Inflammation Research (2019) 68:379–386
https://doi.org/10.1007/s00011-019-01225-z

Inflammation Research

ORIGINAL RESEARCH PAPER

Elucidation of the molecular mechanism underlying the anti-inflammatory activity of an effective and safe bipyrazole-based compound

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Received: 6 July 2018 / Revised: 1 March 2019 / Accepted: 6 March 2019 / Published online: 14 March 2019

Abstract

Introduction Since the synthesis of acetylsalicylic acid by Hoffmann in 1897, new classes of NSAIDs have been introduced; however, their side effects have limited their clinical applications. Consequently, our team has recently synthesized a novel bipyrazole compound that showed a satisfactory efficacy and safety profile. The aim of the current study was to elucidate the molecular mechanism of this bipyrazole compound.

Method The anti-inflammatory efficacy of the compound was assessed using formalin-induced paw edema test. Computer-assisted simulation docking experiments were carried out. Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), tumor necrosis factor-alpha (TNFα), interleukin-1 (IL1) and interleukin-10 (IL10) gene expression were quantified with real-time polymerase chain reaction (RT-PCR) using SYBR Green technology. The samples were taken from the plantar paw of mice after formalin local injection.

Results The efficacy of the bipyrazole compound was similar to that of indomethacin, diclofenac, and celecoxib, as proven by the formalin-induced paw edema. Docking study indicated a superior binding score for the studied compound relative to celecoxib, indomethacin, and diclofenac. RT-PCR assessment revealed a significant decrease in iNOS, COX-2, and TNFα gene expression in the bipyrazole-treated group. Moreover, a reduction in IL1 and nNOS gene expression levels and an increase in IL10 level were detected despite being insignificant compared to the control group.

Conclusion These findings revealed the superiority of the newly synthesized bipyrazole compound not only on the binding site, but also by inhibiting most of the inflammatory mediators including TNF-α.

Keywords Bipyrazole · Non-steroidal anti-inflammatory drugs · Cyclooxygenase-2 (COX-2) · Inducible nitric oxide synthase (iNOS) · Neuronal nitric oxide synthase (nNOS) · Tumor necrosis factor-alpha (TNF-α)

Responsible Editor: John Di Battista.

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Introduction

The era of non-steroidal anti-inflammatory drugs (NSAIDs) dated back to the eighteenth century by the advent of acetylsalicylic acid. Since 3500 years ago, NSAIDs constitute a cornerstone in the treatment of inflammation and pain associated with a variety of medical conditions including rheumatoid arthritis, osteoarthritis and other. The main mechanism of action of NSAIDs was first described in 1971 by Vane and Pipe [1–4]. They demonstrated that NSAIDs inhibit the biosynthesis of prostaglandins by preventing the substrate arachidonic acid from binding to the COX enzyme active site. COX enzyme was subsequently found to exist in several isoforms; the most famous of them is COX-1 which was characterized in 1976 followed by COX-2 isoenzyme that was later discovered in 1991. COX-1 was found to

have a role in numerous physiological functions, including maintenance of normal renal function, mucosal protection of the gastrointestinal tract, and synthesis of pro-aggregatory thromboxane A2 in platelets. On the other hand, COX-2 is believed to have a role in the mediation of pain, inflammation, and fever. Moreover, NSAIDs are known to reduce the production of superoxide radicals, induce apoptosis, inhibit the expression of adhesion molecules, decrease nitric oxide synthase, decrease pro-inflammatory cytokines (e.g., TNF-α, interleukin-1), modify lymphocyte activity, alter cellular membrane functions, and stabilize lysosomal enzymes [5–8]. In fact, NSAIDs increase IL-10, the anti-inflammatory cytokine, while they decrease or have no effect on IL-1, the pro-inflammatory cytokine. On the other hand, NSAIDs increase TNFα production while decreasing PGE2 [9].

Through the growing knowledge of the mechanism of action of NSAIDs, several agents were newly synthesized in order to reduce their side effects. Despite the increasing numbers of new anti-inflammatory agents, most of them are far from ideal. New selective agents carry a larger cardiovascular risk than the older ones which are known for their gastrointestinal side effects [10]. Aiming to find a compatible product to the marketed NSAIDs with better safety profile, researchers have synthesized and analyzed many molecules. For example, in 2010, Mohy El-Din et al. [11] synthesized a new compound containing 4-(3-(4-methylphenyl)-4-cyano-1H-pyrazol-1-yl) benzenesulfonamide with a good anti-inflammatory effect, no ulcerogenic activity and minimal effect on the kidneys. In 2012, Thore et al. synthesized a series of novel ethyl-5-amino-3-methylthio-1H-pyrazole-4-carboxylates. Out of this series, three compounds exhibited significant analgesic and anti-inflammatory activities with quite less ulcerogenic index compared to diclofenac [12]. In the same year, Maddila et al. synthesized a new series of 1,3,4-thiadiazole with pyrazole-3-carboxamides and pyrrole-3-carboxamide. Out of the 12 compounds synthesized, 7 compounds showed potent anti-inflammatory activity comparable to indomethacin. However, Maddila et al.' study lacked safety testing of the synthesized compounds [13]. In 2015, Paprocka et al. demonstrated potential anti-inflammatory activity of two newly synthesized triazole moieties which were comparable to ibuprofen [14].

Recently, our team has proven the efficacy and safety of a novel bipyrazole compound 3-(chlorophenylamino)-2-cyano-*N*-(4-cyano-3-(methylsulfanyl))-1-(phenyl-1H-pyrazol 5-yl)-3(2,3-dihydro-1,5-dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-ylamino)-acrylamide [15], which was given the code (BPA101). In fact, cotton pellet granuloma test after 7 and 30 days of treatment has revealed a better anti-inflammatory effect than diclofenac sodium with good toxicity profile in term of cardiac, renal, gastric and liver toxicity [15]. The aim of the current study was to reassess the efficacy of this new bipyrazole compound and to investigate its binding to COX-2 receptor as well as the involvement of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and tumor necrosis factor-alpha (TNF-α) in its effect.

Materials and methods

Experimental animals and ethical consideration

Male BALB/c mice, weighing 20–25 g, were used. Mice were obtained from the animal house at the Faculty of Pharmacy, Beirut Arab University. The animals were kept in polyacrylic cages and maintained under standard animal housing conditions (temperature 25 ± 2 °C) and relative humidity (40–70%) with dark–light cycles (12/12 h) before and during the experiments. Mice had free access to water ad libitum and standard laboratory chow. All experiments were performed at Beirut Arab University laboratories after obtaining approval from the Investigation Review Board, number 2016A-0043-P-P-0165. Animal care and handling for the research were performed in accordance with the regulations and guidelines stipulated by the Institutional Animal Care and Use Guidelines (IACUG) at Beirut Arab University, Lebanon, certified by the Ministry of Public Health: (1/141).

Drugs, chemicals and kits

The studied compound, BPA101, was synthesized in the pharmaceutical chemistry laboratory, faculty of pharmacy, Alexandria University, Egypt. A concentration of 25.66 mg/kg of BPA101 representing the equieffective dose to 10 mg/kg diclofenac sodium [15] was prepared by dissolving equal volumes of dimethyl-sulfoxide and polyethylene glycol 400 as illustrated by Deb et al. Indomethacin, celecoxib and diclofenac, supplied by Sigma chemicals, Pfizer and Novartis, respectively, were also dissolved in the same solvent to achieve a concentration equivalent to 10 mg/kg, 50 mg/kg, and 10 mg/kg, respectively. These drugs were used as positive controls. Drugs were administered intraperitoneally. Ether supplied by POCH SA, Poland, was used to execute the animals when needed. Formalin supplied by Sigma-Aldrich was used to induce inflammation. For quantitative PCR, TRIzol reagent (Life Technologies, USA), QuantiTect Reverse Transcription kit and 5x FIREPol® EvaGreen® qPCR Mix (no ROX) (Solis BioDyne, Tartu, Estonia) were used.

Formalin-induced paw edema

Formalin-induced mice paw edema was used to confirm the acute anti-inflammatory activity of the tested compound relative to the positive controls. The test was performed

as described by Gerhard Vogel [16]. 1 h after injecting BPA101, indomethacin, celecoxib or diclofenac, mice were challenged with a 200-µl subcutaneous injection of 5%v/v formalin solution into the plantar side of the right hind paw. The paw volume was measured plethysmographically, using Ugo Basile 37140 apparatus, immediately after the injection and 30 min later. The percentage increase in paw volume and percentage inhibition of edema determined by formalin-induced paw edema test are calculated based on the following equations:

$$\text{Percentage increase in paw volume} = \frac{\left(\text{paw volume at } t - \text{paw volume at } t_0\right) \times 100}{\text{paw volume at } t_0},$$

$$\text{Percentage inhibition of edema} = \frac{(\%\ \text{increase in paw volume of the control} - \%\ \text{increase in paw volume of the treated mice}) \times 100}{\%\ \text{increase in paw volume of the control}}.$$

Histopathology of the paw

In order to confirm the inflammatory changes in paws of mice after 30 min of formalin administration, mice were killed. The paws of the group pretreated with vehicle as well as the one pretreated with BPA101 were taken, fixed in 10% buffered formalin solution and stained with hematoxylin and eosin stain.

Molecular mechanism of action

After induction of inflammation and measurement of the paw edema, treated and negative control animals were killed and subplantar muscles were quickly dissected, immediately frozen in liquid nitrogen and stored at − 80 °C.

Total RNA was extracted from euthanized mouse tissues using TRIzol reagent. The yield and quality of total RNA were determined spectrophotometrically using a NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delaware, USA) at λ 260 and 260/280 nm ratio, respectively.

The amount of RNA was quantified by using 5× FIREPol® EvaGreen® qPCR Mix Plus. 1 µg of total RNA was reverse-transcribed into single-stranded complementary DNA (cDNA) by using QuantiTect reverse transcription kit using a random primer hexamer in a two-step RT-PCR reaction in which any genomic DNA contamination was first eliminated using gDNA Wipeout buffer.

The mRNA levels of different inflammatory genes in mouse tissues were determined using 5× FIREPol® EvaGreen® qPCR Mix, no ROX (Solis BioDyne, Tartu, Estonia). Meanwhile, mouse peptidylprolyl isomerase A (PPIA) was used as a housekeeping gene and an internal reference control. Gene-specific PCR primers (Table 1) were designed using Primer Express 3.0 (Applied Biosystems, USA) according to the nucleotide sequence obtained from the Gene Bank. Thermal cycling conditions included initial activation step at 95 °C for 15 min followed by 40–50 cycles of 94 °C for 15 s, 56 °C for 20 s and 72 °C for 30 s. Data acquisition was performed during the extension step. Melting curve analysis of the PCR products was performed to verify their specificity and identity. Rotor-Gene Q thermocycler (Qiagen, USA) collected data automatically and analyzed the value of threshold cycle (Ct).

Mouse interleukin-1 (Il-1), interleukin 10 (Il-10), tumor necrosis factor (TNF-α), neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) mRNA relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. PCR products were confirmed by running on 1.2% agarose gel electrophoresis.

Docking study

Computer-assisted simulation docking experiments were carried out under an MMFF94X force field using Molecular Operating Environment (MOEDock 2009) software, Chemical Computing Group, Montreal, QC. The coordinates from the X-ray crystal structure of COX-2 used in this simulation

Table 1 Different primers and reverse primers for different genes

Genes	Primer	Products size (bp)
PPIA	F:AATGCTGGACCAAACACAAA	116
	R:TTCCACAATGTTCATGCCTT	
IL1	F:ATGGGCAACCACTTACCTATTT	103
	R:GTTCTAGAGAGTGCTGCCTAATG	
IL10	F:TTGAATTCCCTGGGTGAGAAG	95
	R:TCCACTGCCTTGCTCTTATTT	
COX-2	F:CGGACTGGATTCTATGGTGAAA	111
	R:CTTGAAGTGGGTCAGGATGTAG	
TNFα	F:CTACCTTGTTGCCTCCTCTTT	116
	R:GAGCAGAGGTTCAGTGATGTAG	
nNOS	F:GTGAGTGGACAGATGGAAGAAG	101
	R:CCCTAGCACTGATAAGCAGAAG	
iNOS	F:GGAATCTTGGAGCGAGTTGT	99
	R:CCTCTTGTCTTTGACCCAGTAG	

PPIA peptidyl propyl isomerase A, *Il1* interleukin-1, *Il10* interleukin 10, *COX2* cyclooxygenase-2, *TNFα* tumor necrosis factor, *nNOS* neuronal nitric oxide synthase 1, *iNOS* inducible nitric oxide synthase

were obtained from the Protein Data Bank (PDB ID: 3LN1), where the active site is bound to the inhibitor celecoxib. The ligand molecules were constructed using the builder molecule and were energy minimized. The active site of COX-2 was generated using the MOE-Alpha Site Finder, and then ligand was docked within this active site using the MOE Dock. The lowest energy conformation was selected, and the ligand interactions (hydrogen bonding, arene–arene interactions together with other hydrophobic interactions) with COX-2 were recorded.

Statistical analysis

Results were analyzed using the Statistical Package for the Social Science (SPSS®) version 20. Continuous data were expressed as mean ± SEM (standard error of the mean). Analysis of variance (one-way ANOVA) followed by post hoc analysis (Tukey test) was used when appropriate. The difference was considered significant at a p value less or equal to 0.05.

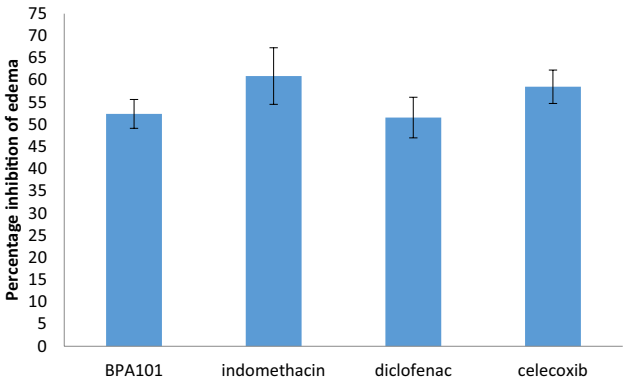

Fig. 1 Percentage inhibition of edema induced by formalin in mice treated with either BPA101, indomethacin, diclofenac or celecoxib

Results

Formalin-induced paw edema

BPA101 showed, in formalin-induced paw edema, a similar anti-inflammatory effect to indomethacin, diclofenac, and celecoxib (Fig. 1).

Histopathological examination of the paw injected with formalin exhibited intense edema with a substantial number of infiltrated inflammatory cells. In contrast, the group of mice pretreated with BPA101 before formalin injection showed no edema as well as low infiltration of inflammatory cells (refer to Fig. 2).

Molecular mechanism of action

Data obtained from real-time PCR revealed that BPA101 inhibited significantly gene expression of iNOS, COX-2, and TNFα compared to the negative control. It also showed a decrease in the gene expression levels of IL1 and nNOS with an increase in IL10 as compared to the control even though it was not significant (Fig. 3).

Docking studies

Docking study was carried out using the enzyme parameters obtained from the crystallographic structure of the complex between COX-2 (PDB ID: 3LN1) with the co-crystallized

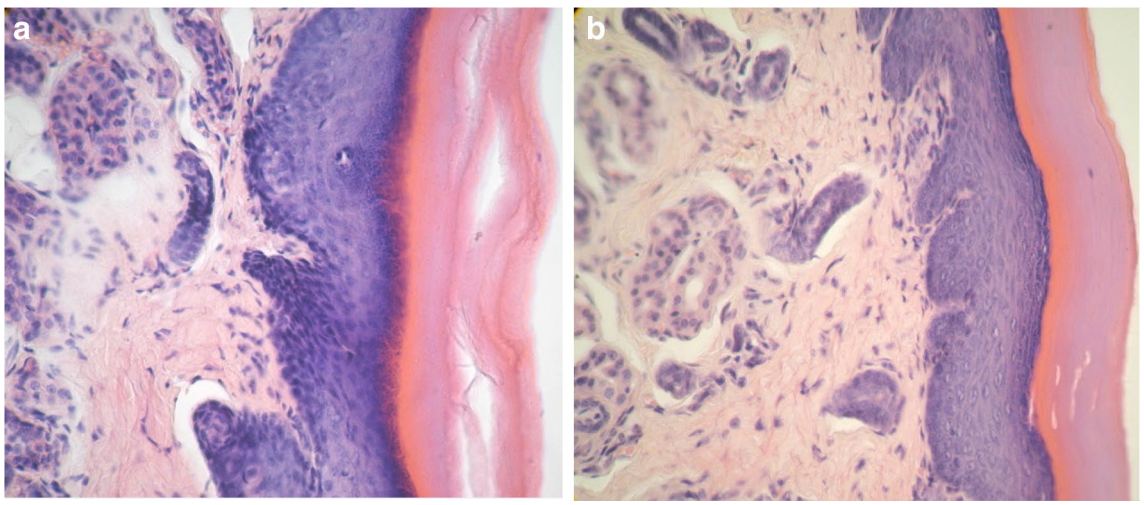

Fig. 2 Histopathological features of hind paw of the mice treated with the vehicle or BPA101 after induction of edema by formalin (**a** control; **b** BPA101)

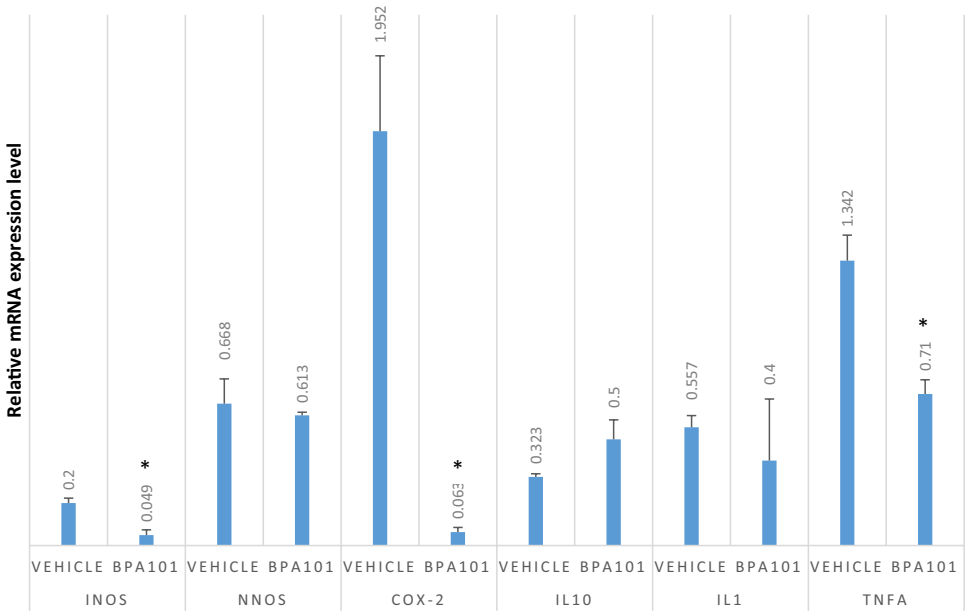

Fig. 3 Gene expression of iNOS, nNOS, COX-2, IL1, IL10 and TNFα in the hind paw of mice receiving BPA101 compared to mice receiving the vehicle ($n=5$) *t* test done to compare between results of compound I and the vehicle. *Denotes significant difference from the control at $p<0.05$

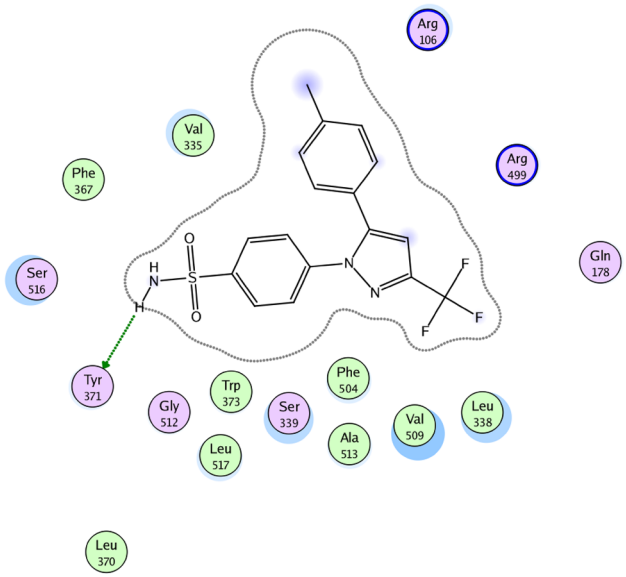

Fig. 4 2D binding mode of the inhibitor (celecoxib) in the binding site of COX-2 receptor (PDB ID: 3LN1) using MOE software

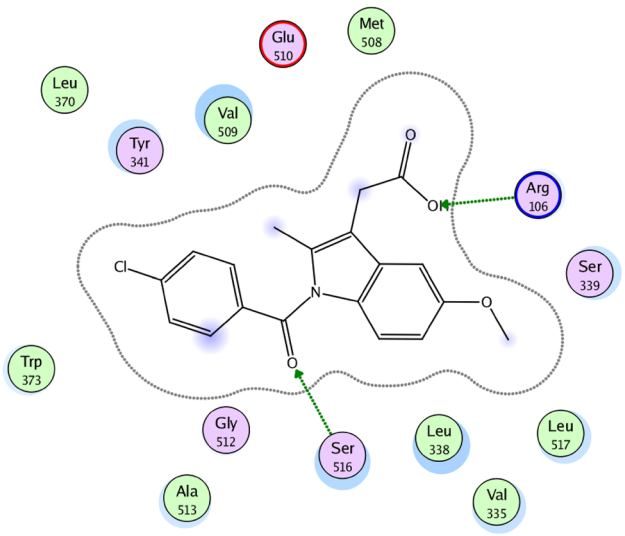

Fig. 5 2D binding mode of the inhibitor (indomethacin) in the binding site of COX-2 receptor (PDB ID: 3LN1) using MOE software

ligand celecoxib [17]. The docking simulation for the ligand was carried out using molecular operating environment (MOE) software supplied by the chemical computing group, Inc., Montreal, QC [18]. Figure 4 showed a binding score equal to (− 6.8381). The X-ray crystal structure revealed that NH_2 group in the ligand is hydrogen bonded to Tyr371. Besides, it showed hydrophobic interactions with Val335, Leu338, Phe367, Leu 370, Trp373, Phe504, Val509, Ala513, and Leu517.

The binding modes for other well-known ligands, namely indomethacin and diclofenac were performed and the following results were obtained. In the case of indomethacin binding mode (Fig. 5), the results revealed a binding score equal to (− 8.0238). The X-ray crystal structure revealed that the OH of the carboxylic group in the ligand is hydrogen bonded to Arg106 while the amide carbonyl oxygen is hydrogen bonded to Ser516. Besides, it showed hydrophobic interactions with Val335, Leu338, Leu370, Trp373, Met508, Val509, Ala513, Leu517.

The binding mode of diclofenac (Fig. 6) showed a binding score of −5.8227. The X-ray crystal structure revealed that the OH of the carboxylic group in the ligand is hydrogen bonded to Arg106 and Tyr341 while its carbonyl oxygen

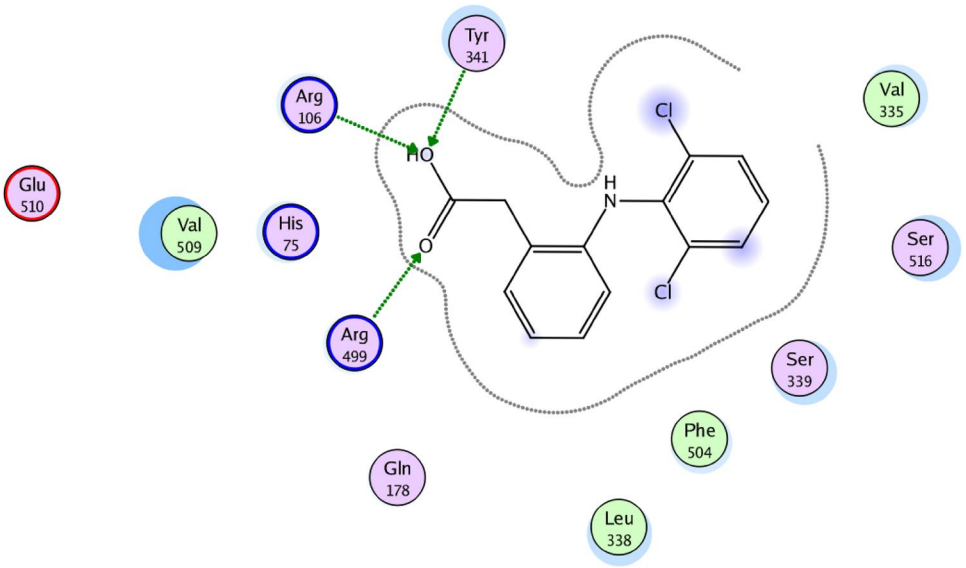

Fig. 6 2D binding mode of the inhibitor (diclofenac) in the binding site of COX-2 receptor (PDB ID: 3LN1) using MOE software

is hydrogen bonded to Arg499. Besides, it showed hydrophobic interactions with Val335, Leu338, Phe504, Val509.

The binding mode of BPA101 indicated a superior binding score (− 11.7090) relative to all ligands used (celecoxib − 6.8381, indomethacin − 8.0238 and diclofenac − 5.8227). Furthermore, it showed hydrogen bonding between the pyrazolo nitrogen and His75, the pyrazolinone oxygen and Ser516. In addition, the cyano nitrogen showed hydrogen bonding with two amino acid residues in the receptor: Ile503 and Phe504 (Fig. 7). Besides, several arene–arene interactions were displayed between the aromatic rings in BPA101 and the receptor binding site including one between the

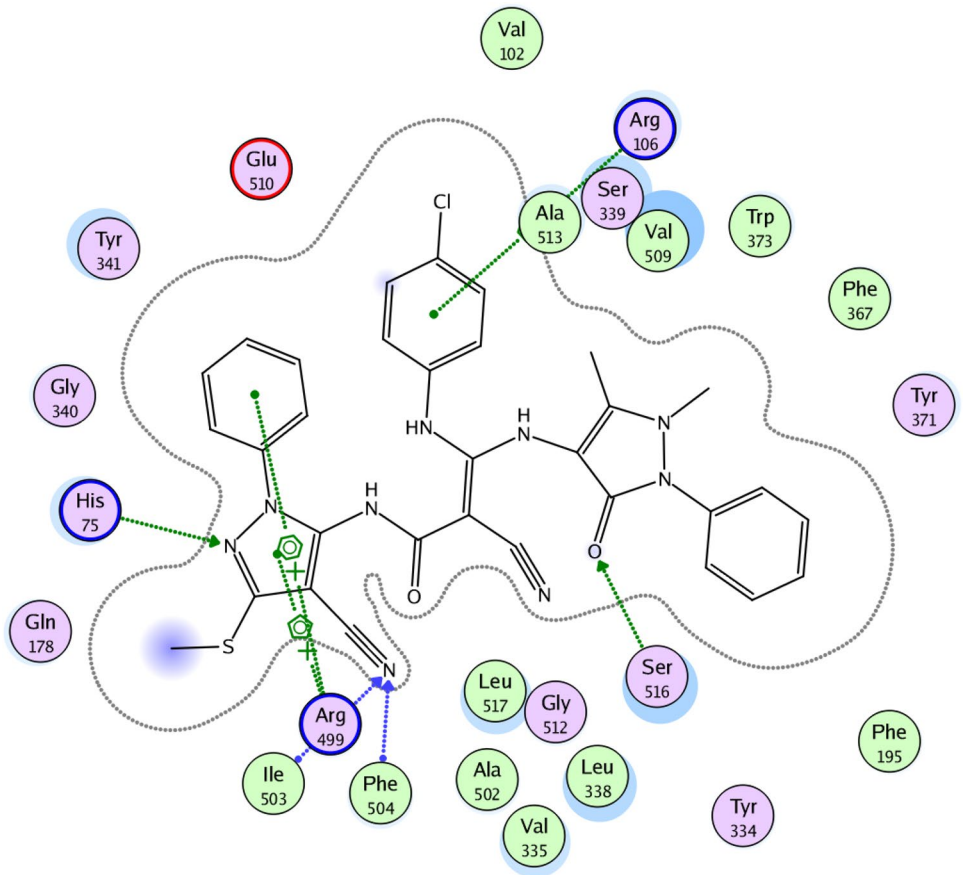

Fig. 7 2D binding mode of BPA101 docked and minimized in the binding site of COX-2 receptor using MOE software

p-chlorophenyl and Arg106, the N1-pyrazolophenyl ring and Arg499 and finally the pyrazolo ring and Arg499. Moreover, it showed hydrophobic interactions with Val102, Phe195, Val335, Leu338, Phe367, Trp373, Ala502, Ile503, Phe504, Val509, Ala513, and Leu517 (Fig. 8).

Discussion

The discovery of the different cyclooxygenase enzymes and their specific role has led to the development of COX-2 selective inhibitors to overcome the GI side effects that accompany the use of traditional NSAIDs. Consequently, rofecoxib, celecoxib, valdecoxib, and etoricoxib were introduced into the market. Nevertheless, rofecoxib and valdecoxib were withdrawn from the market in 2004 and 2005, respectively, due to the increase in cardiovascular side effects risk. On the other hand, celecoxib did not show any increase in the afore-mentioned cardiovascular risk as was observed in the Celecoxib Long-term Arthritis Safety Study (CLASS) trial [3, 5, 10, 19]. Consequently, the recognition of COX-2-selective inhibitors imposed a great challenge for researchers to design and explore alternative drugs with improved potency as well as reduced side effects compared to the available NSAIDs. Several attempts were made to manipulate the ring system fused to the cis-stilbene system, but none was appropriate to reach the market. Recently, our research team synthesized a new series of thiazole derivatives, out of which 3-(chlorophenylamino)-2-cyano-*N*-(4-cyano-3-(methylsulfanyl))-1-(phenyl-1H-pyrazol-5-yl)-3(2,3-dihydro-1,5-dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-ylamino)-acrylamide compound, referred to BPA101, showed potential anti-inflammatory/analgesic effect with adequate hepatic, renal and cardiac safety profile [15]. The anti-inflammatory effect was reassured in this study by the formalin-induced paw edema which proved equal efficacy of BPA101, at a dose of 25.66 mg/kg, to indomethacin (10 mg/kg), diclofenac (10 mg/kg) and celecoxib (50 mg/kg). In fact, a methylsulfonyl group was added to the pyrazole ring instead of sulphonamide or methyl sulphone groups present in celecoxib and etoricoxib, respectively. This has led, as shown by the real-time PCR, to a significant inhibition of the gene expression of iNOS, COX-2, and TNF-α as compared to the negative control. It also showed a decrease in gene expression of IL1 and nNOS and an increase in IL10 as compared to the control. In fact, the inflammatory response is coordinated through the activation of a set of genes, precisely pro-inflammatory cytokines, such as TNF-α, IL1 and other, which activate COX-2 enzyme and thus prostaglandins biosynthesis. These cytokines also accelerate the progression of inflammation through modification of the vascular endothelial permeability and recruitment of effector cells for instance monocytes and neutrophils to the inflammation site. NSAIDs exert their action mainly by inhibiting cyclooxygenase enzymes involved in the inflammation process. BPA101 not only inhibited COX-2, but also suppressed TNFα and IL1 gene expression as opposed to NSAIDs including celecoxib, indomethacin, and diclofenac which increased the level of TNFα as observed in several previous studies [8, 9, 19, 20]. Moreover, NSAIDs did not increase the level of IL10, which is an anti-inflammatory cytokine that is released in order to balance between pro-inflammatory and inflammatory mechanisms, while BPA101

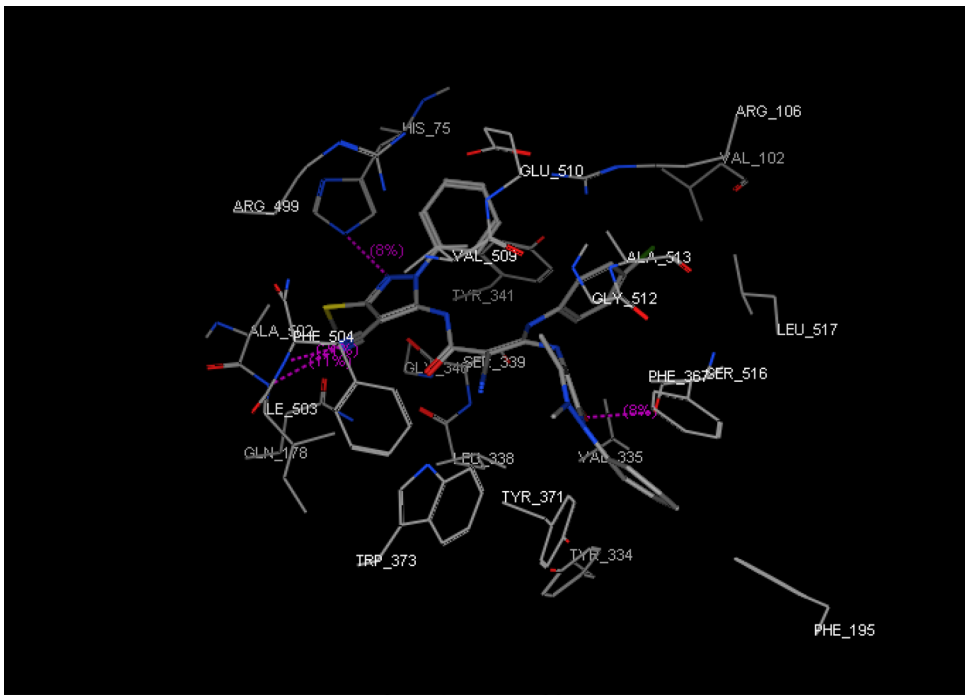

Fig. 8 3D binding mode of BPA101 docked and minimized in the binding site of COX-2 receptor using MOE software

increased it although non significantly. It is noteworthy that most of the biologic anti-inflammatory agents are targeting TNF-α [21]. Accordingly, BPA101 could bear the advantage of a unique multitargeted anti-inflammatory activity. Neutrophils, as well as other cells released during inflammatory reactions, create a cytotoxic environment by releasing noxious chemicals including highly reactive oxygen and nitrogen species. Nitric oxide, one of the members of reactive oxygen species, is synthesized from L-arginine under the influence of nitric oxide synthase (NOS) enzymes. There are three types of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The first two are constitutive while the third is an inducible form synthesized upon cell activation. The expression of iNOS is regulated by cytokines and it acts as cytotoxic or cytostatic to a variety of cells and microorganisms. BPA101 was found to inhibit iNOS significantly but not nNOS as compared to control. In fact, few studies have shown that NSAIDs, including indomethacin, may reduce iNOS and nNOS levels [22–24].

To highlight the efficacy of BPA101 in inhibiting COX-2, a docking study was carried out. It showed that BPA101 binds to COX-2 by hydrogen bonding with His75, Ser516, Il503, and Phen504, sharing Ser516 site with indomethacin. BPA101 also binds, unlike celecoxib, indomethacin, and diclofenac to COX-2 by arene–arene bonds. In fact, *p*-chlorophenyl, found on BPA101, interacts with Arg106, N1-pyrazolophenyl ring with Arg499 and pyrazol ring with also Arg499. These interactions enable BPA101 binding to COX-2 to be superior to all other ligands tested: celecoxib, indomethacin, and diclofenac.

Conclusion

In conclusion, both current molecular and docking studies have proved BPA101 as a potential anti-inflammatory agent possessing good affinity to COX-2 and capability to inhibit TNFα and IL1 which are crucial mediators involved in the inflammatory process and bone matrix degeneration. This unique molecular mechanism is not realized in other marketed NSAIDs.

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