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Molecular effects of nimesulide and aspirin on caspase-3, PPAR-α, and COX-2 gene expression in mice

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Abstract

The characterization of NSAIDs in reducing pain, inflammation, and fever by inhibiting cyclooxygenase (COX) leads to the inhibition of prostaglandin synthesis, which is the primary mechanism; still, there are other mechanisms of NSAIDs to produce these effects. In this study, we report a comparison between nimesulide (NIM) (selective COX-2) and aspirin (ASP) (non-selective COX-2) inhibitors in mice model at the molecular level on the caspase-3, peroxisome proliferation-activated receptors-alpha (PPAR-α) and gene expression of COX-2. Individual ED_{100} doses of NIM and ASP (15.8 and 424.5 mg/kg, i.m.) were injected in mice for 5 consecutive days with and without acetic acid. This study reports new pharmacological mechanisms for NIM and ASP to contribute as anti-inflammatory drugs by other important intracellular mechanisms. NIM outperforms ASP for caspase-3 inhibition in the kidney, liver, and plasma (anti-apoptotic and anti-inflammatory effects). Also, NIM was better than ASP in molecular effects at the PPAR-α reducing inflammation and producing its antinociceptive effects. NIM and ASP in therapeutic doses reduced the COX-2 gene expression in the kidney (down-regulation), which causes the potentiation effect as an anti-inflammatory and analgesic drug. This research provided that NIM outperforms ASP in efficacy for the new mechanisms of NIM and ASP for inhibition of caspase-3 and binding with PPAR-α which worked as an anti-inflammatory action and reduced pain plus the suppression of COX-2 gene expression (down-regulation), which all contributing to reducing inflammation, pain and fever.

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Introduction

Nimesulide (NIM) belongs to the sulfonanilide group (*N*-(4-Nitro-2-phenoxyphenyl)-methanesulfonamide) of nonsteroidal anti-inflammatory drugs with selective cyclooxygenase enzyme (COX-2) inhibition thus preventing the production of prostaglandins that are responsible for producing pain, inflammation, and fever were produced through the COX-2 enzyme induction (1,2). NIM can prevent the inflammatory effects with fewer side effects, for example, gastrointestinal bleeding, ulcers, and renal prostaglandin involvement (3). NSAID inhibition of caspases is COX-independent. This is considered a new anti-inflammatory mechanism (4). Caspases are well-known in cancer, inflammation, arthritis, and neurodegenerative disorders (5). Caspases are a family of genes essential for maintaining homeostasis through regulating cell death and inflammation (5). NIM reduces chondrocyte apoptotic cell death by inhibiting caspase-3. Thus, nimesulide may represent a new preventive option for osteoarthritis by blocking apoptotic events (6). Another study found that NIM plays a role in apoptosis by inhibiting prostaglandins, which modulate cell proliferation (7). Another molecular mechanism by which nimesulide exerts its anti-inflammatory and analgesic effects is by inhibiting peroxisome proliferator-activated receptor alpha (PPAR-α)

and COX-2 mRNA expression, therapeutic concentrations of NIM have treated synovial osteoarthritis in human fibroblasts (8). NIM reduces cytokine-induced COX-2 expression at therapeutic doses (9) and inhibits expression levels of COX-2, both at the mRNA and protein levels (3), so this drug was shown to have anticancer effects in neoplastic pancreatic cells by inhibiting proliferation and inducing apoptosis (10). The efficacy of NIM on the COX-2 gene expression was reported in a previous study, which recorded that NIM inhibited cytokine-induced COX-2 expression and protein at both sub-therapeutic and therapeutic doses (9). The inhibition of COX-2 expression may play a role in tumor development (11). Acetylsalicylic acid, commonly known as aspirin (ASP), has been extensively used as an analgesic and anti-inflammatory medication for about ten decades. It acts by inhibiting the cyclooxygenase enzyme, which suppresses prostaglandin production and leads to relieving inflammation, pain, and fever (12). Recently, it has been recognized that the nuclear receptor peroxisome proliferator-activated receptor-alpha (PPAR-α) acts as a unique receptor of ASP; there are three subtypes of PPARs (PPAR-α, γ, β/ δ) (13). PPAR-α exerts anti-inflammatory effects and inhibits the formation of macrophages by regulating gene expression; this receptor limits inflammation (14) and proved in other research that PPARs exert anti-inflammatory effects in rat models with carrageenan-induced paw inflammation (15). ASP also affects caspase-3 by decreasing rat neuron levels (16). Furthermore, it was found that ASP decreased apoptosis by reducing cleaved caspase-3 levels (17), in regard to ASP, at therapeutic concentrations, blocked COX-2 mRNA. These results propose that ASP produces its anti-inflammatory action by inhibiting the COX-2 induction, thereby suppressing the production of proinflammatory prostaglandins in mice at doses of 10-30 mg/kg orally and reducing human colon cancer by suppressing COX-2 expression (18) in other studies proved that ASP co-administered with cisplatin reduced COX-2 expression by inactivating NF-κB signaling in humans (19).

Our study confirms that NIM and ASP have molecular mechanisms to exert anti-inflammatory and analgesic effects through the actions of drugs on the caspase-3, PPAR-α, and COX-2 gene expression and proved that NIM (selective COX-2 inhibition) is more effective than ASP (non-selective COX) in treating inflammatory conditions. This study aimed to detect the new molecular mechanisms of NIM and ASP in reducing inflammation, thus promoting its effect as an analgesic and antipyretic drug.

Materials and methods

Ethical approve

The study was standardized (the animal use and experimental design) by the care committee affiliated with the College of Veterinary Medicine, Mosul University, with approval code no. UM.VET.2022.076.

Animals

In this study, 78 Swiss albino mice, weighing 24-34 g, males and females, were used throughout the study. Animals were kept in the animal facility at the College of Veterinary Medicine, Mosul University, under standard conditions, including a 14/10 h dark/light cycle with 22±2ºC facility temperature. Food and water were provided according to standard protocols.

Drugs

Nimesulide (10%, Instant Pharmaceuticals, India) and aspirin (pure powder, Sanofi, France) were diluted with normal saline and administered via the intramuscular (i.m.) route at an injection dose of 5 ml/kg.

Blood and tissue collection

Blood samples were collected from the choroid venous sinus of mice using capillary tubes containing EDTA (anticoagulant) (20-23). For the caspase-3 test, the samples were incubated at room temperature for 10-20 minutes while the blood tube was placed at 2-8ºC for 15 minutes within 30 min of collection (in the PPAR-α test). The tubes were centrifuged for 20 minutes at 3000 rpm. The supernatant was collected as plasma samples. Tissue samples were cut, weighed, and frozen at -20ºC. PBS (PH=7.4) was added before homogenization, performed at 4ºC. The supernatant was collected after centrifugation of the samples for 20 minutes at 3000 rpm. The aliquot was then used for the ELISA assay of caspase-3 and PPAR-α.

Measuring the analgesic ED_{50} of NIM and ASP in mice

By using the up-and-down method (24), the ED_{50} of NIM and ASP was determined by using the thermal method (hot plate) at 56ºC (25-28). Determination of the initial doses depended on the preliminary and previous studies (29-32). The decrease and increase in the dose were at a constant value for both drugs (33-39).

Effect of NIM and ASP on apoptosis in mice

The effects of NIM and ASP on apoptosis were compared by measuring the activity of Caspase-3 in plasma, liver, and kidney. The mice were divided into six groups, each containing 5 animals; the control group was injected with normal saline, while the second and third groups were injected with the ED100 of NIM and ASP i.m. (15.8 and 424.5 mg/kg, respectively). The fourth group was injected with 1% acetic acid i.p., and the fifth and sixth groups were injected with NIM+AA and ASP+AA, respectively. All groups were treated for 5 days (one dose daily). Then, 30 minutes after the injection on the fifth day, blood was collected from the eye, and the mice were euthanized. The

liver and kidney were then harvested. The organs were stored at -20ºC for examination in the laboratory using a specific ELISA kit for mouse caspase-3 (Elabscience, USA) (40).

Effect of NIM and ASP on PPAR-α in mice

The comparison between NIM and ASP on the peroxisome proliferator-activated receptor-alpha (PPAR-α) involved 6 groups, each containing 5 mice. The groups were similar to those in the previous experiment. After 5 days of daily administration of the treatments, plasma, liver, and kidney samples were collected as in the previous experiment and examined in the laboratory using a specific ELISA kit for mouse PPAR-α (Elabscience, USA) (40).

The inhibitory effects of NIM and ASP on the COX-2 gene expression in the kidney of mice

In this experiment, we examine the inhibitory effects of NIM and ASP on the COX-2 gene expression using the 6 groups, similar to the previous trial except that it assessed the COX-2 gene expression of the kidney tissue using a specialized kit for COX-2 gene expression in mice (Gena Bioscience, Germany) (40).

mRNA extraction based on kit instructions for determination of COX-2 gene expression

The mRNA extraction for assessment of COX-2 gene expression was conducted according to Archer (40).

Measurement of COX-2 mRNA contents in kidney tissue

The first step in determining gene expression through reverse transcription polymerase chain reaction (RT-PCR) was the quantification of the extracted RNA, which was measured using Qubit™ equipment (Qubit Fluorometer, Invitrogen, USA). A high-sensitivity kit was used for the procedure, and the working solution was in a clean plastic tube. The final volume in each tube was 200 µL. The sample volume was 1-20 µL added to the 180-199 µL working solution, then mixed by vertexing 2 - 3 seconds and incubated at room temperature for 2 minutes. All samples were measured using a Qubit® Fluorometer. The same quantitative procedure for RNA quantification was used to quantify complementary DNA (cDNA).

Expression of COX-2 gene in kidney tissue

The RT-PCR kit (Bioron GmbH, Germany) was used for quantification of COX-2 gene according to Archer (40) and Rao *et al.,* (41) by using RT-PCR apparatus (MiniAmp Plus™ Thermocycler, USA). The RT-PCR *β-actin* Forward / Reverse primer sequences were 5' TTGTGATGGACTCCGGAGAC 3', 5' TGATGTCACGCACGATTTCC 3', respectively and F-5' CCCCTCTCTACGCATTCTGT 3', R-5' TGGCAGAACGACTCGGTTAT 3' for COX-2 gene (Macrogen company, Korea). The PCR threshold standard curve was based on an exponential model of the initial phase of a PCR run where template replication efficiency is constant from cycle to cycle. The electrophoresis (Cleaver Scientific, UK) was achieved on 2% agarose gel and the RT-PCR bands were 202 bp COX-2 cDNA and 186 bp *β-action* cDNA.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 20 was used in statistical analysis. One-way analysis was the statistical test applied for parametric data, which was accomplished by comparing means. Further analysis was done using an unpaired T-test to compare the mean of the two resembling groups (42). The significance was at $P<0.05$.

Results

Measuring the analgesic ED_{50} of NIM and ASP in mice

The dose of NIM injected i.m. for mice causing the analgesic response in 50% of the experimental animals was 7.9 mg/kg, and for ASP was 212.23 mg/kg.

Effect of NIM and ASP on apoptosis in mice

NIM and ASP significantly inhibited caspase-3 in the kidney, liver, and plasma in mice compared with control groups of normal saline (negative group) and acetic acid (positive group). These results reveal the effects of NIM and ASP on apoptosis because the inhibition of caspase 3 reduced apoptosis (cell proliferation), and NIM was more effective than ASP in inhibiting caspase-3 in the ED_{100} dose (Table 1).

Table 1: Concentration of caspase-3 in mice

Groups	Kidney	Liver	Plasma
Saline	2.83±0.15	3.70±0.46	3.93±0.80
NIM + NS	2.65±0.17	2.34±0.27*	3.56±0.19
ASP + NS	2.76±0.32	3.43±0.20[a]	3.80±0.25
Acetic acid	3.43±0.18*	4.04±0. 23	4.37±0.25*
NIM + AA	2.45±0.13+	2.37±0.36+	3.41±0.36+
ASP + AA	2.58±0.20+	2.48±0.17+	3.78±0.18

Numbers were as mean±Std.E. 5 mice/group. $P<0.05$. *mean differs significantly from the normal saline group. + means differs significantly from the acetic acid group. a mean differs significantly from the NIM group in the same treatment paradigm.

Effect of NIM and ASP on PPAR-α in mice

Effects of NIM and ASP on the PPAR-α –mediated induction of COX-2 expression were inhibited by therapeutic concentrations ED_{100} of NIM and ASP, which were injected i.m. and measured in kidney, liver, and plasma by ELISA kit. NIM and ASP acted as ligand dependent receptor activators, inhibiting the PPAR-α (dependent- transactivation of the target genes belonging to COX-2), as in table 2.

Table 2: Concentration of PPAR-α in mice

Groups	Kidney	Liver	Plasma
Saline	376.6±27.1	365.2±20.08	18.67±4.7
NIM + NS	353.0±26.7*	361.0±23.9*	9.9±1.5*
ASP + NS	346.1±3.8*	360.8±8.5*	9.7±1.3*
Acetic acid	349.8±25.2	363.1±24.7	16.0±2.6
NIM + AA	312.5±4.9^{+}	352.2±11.9^{+}	5.8±0.3^{+}
ASP + AA	315.8±23.8^{+}	356.4±20.4^{+}	5.1±0.9^{+}

Numbers were as mean±Std.E. 5 mice/group. P<0.05. *mean differs significantly from the normal saline group. + means differs significantly from the acetic acid group.

Effects of NIM and ASP on the COX-2 gene expression

NIM and ASP affected the COX-2 gene expression by decreasing the gene expression of COX-2 in comparison with positive and negative control groups after treating the mice for 5 days (one dose daily), as in table 3 and figures 1-3.

Table 3: concentration of COX-2 gene in mice

Groups	Kidney
Saline	417±30.90
NIM + NS	412±3.61
ASP + NS	410±14.73
Acetic acid	468±48.12
NIM + AA	390±4.04
ASP + AA	400±22.50

Numbers were as mean±Std.E. 3 mice/group.

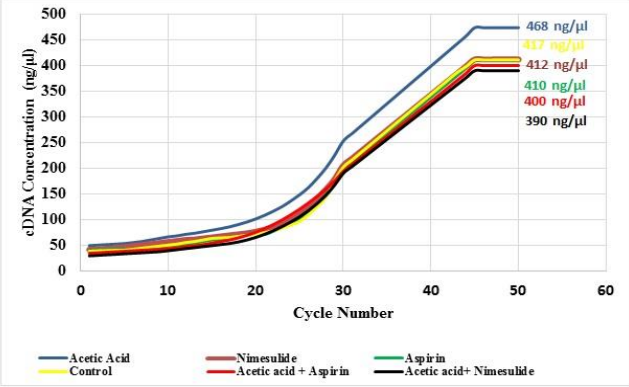

Figure 1: Comparison of the effectiveness between NIM and ASP on COX-2 gene expressions in the kidney tissue of mice using quantitative RT-PCR.

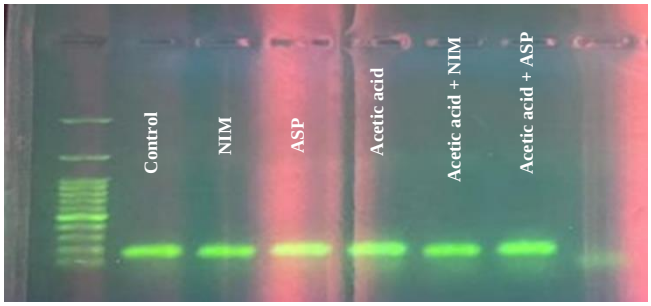

Figure 2: Positive ß-*actin* located in lanes 1-2 (186 bp), positive COX-2 gene (202 bp) located in lanes 3-6, and negative control in lane 7. Lane M: 100 bp DNA ladder.

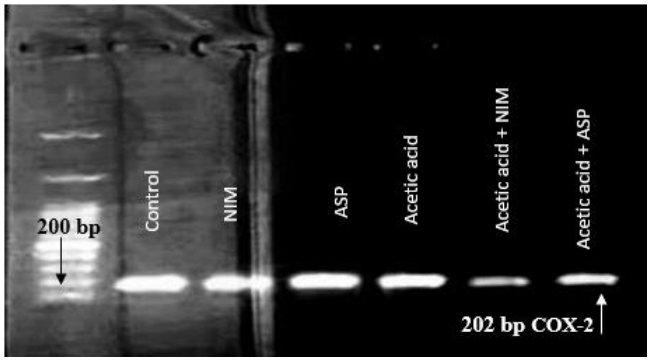

Figure 3: Kidney tissue COX-2 cDNA, RT-PCR product electrophoresis.

Discussion

The study aimed to estimate the drug comparison between NIM (selective COX-2 inhibitor) and ASP (non-selective COX inhibitor) at the level of effects on the caspase-3, PPAR-α, and COX-2 gene expression; in previous studies, there were conflicting results about effects of NIM and ASP on caspase-3 and PPAR-α. Therefore, there was confusion about the effects of these drugs and the molecular mechanisms, so this report explored the anti-inflammatory mechanism of NIM and ASP COX-independent to show other behaviors of NSAID as anti-inflammatory drugs, Caspases are members of cysteine-aspartic protease, it plays a vital role in apoptosis and inflammation (40). When activation initiator caspases will be proteolytically activated and then activate executioner caspases (caspase-3, 6, and 7), which leads to immunological cell death ended by apoptosis (42). Caspase has a role in cancer, inflammation, arthritis, cardiovascular disease, and neurodegenerative disorders (43). NSAIDs reduction of caspases is action COX-independent, representing a novel anti-inflammatory mechanism in humans (44). A previous study proved that NIM decreased cleavage of caspase 3 in 5 days after being treated with the drug in humans (45), which was similar to the result found in this research. So, the

inhibition of caspase-3 leads to the inhibition of apoptosis, which is useful in treating cancer. ASP was recorded in the previous study to reduce etoposide-induced caspase-3 activation in hepatocellular carcinoma, and ASP has an anti-apoptotic effect by blocking caspase 3 in humans (46). Also, other research reported that ASP showed significantly decreased expression of TNF-α, caspase-3, and apoptotic index (47), and these results are similar to what emerged from this research, where ASP decreases caspase-3, which is responsible as executioner caspase to generate apoptosis.

Peroxisome proliferator-activated receptors (PPARs) were known over ten years ago and were categorized as nuclear receptors. 3 PPAR subtypes have been discovered as PPAR-*α, β/δ, γ*, and the diverse PPAR subtypes have been revealed to play vital roles in important disorders like obesity, atherosclerosis, diabetes, fertility, and cancer. Most studies focus on the role of PPARs in inflammatory manners. Many studies have shown that agonists of PPAR-*α* and PPAR-*γ* produce anti-inflammatory effects both *in vivo* and *in vitro*. Using the carrageenan-induced paw edema model for inflammation, a recent study presented that these agonists affect the initiation phase but not the late phase of the inflammatory process (15). PPAR-α has a role in inflammation by modulating inflammation. This is evidenced by the proposal of Leukotriene B4 (LTB4), a powerful chemotactic inflammatory eicosanoid (48). The binding to PPAR-*α* activates the transcription of genes of the *ω*- and *β*-oxidation pathways that can trigger the catabolism of LTB4 itself (49). Activation of PPAR-*α* by the NSAIDs donates to these medications' anti-inflammatory, antipyretic, and analgesic properties by activating the oxidative pathways involved in catabolizing the eicosanoids (50). Reduction of the proinflammatory molecules synthesis like IL-6 and prostaglandins seems to participate in PPAR-*α*-mediated anti-inflammatory effect through reduced activity of NF-*κ*B (51). A previous study found that NIM inhibited PPAR-α and *γ* isoforms agonist stimulation of the COX-2 expression and synthesis in humans (8).

This result agrees with our study on the effects of NIM on the PPAR-α by inhibiting it. The effects of ASP on PPAR-*α* in humans and mice registered in the previous study mentioned that a high dose of ASP reduced the expression of PPAR-α, suggesting a novel pharmacologic effect of ASP COX-independent (52). This also converges with our study, and these effects on the PPAR-α were given another mechanism as an anti-inflammatory-influenced, influenced un-depending on COX-2 inhibitor. With regards to the effects of NIM and ASP on gene expression of COX-2, in a previous study, NIM significantly decreased COX-2 gene expression in different doses 100,200, 400µmol/L led to significantly decreased pancreatic cancer group than those in the control group in humans (11), Also in other research, NIM caused down-regulation of the survivin and COX-2 expressions (at mRNA and protein levels) in FaDu cells that show a significant role in NIM-induced growth inhibition of hypopharyngeal carcinoma in human (53). Also, NIM was reported to significantly reduce COX-2, survivin, and PCNA expression levels at both the protein and mRNA levels in nude mice to treat laryngeal squamous cell carcinoma (3). Other research has proved that ASP suppressed COX-2 expression in humans and mice and has been useful in reducing human colon cancer (18). The recent study mentioned that molecular mechanisms of ASP seem to contribute to tumor suppression by inhibiting COX-2 gene expression to suppress the production of elevated neoplastic prostaglandins and dysregulation of the NF-κB signaling pathway, which produced inhibiting gene expression of COX-2 (19). Another study observed that ASP caused downregulation in the expression of COX-2, suggesting that COX-2 plays an important role in colorectal cancer (54). This report found that NIM and ASP suppression of COX-2-gene expression in different levels, ASP had more effected than NIM on COX-2 gene expression in the negative group, still in the positive group (inducible inflammation) by acetic acid, NIM outperformed ASP in inhibition of the COX-2 gene expression because of the action of NIM (selective COX-2 inhibitor) appeared in inflammation or inducible inflammation when was injected NIM with AA. It is worth noting that for the first time, this research mentions the annotation of the COX-2 gene, whereas no annotation has been found in the gene bank of COX-2 in mice yet, so no exon or intron region has been established.

Conclusions

This research provided that NIM outperforms ASP in efficacy for the new mechanisms of NIM and ASP for inhibition of caspase-3 and binding with PPAR-α, which worked as an anti-inflammatory action and reduced pain. Plus, the suppression of COX-2 gene expression (down-regulation) all contributed to reducing inflammation, pain, and fever.

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Conflict of interest

The authors declare there is no conflict of interest.

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التأثيرات الجزيئية للنيميسولايد والاسبرين على أنزيم الكاسبيز-٣، مستقبلات البيروكسيسوم المنشط الفا والتعبير الجيني لأنزيم الأكسدة الحلقية-٢ في الفئران

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الخلاصة

تتميز مضادات الالتهاب غير الستيرويدية بتقليل الألم والالتهاب وخافض للحرارة عن طريق تثبيط إنزيم الأكسدة الحلقية الذي يؤدي إلى تثبيط تخليق البروستوكلاندين المسؤول عن الألم الالتهابي، وكانت الآلية الأساسية لعلاج حالات الالتهاب، ولكن كانت هناك آليات أخرى لعلاج الألم. في هذه الدراسة نورد مقارنة بين دواء النيميسولايد (المثبط الانتقائي) والأسبرين (المثبط غير الانتقائي) لأنزيم الأكسدة الحلقية-٢ في نموذج الفئران عند مستوى التأثيرات الجزيئية على أنزيم الكاسبيز-٣، مستقبلات البيروكسيسوم المنشط-الفا فضلا عن التعبير الجيني لأنزيم الأكسدة الحلقية-٢. تم حقن الفئران بجرعة فردية من كل من النيميسولايد والأسبرين ١٥,٨ و ٤٢٤,٥ ملغم/كغم، في العضل لمدة ٥ أيام متتالية مع أو بدون حمض الخليك. سجل لدينا هدفا دوائيا جديدا للنيميسولايد والأسبرين للمساهمة كأدوية مضادة للالتهابات من خلال آليات مهمة أخرى داخل الخلايا، إذ تفوق النيميسولايد على الأسبرين في تأثيره المثبط لأنزيم الكاسبيز-٣ في الكلى والكبد والبلازما والذي تم تسجيله في هذه الدراسة ويؤدي إلى تقليل الالتهاب، كما كان النيميسولايد افضل من الأسبرين في التأثير التثبيطي على مستقبلات البيروكسيسوم المنشط-الفا مما يؤدي إلى تقليل الالتهاب وعمل كل من النيميسولايد والأسبرين وبالجرع العلاجية على التقليل من التعبير الجيني لأنزيم الأكسدة الحلقية-٢ في الكلى وتسببت في تقليل الالتهاب. قدم هذا البحث آلية جديدة لكل من النيميسولايد والاسبرين عن طريق تثبيط الكاسبيز-٣ ومستقبلات البيروكسيسوم المنشط-الفا والذي يعمل كمضاد للالتهابات ويقلل الألم بالإضافة إلى التقليل من التعبير الجيني المستحث لأنزيم الأكسدة الحلقية-٢ (التقليل من عدد المستقبلات)، والتي تساهم جميعها في تقليل الالتهاب والألم والحمى.