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Quinazolinone based hydroxamates as anti-inflammatory agents

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ABSTRACT

*Five thioether-linked hydroxamate/quinazolinone hybrid structures were synthesized and tested for their anti-inflammatory activities. The obtained results indicated that compounds **7a-c** and **7e** showed the inhibition on LPS-stimulated NO production with the IC_{50} values ranging from 58.03 to 66.19 µM. Molecular docking results showed that all synthesized compounds displayed affinity towards the 5-LOX, MK2, P2Y12, 15-PGDH, and DNA polymerase receptors based on the observed low binding energies and interactions with the key amino acids in the binding sites of the enzymes. Noticeably, compound **7e** exhibited as a potential compound targeting six receptors including 5-LOX, MK2, mPGES-1, P2Y12, 15-PGDH, and DNA polymerase receptors.*

1. INTRODUCTION

Inflammation is a normal defense mechanism by the body's immune system in response to toxins, infectious pathogens, and local injury (Dkhil et al., 2018). However, persistent and untreated inflammation may lead to the development of certain diseases. Therefore, anti-inflammatory agents may be helpful in the management of inflammatory disorders (Moilanen, 2014). 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 signaling pathways such as cyclooxygenases (COXs) (Vane et al., 1998; Smith et al., 2000), lipoxygenases (LOXs) (Kühn & O'Donnell, 2006), protein kinase MK2 (Kotlyarov et al., 1999; Gaestel, 2006) 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Tai et al., 2011), prostaglandin E synthase-1 (Samuelsson et al., 2007), P2Y receptors (Kügelgen & Wetter, 2000), and DNA polymerase (Ishida et al., 2011). Thus, inhibition of these enzymes may be a valuable treatment for inflammatory conditions.

Recently, the combination of two or more pharmacophores of bioactive scaffolds to form hybrid analogues with improved potency has been extensively applied for the development of new drugs or drug candidates. The presence of two or more biologically active pharmacophores in one unit is expected to enhance the biological activity as well as increase its ability to interact with more than one biological target (Kerru et al., 2017).

Nitrogen-based heterocycles, especially 4(3*H*)-quinazolinone (also named as quinazolinone) and its derivatives have attracted great attention in the field of medicinal chemistry due to their diverse biological activities including anti-cancer (Chandrika et al., 2008; Wdowiak et al., 2021), anti-microbial (Kuyper et al., 1996), anti-convulsant (Archana et al., 2002; Georgey et al., 2008), anti-malarial (Verhaeghe et al., 2008), anti-tumor (Abdel Gawad et al., 2010), and anti-inflammatory (Alagarsamy et al., 2007). On the other hand, the hydroxamate function, formally known as *N*-hydroxy-*N*-alkylamide, is found in various naturally occurring compounds and plays a key role in the

iron acquisition mechanisms of microorganisms. Over the last few years, compounds containing hydroxamate function have been studied for many therapeutic applications including metal detoxification, anticancer, antioxidant, antibacterial and antifungal, antitumor, especially anti-inflammatory activities (Bertrand et al., 2013; Chen et al., 2015). Hybrid analogues containing 4(3*H*)-quinazolinones and hydroxamates have recently been reported as potent HDACs inhibitors for the treatment of cancer (Yang et al., 2016; Osipov et al., 2020; Bui et al., 2022) or Alzheimer's disease (Yu et al., 2013). However, to the best of our knowledge, up to now, there has no report in the literature on the anti-inflammatory activity of 4(3*H*)-quinazolinone based hydroxamates.

In the view of the facts mentioned above and also in an attempt to develop new potential anti-inflammatory agents, herein we report our initial results on the anti-inflammatory activity of five 4(3*H*)-quinazolinone based hydroxamate derivatives. Molecular docking studies were carried out to understand their activity.

2. MATERIALS AND METHODS

2.1. Materials

Reactions were monitored by thin-layer chromatography on 0.2-mm pre-coated silica gel 60 F_{254} plates (Merck). ^{1}H-NMR and ^{13}C-NMR spectra were measured with Bruker Avance 400 MHz, Bruker Avance 500 MHz, and Bruker Avance 600 MHz spectrometers. Mass spectrometry data were recorded on an 1100 series LC-MSD-TrapLS Agilent spectrometer, and HR-ESI-MS observation was performed on a Bruker MicrOTOF-Q mass spectrometer. FT-IR was conducted using the KBr pellet method with a Thermo Nicolet 6700. Chemical shifts are given in parts per million relative to tetramethylsilane (Me_4Si, $\delta = 0$); *J* values are given in Hz. General procedures for the synthesis of the tested compounds can be found in the literature (Bui et al., 2022).

2.2. Nitric oxide assay

Briefly, RAW264.7 cells were grown in Minimum Essential Medium (α-MEM) supplemented with 1% penicillin-streptomycin solution and 10% Fetal Bovine Serum (FBS). When the cells reached about 70% confluency, they were harvested and diluted into fresh medium. Cell concentration was adjusted to 4 × 106 cells/mL. A total of 100 µL of cell suspension was seeded into a 96-well culture plate (4 × 104 cells/well) and incubated for 24 h at 37°C, in a humidified atmosphere containing 5% CO_2. Subsequently, 50 µL aliquots of 400 ng/mL of LPS and five different concentrations (50, 25, 12.5, 6.25, and 3.13 µM) of the isolated compounds and the positive control, L-*NMMA* were added to each well and incubated for 24 h. The supernatant (100 µL) from each well was transferred to another 96-well culture plate and combined with 100 µL of the Griess reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylethylene diamine dihydrochloride in 2.5% H_3PO_4), and then the reactions proceeded for 15 min at room temperature. The absorbance was recorded at 540 nm, using an SH-1200 microplate reader (Corona Electric). L-*NMMA* was used as a positive control. The percentages of NO inhibition were calculated by the following formula: % NO inhibition = 100 × [{Abs(control) – Abs(sample)}/Abs(control)]; where Abs(control) and Abs(sample) are the absorbances of the control group treated with LPS alone and the absorbance of the samples.

2.3. Molecular docking studies

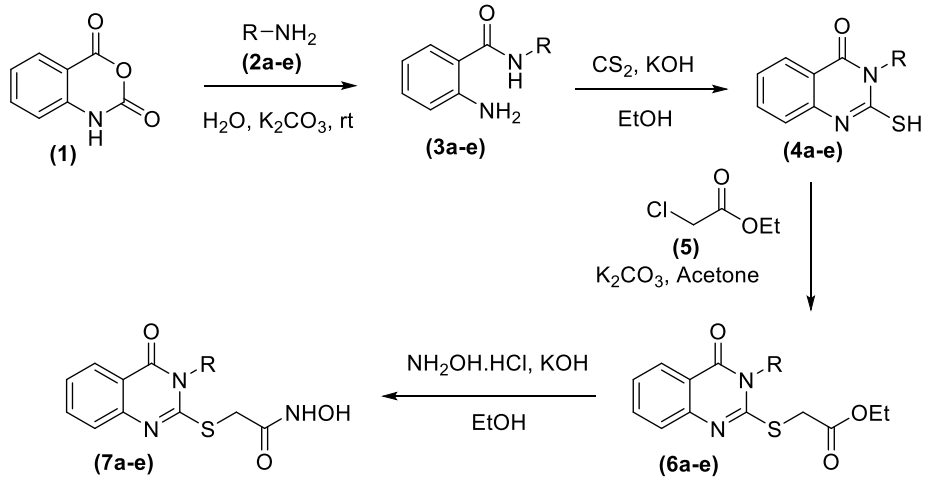
The ligands were optimized within the DFT at the B3LYP/6-31g(d,p) level by Gaussian programs (Frisch et al., 2009). The three-dimensional (3D) crystal structures of the cyclooxygenases-1 (ID PDB: 4O1Z), cyclooxygenases-2 (ID PDB: 4M11) 5-lipoxygenases (ID PDB: 3V99), protein kinase MK2 (ID PDB: 2JBP), 15-hydroxyprostaglandin dehydrogenase (ID PDB: 2GDZ), prostaglandin E synthase-1 (ID PDB: 5T37), P2Y receptors (ID PDB: 4NTJ), and DNA polymerase (ID PDB: 4MJQ) 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 (Kramer et al., 1999) 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. RESULTS AND DISCUSSION

3.1. Chemistry

The four-step synthetic pathway towards the desired hybrid quinazolinone/hydroxamate derivatives bearing thioether linkage (**7a-e**) is presented in Figure 1. As described previously in the literature

(Bui et al., 2022), starting from the commercially available isatoic anhydride (**1**), ring opening was taken place by appropriate amines (**2a**-**e**) under mild basic conditions to provide the corresponding 2-aminobenzamides (**3a**-**e**). Condensation reaction between (**3a**-**e**) and carbon disulfide was then conducted using a strong base as KOH to afford the quinazolinone core structures (**4a**-**e**) with the mercapto moiety being incorporated at the C-2 position of the heterocycle. The mercapto function was then further elaborated by introducing an ester substituent through a SN_2 nucleophilic substitution reaction with ethyl 2-chloroacetate (**5**) under mild basic conditions (K_2CO_3 in acetone) to provide thioether linkage containing quinazolinones (**6a**-**e**). The total yields over three steps were quite high (48-82%). Finally, aminolysis of the ester moiety took place by hydroxylamine hydrochloride to accomplish the desired hybrid structures quinazolinone/hydroxamic acid (**7a**-**e**). The total yields of the whole process (four steps) were moderate to good (14-69%). The structures of all synthesized compounds (**7a**-**e**) were firmly characterized based on reliable spectroscopic data including IR, HR-MS and NMR (Bui et al., 2022).



7a: R = $CH_2C_6H_5$, total yield 32%
7b: R = CH_2-4-F-C_6H_4, total yield 14%
7c: R = CH_2-3-F-C_6H_4, total yield 14%
7d: R = CH_2-4-CH_3-C_6H_4, total yield 69%
7e: R = CH_2-2-NO_2-C_6H_4, total yield 20%

Figure 1. Developed synthetic pathway towards hybrid quinazolinone/hydroxamate derivatives bearing thioether linkage

3.2. Anti-inflammatory 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 free oxygen radical and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (Moncada & Higgs, 1991; Alderton et al., 2001; Bogdan, 2001; Dawn & Bolli, 2002). Inhibition of NO production, therefore, may be beneficial for the treatment of inflammatory disease (Kröncke et al., 1998; Bogdan, 2001; Aktan et al., 2003).

The anti-inflammatory activities and cytotoxic effects of compounds **7a**-**e** were evaluated by monitoring the NO inhibition activity and cell viability of LPS-induced RAW264.7 cells, respectively. The results are presented in Table 1 and Table 2.

The obtained data in Table 1 indicated that compounds **7a**-**c** and **7e** displayed the inhibition on LPS-stimulated NO production with the IC_{50} values ranging from 58.03 to 66.19 μM, with the best result being observed for compound **7a** (IC_{50} = 58.03 μL) that was closely comparable with the control. Furthermore, compound **7a** did not show any significant effect on cell proliferation at all tested concentrations. The obtained results could serve as the starting points for further structural design and modification to obtain lead compounds with potential anti-inflammatory activities.

Table 1. NO inhibition activity

Samples	Concentration (μM)					IC_{50} (μM)
	6.25	12.5	25	50	100	
7a	15.17 ± 3.29	21.26 ± 3.35	28.97 ± 1.72	47.82 ± 0.20	75.29 ± 2.11	58.03 ± 2.35
7b	13.45 ± 0.00	18.05 ± 1.21	28.74 ± 1.31	44.72 ± 1.39	71.04 ± 0.00	63.00 ± 1.02
7c	13.79 ± 0.60	20.46 ± 0.87	27.24 ± 1.24	42.30 ± 0.87	72.18 ± 0.80	63.08 ± 0.27
7d	14.37 ± 0.84	19.08 ± 0.91	28.02 ± 1.37	43.24 ± 0.42	62.80 ± 0.55	70.59 ± 1.06
7e	9.78 ± 3.32	17.63 ± 2.81	24.52 ± 2.72	37.20 ± 2.21	72.46 ± 1.88	66.19 ± 3.35
L-NMMA	28.74 ± 1.51	40.70 ± 0.91	52.78 ± 1.11	61.96 ± 0.63	68.80 ± 0.00	37.11 ± 1.88

Table 2. Percentage of cell viability

Samples	Concentration (μM)				
	6.25	12.5	25	50	100
7a	114.82 ± 0.31	123.40 ± 3.92	120.79 ± 2.87	120.65± 3.91	105.48 ± 7.98
7b	95.45 ± 3.68	93.63 ± 2.36	89.86 ± 4.68	80.80 ± 1.41	77.20 ± 2.41
7c	111.22 ± 1.76	110.75 ± 2.52	110.93 ± 2.66	109.99 ± 2.39	109.78 ± 2.02
7d	102.03 ± 3.96	103.58 ± 0.58	97.75 ± 1.56	90.33 ± 0.78	52.10 ± 1.74
7e	117.08 ± 2.74	120.30 ± 4.31	119.67 ± 2.13	118.22 ± 2.11	98.90 ± 8.34
L-NMMA	109.22 ± 1.14	112.59 ± 2.07	115.03 ± 1.80	116.31 ± 1.26	110.59 ± 0.82

3.3. Docking results

Molecular docking is a fast and efficient computational method to predict the bioactive compounds to a specific protein or reversely predict the target proteins for one bioactive compound (Lee et al., 2016). In the aim to foresee the possible interactions of the synthesized compounds with the 5-LOX, COX-1, COX-2, MK2, mPGES-1, $P2Y_{12}$, 15-PGDH, and DNA polymerase, the molecular modeling simulations of **7a-e** into the binding pockets of the enzymes were performed. The study was based on the hypothesis that the 2-mercaptoquinazolinone core structure of **7a-e** possesses the ability to interfere with the active sites of the above target receptors and induces the inhibition of inflammation (Rajput & Singhal, 2013; Chaitanya et al., 2014).

The docking protocol was carried out by firstly redocking with the co-crystallized ligands into the receptors (Table 3 and Figure 2). The results showed that the root-mean-square-deviation (RMSD) values are all less than 2 Å, indicating the reliability of the docking model.

Table 3. Re-docking of bioactive co-crystallizers in the proposed sites of the receptors

Target	ID	Co-crystallized inhibitor	RMSD (Å)[g]
5-LOX	3V99	Arachidonic acid[a]	-
COX-1	4O1Z	MXM[b]	1.1807
COX-2	4M11	MXM[b]	1.1477
MK2	2JBP	P4O[c]	0.9887
mPGES-1	5T37	758[d]	1.9987
$P2Y_{12}$	4NTJ	AZJ[e]	0.8092
15-PGDH	2GDZ	-	-
DNA polymerase	4MJQ	27R[f]	1.5197

Reference ligands and ID of receptors were named according to the RCSB protein data bank. [a]Substitution for diclofenac as previously described by Srivastava et al.,2016. [b]Meloxicam. [c]2-(2-quinolin-3-ylpyridin-4-yl)-1,5,6,7-tetrahydro-4H-pyrrolo[3,2-c]pyridin-4-one. [d]2-chloro-5-{[(2,2-dimethylpropanoyl)amino]methyl}-N-(1H-imidazol-2-yl)benzamide. [e]ethyl 6-{4-[(benzylsulfonyl)carbamoyl]piperidin-1-yl}-5-cyano-2-methylpyridine-3-carboxylate. [f]Bromfenac; (-) Without a co-crystallization inhibitor or no RMSD value was calculated. [g]RMSD values were calculated using LeadIT software.

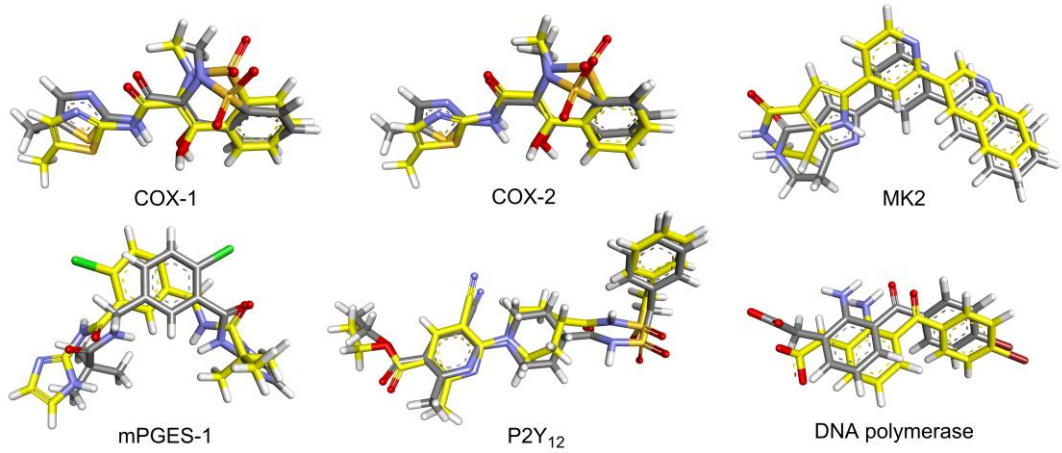


Figure 2. Re-docking protocols of inhibitors at the binding pockets of the receptors

re-docking ligand-yellow, co-crystallized ligand-gray

The virtual screening was then conducted. The results showed that all the tested compounds exhibited the binding ability towards 5-LOX, COX-2, MK2, $P2Y_{12}$, 15-PGDH, and DNA polymerase receptors, with the binding energies lower than -10 kJ/mol, but with poor selectivity on the COX-1 receptor (Table 4). Specifically, compounds **7a**, **7b**, **7d,** and **7e** demonstrated the binding energies of -19.7423, -20.2687, -20.3706, -24.2066, and -19.6698 kJ/mol, respectively, on the 5-LOX receptor, which were lower than that of the control diclofenac. Noticeably, compounds **7e** showed the highest potency on 5-LOX, mPGES-1, $P2Y_{12}$, 15-PGDH, and DNA polymerase receptors, with the predicted binding energy being lower than that of the controls. Most of the tested compounds formed interactions with key amino acid residues such as His372, His367, His550, Val671, and Phe177 at the binding site of 5-LOX. Three histidines (372, 367, and 550) formed polar hydrogen interactions with -C=O group of hydroxamate in a similar way as the nitro group of diclofenac (Figure 2). In particular, the nitro group of **7e** showed polar interactions with Phe177, while the remaining compounds showed only hydrophobic interactions. This may be the cause for the observed lower binding energy of **7e** at the binding site of 5-LOX compared to other compounds. Compounds **7b**, **7d**, and **7e** were not successfully docked into the hydrophobic binding site of COX-1, which showed the positive values of the binding energy, suggesting unfavorable configuration for the targeted COX-1 receptor.

Table 4. Multitargeted molecular docking protocol of the synthesized compounds

Ligand	Targeted molecular docking (kJ/mol)							
	5-LOX	**COX-1**	**COX-2**	**MK2**	**mPGES-1**	**$P2Y_{12}$**	**15-PGDH**	**DNA polymerase**
7a	-19.7423	-9.1671	-12.2080	-20.1827	-7.4103	-23.3960	-29.1612	-11.5204
7b	-20.2687	ND	-12.2469	-19.1724	-7.9871	-21.0091	-30.4524	-11.5775
7c	-17.8774	-10.3131	-12.8021	-17.8822	-4.4931	-21.0668	-30.3931	-12.4451
7d	-20.3706	ND	-11.1842	-18.2803	-2.9603	-20.2148	-27.7067	-11.5551
7e	-24.2066	ND	-12.7692	-22.4516	-13.1185	-32.1360	-31.9803	-15.4975
Diclofenac	-19.6698							
Meloxicam		-20.5138	-21.4030					
P4O				-24.1858				
758					-8.6743			
AZJ						-30.4465		
13[a]							-30.6314	
Bromfenac								-13.0852

[a]Piperidin-1-yl(1-(m-tolyl)-1H-benzo[d]imidazol-5-yl)methanone; ND: not docked; Reference ligand names were used according to the RCSB protein data bank.

Microsomal prostaglandin E synthase-1 (mPGES-1) is believed to be the main mediator of pain and inflammation. The selective mPGES-1 inhibitor may offer analgesic properties. Compound **7e** was predicted to be a potential inhibitor for this receptor based on the observed lower binding energy compared to the control 758, a known mPGES-1 inhibitor (Partridge et al., 2017). Furthermore, the key amino acid Arg126 was found to simultaneously form polar interactions with the nitro and hydroxamate groups of **7e** (Figure 3). Other interactions with Ser127 and Asn74 were also observed. These results are similar to the previously published docking model. The compound 758 showed direct interactions with the amino acids Asn74, Ser127, and Thr131, which were in accordance with the *in vitro* mPGES-1 inhibition results with an IC_{50} value of 0.241 µM (Partridge et al., 2017).

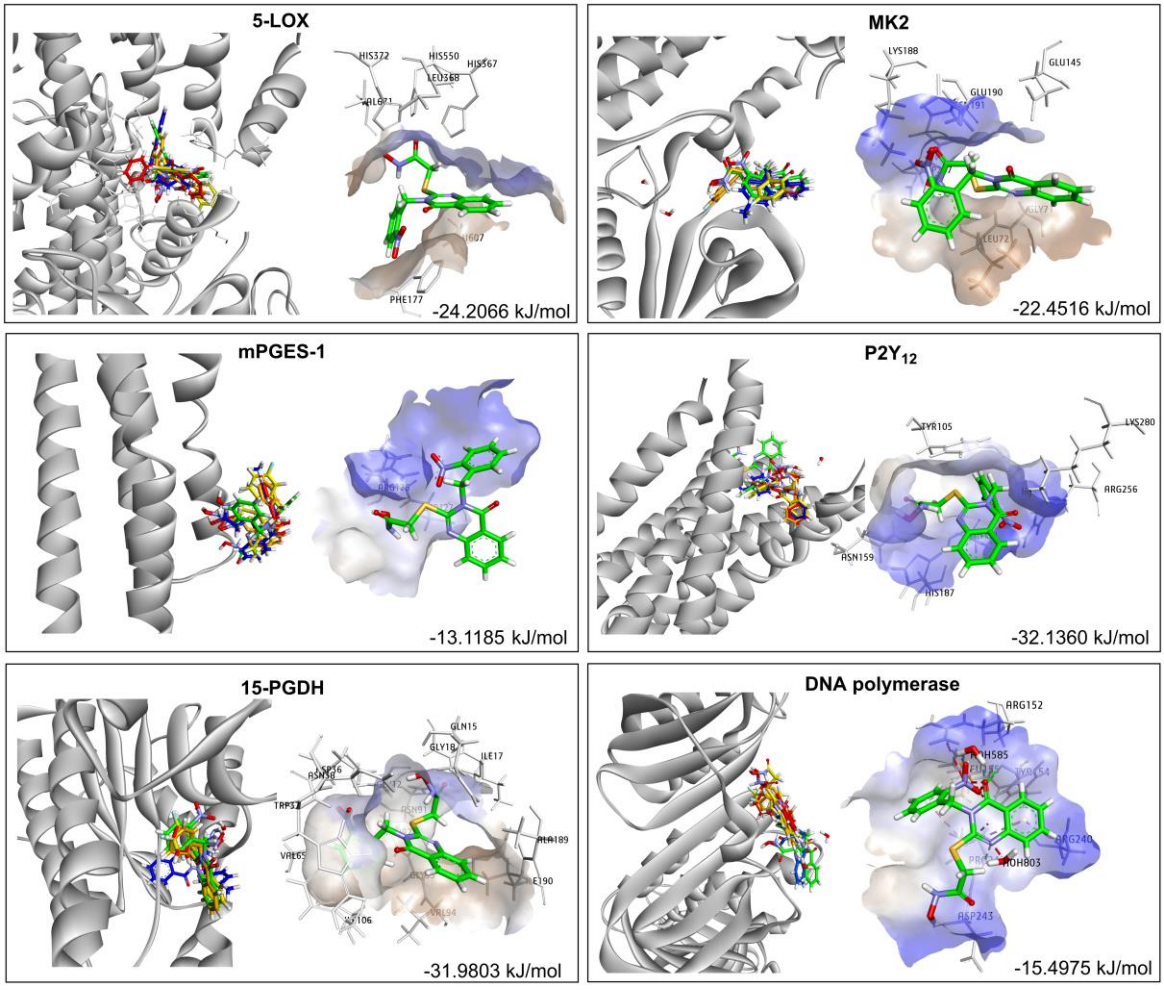


Figure 3. Molecular docking results of compounds 7a (red), 7b (orange), 7c yellow), 7d (blue), and 7e (green) at the active sites of the receptors (left) and interaction of the best compound 7e for each receptor (right). Blue surfaces indicate hydrophilicity and brown surfaces indicate hydrophobicity

Protein kinase MK2 is a member of the p38 MAP kinase pathway, which is a key regulator of the inflammatory process. In 2007, Hillig et al. reported the first structure of MK2 in complex with P40, a high affinity MK2 inhibitor with an IC_{50} value of 8.5 nM (Hillig et al., 2007). The docking results of **7a-e** into MK2 showed that the hydroxamate groups formed hydrogen bonds with Asp207 at the binding site in a similar way as described for compound P40. Furthermore, hydrogen interactions were also observed between the ligand and amino acids such as Leu72, Lys188, and Asn191. These binding mode would open avenues for further optimization through structure-based design and development of promising MK2 inhibitors.

In the case of 15-PGDH receptor, all tested compounds indicated a favorable binding mode at the binding pocket site of the enzyme, with the binding energy around -30 kJ/mol, which was comparable to the control compound 13, a known 15-PGDH inhibitor with the IC_{50} value of 56 nM (Niesen et al., 2010). In contrast to the binding mode of compound 13, compounds **7a-e** showed a more hydrophilic rather than hydrophobic binding mode. Several interactions have been observed between the hydroxamate groups and the hydrophilic amino acids Gln15, Asp36, and Asn91. The heterocyclic quinazolinone was oriented at the hydrophobic pocket site (Val94, Ala189, and Ile190). Noticeably, compound **7e**, with the lowest binding energy (-31.9803 kJ/mol) among the tested compounds, had the phenyl substituent of the quinazolinone being oriented to the adjacent hydrophobic site (Trp37, Val65, Ala92, and Ile106) and the nitro group participated in hydrogen interaction with the hydrophobic amino acid Trp37.

Several generations of $P2Y_{12}$-targeted antithrombotic drugs have been developed due to the critical functional role of this enzyme in platelet aggregation (Du & Liu, 2014). In 2014, the structure of the human $P2Y_{12}$ receptor in a complex with an antithrombotic drug (ligand AJN, IC_{50} = 9 nM) was reported by Zhang (Zhang et al., 2014). The AZJ was found to accommodate the hydrophobic pocket Val102, Val190, Tyr105, Tyr109, Tyr194, Phe252, Arg256, Tyr259, and Leu276, with the predicted binding energy of -30.4465 kJ/mol. Our docking studies showed that compounds **7a-e** formed polar hydrogen interactions with Arg256 in a similar way as AZJ. Especially, compound **7e** displayed as the most potential compound with the binding energy of -32.1360 kJ/mol, which was lower than that of the control AZJ. Furthermore, **7e** was oriented in a hydrophilic pocket and surrounded by key amino acids such as Tyr105, Tyr109, Asn191, and Phe252. Hydrogen interactions with amino acids His187, Asn159, Asn191, and Lys280 were also observed.

Some non-steroidal anti-inflammatory drugs (NSAIDs) have antibacterial properties. Targeting the DNA replication machinery, therefore, is a validated strategy for useful bacterial antibiotics (Yin et al., 2014). In this study, the tested compounds **7a-e** were docked into the *Escherichia coli* DNA polymerase III β subunit using bromfenac as the reference. Bromfenac is a known nonsteroidal anti-inflammatory drug, which inhibited the *E. coli* DNA polymerase III β subunit with the K_i value of 193 μM (IC_{50} = 328 uM) (Yin et al., 2014). The docking results indicated that compounds **7a-e** interacted with subsite I of the hydrophobic pocket in a manner similar to bromfenac. Compound **7e** had the lowest free binding energy (-15.4975 kJ/mol) compared to other compounds and was lower than that of the reference bromfenac (-13.0852 kJ/mol). In addition, this compound formed three hydrogen bonds at the active site (NO_2-Tyr154, NO_2-Arg152, CONHOH-Asp243). Tyr154 and water molecules (HOH585) showed the same binding configuration with the nitro group as in the case of bromfenac, while the phenyl substituent and the quinazolinone ring oriented into the hydrophilic pocket (Arg240, Pro242, and Thr172).

In summary, docking-based virtual screening results indicated that compounds **7a-e** showed affinity towards 5-LOX, MK2, $P2Y_{12}$, 15-PGDH, and DNA polymerase receptors based on the observed low binding energies and interactions with the key amino acids in the binding sites of the enzymes. Noticeably, compound **7e** exhibited as a potential compound targeting six receptors including 5-LOX, MK2, mPGES-1, $P2Y_{12}$, 15-PGDH, and DNA polymerase receptors. These results in combination with the *in vitro* NO inhibition activity would be good references for further structure-based design and synthesis of active agents which can be used for the treatment of inflammatory disorders.

4. CONCLUSIONS

Five thioether-linked quinazolinone/hydroxamate hybrid derivatives **7a-e** have been successfully synthesized in moderate to good total yields. The synthesized compounds exhibited moderate *in vitro* NO inhibition activities. Docking-based virtual screening results indicated that compounds **7a-e** showed affinity towards 5-LOX, MK2, $P2Y_{12}$, 15-PGDH, and DNA polymerase receptors. Noticeably, compound **7e** exhibited as a potential compound targeting six receptors including 5-LOX, MK2, mPGES-1, $P2Y_{12}$, 15-PGDH, and DNA polymerase receptors. These results indicate that quinazolinone conjugated hydroxamates are expected as potent candidates for discovery and development of anti-inflammatory drugs.

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