# IN VITRO AND IN SILICO EVALUATIONS OF ANTI-INFLAMMATORY ACTIVITY OF 1,3,4-OXADIAZOLE DERIVATIVES

*Đến tòa soạn 09-02-2023*

**Nguyen Phu Quy,[1,2] Do Minh Kiep,[3] Nguyen Cuong Quoc,[1] Tran Quang De,[1] Hiroyuki Morita[3] and Bui Thi Buu Hue*[,1]**

[1] *College of Natural Sciences, Can Tho University*

[2] *Faculty of Pharmacy and Nursing, Tay Do University*

[3] *Institute of Natural Medicine, University of Toyama, 2630-Sugitani, Toyama 930-0194, Japan*

*Email: btbhue@ctu.edu.vn

## TÓM TẮT

# ĐÁNH GIÁ HOẠT TÍNH KHÁNG VIÊM IN VITRO VÀ IN SILICO CỦA CÁC DẪN XUẤT 1,3,4-OXADIAZOLE

*Mười dẫn xuất 1,3,4-oxadiazole được tiến hành đánh giá hoạt tính kháng viêm. Kết quả cho thấy các hợp chất **4c-d**, và **5** thể hiện hoạt tính ức chế sản sinh NO sử dụng mô hình tế bào RAW264.7 bị kích thích bởi tác nhân LPS với giá trị $IC_{50}$ trong khoảng từ 58.04 đến 85.31 µM. Kết quả docking phân tử cho thấy các hợp chất **4c-d**, và **5** thể hiện ái lực với các enzyme iNOS và nNOS dựa trên giá trị năng lượng liên kết thấp và các tương tác với các amino acid thiết yếu tại các tâm hoạt động của các enzyme.*

***Từ khóa**: Docking phân tử, hoạt tính kháng viêm, 1,3,4-oxadiazole.*

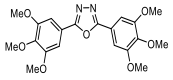
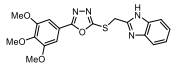
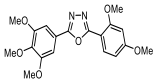
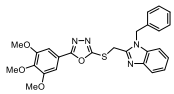
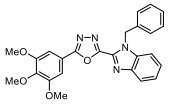
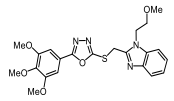
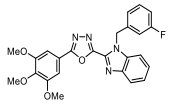
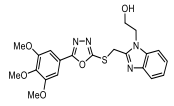
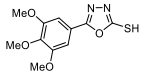
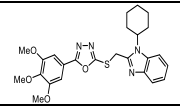
## 1. INTRODUCTION

Anti-inflammatory is the property of a substance or treatment that reduces inflammation in the body (redness, swelling, and pain) by blocking certain substances in the body that cause inflammation. They are used to treat many different conditions. It has been found that the anti-inflammatory activity of many agents may originate mainly from their ability to inhibit some of the key enzymes involved in inflammation and/or cell signalling pathways such as cyclooxygenases (COXs), lipoxygenases (LOXs), and nitric oxide synthase (NOS) [1]. Thus, inhibition of these enzymes may be a valuable treatment for inflammatory conditions.

The 1,3,4-oxadiazole molecule serves as a scaffold for arranging pharmacophores to create effective and selective drugs. The toxophoric moiety (N–C–O) of 1,3,4-oxadiazole, emphasizing its significance as a possible biodynamic molecule [2] has been extensively researched and reported for biological activities, especially anti-inflammatory action [3].

We have previously reported the effective synthesis and cytotoxicity evaluation of two series of 2-substituted benzimidazole conjugated 1,3,4-oxadiazole derivatives [4]. The results showed that, 14 compounds demonstrated consistent to stronger cytotoxicities compared to the control 5-FU towards the tested cell lines including HeLa, MCF-7 and A549, with the $IC_{50}$ ranging from 2.7 to 38 $\mu$M; and 10 compounds showed very low activity against the tested cancer cell lines (Table 1).

*Table 1. Non-cytotoxic 1,3,4-oxadiazole against the HeLa, MCF-7 and A549 cell lines*

| Comp. | Structure | Comp. | Structure |
|---|---|---|---|
| **4a** |  | **7a** |  |
| **4b** |  | **7b** |  |
| **4c** |  | **7c** |  |
| **4d** |  | **7d** |  |
| **5** |  | **7e** |  |

In an attempt to develop new potential anti-inflammatory agents, herein we report our initial results on the anti-inflammatory activity of the above non-cytotoxic compounds **4a-d**, **5** and **7a-e**. Molecular docking studies were carried out to understand their activity.

## 2. MATERIALS AND METHOD

### 2.1. Materials

RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin G sodium (100 units/mL), streptomycin sulfate (100 μg/mL), amphotericin B (0.25 μg/mL), and 10% fetal bovine serum (FBS). Griess reagent was purchased from Sigma – Aldrich. The absorbance was taken at 540 nm using a Multiscan plate reader (Genios, Tencan).

### 2.2. Assay for inhibition of NO production

Nitrite concentration in the medium was measured as an indicator of nitric oxide production according to the Griess reaction method. Each nitrite standard and sample were assayed in triplicate. A freshly prepared standard curve was used each time the assay was performed. In brief, $1\times10^5$ RAW264.7 cells were seeded in 24-well plates, incubated for 24 h and pre-treated with the indicated concentrations (0, 25, 50, 75, 100 μg/mL) of the assay compounds for another 30 min, then challenged with LPS (0.5 μg/mL) for an additional 18 h. 100 μl of cultured medium and Griess reagent (1% sulfanilamide in 5% phosphoric acid and naphtylethylenediamine dihydrochloride 0.1% in distilled water) were mixed and incubate the plate at room temperature for 10 min, the absorbance at 540 nm was determined with a microplate reader and the absorption coefficient was calibrated using a standard solution of sodium nitrite. For positive control studies, 10 μg/mL L-*N*-Methylarginine (L-*N*MMA) was used.

### 2.3. Molecular docking studies

The ligands were optimized within the DFT at the B3LYP/6-31g (d,p) level by Gaussian programs. The three-dimensional (3D) crystal structures of iNOS (ID: 4CX7) and nNOS (ID: 6AV2) were obtained in the PDB format from RCSB. All water molecules (outside of the active site), small molecules, and co-crystallized ligand were deleted by Discovery Studio Visualizer program for docking protocol. The molecular docking study utilizes Flexx with assuming a rigid structure of protein and considering the conformational space of the ligands to analyze the inductive effect of the hybrid compounds. The binding sites include key amino acids within radius 7.00. The outputs of the docking studies were analyzed using Discovery Studio and the computer with core i7, 3.2 GHz processor.

## 3. RESULT AND DISCUSSION

### 3.1. Anti-inflamatory studies

Nitrogen oxide (NO), an important host defense effector in the immune system, is an important biological mediator in the living organism. However, the overproduction of NO which is catalyzed by iNOS, a soluble enzyme, is cytotoxic. On the other hand, NO is a free oxygen radical and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders. Inhibition of NO

production, therefore, may be beneficial for the treatment of inflammatory disease [5].

The 1,3,4-oxadiazoles (**4a-d**) and (**7a-e**) were evaluated for the inhibition on NO production in LPS-stimulated RAW264.7 macrophage cells (Table 2).

*Table 2. Synthesis and NO inhibition of 1,3,4-oxadiazole hybrids*

| Comp. | $IC_{50}$ ($\mu$M)[a] | Comp. | $IC_{50}$ ($\mu$M)[a] |
|---|---|---|---|
| **4a** | > 100 | **7a** | > 100 |
| **4b** | > 100 | **7b** | > 100 |
| **4c** | 85.31 ± 0.83[b] | **7c** | > 100 |
| **4d** | 65.90 ± 0.67[b] | **7d** | > 100 |
| **5** | 61.64 ± 1.16[b] | **7e** | > 100 |
| | | L-*N*MMA[c] | 40.38 ± 1.63[b] |

[a] $IC_{50}$, the half-maximal inhibitory concentration. [b] Data are performed as mean ± SD of three independent experiments. [c] L-*N*MMA was used as a positive control.

The results in Table 2 indicated that compounds **4c-d,** and **5** showed weak to moderate NO inhibitory activity in LPS-induced RAW264.7 macrophage cell with the $IC_{50}$ values ranging from 58.04 to 91.61 $\mu$M, which were comparable to the positive control, L-*N*MMA ($IC_{50}$ = 40.38 ± 1.63 $\mu$M). In contrast, compounds **4a-b** and **7a-e** did not exhibit the inhibition on NO production toward LPS-stimulated RAW264.7 cells.

### 3.2. Docking results

Molecular docking is an important tool for the development of drug discovery, which can be used to elucidate the binding modes of inhibitor-receptor complexes. Therefore, the docking protocols on the proteins iNOS (ID: 4CX7) and nNOS (ID: 6AV2) were performed to shed some light on the binding mode of the three selected compounds (**4c-d**, and **5**) that gave NO inhibition activities. Before docking protocols were made, redocking of the co-crystallized ligands on two crystal structures was carried out to account for protein docking model evaluation. The results showed that, in both cases, the root-mean-square deviation (RMSD) values for the ligands were less than 2 Å, which proved the reliability of the used docking protocol.

The docking results of the three selected compounds are presented in Table 3. The results indicated that, compounds **4c-d**, and **5** showed binding to iNOS and nNOS receptors based on their ability to interact with key amino acids at the active sites of the receptors. The three tested compounds showed hydrophobic interactions with the heme group, in which the phenyl moieties of oxadiazole being oriented underneath the Fe atom of the heme group favoring a π–cation interaction (Figrure 1).

*Table 3. The binding energy and H-bonds of compounds at the active site of iNOS and nNOS*

| Compd. | Target | | | |
|---|---|---|---|---|
| | iNOS | | nNOS | |
| | BE | Key residues | BE | Key residues |
| **4c** | -22.0772 | Glu377, Tyr373 | -15.2395 | Arg608, Arg486 |
| **4d** | -25.8999 | Glu377, Arg388, Tyr373 | -17.2343 | Arg608, Arg486 |
| **5** | -19.4055 | Tyr373, Gln263, Glu377, | -11.7831 | Arg608, Arg486, Ala502 |
| L-NMMA[a] | -29.4375 | Hem550, Agr388 | -24.4860 | Arg608 |
| S71[b] | -35.5547 | Hem550, Trp372, Glu377, Met120 | - | - |
| BY7[c] | - | - | -16.8839 | Hem801, Trp592, Glu597 |

*BE: Binding energy (kJ/mol); [a]Control; [b]Co-crystallized ligands on iNOS crystal structures (RMSD value for redocking of 0.6492 Å); [c]Co-crystallized ligands on nNOS crystal structures (RMSD value for redocking of 1.2086 Å)*

For the iNOS and nNOS receptor, the configurational poses showed the interactions between the tested compounds with the key catalytic residues, as previously reported for S71

and BY7 inhibitor ($K_i$ = 6.6 µM, and $K_i$ = 0.541 µM, respectively) [6]. However, the compounds did not show direct H-bonds to the heme group of receptors. This is likely the cause of the rather poor activity of the selected compounds observed in the *in vitro* NO inhibition assays. Compound **4d** exhibited the best interaction compared to the remaining compounds. Specifically, compound **4d** was stabilized in the binding pocket by hydrogen bonds with key amino acids (Tyr373, Glu377, Arg388), and some hydrophobic interactions with Val352, Pro350, Met120, Trp463 and Gln283 at the active site of iNOS receptor. The remaining compounds showed interactions with at least two key amino acids among Gln263, Tyr347, Asp382, Tyr373 and Arg388 at the active site [7]. Regarding the binding poses on the nNOS, the docking results showed that the amino acids Glu597 and Arg608 in the binding pocket play an important role in determining the ability of the tested compounds to bind to the target nNOS. Compound **4d** also showed interactions with two key amino acids (Arg608 and Arg486). The benzimidazole nucleus was found to locate in the hydrophobic pocket and interacted with Pro570, Glu597, Val572 and Ser482. In summary, both the *in vitro* NO inhibitory activity and the molecular docking results confirm the activity of the tested compounds, which may be of great importance in future studies for the development of anti-inflammatory agents.

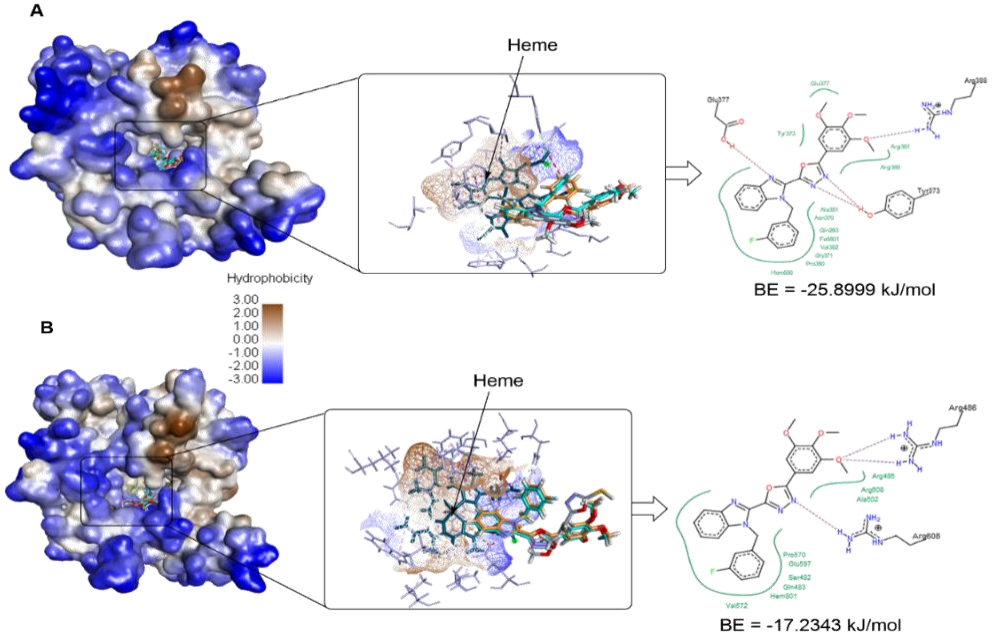



*Fig.1. Binding modes of compounds 4c (cyan), 4d (organ), and 5(gray) at the active sites of the iNOS (A) and nNOS (B) receptors. 2D interaction diagrams of compound 4d for each receptor (right).*

## 4. CONCLUSIONS

The 1,3,4-oxadiazole derivatives **4c-d** and **5** suppressed NO production in LPS-stimulated RAW264.7 macrophages. Docking-based virtual screening results showed affinity of these compounds with the iNOS and nNOS receptors. These results indicated that the structures **4c-d** and **5** would be considered as promising starting structures for further optimization towards pharmacological agents for the treatment of inflammatory conditions.

## REFERENCES

[1] Jin, S. E., Kim, O. S., Yoo, S. R., Seo, C. S., Kim, Y., Shin, H. K., Jeong, S. J, (2016). Anti-inflammatory effect and action mechanisms of traditional herbal formula Gamisoyo-san in RAW 264.7 macrophages. *BMC. Complementary and Alternative Medicine*, **16(1)**, 1-11.

[2] Somani, R. R., & Bhanushali, U. V, (2011). Synthesis and evaluation of antiinflammatory, analgesic and ulcerogenic potential of NSAIDs bearing 1,3,4-oxadiazole scaffold. *Indian Journal of Pharmaceutical Sciences*, **73(6)**, 634.

[3] Bala, S., Kamboj, S., Saini, V., & Prasad, D. N, (2013). Anti-inflammatory, analgesic evaluation and molecular docking studies of N-phenyl anthranilic acid-based 1,3,4-oxadiazole analogues. *Journal of Chemistry*, **2013(3),** 1-6.

[4] Quy, N. P., Hue, B. T. B., Do, K. M., Quy, H. T. K., De, T. Q., Phuong, T. T. B., ... & Morita, H, (2022). Design, Synthesis and Cytotoxicity Evalufation of Substituted Benzimidazole Conjugated 1, 3, 4-Oxadiazoles. *Chemical and Pharmaceutical Bulletin*, **70(6)**, 448-453.

[5] Aktan, F., Henness, S., Roufogalis, B. D., Ammit, A. J, (2003). Gypenosides derived from Gynostemma pentaphyllum suppress NO synthesis in murine macrophages by inhibiting iNOS enzymatic activity and attenuating NF-κB-mediated iNOS protein expression. *Nitric Oxide*, **8(4)**, 235–242.

[6] Do, H. T., Wang, H. Y., Li, H., Chreifi, G., Poulos, T. L., Silverman, R. B, (2017). Improvement of cell permeability of human neuronal nitric oxide synthase inhibitors using potent and selective 2-aminopyridine-based scaffolds with a fluorobenzene linker. *Journal of medicinal chemistry*, **60(22)**, 9360-9375.

[7] Fedorov, R., Hartmann, E., Ghosh, D. K., & Schlichting, I, (2003). Structural Basis for the Specificity of the Nitric-oxide Synthase Inhibitors W1400 and N ω-Propyl-l-Arg for the Inducible and Neuronal Isoforms. *Journal of Biological Chemistry*, **278(46)**, 45818-45825.