

Original Research Article

Design, synthesis and molecular docking studies of novel azo-linked 1,3,4-oxadiazole derivatives for targeting C-Met tyrosine kinase receptor as a possible anticancer agent

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Abstract

Purpose: To synthesize and characterize azo derivatives containing the bioactive oxadiazole moiety.

Methods: A novel series of azo-linked 1,3,4-oxadiazole derivatives (compounds 3a - 3d) targeting C-Met tyrosine kinase receptor was prepared in a three-step procedure, and cell viability was assessed using an MTT assay against hepatocellular carcinoma (HCCL) cell lines, with crizotinib as a reference drug.

Results: Molecular docking scores against the C-Met tyrosine receptor ranged from -5.75 to -8.47 kcal/mol. It is important to note that compounds 3a - 3d were predicted to have better binding affinities than the reference inhibitor, crizotinib, with docking values of -6.80 kcal/mol, and thus are potential C-Met inhibitors. These docking findings were also supported by in vitro cytotoxicity HCCL assays. The mean inhibition percentages were as follows: crizotinib (64.33 %); compound 3a (72.42 %); compound 3b (70.14 %); compound 3c (73.54 %); and compound 3d (78.53 %).

Conclusion: Comparison of the synthesized compounds with crizotinib has shown that all were more cytotoxic, with compound 3d being the most active. All findings indicate that the synthetic compounds possess considerable promise as anticancer agents targeting C-Met tyrosine kinase receptors and warrant further biological and pharmacological studies.

Keywords: Azo derivatives, 1,3,4-Oxadiazole, C-Met tyrosine kinase, Molecular docking, Cytotoxicity assay

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INTRODUCTION

Cancer is one of the fastest-growing diseases, and it has been ranked as the leading cause of death in industrialized nations. Meanwhile, it is second only to cardiovascular disease in terms of development. The number of new cancer cases to be diagnosed annually is expected to reach 21

million by 2030 [1]. The increasing cancer burden worldwide has prompted considerable research to develop effective, novel, and selective anticancer agents that can inhibit tumor cell growth or suppress tumor cell proliferation.

The major limitation of standard chemotherapy has been the mode of non-selectivity, such that

these drugs tend to induce destruction to both normal and malignant cells. By contrast, targeted therapies are developed to block essential biochemical pathways or oncoproteins that are critical to tumor cell proliferation and survival, thereby targeting cancer cells while leaving normal tissue intact. One of the most recent developments in this field is the inhibition of oncogenic proteins, as well as the mechanisms that trigger their degradation, including small interfering RNA (siRNAs) for controlling gene expression and pharmacological products that regulate post-translational modifications [2].

Receptor tyrosine kinases are critical in tumor development, particularly in gastric, esophageal, colorectal, and pulmonary cancers [3]. Various small-molecule inhibitors have been developed to selectively target tyrosine kinases (TKIs). However, resistance mechanisms, such as EGFR mutations and c-Met overexpression, often reduce the clinical efficacy of these drugs by decreasing drug binding or altering downstream signaling pathways, allowing tumors to survive despite treatment [4]. These drug resistance mechanisms usually decrease drug binding or disrupt downstream signaling pathways, allowing tumors to survive despite treatment. Some possible solutions to address EGFR-TKI resistance include the development of new, irreversible TKIs, anti-EGFR monoclonal antibodies, combination therapy, and oncoprotein degradation agents.

The 1,3,4-oxadiazole derivatives have garnered considerable attention as potential anticancer compounds due to their diverse biological activities [5]. Oxadiazole derivatives exhibit a wide range of biological activities, including antiviral, antimicrobial, anti-inflammatory [6], antioxidant [7], and antidiabetic [8,9] effects. Many compounds derived from oxadiazole derivatives have advanced to clinical use. Crucially, 1,3,4-oxadiazole derivatives are promising candidates for developing new medications to overcome TKI resistance, as they are chemically simple to handle and yield effective results.

The FDA approved crizotinib, a pyridine-based chemotherapeutic drug (Figure 1), for use in treating lung cancer in 2011. One of the most potent c-Met inhibitors is crizotinib, which has been approved for the treatment of non-small cell lung cancer [10,11]. Compared with traditional chemotherapy, targeted therapies often exhibit enhanced effectiveness and reduced toxicity due to their selective mechanisms of action [12]. However, acquired resistance resulting from additional mutations or alternative signaling

pathways frequently limits their long-term clinical success.

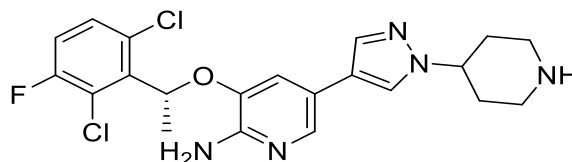


Figure 1: Chemical structure of crizotinib

With the growing global cancer problem and the persistent challenge of resistance to current TKIs, there is a pressing need for novel, selective anticancer agents that can overcome these limitations. 1,3,4-oxadiazole derivatives exhibit significant structural flexibility and biological activity, making them promising candidates for next-generation TKIs. Therefore, the present study was designed to synthesize and characterize oxadiazole derivatives, evaluate their *in vitro* anticancer activity in hepatocellular carcinoma cells compared to crizotinib, and investigate their molecular interactions with tyrosine kinase targets (particularly c-Met and ALK) using molecular docking simulations. The ultimate objective is to identify oxadiazole-based lead compounds with the potential to serve as selective and effective agents against TKI-resistant cancers.

METHODS

Diisopropyldiazene-1,2-dicarboxylate and carbon disulfide (Sigma-Aldrich, USA), hydrazine hydrate (Scharlau, Spain), potassium hydroxide and sodium acetate (Merck, Germany) were acquired from commercial suppliers and used without additional purification. Structural characterization was carried out using Nuclear Magnetic Resonance spectroscopy (^1H NMR and ^{13}C NMR) on a Bruker Biospin GmbH spectrometer operating at 400 and 100 MHz, respectively. Fourier-transform infrared (FT-IR) spectra were recorded using a PerkinElmer Spectrum 27 instrument (Bruker, Germany).

Preparation of compound 2

Compound 2 was synthesized via ester hydrazinolysis followed by cyclization to the corresponding 1,3,4-oxadiazole as described in the literature [13–18], 0.97 mL of hydrazine hydrate (0.02 mol) was dissolved in 50 mL of anhydrous ethanol (R.B.F). Then, under reflux conditions, 2.02 mL of diisopropyldiazene-1,2-dicarboxylate (0.01 mol) was added dropwise. The progress reaction was monitored by thin-layer chromatography (TLC) with a (4:6) hexane: ethyl acetate mixture as eluent, resulting in

compound 1 (5-hydrazinecarbonylcarbazone) as previously reported [1]. To obtain a solid precipitate, the solvent was gently evaporated at a moderate temperature. To produce white needle-shaped crystals (m.p. 142 - 144 °C), the crystals were collected by filtration, dried, and then purified using an appropriate solvent. Next, potassium hydroxide (1.06 g, 0.02 mol) was dissolved in anhydrous ethanol (50 mL), and carbon disulfide (CS₂; 1.23 mL, 0.02 mol) was slowly added at 0 °C. The flask was heated in reflux mode for 65 hours. Hydrogen sulfide (H₂S) development was also observed using lead acetate paper, which turned black, indicating the formation of lead sulfide. Thereafter, reduced pressure was used to remove the solvent, and the participants were solidified using 10 % hydrochloric acid (HCl), followed by filtration and water washing. The final solid material was crystallized using ethanol to produce compound 2. The characterization of compound 2 is as follows:

- New band absorption appeared at 3204 cm⁻¹ (NH tautomer/SH), 1609 cm⁻¹ (C=N of oxadiazole ring), 1477 cm⁻¹ (asymmetric C-O-C), and 1350 cm⁻¹ (symmetric C-O-C C=N) in the FT-IR spectrum.

- Singlet signals for the NH tautomeric proton (SH) at δ 14.14 ppm, equivalent to the tautomeric SH proton, were visible in the ¹H-NMR spectrum. Furthermore, the ¹³C-NMR spectrum displays signals corresponding to the oxadiazole ring at δ 181.11 (C=N-N=N-) and 161.94 (C=N-NH).

Synthesis of compounds 3a - 3d

Compound 2 (2.3 g, 0.01 mol) and sodium acetate (CH₃COONa; 1.64 g, 0.02 mol) were combined in 100 mL of 100 % ethanol and heated for 15 min. Subsequently, the reaction mixture was refluxed for 4 hours after the addition of the alkyl halides (0.02 mol). TLC was used to monitor the reaction's progress with a 7:3 hexane: ethyl acetate eluent. After filtering and water washing, the solid precipitate was recrystallized from ethanol. The physicochemical characteristics of the synthetic chemicals are presented in Table 1, labeled 3a - 3d. The following are each compound's spectroscopic characteristics:

- For compound 3a, the ¹H-NMR spectrum showed a singlet signal for the 6H, CH₃ proton at δ 2.21 ppm, and the ¹³C-NMR spectrum revealed signals at δ 150.22 (C=N-N=N-), 141.59 (C=N-CH₂), and 27.50 ppm (CH₃).

- For compound 3b, the ¹H-NMR spectrum displayed signals at δ 0.86-0.91 ppm (t, 6H, CH₃), δ 1.33 - 1.40 ppm (m, 2H, CH₃), and δ 2.95 - 3.01 ppm (m, 2H, CH₃), and the ¹³C-NMR spectrum recorded signals at δ 165.77 (C=N-N=N-), 149.67 (C=N-CH₂), 56.80 (CH₂), 28.88 (CH₂), and 20.31 ppm (CH₃).

- For compound 3c, the ¹H-NMR spectrum showed multiplets at δ 0.95 - 0.77 ppm (t, 6H, CH₃), δ 1.30 - 1.20 ppm (m, 2H, CH₃), δ 1.44 - 1.32 (m, 2H, CH₃) ppm, δ 1.56 - 1.60 ppm (m, 2H, CH₃), δ 3.25 - 3.28 ppm (m, 2H, CH₃), and the ¹³C-NMR spectrum revealed signals at δ 165.57 (C=N-N=N-), 155.89, (C=N-CH₂), 51.02 (CH₂), 36.48 (CH₂), 27.50 (CH₂), 36.48 (CH₂), 21.61 (CH₃), and 16.24 ppm (CH₃).

- For compound 3d, the ¹H-NMR spectrum displayed signals at δ 4.51 ppm (s, 2H, CH₂) and δ 6.65 - 7.41 ppm (m, 2H, CH₃), and the ¹³C-NMR spectrum revealed signals at δ 154.54 (C=N-N=N-), 153.59 (C=N-CH₂), and 35.41 (CH₂).

Molecular docking protocol

The Molecular Operating Environment (MOE) 2019.0101 (Chemical Computing Group Inc., Canada) was used for molecular docking. The crystal structure of the c-Met tyrosine kinase receptor (PDB ID: 7B43) was obtained from the RCEB Protein Data Bank (<https://www.rcsb.org>) and created by modifying protonation states, deleting water molecules, and adding a hydrogen atom. ChemDraw was used to sketch the ligands (compounds 3a - 3d and crizotinib). These were then converted to 3D structures and energy-minimized using the MMFF94 force. Docking was performed at the ATP-binding site of the c-Met tyrosine kinase receptor using the Triangle Matcher placement method and the London dG scoring function, followed by force-field-based minimization. Binding affinities and interaction profiles were analyzed, and the reliability of the docking protocol was confirmed by redocking the co-crystallized ligand, ensuring a root mean square deviation (RMSD) of ≤ 2.0 Å.

MTT cell viability assay

The cytotoxicity of the produced compounds (3a - 3d) and crizotinib was evaluated in 96-well microplates using the MTT test. Hepatocellular carcinoma cell lines (HCCLs) were planted (1 x 10⁴ cells/well) and incubated for 24 hours. After producing a confluent monolayer, the wells were treated with compounds 3a - 3d and crizotinib (1000 µg/mL each) for 72 hours. After discarding the growth medium, add 28 µL of 2 mg/mL MTT

solution to each well and incubate for 2 hours at 37 °C to generate formazan crystals. After discarding the solution, the crystals were dissolved in dimethyl sulfoxide (100 µL) with gentle agitation for 15 minutes at 37 °C. A microplate reader was used to determine the absorbance at 620 nm. The results of six replicates were shown as the mean $\pm$ standard deviation of the inhibition rate and calculated using Eq 1 and 2.

$$\text{Proliferation rate (PR \%)} = (B/A)100 \dots\dots (1)$$

$$\text{Inhibition rate (IR \%)} = 100 - \text{PR} \dots\dots\dots (2)$$

where A and B are the absorbance of the untreated control and treated wells, respectively.

Statistical analysis

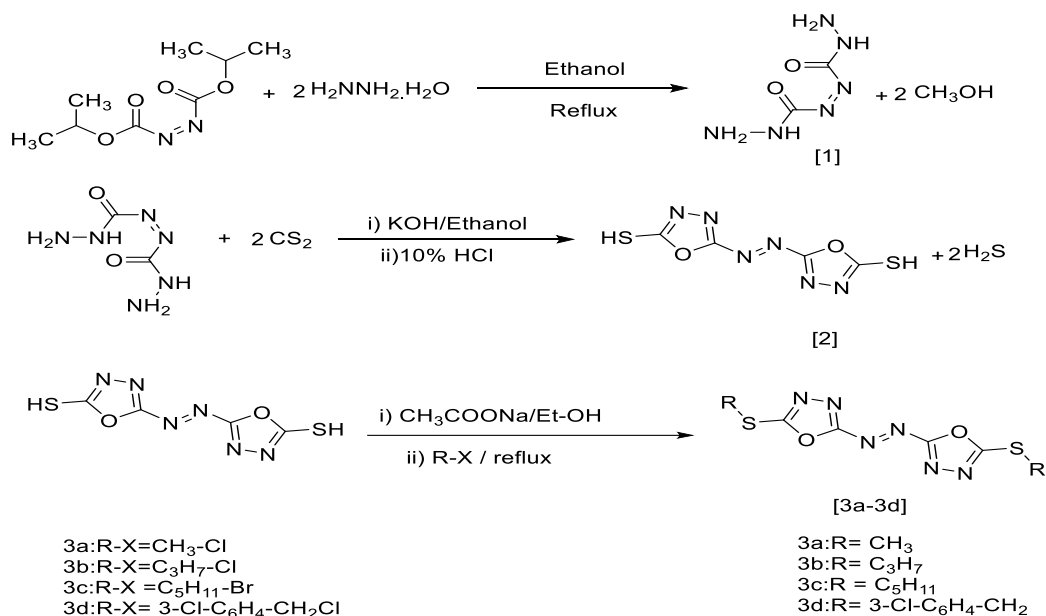
All analyses were run in SigmaStat version 19.0 (Chicago, Illinois, USA). Results are reported as mean $\pm$ standard deviation (SD) for six replicates per group (n = 6). One-way ANOVA was used to identify significant main effects. Means were compared using a Tukey HSD test to identify significant pairwise differences. The alpha level was set at < 0.05, which was considered significant. The assays were utilized to compare

cytotoxicity between the produced compounds and crizotinib.

RESULTS

Synthesis of compounds 3a - 3d, identified as 5-((5-(alkylthio)-1,3,4-oxadiazol-2-yl)amino)-1,3,4-oxadiazole-2-thioalkyl, was achieved in a method involving three steps (Scheme 1). Initially, reacting diisopropyldiazene-1,2-dicarboxylate with hydrazine hydrate resulted in the production of 5-hydrazinecarbonylcarbazone (compound 1). In the next step, carbon disulfide was used to cyclize compound 1, assisted by potassium hydroxide, to produce 5,5-phenyl-1,3,4-oxadiazole-2-thiol (compound 2). Finally, mixing two moles of alkyl halides with compound 2 in the presence of sodium acetate resulted in a new 5-((5-(alkylthio)-1,3,4-oxadiazol-2-yl)amino)-1,3,4-oxadiazole-2-thioalkyl (compounds 3a - 3d).

The synthesized compounds (3a - 3d) were pale yellow solids with melting points ranging from 214 to 230 °C and yields of 83 - 86 %, corresponding to an increase in molecular weight from 285 to 451 (Table 1).



Scheme 1: Multi-step reaction for the preparation of 5-((5-(alkylthio)-1,3,4-oxadiazol-2-yl)amino)-1,3,4-oxadiazole-2-thioalkyl (compounds 3a - 3d)

Table 1: Physicochemical properties and molecular formula of target compounds 3a - 3d

Comp no	Mol formula	Mol Wt	M.P (°C)	Yield (%)	Color
3a	C ₆ H ₆ N ₆ O ₂ S ₂	258	214-216	83	Pale Yellow
3b	C ₁₀ H ₁₄ N ₆ O ₂ S ₂	314	216-218	86	Pale Yellow
3c	C ₁₄ H ₂₂ N ₆ O ₂ S ₂	382	222-224	84	Pale Yellow
3d	C ₁₆ H ₈ Cl ₂ N ₆ O ₂ S ₂	451	228-230	86	Pale Yellow

Molecular docking results

Docking tests were carried out to determine the binding affinities of the newly targeted prepared compounds 3a - 3d for the c-Met tyrosine kinase receptor, using crizotinib as a reference inhibitor (Table 2). The docking score of compound 3a was -5.75 kcal/mol, representing a moderate binding affinity. Simultaneously, the docking score of compound 3b was better than that of compound 2a, recording a -6.16 Kcal/mol. Additionally, the improved score for compound 3c was -6.90 Kcal/mol. Remarkably, compound 3d had the most favorable score of -8.47 Kcal/mol, demonstrating superior binding interactions.

Table 2: Docking scores of synthesized compounds (3a - 3d) and the reference compound with c-Met tyrosine

Compound	Lowest Binding Energy (kcal/mol)
3a	-5.75
3b	-6.16
3c	-6.90
3d	-8.47
Crizotinib	-6.80

The key molecular interactions included hydrogen bonding, π - π stacking, π -sulfur interactions, and hydrophobic (alkyl and van der Waals) contacts. The docking interactions are shown in Figure 2.

Cytotoxicity on hepatocellular carcinoma cell lines (HCCLs)

The cytotoxicity of novel compounds (3a - 3d) demonstrated anticancer activity in HCCLs. Compared with crizotinib, the synthesized compounds showed mean inhibition rates ranging from 70.42 to 78.53 %, which were superior to that of crizotinib (64.33 %). The detailed results of the cytotoxicity assay for compounds (3a - 3d) are shown in Table 3 and Figure 3. The data showed that all synthesized compounds exhibited significantly greater cytotoxicity than crizotinib ($p < 0.01$ to $p < 0.001$).

DISCUSSION

The design and synthesis of novel bioactive compounds are crucial in modern medicinal chemistry, particularly in the development of targeted treatments for cancers such as hepatocellular carcinoma. In this study, a series of symmetrical heterocyclic derivatives, 5-((5-(alkylthio)-1,3,4-oxadiazol-2-yl)amino)-1,3,4-oxadiazole-2-thioalkyl (compounds 3a - 3d), was prepared using a straightforward three-step process. The 1,3,4-oxadiazole present in these compounds is a heterocycle recognized for its diverse pharmacological activities, expanding the biological importance of these compounds [19,20].

Structurally, the synthetic compounds were obtained in good yields (83 - 86 %), demonstrating the efficiency and reliability of the procedure. The rising melting points and intermolecular forces in compounds 3a - 3d were correlated with increased molecular rigidity and stronger intermolecular interactions, suggesting that size and polarity affect their physical properties. Additionally, alkylthio functionality increases lipophilicity, thereby enhancing cellular uptake and bioavailability by increasing membrane permeability. Also, the diazene linking group is electron-rich and can thus be used in DNA interactions, enhancing the biological activity of those compounds [13,19]. Previous studies have shown that the central ring of the oxadiazole chemical structure is the main determinant of their biological activity, thereby improving their metabolic and pharmacokinetic properties and augmenting their therapeutic potential [21].

Molecular docking was used to determine the binding affinities of compounds 3a - 3d with the c-Met tyrosine kinase receptor, which is essential in the pathogenesis of several cancer types, including hepatocellular carcinomas (HCCs) [22]. The findings showed that 3d had the highest binding affinity (-8.47 kcal/mol), which is better than that of the clinically approved c-Met inhibitor, crizotinib (-6.80 kcal/mol), under the same conditions.

Table 3: Comparative cytotoxicity of crizotinib and synthesized compound (3a - 3d) at 1000 µg/mL on HCCLs

Compound	Crizotinib	3a	3b	3c	3d
Mean absorbance	0.053	0.048	0.053	0.053	0.053
Mean inhibition (%)	64.332	72.424	70.138	73.541	78.532
Standard Deviation	1.630	1.64	2.92	2.897	3.68
P-value (Tukey HSD)	-	0.002	0.008	<0.001	<0.001
Significance	-	**	**	***	***

Note: Data are based on six technical replicates (n = 6). ** $P < 0.01$, *** $p < 0.001$ vs crizotinib

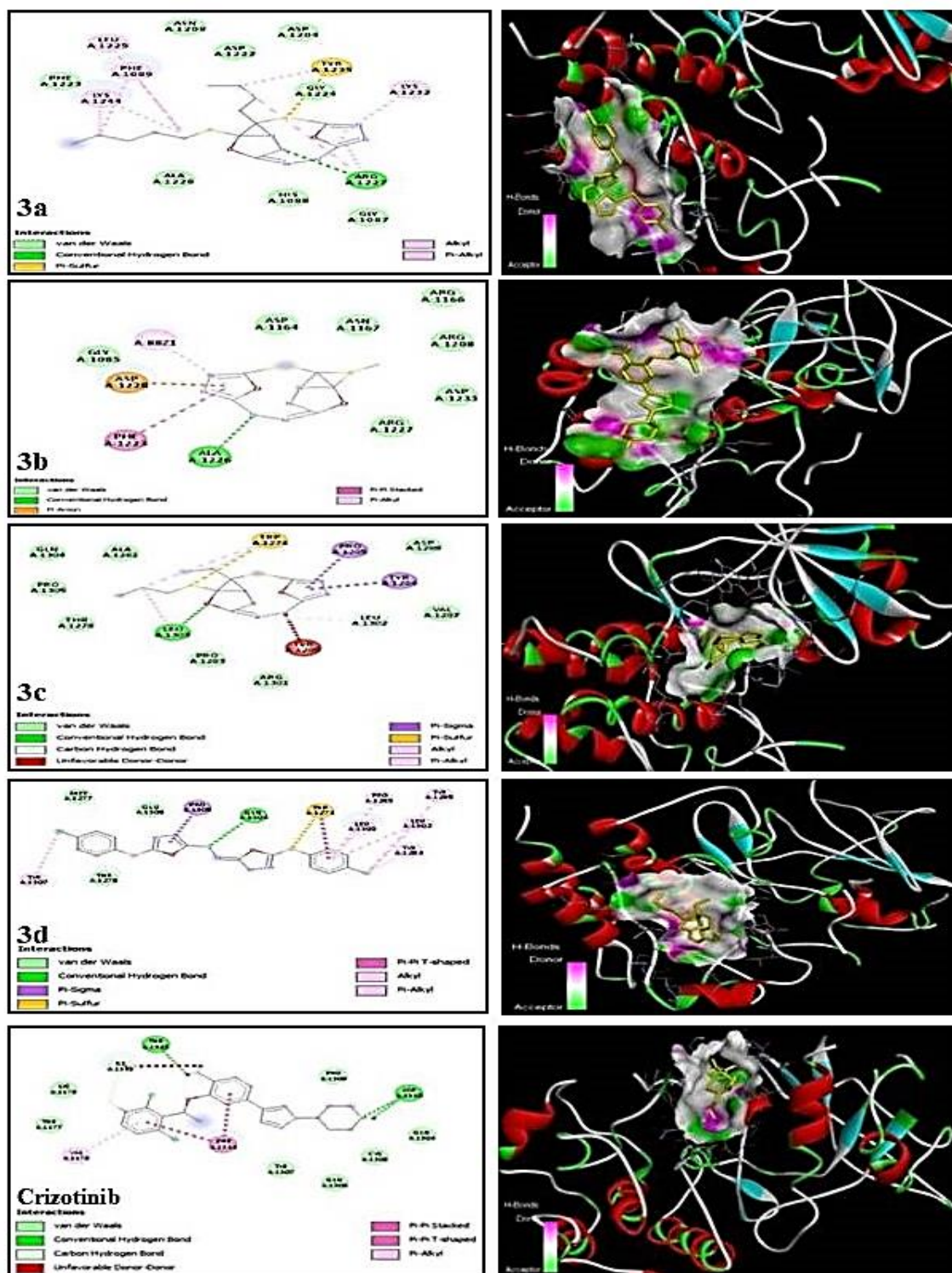


Figure 2: Visualization of two-dimensional and three-dimensional Binding and interactions of molecules with c-Met tyrosine kinase and the synthesized compounds (3a, 3b, 3c, and 3d), as well as the reference inhibitor compound (crizotinib). Two-dimensional (left panels) and three-dimensional (right panels) images

This excellent affinity is due to a deal of interactions, a hydrogen bond with the leucine residue, a carbon-hydrogen bond with arginine, a O-O sulfur interaction with tyrosine, as well as a hydrophobic contact at the receptor active site, as observed in earlier studies [20,23]. These

peculiarities can be enhanced by encouraging the compound to bind to the active site of c-Met tyrosine receptors, as proposed in the study [13], or by other mechanisms.

The cytotoxicity of the novel compounds in vitro on HCCLs was tested. All compounds showed potent inhibition relative to the reference compound, crizotinib. Compound 3d was the most active, with 78.53 % inhibition of HCCL viability, followed by 3c (75.87 %), 3a (72.66 %), and 3b (70.14 %). On the contrary, the inhibition rate by crizotinib (64.33 %) under identical conditions was lower, indicating that the synthesized compounds exhibit favorable binding affinity and are possible candidates for cancer therapy, as earlier studies have examined the effects of oxadiazole derivatives [24,25]. Moreover, it seems that oxadiazole's best cytotoxicity can be explained by the presence of sulfonamide links in the compound, which increase its contact with the biological target. Also, the bulky and halogenated aromatic functional groups of compound 3d might undergo cellular uptake and membrane permeability, and enhance noncovalent interactions, including π - π stacking and halogen bonding, resulting in accumulation in cancer cells and thus promoting the process, as observed in the literature [26].

The structure-activity relationship (SAR) investigation revealed that lipophilic alkylthio groups enhance membrane permeability and hydrophobic interactions at the receptor-binding active site. In addition, the thick layers on the phenyl rings, especially in compound 3d, enable π - π and π -sulfur stacking interactions that are critical for inhibiting the kinase. The diazene linker also provides some rigidity and planarity to the molecule, thereby allowing optimal alignment at the active site and potentially increasing the efficiency of DNA intercalation [13]. All these structural aspects may improve bioavailability and selectivity against the target, warranting further studies to optimize therapeutic activity.

Even though the outcomes are encouraging, it remains unclear how the identified cytotoxicity is achieved. Heterocyclic systems containing, in particular, 1,3,4-oxadiazole and diazene functional groups exhibit multiple pathways, suggesting that multiple biological pathways can mediate the anticancer effect. Such processes involve the inactivation of receptor tyrosine kinases (RTKs), including c-Met and other similar targets (ALK and EGFR), as well as DNA intercalation, resulting in replication stress, mitochondrial-mediated apoptosis, and oxidative damage via reactive oxygen species. Further studies are required to verify these mechanisms by conducting large-scale RTK inhibition tests, cell cycle and apoptosis analyses, and caspase-3 and -9 activity assays. It will also need in vivo models to determine pharmacokinetics, biodistribution, and therapeutic efficacy.

Assessment of metabolic stability and drug absorption, distribution, metabolism, and toxicity (ADME/Tox) will also be required to evaluate drug-like properties. The future of medicinal chemistry should focus on the development of prodrug analogues to enhance solubility and bioavailability, innovative delivery systems and tumor-targeting moieties to enhance tumor selectivity and reduce off-target effects, and on developing these compounds as anticancer agents.

CONCLUSION

The 5-((5-(alkylthio)-1,3,4-oxadiazol-2-yl)amino)-1,3,4-oxadiazole-2-thioalkyl has been synthesized via a short cyclization reaction. The synthesized compound demonstrates strong anticancer properties. Molecular docking results indicated that compound 3d exhibits the best binding affinity (-8.47 kcal/mol) to c-Met tyrosine kinase receptor than crizotinib (-6.80 kcal/mol). Compound (3d) showed significant cytotoxicity against the HCCLs, suggesting that these new compounds may serve as cancer agents. Furthermore, the findings suggest that the oxadiazole-based diazenes, particularly compound 3d, have significant potential as lead candidates for the development of c-Met tyrosine kinase inhibitors in cancer therapy. To further validate their therapeutic promise, in vivo studies, pharmacokinetic evaluations, and ADME profiling will be critical for confirming their therapeutic uses.

DECLARATIONS

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Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript polishing purposes (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions. All intellectual contributions, study analyses, and interpretations

reported in the manuscript are the original work of the authors.

Availability of data and materials

This paper contains all the data that were produced or examined during this investigation.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. F.M.H., B.A.H., M.M.A., and H.J.K. worked on the design, synthesis, molecular docking, and cytotoxicity testing of new 5-((5-(alkylthio)-1,3,4-oxadiazol-2-yl)amino)-1,3,4-oxadiazole-2-thioalkyl derivatives that target the C-Met tyrosine kinase receptor in resistant HCCLs. F.M.H. wrote the original draft, which was then corrected and finalized by H.J.K., who also produced the latest version for submission. All authors approved the final manuscript.

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