ethanol, NaCl, K and KL were administered by gavage. The animals were randomly divided into groups of 6. On day 7 all animals were sacrificed. Their brains and blood were collected for laboratory tests.

There were no statistically significant differences in the concentration of IL-10 in the central nervous system between the study groups. We observed a positive correlation between brain IL-10 levels and body weight, also a correlation between brain IL-6 and brain weight. The level of IL-10 in the brain correlated with the concentration of IL-6 of all animals.

Conclusions

KL and K have some immunomodulating effect on the brain.

There is a positive correlation between the level of IL-6 and IL-10 in rat brains.

Keywords: immunomodulation, ketoprofen, ketoprofen lysine salt, interleukin 10, interleukin 6.

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INTRODUCTION

Ketoprofen (K) is a non-steroidal antiinflammatory drug (NSAID) widely used for acute pain [1]. There is a new preparation: ketoprofen lysine salt (KL). KL is characterized by higher solubility compared to K. KL's more rapid gastrointestinal absorption in comparison to K is emphasized [2]. There is data suggesting that KL has an increased gastric tolerance *in vitro* [3,4]. In an animal model of alcohol induced gastric mucosa damage KL had an increased gastrointestinal and renal tolerability compared to K [2]. The new polymorphic form of KL *in vivo* showed significantly higher absorption and different pharmacokinetics than commercial KL [5].

Interleukin 6 (IL-6) is elevated in majority of inflammatory conditions. The cytokine is involved not only in inflammation and infection responses but also in the regulation of metabolic, regenerative, and neural processes [6]. It plays a role in activation of some regulatory and immunologic pathways. Acute inflammation is characterized by infiltration of neutrophils. With time they are replaced by monocytes and T cells. In this way tissue damage from reactive oxygen-species (ROS) is prevented. Endothelial cells and other vascular elements are activated by microbial products, interleukin 1 β (IL-1) and tumor necrosis factor α (TNF α), produce various chemokines like IL-6. It leads to the attraction of neutrophils. Proteolytic processing of the IL-6R from neutrophils subsequently leads to IL-6 *trans-signalling* in resident tissue cells. This switches from neutrophil to monocyte recruitment [7,8,9].

There is data suggesting that neither K nor KL are able to reduce the increased expression of IL-6 in the course of neuroinflammation and neuropathic pain [5].

It was observed by Matthews et al. that mice in the absence of IL-6 develop glucose intolerance and insulin resistance. The IL-6^{-/-} mice exhibited signs of liver inflammation [10]. When the IL-6R gene was deleted in hepatocytes, mice developed a reduction in insulin sensitivity and glucose tolerance [11]. Patients receiving the IL-6R neutralizing drug (tocilizumab), increased body weight, developed hypertriglyceridemia and hypercholesterolemia during the treatment period. This shows a link between blockade of IL-6 and impaired metabolic homeostasis [12]. Transgenic mice which overexpressed IL-6 and sIL-6R, showed massive stimulation of gp130 signalling. They were smaller than wild type mice and did not develop visible fat [13]. Considering cited results on mice lacking the hepatic IL-6R (since hepatocytes express membrane bound IL-6R), it can be suspected that the effect of IL-6 on hepatic control of insulin sensitivity and glucose tolerance is mediated by IL-6 [14].

In an *in vivo* study it was recorded that IL-6 with sIL-6R sensitized rat skin nociceptors to heat. Researchers observed that the heat-activated ionic current was modulated by IL-6 in rat sensory neurons [15]. Experiments conducted on mice lacking IL-6 showed that this cytokine is crucial for the perception of pain. The mice did not develop thermal hyperalgesia [16]. Murphy et al. studied how nerve damage caused severe chronic neuropathic pain that was not relieved by the use of NSAIDs or opioids. Researchers demonstrated in an experiment conducted on rodents that the expression of

IL-6 mRNA on sensory neurons determined the perception of neuropathic pain. In animals with a mutation that did not allow the production of IL-6, despite nerve damage, skin hypersensitivity to thermal or pressure stimuli did not occur. A decrease in the level of substance P and galanin was found in the central sensory projections of neurons. The opposite phenomenon was observed in wild-type mice. Researchers found that intrathecal injection of IL-6 induced accelerated galanin synthesis in neurons, especially in the dorsal root ganglia of the lumbar spine. In this way, it was confirmed that IL-6 participates in the pathogenesis of neuropathic pain and significantly affects the level of neuropeptides mediating the conduction of pain stimuli: galanin and substance P [16].

Sheppard et al. proved that the administration of an IL-6-neutralizing drug tocilizumab, reduces the activity of arthritis and the level of pain experienced in connection with the disease [17].

Interleukin10 (IL-10) is a pleiotropic cytokine. IL-10 is a potent anti-inflammatory and immunosuppressive molecule. IL-10 acts as a regulator of lymphoid cells. IL-10 inhibits activation of many cytokine synthesis. It also regulates macrophages' functions. This way IL-10 can be considered a significant suppressor of NK cells, macrophages as well as T cells. It was also found that IL-10 is able to regulate proliferation of B cells and influence their differentiation [18]. In development of numerous diseases IL-10 deregulation was recorded: neuropathic pain, Parkinson's disease, Alzheimer's disease, osteoarthritis, rheumatoid arthritis, psoriasis, systemic lupus erythematosus, type 1 diabetes, inflammatory bowel disease, and allergy [19].

Alcohol is a neurotoxin. Its' use apart from toxic effects produces also neuroinflammation [20]. Both alcohol and NSAIDs increase the risk of inflammation and stomach ulcers. K as a non-selective cyclooxygenase inhibitor, inhibits both constitutive cyclooxygenases and those induced by inflammation. K inhibits the transfer of leukocytes from the vascular bed to tissues affected by inflammation and inhibits elastase released by neutrophils in the inflammatory focus. It is known that K reduces the submucosal flow of blood in the stomach and reduces the amount of secreted gastric juice, but at the same time it does not reduce the amount of hydrogen ions secreted into the stomach lumen, which significantly increases the risk of inflammation, ulceration, and even bleeding of the upper gastrointestinal tract [21]. Chronic gastritis can lead to reduced food intake and weight loss.

Alcohol consumption remains high in Poland and other Central European countries. In 2021, the average *per capita* alcohol consumption was 9.7 liters [22]. The predominant style of alcohol consumption in central and eastern Europe is binge drinking [23]. This applies to both men and women. Many people use NSAIDs to relieve headaches the day after an alcohol overdose, thus increasing the risk of damage to the stomach lining.

Alcohol abuse and *Helicobacter pylori* infection are two independent risk factors for the development of stomach cancer. Aziz et al. found that IL-10 inhibits glucose uptake and glycolysis. If IL-10 is lacking in CD8+ cells, damaged mitochondria accumulate. Therefore, the production of interleukin 1- β (IL1 β) increases. Under such conditions, IL-1 β promotes *H. pylori* infection. IL-1 then facilitates the division of cancer cells. If a patient chronically abuses alcohol and is infected with *H. pylori*,

IL-10 is inhibited. This is associated with impaired functioning of CD8+ cells [24].

Attempts are being made to create other new combination preparations based on K. For example, a combination of gabapentin and KL. Physicians treating chronic pain, i.e. pain lasting longer than 3 months, are looking for adjuvants that help improve pain control. Gabapentin may be such an adjuvant. There is hope for it, especially in cases of neuropathic pain. Aramini et al. performed co-crystallization to obtain KL-gabapentin. This new product was tested in rats in a carrageenan-induced neuropathic inflammatory pain model. After carrageenan injection, the level of IL-6 in the spinal cord was examined. Since the level of pro-inflammatory cytokine was high in the central nervous system, there must have been an acute inflammatory reaction there. KL-GABA c-crystal had an effective analgesic and anti-inflammatory effect on the tested animals. However, the administration of the drug did not significantly affect the level of IL-6 [25].

AIM

The aim of the study was to find out what is the effect of K and KL on IL-6 and IL-10 levels in the brain after ethyl alcohol intoxication in a rat model and if they have any immunomodulating effect on the brain.

MATERIALS AND METHODS

K (Ketonal) solution for injection manufactured by Sandoz GmbH, Wien, Austria was purchased in ampoules 50mg/mL. KL (Ketonal Sprint) in the form of granules (Sandoz GmbH, Wien, Austria) was used. In order to prepare solution 0.9% NaCl was used (B. Braun, Melsungen AG, Hessen, Germany). Ethanol (95% v/v Spirytus) was purchased from Polmos (Lublin, Poland). The 50% v/v ethyl alcohol solution was prepared with 0.9% NaCl. The dose of alcohol was intended to produce the model of binge drinking in an adult human. The doses of K and KL used are respective to the recommended painkilling doses for humans.

The experiment was carried out on 36 young non-pregnant female Wistar rats of body mass 190-205g at the beginning of the experiment. Animals were bred at the Experimental Medicine Centre (EMC) at the Medical University of Lublin (Lublin, Poland). The herd originated from Charles River Laboratories (Cologne, Germany). The rats were 7 weeks old on day 1 of the experiment. The procedures were conducted according to the European law at the EMC. Standard laboratory conditions with relative air humidity of 55–60%, air temperature of 21–22 °C and a 12-hour light/dark cycle prevailed in the EMC. The animals had free access to water (sterilized with ultraviolet rays) and rodent feed purchased from Altromin International (Lage, Germany). The ethanol, NaCl, K and KL were administered by gavage through a gastric tube. The animals were randomly divided into groups of 6:

Group 1. on day 1 was administered 50% ethanol at the dose of 5mL/kg b.w.

Group 2. on day 1 was administered 0.9% NaCl at the dose of 5mL/kg b.w.

Group 3. on day 1 was administered 0.9% NaCl (5mL/kg b.w.) and K at the dose of 8mg/kg b.w. on days 2-6.

Group 4. on day 1 was administered 50% ethanol (5mL/kg b.w.) and K at the dose of 8mg/kg b.w. on days 2-6.

Group 5. on day 1 was administered 9% NaCl (5mL/kg b.w.) and KL at the dose of 12.8mg/kg b.w. on days 2-6.

Group 6. on day 1 was administered 50% ethanol (5mL/kg b.w.) and KL at the dose of 12.8mg/kg b.w. on days 2-6.

On day 7 all animals were decapitated with a guillotine. No anaesthetic was used in order to prevent possible interactions with laboratory tests' results and brain structure. The body and brain mass were measured. In order to determine blood counts 200µL of blood was drawn for EDTA. We used an automatic hematological analyzer. The rest of blood was allowed to clot. Then it was centrifuged. Laboratory tests were done using an Erba Mannheim XL-60 biochemical analyser (Mannheim, Germany). Brains were dissected and weighed. We used a weighing scale model SBS-LW-500/10 manufactured by Steinberg Systems of weighing range 0.01g to 500g (Berlin Germany). The brains were carefully removed immediately after the decapitation and immersed in cooled (2–8 °C) saline to remove blood. The brains were washed with a 20 µL injection solution. The concentration of IL-10 and IL-6 was measured by a ready-to-use sandwich enzyme immunoassay (ELISA) diagnostic kit dedicated to rats' tissues (ELISA Kits for IL-10, IL-6, Cloud-Clone Corp., Katy, TX, USA). All procedures were conducted according to the manufacturer's instructions.

The data were analysed using IBM SPSS Statistics v.25 software. Comparisons between the groups were made using the Kruskal-Wallis test. Correlations between quantitative variables were found using Spearman's correlation coefficient rho. The significance level was $p < 0.05$.

The project was approved by the Local Ethical Committee for Animal Experiments in Lublin (70/2021 issued on 8 NOV 2021).

RESULTS

The mean ($\pm$ SD) IL-6 concentration in the brains was 385.6 $\pm$ 72 pg/mL in group 1, 394.66 $\pm$ 67 in group 2, 415.33 $\pm$ 49.31 in 3, 362.66 $\pm$ 73.28 in 4, 394 $\pm$ 56.64 in 5 and 319.16 $\pm$ 77.38 in 6. The IL-10 concentrations were: 133.2 $\pm$ 39.75pg/mL in group 1, 136.33 $\pm$ 34.62 in 2, 135.5 $\pm$ 18.93 in 3, 92.33 $\pm$ 32.5 in 4, 131.29.88 in 5 and 130.5 $\pm$ 12.2 in 6. The concentration of IL-10 in rat brains was lower than IL-6 concentration in all the study groups. The use of K or KL did slightly reduce the levels of IL-6 and IL-10 in rats previously intoxicated with ethanol. There was no statistically significant differences between groups in blood morphology, biochemistry parameters and in IL-6 and IL-10 concentrations in the brains when analysed with Kruskal-Wallis' test (Table 1.). A significant positive correlation between the level of IL-6 and IL-10 in rat brains was found in Spearman's rank-order correlation coefficient test.

Spearman's rank correlation coefficients are a non-parametric measure of the statistical relationship between random variables. The result is marked with the Greek letter rho. It takes values from -1 to +1. A score of +1 means that an increase in one value always leads to an increase in the other. Since no statistically significant differences were observed between the groups, when

examining Spearman's rank correlation coefficients between the results obtained in the studied groups, we put all the results from group 1,2,3,4,5,6 into one set. This means that we examined the correlation between IL-6 and IL-10 in all tested animals. The obtained result does not show any correlation for the control group, nor for the groups consuming alcohol, neither K nor KL. According to our knowledge, this is the best way to look for correlations between two parameters.

We tried to differentiate if K or KL had more influence on IL-6 and IL-10 in rat brains. Spearman's rank correlation coefficient was calculated for groups 1,2,3,4, where groups 3 and 4 received K and rho was 0.522; $p < 0.001$.

It means that the correlation between IL-6 and IL-10 in rat brains in control groups (treated with NaCl or ethanol) and exposed to K (after NaCl or ethanol) was very strong. Spearman's rank correlation coefficient for groups 1,2,5,6 where groups 5 and 6 received KL was: rho

=0,278; $p=0.2$. It means less positive correlation than in case if K.

Correlations between blood morphology and biochemical parameters were also detected (pooling results from groups 1,2,3,4,5,6). There is a correlation between brain IL-10 level and body mass (rho 0.454), IL-6 in the brain and brain mass (rho 0.364), lymphocyte % and serum albumin (rho 0.528), haemoglobin concentration and lymphocyte% (rho 0.46), serum creatinine and white blood cell count (rho 0.34), white blood cell count and platelet count (rho 0.585), erythrocyte count and haemoglobin concentration (rho 0,768).

There was a negative correlation between serum glucose concentration and IL-10 concentration in the brains (rho-0.386), white blood cell count and serum albumin concentration (rho -0.369), platelet count and % lymphocytes (rho -0.354) and platelet count vs serum albumin concentration (rho -0.408).

Tab.1

Results of the laboratory tests. SD-standard deviation, n=6.

Mean $\pm$ SD	Group 1. 50% ethanol	Group 2. 0.9% NaCl	Group 3. 0.9% NaCl (on day 1) and K (on days 2-6)	Group 4. 50% ethanol (on day 1) and K (on days 2-6)	Group 5. 9% NaCl (on day 1) and KL (on days 2-6)	Group 6. 50% ethanol (on day 1) and KL (on days 2-6)
body mass on day 7 [g]	215.67 $\pm$ 21.20	207.83 $\pm$ 12.45	213.00 $\pm$ 16.42	195.00 $\pm$ 7.51	210.00 $\pm$ 20.77	210.00 $\pm$ 17.49
brain mass [g]	1.64 $\pm$ 0.09	1.71 $\pm$ 0.08	1.75 $\pm$ 0.09	1.68 $\pm$ 0.07	1.76 $\pm$ 0.06	1,61 $\pm$ 0.08
IL-6 concentration in the brains [pg/mL]	385.6 $\pm$ 72	394.66 $\pm$ 67	415.33 $\pm$ 49.31	362.66 $\pm$ 73.28	394 $\pm$ 56.64	319.16 $\pm$ 77.38
IL-10 concentration in the brains [pg/mL]	133.2 $\pm$ 39.75	136.33 $\pm$ 34.62	135.5 $\pm$ 18.93	92.33 $\pm$ 32.5	131.29.88	130.5 $\pm$ 120.2
lymphocytes [% of leucocytes]	76 $\pm$ 5	83.16 $\pm$ 7	54.83 $\pm$ 8	55.83 $\pm$ 7	67.5 $\pm$ 5	63 $\pm$ 8
serum albumin level [g/dL]	4.27 $\pm$ 0.07	4.48 $\pm$ 0.55	3.85 $\pm$ 0.66	3.64 $\pm$ 0.26	3.72 $\pm$ 0.67	3.89 $\pm$ 0.28
serum glucose concentration [mg/dL]	93.33 $\pm$ 0.79	101.33 $\pm$ 0.48	101.67 $\pm$ 2.43	107.50 $\pm$ 1.78	95.00 $\pm$ 1.24	113.50 $\pm$ 1.60
serum creatinine concentration [mg/dL]	0.21 $\pm$ 0.02	0.22 $\pm$ 0.03	0.22 $\pm$ 0.03	0.22 $\pm$ 0.02	0.23 $\pm$ 0.04	0.21 $\pm$ 0.02

Tab.1 ont.

Results of the laboratory tests. SD-standard deviation, n=6.

white blood cell count [/ μ L]	8526.67 $\pm$ 4637.64	9670.00 $\pm$ 3565.55	9110.00 $\pm$ 2019.63	12533.33 $\pm$ 3021.81	11758.00 $\pm$ 932.72	11200.00 $\pm$ 2608.52
erythrocyte count in the peripheral blood [/ μ L]	7196666.67 $\pm$ 257940.56	7860000.00 $\pm$ 253219.27	6853333.33 $\pm$ 1302868.63	7170000.00 $\pm$ 882201.79	6328000.00 $\pm$ 3180073.9	6235000.00 $\pm$ 2784878.81
haemoglobin concentration [g/dL]	15 $\pm$ 0.79	15.87 $\pm$ 0.48	13.70 $\pm$ 2.43	14.30 $\pm$ 1.78	15.54 $\pm$ 1.24	14.78 $\pm$ 1.60
platelet count in the blood [/ μ L]	987000 $\pm$ 291.151	933500 $\pm$ 180737	1098000 $\pm$ 272181	1287000 $\pm$ 84230	1141400 $\pm$ 199938	1189333 $\pm$ 98382

DISCUSSION

The role of IL-6 in the control of metabolism has been suspected based on several observations. Adipocytes of obese individuals secrete IL-6. This activity is correlated with the adipocyte volume [26]. Therefore, obesity was regarded as a state of chronic, low-grade inflammation [27].

IL-6 suppresses appetite, especially in people with cancer or inflammatory diseases [28], but not in healthy ones [29]. Elevated IL-6 level correlates with fatigue in elderly patients [30]. Alcohol consumption has deleterious effect on the immune system and affects production of IL-1, IL-6 and other proinflammatory cytokines [31].

Martins et al. compared the effect of codeine, K, tramadol and a combination of these drugs on the severity of postoperative pain in an animal model. Researchers measured the levels of glucose, cortisol and IL-6 in the blood serum of the studied dogs. The measurements were taken before the surgery and then 1, 2, 3, 4, 5 and 24 hours after. All drugs had an effective analgesic effect. After K and codeine, an increase in serum glucose concentration was observed, but there were no changes in IL-6 concentration [32]. The researchers concluded that NSAIDs did not necessarily affect the concentration of pro-inflammatory cytokines.

In our study none of the groups was significantly obese, however group 6 had the highest mean glucose concentration and the IL-6 brain concentration in this group was lower than in group 1, group 2, group 3 and 4 and group 5. We did not notice differences in the amount of animal feed consumed between the study groups. However alcohol adds extra calories [33]. Kuczyńska demonstrated in male rat model that KL has no gastroprotective properties if the stomach mucosa is

damaged by alcohol [34]. In medical practice, when using K or other NSAIDs, we use proton pump inhibitors, especially in patients with risk factors for gastritis and gastrointestinal bleeding. Researchers are still looking for new substances with as few side effects as possible that will effectively protect the gastric mucosa of patients treated with NSAIDs.

Arab et al. investigated how morin (a flavonoid) can protect against K-induced gastric damage. Among promising gastroprotective properties and immunomodulating effects it was recorded that it increased the IL-10 level [35].

Alcohol consumption affects the expression of brain neurotrophic factor (BDNF) in the striatum, cerebral cortex and hippocampus. Chronic alcohol abuse damages neurons and triggers a cascade of inflammatory processes in the body, resulting in apoptosis and neurodegeneration [36]. Schunck et al. conducted research on alcohol-dependent rats. The ethanol administration was then discontinued, and the animals studied were models of withdrawal from this neurotoxin. The hot plate test was performed in these rats during the period of alcohol abstinence. The IL-10 concentrations were also measured in the prefrontal cortex, hippocampus, and brainstem. Alcohol withdrawal resulted in a significant increase in the level of IL-10 in brain structures in the experimental rats compared to the control group. The researchers showed that alcohol withdrawal had an analgesic effect and increased the pain threshold. The authors of the study suggest that the antinociceptive effect in this case results from the increased level of IL-10 in the brain [36].

For medical practice results obtained in humans are more reliable than ones obtained in the course of experiments conducted on animals. Murakami et al. in 46 patients with alcoholic cirrhosis measured the

concentrations of IL-6 and IL-10 in their blood sera. Authors observed that IL-6 concentration was higher in patients who died compared to those who survived due to liver cirrhosis. In their opinion high serum IL-6 concentration on admission to hospital is a bad prognostic sign meaning high risk of patient's death during hospitalization due to liver cirrhosis caused by alcohol abuse [37].

In the study by Tablov et al. it was hypothesized that K would increase the release of IL-6 and IL-10. The study was conducted on a group of 40 patients after hysterectomy. Some of them received an opioid and the rest received K intravenously with an opioid. IL-6 and IL-10 levels were measured in blood samples taken three times: before surgery, 1 day after surgery and 3 days after surgery. It was observed that the IL-6 level increased 1 day after the surgery and then decreased to the preoperative value 3 days after the hysterectomy. It was different in the case of IL-10, because the level of this cytokine did not change significantly during the study. The authors found that both types of painkilling therapy similarly affect the immune response after hysterectomy [38].

Dexketoprofen is an enantiomer of K [39]. Piirainen et al. in their study, they compared dexketoprofen and etoricoxib in terms of pharmacokinetics and pharmacodynamics. They compared the concentrations of these drugs in plasma and cerebrospinal fluid. The study was conducted on a group of 24 patients after hip arthroplasty. Half of the patients received dexketoprofen intravenously as an analgesic after surgery. The remaining patients received etoricoxib orally. In addition to drug concentrations IL-6 and IL-10 levels were also determined in blood and cerebrospinal fluid. The authors did not record significant differences between the investigated products in means of their influence on inflammation in the postoperative period [40].

Al-Hakeim et al. in the study conducted with patients with depressive mood disorders showed that treatment with sertraline and K normalizes IL-6 level [41].

Our team previously investigated the effects of ethanol, K and its' lysine salt on male rats. No abnormalities were found in the histopathological examination of the brains of experimental animals, but some changes were noted in blood biochemical tests and blood counts, similarly to our present study [42].

In our present study, there were no statistically significant differences in the concentration of IL-10 in the central nervous system between the study groups. However, the time interval between alcohol poisoning and brain examination in our experiment was 5 days. We observed a positive correlation between brain IL-10 levels and body weight and a correlation between brain IL-6 and brain weight. In our experiment, the level of IL-10 in the brain correlated with the concentration of IL-6 of all animals. From the statistical point of view, in order to search for a correlation between IL-6 and IL-10 in the brains of the examined animals, it was decided to analyze the results of these cytokines from all groups together in Spearman's rank correlation coefficient test.

The limitation in our study results interpretation is that very few studies were conducted in a similar model of ethanol intoxication and NSAIDs' use afterwards, what made our discussion imperfect. Searching databases for results of similar studies yielded a very small number of studies cited above.

CONCLUSIONS

KL and K have some immunomodulating effect on the brain.

There is a positive correlation between the level of IL-6 and IL-10 in rat brains.

Conflict of Interest Statement: Authors declare no conflict of interest.

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