Synthesis and cytotoxic activity evaluation of some new 1, 3, 4-oxadiazole, 1, 3, 4-thiadiazole and 1, 2, 4-triazole derivatives attached to phthalimide

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Abstract

Background and purpose: In the last few decades, nitrogen-rich heterocyclic compounds such as 1, 3, 4-thiadiazoles, 1, 2, 4-triazoles and 1, 3, 4-oxadiazoles have received considerable attention because of their notable biological properties, especially cytotoxic effects. The small molecules of mentionedazole derivatives revealed very intensive antitumor activity. In addition, phthalimide-thiadiazole and naphthalimide-triazole hybrid derivatives have shown remarkable cytotoxic effects. According to these observations, some of the hybrid derivatives containing the phthalimide-five-membered azoles were prepared in three steps in this research.

Experimental approach: The thiol group of azoles was treated with ethyl chloroacetate which was followed by a reaction with hydrazine hydrate to provide acid hydrazide derivatives. Subsequently, the corresponding acid hydrazides were utilized to prepare the final derivatives through the reaction with phthalic anhydride. Cytotoxic activity of final compounds was evaluated against MCF-7 and HeLa cell lines using MTT assay.

Findings/Results: Compound 3d containing two phthalimide moieties in its structure showed a significant improvement in cytotoxic activity with an IC50 value of 29 µM against HeLa cell line. Compounds 3a-3c showed less cytotoxic effects against both cell lines.

Conclusion and implications: The combination of the thiadiazole nucleus with two phthalimide structures increased the cytotoxic activity against the HeLa cell line. This increase in cytotoxic activity is probably due to its being more lipophilic characteristic and interaction of this derivative with the biological targets of two directions.

Keywords: Cytotoxic activity; Phthalimide; 1, 3, 4-Oxadiazole; 1, 3, 4-Thiadiazole; 1, 2, 4-Triazole.

INTRODUCTION

Cancer treatment is still a major public health concern and a situation demanding serious investigation for more safe and effective chemotherapeutics, especially in view of metastasis and drug resistance development. Therefore, the development of novel anticancer agents has remained a major challenge in the field of cancer chemotherapy (1,2).

Five-membered heterocyclics are used as a pivotal scaffold of many compounds in medicinal chemistry due to their similarity with biologically active compounds within our body and provide a wide range of biological activities such as anticonvulsant, anticancer, antimicrobial, and anti-inflammatory effects (2,3). In this family, azoles, including 1, 3, 4-oxadiazoles, 1, 3, 4-thiadiazoles and 1, 2, 4-triazoles have been involved as “privileged” construction in plentiful therapeutic areas particularly anticancer chemotherapy (2-9). Literature survey indicated that a minor alteration in the structure of 1,3,4-oxadiazoles, 1,2,4-triazoles and 1,3,4-thiadiazines can lead to changes in their biological activities (10).
During recent years, some small molecules containing the aforementioned azoles were synthesized as potential anticancer agents (7,9,11-13). Structure-activity relationship studies have indicated that a basic scaffold of 1, 3, 4-oxadiazoles is necessary for the broad spectrum cytotoxic activity towards diverse cell lines (14). 1,3,4-Oxadiazole is bioisosteres of amides and esters, which can participate in hydrogen bonding interactions with the receptors (2,6). Mono and di-substituted 1, 3, 4-oxadiazoles have indicated remarkable antitumor activities (2). The anticancer mechanisms for oxadiazoles are related to the inhibition of different growth factors, enzymes, and kinases (2,14). Zhang et al. synthesized new 1,3,4-oxadiazole derivatives containing pyridine and acyl hydrazone moieties as potential telomerase inhibitors (15). Some of the compounds bearing 1, 2,4-triazole moiety are approved as anticancer drugs (7,16). Mercapto substituted 1,2,4-triazole have also shown antitumor properties (16).

The sulfur atom of thiaadiazole (another important five-membered azole) can improve liposolubility of 1, 3, 4-thiadiazole which in turn leads to good tissue permeability (12). Some 1,3,4-thiadiazole derivatives have already been synthesized as kinase inhibitors with potential anticancer activity (17). Many differently substituted 1, 3, 4-thiadiazole and 1, 2, 4-triazole derivatives, in particular, 2-alkythio derivatives have shown anticancer activities (7,16,18,19).

On the other hand, phthalimides, have exhibited remarkable pharmacological effects, including anti-inflammatory, antimycobacterial, analgesic, anticonvulsant, and anticancer activities (20-23). Some derivatives of thiaadiazole and triazole containing phthalimide pharmacophore were reported as anticancer agents (24-26). The synthesis of some derivatives of naphthalimide substituted triazole with a high affinity towards DNA has been reported. The enhanced cytotoxicity has been attributed to the presence of the triazole nucleus and planarity of naphthalimide (24). Kushwaha et al. prepared some of the different heterocycle substituted phthalimide derivatives including 1,3,4-thiadiazine, pyrazole, thiazole, and thiazoline with potnet cytotoxic activities (25).

In light of the therapeutic importance of these azoles and phthalimide, a series of novel hybrid molecules containing 1,3,4-oxadiazole, 1,3,4-thiadiazole, or 1,2,4-triazole bearing phthalimide moiety were synthesized and evaluated against two cancer cell lines. The combination of different heterocyclic with phthalimide may show a synergistic effect. The introduction of different azoles in the structure of the compound may increase the interaction of these molecules with the biological targets translating to enhanced therapeutic effect.

**MATERIALS AND METHODS**

**Instrumentation**

Reagents and starting materials were bought from commercial suppliers like Merck (Germany) and Aldrich (USA) companies. Thin-layer chromatography (TLC) was conducted on aluminum-based silica gel 60 F254 plates (Germany). Proton nuclear magnetic resonance (HNMR) spectrometer (Bruker 400 MHz, Germany) was used for recording chemical shifts expressed as (ppm) with tetramethylsilane (TMS) as internal standard. Infrared (IR, KBr discs) was obtained with a WQF-510 Fourier-transform (FT)-IR spectrophotometer (China). Melting points was obtained using electrothermal 9200 melting point apparatus (England) and are uncorrected. Mass spectra were performed on Agilent Technologies 5975C mass spectrometer (USA).

**Synthesis of ethyl 2-(5-(4-chlorophenyl)-1, 3, 4-oxadiazol-2-ythio) acetate (1a)**

Compound 5-(4-chlorophenyl)-1, 3, 4-oxadiazole-2-thiol (0.02 mol) was dissolved in dry acetone (20 mL). Anhydrous potassium carbonates (0.02 mol) and ethyl chloroacetate (0.02 mol) were added to the above solution and the mixture was stirred for 2 h at room temperature. The reaction mixture was filtered off and the organic solution was concentrated in vacuo to give a crude product which was crystallized from methanol. (15,19) (Scheme 1).
Synthesis of ethyl 2-(4-methyl-4H-1, 3, 4-triazol-3-ylthio) acetate (1b)

Compound 4-methyl-4H-1, 2, 4-triazole-3-thiol (0.02 mol) was dissolved in dry acetone (10 mL). The mixture was basified by anhydrous potassium carbonates (0.02 mol) then, ethyl chloroacetate (0.02 mol) was added and the reaction contents were reflux for 24 h and monitored by TLC. The precipitates were collected through filtration and the organic solution was concentrated in vacuo to give a crude product which crystallized by chloroform (5,27,19) (Scheme 1).

Synthesis of 2-(5-(4-chlorophenyl)-1, 3, 4-oxadiazole-2-ylthio) acetohydrazide (2a)

The ester 1a (0.01 mol) was dissolved in methanol (15 mL) and (0.1 mol) 80% hydrazine hydrate was added followed by stirring for 3-4 h at room temperature. TLC was processed to monitor the reaction completion. The product was acquired by the addition of excess cold water and separated by filtration, washed by n-hexane, and dried (28,29) (Scheme 1).

Synthesis of 2-(4-methyl-4H-1, 2, 4-triazol-3-ylthio) acetate (2b)

A mixture of the ester 1b (0.01 mol) and hydrazine hydrate (0.02 mol) in ethanol (20 mL) was stirred at room temperature for 10 h and then filtered off (30)(Scheme 1).

Synthesis of (5-(4-chlorophenyl)-1, 3, 4-oxadiazole-2-ylthio)-N-(1, 3-dioxoisoindolin-2-yl) acetamide and N-(1, 3-dioxoisoindolin-2-yl)-2-(4-methyl-4h-1, 2, 4-triazol-3-ylthio) acetamide (3a,3b)

A mixture of the acid hydrazide (2a or 2b; 0.01 mol), phthalic anhydride (0.02 mol) in glacial acetic acid (20 mL) was heated under reflux for 4-5 h (31). The precipitate was filtered and 3a recrystallized from methanol and 3b recrystallized from methanol, then petroleum ether/chloroform (2/1) (Scheme 1).

Synthesis of ethyl 2-(5-amino-1, 3, 4-thiadiazole-2-ylthio) acetate (1c)

2-Amino-5-mercapto-1,3,4-thiadiazole (0.02 mol) was dissolved in absolute ethanol (20 mL) and treated with potassium hydroxide (0.02 mol). The mixture was stirred at room temperature for 15 min. Then, ethylchloroacetate (0.02 mol) was added and the mixture was refluxed for 2 h. After completion of the reaction, the solvent was evaporated under reduced pressure. Extraction was carried out with chloroform and water. Chloroform was evaporated and the remaining solid was recrystallized from ethanol and collected as white crystal (32,33) (Scheme 2).
Synthesis and cytotoxic evaluation of azole derivatives

Scheme 2. General procedure applied for the synthesis of the target compounds (3c and 3d). (i), ClCH$_2$COOEt, KOH, ethanol; (ii), NH$_2$NH$_2$; (iii), phthalic anhydride, glacial acetic acid, reflux.

Synthesis of 2-(5-amino-1, 3, 4-thiadiazole-2-ylthio) acetohydrazide (2c)

A mixture of compound 1c (0.01 mol) and hydrazine hydrate (0.02 mol) in ethanol (20 mL) was refluxed for 3 h. The obtained precipitate was filtered, washed with cold water, dried and recrystallized from ethanol to get compound 2c (22) (Scheme 2).

Synthesis of 2-(5-amino-1, 3, 4-thiadiazole-2-ylthio)-N-(1,3-dioxoisoinolin-2-yl)acetamide (3c)

A mixture of acid hydrazide (2c, 0.01 mol), phthalic anhydride (0.01 mol) in glacial acetic acid (20 mL) was heated under reflux for 4-5 h (31). The precipitate was filtered and recrystallized from ethanol then, petroleum ether/chloroform (2/1) (Scheme 2).

Synthesis of N-(1, 3-dioxoisoinolin-2-yl)-2-(5-(1, 3-dioxoisoinolin-2-yl)-1,3,4-thiadiazol-2-ylthio)acetamide (3d)

A mixture of the acid hydrazide (2c, 0.01 mol), phthalic anhydride (0.02 mol) in glacial acetic acid (20 mL) was heated under reflux for 4-5 h. The obtained precipitate was filtered and crystallized from ethanol then, petroleum ether/chloroform (2/1) (31) (Scheme 2).

Cytotoxicity assay

The cancerous cell lines; MCF-7 (breast cancer) and HeLa (cervical cancer), were received from Pasture Institute (Tehran, I.R. Iran) and were grown in Roswell Park Memorial Institute (RPMI) 1640 which was supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 5% v/v fetal bovine serum (FBS). After 2-3 subcultures, cells were cultured in a 96-well plate at a concentration of (5 × 10$^4$ cells/mL) and incubated for 24 h. Then, the cells were treated with various concentrations of the synthesized compounds. Paclitaxel (180 µg/mL) and dimethyl sulfoxide (DMSO, 1%) were regarded as the positive and negative controls, respectively.

Incubation was performed in a humidified atmosphere condition including 5% CO$_2$ and 37 °C for 48 h. After 48 h exposure period, 20 µL of MTT dye (5 mg/mL) was added to each well and incubated for another 3 h at the same condition. DMSO (150 µL per well) was used to dissolve obtained formazan crystals and absorbance was recorded at 570 nm using an ELISA plate reader (34,35). The experiments were accomplished in triplicate. Analysis of variance (ANOVA) and Tukey test was used to obtain the differences between groups with negative control.

Cell viability was calculated using the following equation:

\[
\text{Cell Survival(\%)} = \frac{\text{MA of drug treated wells} - \text{MA of blank}}{\text{MA of negative control} - \text{MA of blank}} \times 100
\]

where, MA is mean absorbance.

Statistical analysis

The data are expressed as mean ± SD. The experiments were accomplished in triplicate.
Analysis of variance (ANOVA) and Tukey post-hoc test was used to obtain the differences between the groups with negative control. A P value less than 0.05 was considered statistically different.

RESULTS

Chemistry

Ethyl 2-(5-(4-Chlorophenyl)-1, 3, 4-oxadiazo-2-thylthio) acetate (1a)

Yield: 80%, white solid, m.p. 83-85 °C (lit: 85-86 °C (36)); IR (KBr, v_max cm⁻¹): 2938 (C-H), 1738 (C=O), 1119 (C-O); ¹H NMR: (400 MHz; CDCl₃): δ 7.88 (2H, d, J = 8 Hz, H-Ar), 7.40 (2H, d, J = 8 Hz, H-Ar), 4.19 (2H, q, J = 4 Hz, OCH₃), 4.04 (2H, s, CH₂), 1.23 (3H, t, J = 4 Hz, CH₃).

2-(5-(4-Chlorophenyl)-1, 3, 4-oxadiazo-2-thylthio) acetohydrazide (2a)

Yield: 75%, white solid, m.p. 169-171 °C (lit: 178-180 °C (37)); IR (KBr, v_max cm⁻¹): 3299 (NH), 3300 (NH), 1685 (C=O). NMR: (400 MHz; DMSO-d₆): 9.28 (1H, s, NHCO), 7.32 (2H, s, H-Ar), 2.95 (2H, m, H-CH₂). MS: m/z: 205 (M⁺); calculated M.W. 205 g/mol.

(5-(4-Chlorophenyl)-1, 3, 4-oxadiazo-2-thiolyl)-N-(1, 3-dioxoisoindolin-2-yl) acetamide (3a)

Yield: 65%, white solid, m.p. 132-135 °C; IR (KBr, v_max cm⁻¹): 3295 (NH), 3300 (NH), 1685 (C=O).

(5-(4-Chlorophenyl)-1, 3, 4-oxadiazo-2-thiolyl)-N-(1, 3-dioxoisoindolin-2-yl) acetamide (3b)

Yield: 54%, white solid, m.p. 225 °C (decomposed); IR (KBr, v_max cm⁻¹): 3295 (NH), 1737, 1699 (C=O); ¹H NMR: (400 MHz; DMSO-d₆): 11.01 (1H, s, NHCO), 8.60 (1H, s, H-triazole), 7.97-7.92 (4H, m, H-phthalic), 4.06 (2H, s, CH₂), 3.61 (3H, s, CH₃); MS (m/z): 317 (M⁺); calculated M.W. 317.2 g/mol.

N-(1, 3-dioxoisoindolin-2-yl)-2-(4-methyl-4H-1, 2, 3-triazole-3-ylthio) acetamide (3c)

Yield: 57%, white solid, m.p. 82-83 °C (lit: 83-84 °C (38)); IR (KBr, v_max cm⁻¹): 3396 (NH₂), 3280 (NH₂), 2923 (C-H), 1735 (C=O), 1300 (C-O) cm⁻¹.

2-(5-Amino-1, 3, 4-thiadiazole-2-thylthio) acetohydrazide (2c)

Yield: 53.78%, white solid, m.p. 150-151 °C (lit: 150-151 °C (39)); IR (KBr, v_max cm⁻¹): 3300, 3274, 3029 (NH₂, NH), 1685 (C=N), 1648 (C=O) cm⁻¹; ¹H NMR: (400 MHz; DMSO-d₆): 9.28 (1H, s, NHCO), 7.32 (2H, s, NH₂), 4.31 (2H, s, CH₂), 3.71 (2H, s, NH₂). MS (m/z): 205 (M⁺); calculated M.W. 205 g/mol. 2-(5-Amino-1, 3, 4-thiadiazole-2-thylthio)-N-(1, 3-dioxoisoindolin-2-yl) acetamide (3c)

Yield: 30%, white solid, m.p. 265 °C (decomposed); IR (KBr, v_max cm⁻¹): 3300, 3274 (NH₂, NH), 1745 (C=O, 8.15-8.13 (2H, m, H-phthalic), 8.09-8.06 (2H, m, H-phthalic), 7.71 (2H, s, NH₂), 3.41 (2H, s, CH₂); MS (m/z): 335 (M⁺), 292; calculated M.W. 335.3 g/mol.

N-(1, 3-dioxoisoindolin-2-yl)-2-(5-(1, 3-dioxoisoindolin-2-yl)-1, 3, 4-thiadiazole-2-thylthio) acetamide (3d)

Yield: 50 %, white solid, m.p. 267 °C (decomposed); IR (KBr, v_max cm⁻¹): 3400 (NH), 3110 (C-H, Ar), 1785, 1749 (C=O); ¹H NMR: (400 MHz; DMSO-d₆): 11.16 (1H, s, NHCO), 8.07-8.05 (2H, m, H-phthalic), 7.98-7.95 (6H, m, H-phthalic), 4.42 (2H, s, CH₂); MS (m/z): 465 (M⁺), calculated M.W. 465 g/mol.

Cytotoxic assay

Final compounds were assessed for their cytotoxic effects on two human cancer cell lines, MCF-7 and HeLa. Cytotoxic activity results were presented in Table 1 and Fig. 1. Significant differences (P ≤ 0.001) in viability compared to the negative control were observed on both cell lines for final compounds. None of the compounds were found as effective as paclitaxel.

Table 1. The IC50 of the final compounds against MCF-7 and HeLa cell lines.

| Compounds               | IC50 (µM) MCF-7 | IC50 (µM) HeLa |
|-------------------------|----------------|----------------|
| 3a                      | 87 ± 3.5       | 77 ± 3.3       |
| 3b                      | 86 ± 3.9       | 71 ± 3.6       |
| 3c                      | 92 ± 3.3       | 67 ± 4.1       |
| 3d                      | 73 ± 3         | 29 ± 2.9       |
| Paclitaxel              | 5.25-11.03 (µg/mL) | 7.76 (µg/mL) |
|                         | 6.1 (µM)       | 9.08 (µM)      |
**Synthesis and cytotoxic evaluation of azoles derivatives**

**DISCUSSION**

In the light of reported cytotoxic activity of azoles (7,9,40), cyclic imides, and conjugated azole-imide (24,25), and in continuing efforts to develop potent cytotoxic agents, some phthalimide-based azoles including S-CH$_2$CONH-linker were designed and prepared after exploring molecular hybridization approaches in a three-step procedure as depicted in Schemes 1 and 2. Molecular hybridization is one of the useful approaches in the design of new therapeutic compounds. One important consideration in hybrid compound design is the choice of a linker, as the nature of the linker and the distance between the pharmacophores can play an essential role (41). The choice of these structures was in accordance with the fact that the conjugated azole-phthalimide and 2-alkythio derivatives of azoles (18) showed anticancer effects in the previous studies (24,25).

The thiol group of azoles was alkylated using ethyl chloroacetate. 2-Alkylthio derivatives of azoles are important compounds used as key intermediates in organic and inorganic synthesis (36). Corresponding 2-alkylthio derivatives of azoles were treated with hydrazine hydrate to provide acid hydrazide derivatives, which were followed by a reaction with phthalic anhydride to obtain final products. The formation of final compounds was confirmed by recording their IR, $^1$HNMR, and mass spectra. IR analysis of final compounds showed the peaks at 3295, 3300, 3274, and 3400 cm$^{-1}$, which were due to the NH groups. The absorption bands at 1699,
1736, 1737, 1745, 1749, and 1785 cm\(^{-1}\) were due to the C=O groups. The \(^1\)HNMR spectra of compounds in DMSO-\(d_6\) solvent showed a singlet peak around 11 ppm, which was attributed to the NH proton of amide groups. All protons belonging to the phthalic ring appeared at ranges of \(\delta 8.15\)-7.92 ppm as multiple signals. The protons of the methylene linker appear as a singlet at ranges of 4.52-3.41 ppm. In compound 3a, two doublet peaks at regions 7.80 and 7.54 ppm were assigned to protons of the Cl-phenyl ring attached to the oxadiazole ring.

In compound 3b, the characteristic peak of the triazole was observed as a singlet at 8.60 ppm and one singlet peak corresponding to the three protons of methyl substitution appeared at 3.61.

All the final products were subjected for evaluation of cytotoxic effects against MCF-7 and HeLa cell lines using MTT-colorimetric assay. Cytotoxic results indicated that oxadiazole and triazole derivatives containing phthalimide moiety exhibited relatively similar cytotoxic activity against both cell lines.

The IC\(_{50}\) results rendered the thiadiazole derivatives 3c and 3d to be more effective against the HeLa cell line as compared to the other azoles in this study. The structure of intermediate 2c allows realizing change in two directions of the thiadiazole ring. Thiadiazole derivative 3d containing two phthalimide moieties in structure with IC\(_{50}\) value of 29 \(\mu\)M and 73 \(\mu\)M showed the highest activity against HeLa and MCF-7, respectively.

The presence of the second phthalimide in the structure of compound 3d caused more lipophilic characteristics compared to the 3c derivative which may be a logical reason for the improvement of its cytotoxic effect. In addition, it may enhance the interaction of this derivative with the biological targets.

IC\(_{50}\) results determined that in these series of azole derivatives the type of heterocyclic ring plays an important role in developing the cytotoxic properties as seen in thiadiazole derivatives compared to oxadiazole and triazole.

According to the results, it can be inferred that the cytotoxic effects of the tested compounds were concentration-dependent and the compounds with higher concentrations had more cytotoxic effects. Tested compounds exhibited significant differences in cell viability compared to the negative control on both cell lines which is presented in Fig. 1.

**CONCLUSION**

In summary, the novel derivatives of five-membered azoles bearing phthalimide moiety were synthesized in three steps and their \textit{in vitro} cytotoxic activities were assayed against MCF-7 and HeLa cell lines. Results of IC\(_{50}\) demonstrated that oxadiazole and triazole-phthalimide derivatives had the lowest cytotoxic against both cell lines and compound 3d with two phthalimide moieties in structure exhibited much better cytotoxic activity against the HeLa cell line. It can be concluded that the HeLa cell line is more susceptible than MCF-7 to compound 3d.

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**Conflict of interest statement**

The authors declared no conflict of interest in this study.

**Authors’ contribution**

E. Jafari contributed to the conception and design of the work, conducting the study, analyzing the data, drafting and revising the manuscript. F. Hassanzadeh contributed to the conception of the work, analyzing the data, revising the manuscript. F. Shojaei performed the experiments and analyzing the data. H. Sadeghi-Aliabadi contributed to the conception of the work, conducting the study, revising the manuscript. All authors agreed with all aspects of the work.

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