



Synthesis and exploration of a novel chlorobenzylated 2-aminothiazole-phenyltriazole hybrid as migratory inhibitor of B16F10 in melanoma cells

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ABSTRACT

In the study presented here, a novel chlorobenzylated bi-heterocyclic hybrid molecule (7) was synthesized and its structural confirmation was carried out by IR, ¹H-NMR, ¹³C-NMR and CHN analysis data. This compound 7 was subjected to biological study with B16F10 mouse melanoma cells. The anti-proliferative results showed that 7 showed no significant toxicity at concentrations ranging of 0–44 μM. The treatment of B16F10 cells with 7 at aforementioned concentration range indicated that migration of cells was significantly lower than that of the control cells in a dose dependent manner. The possible migration inhibitory effect of these melanoma cells was further evaluated through gelatinolytic activity of MMP-2 and MMP-9 secreted from B16F10 cells. It was inferred from our results that 7 was not affecting the expression and activity of these enzymes. Some other zinc-dependent matrix metalloproteinases (MMPs) were involved in the inhibitory progression. Taken together, compound 7 inhibited migrations of B16F10 mouse melanoma cells. Therefore, it may deserve consideration as a potential agent for the treatment of cancer.

1. Introduction

Thiazole is an aromatic five membered ring heterocyclic compound containing both sulfur and nitrogen atom in the ring. It is widely found in many bioactive natural products like vitamin B1 (thiamine) and penicillin. It has an important component effect of the pharmacophore of an enormous number of medicinal molecules. Therefore, the derivatives of thiazole have attracted a pronounced deal of interest in the pharmaceutical field. Their biological activity evaluation shows that they have anthelmintic, antifungal, antiprotozoal, antitubercular, antibacterial, anesthetic, sedative, cardiogenic and anti-inflammatory properties [1]. Among the thiazoles, 2-aminothiazoles are important heterocyclic amines and their derivatives also represent a devastating and swift developing field in modern heterocyclic chemistry. From literature it has been anticipated that 2-aminothiazoles play a vibrant role as medicinal agents [2].

Thiazoles are five membered heterocyclic compounds consisting of

two carbon and three nitrogen atoms. They are found in two isomeric forms, one is 1,2,3-triazole, known as 'osotriazole' and other is 1,2,4-triazole, recognized generally as 'triazole'. From last two decades 1,2,4-triazoles have a great contribution in pharmaceutical chemistry, due to its enormous activities, pharmacokinetic outlines, diminutive poisonousness and good pharmacodynamic [3]. Their derivatives are regarded as imperative compounds in industrial, agricultural, and biological applications. Triazoles are known as antimalarial, antimicrobial, anticancer, anti-inflammatory and antidepressant agents. Furthermore, the majority of triazoles are reported as environmentally friendly materials [4]. Depending on the substituent type, derivatives of 1,2,4-triazole have a high potential biological activities, such as antifungal, antibacterial, anticonvulsant and antipsychotic. Some also show antitubercular, urease inhibition and antioxidant activities [5,6].

As one of the leading cause of morbidity and mortality worldwide, cancer is a major public health problem [7]. In order to combat with cancer, a great deal of attraction has been focused on the design and

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synthesis of new antitumor drugs with high efficiency and low toxicity to healthy cells and tissues [8]. Several zinc-dependent matrix metalloproteinases (MMPs) are known to play crucial roles in the turnover of extracellular matrix (ECM) and the associated destruction of articular cartilage in osteoarthritis [9]. MMPs are elevated in several cell types of cancers and their overexpression has been associated with poor prognosis [10]. MMP-2 and MMP-9 could play a role in remodeling and degradation of ECM during physiological and pathological processes [11]. Another study also showed that in ethanol on mice ulcer model PCMS down regulates both pro- & active- MMP9 [12].

Multiple thiazole and triazole based derivatives are used in pharma industry to check their toxicity and lead activity against particular targets [13,14]. The diversified bioactivities of thiazole and triazole moieties prompted us to synthesize a new hybrid bi-heterocyclic molecule encompassing a chlorobenzyl group in one skeleton to explore its anti-proliferative and migratory inhibiting potential of tumor cells.

2. Results and discussion

2.1. Chemistry

The designed chlorobenzylated bi-heterocyclic molecule was synthesized in a multi-step protocol as described in Scheme 1. The synthesis was geared up by refluxing ethyl 2-(2-amino-1,3-thiazol-4-yl)acetate (1) with methanol and hydrazine hydrate to get 2-(2-amino-1,3-thiazol-4-yl)acetohydrazide (2). The hydrazide 2 was further refluxed with phenyl isothiocyanate in methanol to obtain a solid intermediate compound, 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-N-phenyl-1-hydrazinecarbothioamide (4) which was further cyclized to 5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazole-3-thiol (5). This nucleophile 5 was dissolved in DMF, one pinch of LiH was added and this mixture was stirred for 15–20 min to activate its mercapto position. Finally, its nucleophilic substitution reaction was carried out with equimolar amount of 4-chlorobenzyl chloride (6) to achieve the designed bi-heterocyclic hybrid compound 7.

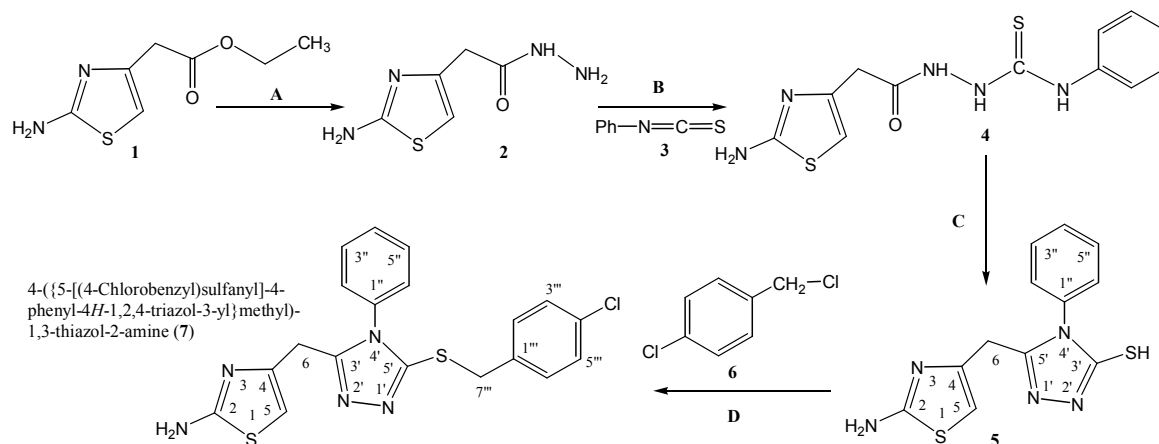
For the benefit of reader, the structural analysis of 7 is discussed hereby. The molecule 7 was synthesized as yellow amorphous solid having melting point of 96–97 °C. Its molecular formula, C₁₉H₁₆ClN₅S₂, was established by CHN analysis data, by counting the number of protons in its ¹H-NMR spectrum and by counting the number of carbon resonances in its ¹³C-NMR spectrum. Various functionalities in the molecule were ascribed by its IR spectrum, whereby, the characteristic peaks appeared at ν3348 (N–H stretching), 3042 (C–H stretch of aromatic ring), 2922 (–CH₂– str.), 1578 (C=C stretch of aromatic ring), 1522 (C=N stretch), 1162 (C–N–C bond stretch), and 614 cm⁻¹ (C–S

stretch). In its ¹H-NMR spectrum, the phenyl ring attached to the nitrogen (4') atom of 1,2,4-triazole heterocyclic ring was identified through two multiplets in the aromatic region at δ 7.50–7.47 (m, 3H, H-3', H-4' & H-5') and 7.21–7.20 (m, 2H, H-2'' & H-6''). The 4-chlorobenzyl moiety attached to the sulfur atom in the molecule was clearly represented by an A₂B₂ spin system specified by the two *ortho*-coupled doublets in aromatic region at δ 7.35 (br.d, *J* = 8.7 Hz, 2H, H-2''' & H-6'''), and δ 7.33 (br.d, *J* = 8.7 Hz, 2H, H-3''' & H-5'''), along with a benzylic methylene singlet at δ 4.30 (s, 2H, CH₂-7'''). The 2-amino-1,3-thiazol-4-yl heterocycle was characterized by two singlets at δ 6.85 (s, 2H, H₂N-2), and δ 5.82 (s, 1H, H-5), while a singlet δ 3.76 (s, 2H, CH₂-6) was assignable to a methylene group connecting the two heterocycles in the molecule. The ¹H-NMR spectrum of this molecule has been shown in Fig. 1(a) while its expanded aromatic region has been shown in Fig. 1(b).

All these assignments were also substantiated by its ¹³C-NMR spectrum (Fig. 2) which exhibited overall fifteen carbon resonances due to some symmetrical carbon atoms in the molecule. The 2-amino-1,3-thiazol-4-yl heterocycle was clearly signified by two quaternary signals at δ 168.69 (C-2), and 146.11 (C-4), along with a methine signal at δ 102.67 (C-5). Similarly, the other heterocycle i.e. (1,2,4-triazol-5-yl) sulfanyl was also signified by two quaternary signals at δ 153.97 (C-3') and 149.80 (C-5') while a methylene connecting the two heterocycles (4-position of the former heterocycle with 3'-position of the latter heterocycle) was obvious at δ 28.05 (C-6). The 4-chlorobenzyl moiety was also apparent with two quaternary signals at δ 136.93 (C-4''') and 132.48 (C-1'''), in addition to two symmetrical methine duplets at δ 131.27 (C-3''', C-5''') and δ 128.81 (C-2''', C-6''') while the benzylic methylene connecting it to the sulfur atom was characteristic at δ 35.93 (C-7'''). The phenyl group attached to the nitrogen (4') atom of 1,2,4-triazole ring was distinctive by a quaternary signal at δ 133.43 (C-1'') and three methine resonances at δ 130.16 (C-4''), 129.92 (C-3'' & C-5'') and 127.64 (C-2'' & C-6''). So, on the basis of aforesaid cumulative evidences, the structure of 7 was confirmed and it was named as 4-({5-[(4-chlorobenzyl)sulfanyl]-4-phenyl-4H-1,2,4-triazol-3-yl)methyl}-1,3-thiazol-2-amine.

2.2. Biochemical properties and Lipinski's rule of five (RO5) validations

The biochemical properties and RO5 analysis are significant approaches in to evaluate the lead compounds. The predicted results showed that, the designed ligand 7 exhibited molecular mass, HBA and HBD values less than standard such as < 500 (g/mol), < 10 and < 5, respectively. However, only logP valued was exceeded from standard value (< 5) (Table 1). The hydrogen-bonding capacity has been



Scheme 1. Outline for the synthesis of 4-({5-[(4-chlorobenzyl)sulfanyl]-4-phenyl-4H-1,2,4-triazol-3-yl)methyl}-1,3-thiazol-2-amine. **Reagents & Conditions:** (A) MeOH/N₂H₄·H₂O/refluxing for 2 h. (B) MeOH/3/refluxing for 1 h. (C) The ppt. of 4 dissolved in 10% NaOH/filtration/acidification of filtrate in cold state to get ppt. of 5. (D) DMF/LiH/stirring for 12 h.

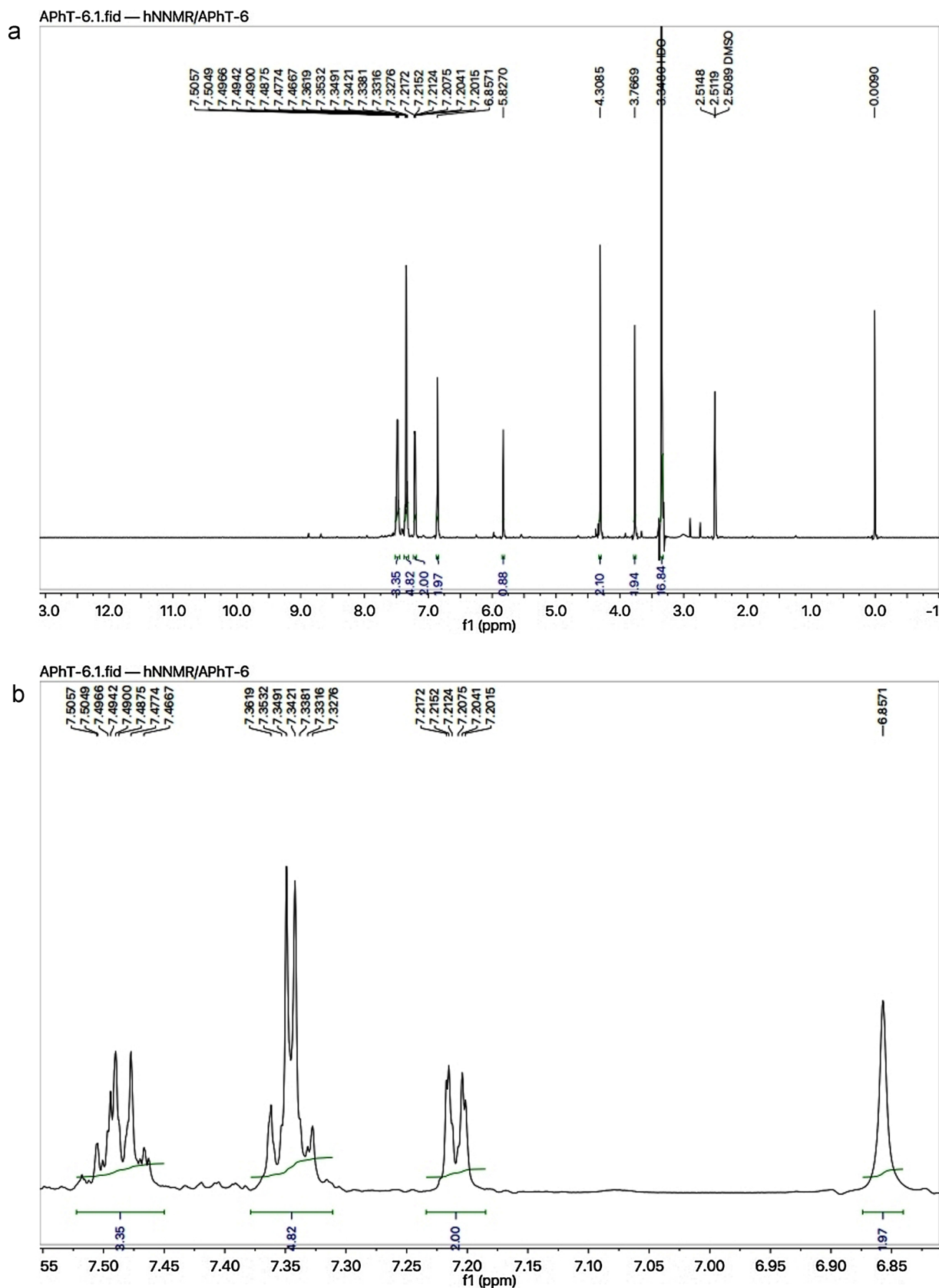


Fig. 1. a). ¹H-NMR spectrum of 7. b). Expanded aromatic region of ¹H-NMR spectrum of 7.

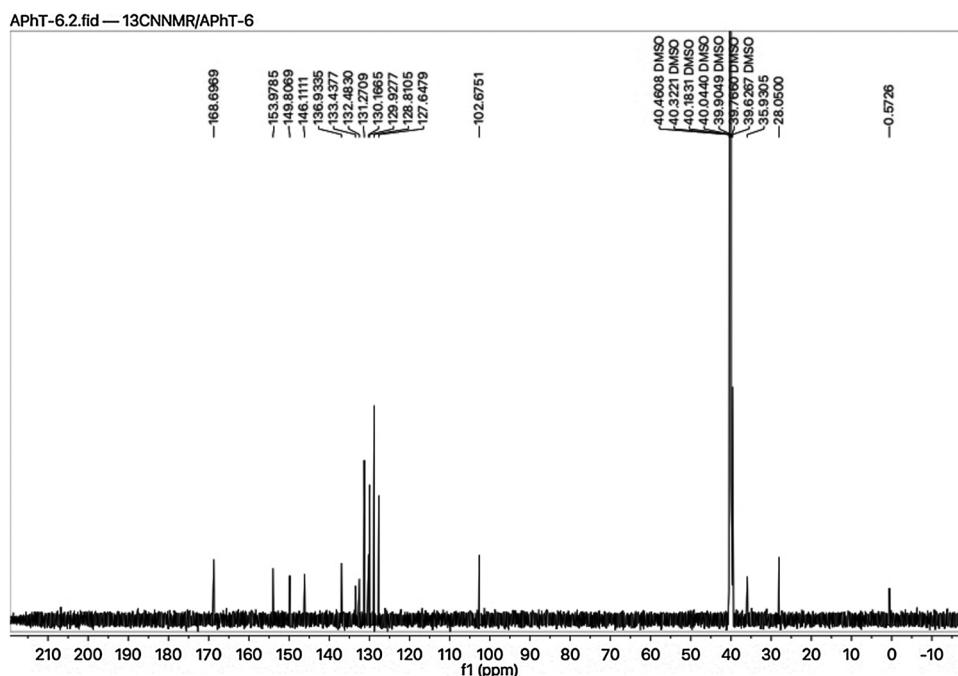


Fig. 2. ^{13}C -NMR spectrum of 7.

Table 1

Predicted biochemical properties of compound 7.

Properties	Values
Molecular weight (g/mol)	423
HBA	05
HBD	02
Mol LogP	5.25
Mol PSA	56
Mol Vol	348
Drug Score	0.23

considered as significant parameter for drug permeability. The exceeded values of HBA and HBD result in poor permeation, but in our case these values were within the specified range and satisfactory. Polar surface area (PSA) is considered as good descriptor for characterizing the drug absorption, including intestinal absorption, bioavailability and blood-brain barrier penetration. Our results showed that compound 7 possessed $< 140 \text{ \AA}^2$ PSA values. Another significant parameter is drug score. Our predicted results showed that 7 possessed positive value which indicated its considerable drug like behavior (Table 1).

2.3. Biological activities

The effect of bi-heterocyclic compound 7 on cell viability of B16F10 cells was examined, whereby the MTT assay indicated that this molecule showed no significant toxicity at concentrations ranging of 0–44 μM (Fig. 3a). These results disclosed that compound 7 did not have anti-proliferative effect.

Metastasis is a hallmark and their first step is the migration of cancer cells away from the primary tumor [15]. Hence, the investigation for migration inhibitory agent has been needed for cancers therapy. Many reports indicated that expression and activity of MMP-2 and MMP-9 are related with the migration of a variety of cancer cells and the inhibition of their activation is important for the regulation of migration [18]. Therefore, B16F10 cells were treated with designed molecule 7 (0–44 μM) to study its effect on the migration of cells. From the results, it was inferred that migration of cells treated with compound 7 was significantly lower than that of the control cells in a dose

dependent manner (Fig. 3b). These results exhibited that compound 7 has anti-migration activity and it is proposed that this molecule possesses a potential as an anti-migratory agent.

The invasion and metastasis of cancer requires various physiological changes such as the expression of proteolytic enzymes such as MMPs, which are involved in the degradation of the ECM. The possible migration inhibitory effect of B16F10 cells was further evaluated through their effect on activity of matrix metalloproteinase-2 (MMP-2) and MMP-9, which are critical enzymes for the invasive or migration of tumor cells (Fig. 4).

Gelatinolytic activity of MMP-2 and MMP-9 secreted from B16F10 cells was evaluated with gelatin zymography, which was carried out with compound 7 stimulated conditioned medium of B16F10 cells. It was observed that treatment of this compound to the cells did not affect on expression and activity of MMP-2/-9 (Fig. 4).

So, it was postulated from our results that compound 7 inhibited the migration of cells in a dose dependent manner. Therefore, compound 7 might deserve consideration as a potential agent for the prevention of cancer metastasis. However, these inhibitory effects on migration of cells are not regulated by MMP-2 and -9 activities. These results suggested that there is a possibility that another MMPs, rather than MMP-2 and -9, are involved in this inhibitory progression of compound 7 on migration of B16F10 cells. A further study is needed to ascertain the regulatory mechanisms about which MMPs are related with the effects of compound 7 on migration. In addition, *in vivo* testing and clinical trial of compound 7 on cancer patients is recommended for future studies.

3. Conclusion

The structure of the newly synthesized chlorobenzylated 2-aminothiazole-phenyltriazole hybrid was corroborated thoroughly by the spectral analysis, and this compound exhibited suitable anti-migration activity in B16F10 melanoma cells. So, this bi-heterocyclic medicinal scaffold can find its utilization as a potential agent for the prevention of cancer metastasis.

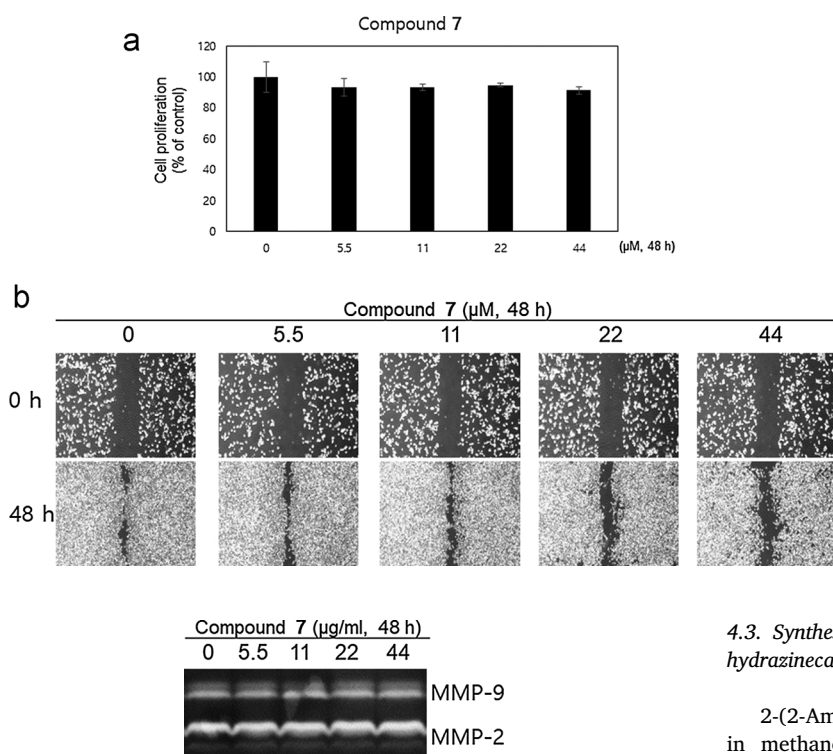


Fig. 4. Cells were treated with compound 7 under diverse concentrations (0, 5.5, 11, 22, and 44 μM) for 48 h. MMP activity was determined by gelatin zymography. The data represented three similar experiments.

4. Experimental part

4.1. General

Analytical grade chemicals were purchased from Sigma Aldrich & Alfa Aesar (Germany) and solvents of analytical grades were supplied by local suppliers. By using open capillary tube method, melting points were taken on Griffin and George apparatus and were uncorrected. By using thin layer chromatography (with ethyl acetate and *n*-hexane (30:70) as mobile phase), initial purity of compounds was detected at 254 nm. Elemental analyses were performed on a Foss Heraeus CHN-O-Rapid instrument and were within $\pm 0.4\%$ of the theoretical values. IR peaks were recorded on a Jasco-320-A spectrometer by using KBr pellet method. $^1\text{H-NMR}$ spectra (δ , ppm) were recorded at 600 MHz ($^{13}\text{C-NMR}$ spectra, at 150 MHz) in $\text{DMSO-}d_6$ using the Bruker Advance III 600 Ascend spectrometer using BBO probe. The abbreviations used in interpretation of $^1\text{H NMR}$ spectra are as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; br.t, broad triplet; m, multiplet; dist. distorted.

4.2. Synthesis of 2-(2-amino-1,3-thiazol-4-yl)acetohydrazide (2)

Ethyl 2-(2-amino-1,3-thiazol-4-yl)acetate (**1**; 1 g, 0.0054 mol.) in 10 mL methanol and hydrazine monohydrate (80%; 0.5 mL) was taken in a 50 mL round bottom flask. The reaction mixture was stirred for 45 min at room temperature (RT). After absolute conversion, the acid hydrazide was obtained by distilling methanol off from the reaction mixture. The precipitates were filtered, washed with cold *n*-hexane and air-dried to get pure 2-(2-amino-1,3-thiazol-4-yl)acetohydrazide (**2**, 0.71 g, 0.0041 mol).

Fig. 3. a) Cells were treated with compound 7 under diverse concentrations (0, 5.5, 11, 22, and 44 μM) for 48 h. Cell viability was determined by MTT assay. Data are presented as the mean \pm SD of at least three independent experiments. *, $P < 0.05$. **b)** Cells were treated with molecule 7 under diverse concentrations (0, 5.5, 11, 22, and 44 μM) for 48 h. Cell monolayers were wounded using a sterile 10 μL micropipette tip, and the remaining cells were incubated in medium containing 0, 5.5, 11, 22, and 44 μM compound 7 for 48 h. At the indicated time after scraping, the wound areas were photographed. The data represented three similar experiments.

4.3. Synthesis of 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-*N*-phenyl-1-hydrazinecarbothioamide (4)

2-(2-Amino-1,3-thiazol-4-yl)acetohydrazide (**2**; 0.71 g, 0.0041 mol.) in methanol (10 mL) was dissolved by heating and phenyl isothiocyanate (**3**, 0.13 mol.) was added subsequently. Mixture was refluxed for one hour. On completion of reaction, precipitates of 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-*N*-phenyl-1-hydrazinecarbothioamide (**4**) were obtained which were filtered and dried (1.32 g) to use further.

4.4. Synthesis of 5-[2-(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4*H*-1,2,4-triazole-3-thiol (5)

The intermediate, 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-*N*-phenyl-1-hydrazinecarbo-thioamide (**4**; 1.32 g, 0.0041 mol.), was dissolved in 10% NaOH (5 mL) and the solution was filtered. The filtrate so obtained was acidified with conc. HCl in cold stated to get the precipitates of desired cyclized product **5** (1.1 g).

White crystalline solid; Yield: 92%; m.p. 233–234 $^\circ\text{C}$; Mol. Formula: $\text{C}_{12}\text{H}_{11}\text{N}_5\text{S}_2$; Mol. Mass.: 289 g mol^{-1} ; IR (KBr, ν , cm^{-1}): 3321 (N–H str.), 3040 (C–H str. of aromatic ring), 2912 (–CH₂– str.), 1560 (C=C str. of aromatic ring), 1512 (C=N str.), 1145 (C–N–C bond str.), 609 (C–S str.); $^1\text{H-NMR}$ (600 MHz, $\text{DMSO-}d_6$, δ , ppm): 13.73 (s, 1H, HS-3'), 7.47–7.46 (m, 3H, H-3'', H-4'' & H-5''), 7.28–7.27 (m, 2H, H-2'' & H-6''), 6.88 (s, 2H, H₂N-2), 5.85 (s, 1H, H-5), 3.67 (s, 2H, CH₂-6); $^{13}\text{C-NMR}$ (150 MHz, $\text{DMSO-}d_6$, δ , ppm): 168.78 (C-2), 150.05 (C-5'), 150.58 (C-3'), 144.86 (C-4), 134.17 (C-1''), 129.70 (C-4''), 129.49 (C-3'' & C-5''), 128.63 (C-2'' & C-6''), 103.10 (C-5), 28.60 (C-6). Anal. Calc. for $\text{C}_{12}\text{H}_{11}\text{N}_5\text{S}_2$ (289.38): C, 49.81; H, 3.83; N, 24.20. Found: C, 49.76; H, 3.79; N, 24.17.

4.5. Synthesis of 4-({5-[2-(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4*H*-1,2,4-triazol-3-yl}methyl)-1,3-thiazol-2-amine (7)

5-[2-(2-Amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4*H*-1,2,4-triazole-3-thiol (**5**; 0.2 g) was dissolved in DMF (3 mL) contained in a 100 mL round bottom flask at room temperature. One pinch of LiH was added to this solution and it was stirred for 15–20 min. Then, 4-chlorobenzyl chloride (**6**) was added in equimolar amount and mixture was stirred for 12 h. A single spot of product on TLC showed the completion of a reaction. The reaction mixture was quenched with ice cold water (50 mL). The respective targeted derivative, **7** (0.23 g), was collected through filtration in purified form.

Yellow amorphous solid; Yield: 90%; m.p.: 96–97 $^\circ\text{C}$; Mol. Formula:

C₁₉H₁₆ClN₅S₂; Mol. Mass.: 413 g mol⁻¹; IR (KBr, ν, cm⁻¹): 3348 (N–H str.), 3042 (C–H str. of aromatic ring), 2922 (–CH₂– str.), 1578 (C=C str. of aromatic ring), 1522 (C=N str.), 1162 (C–N–C bond str.), 614 (C–S str.); ¹H-NMR (600 MHz, DMSO-*d*₆, δ, ppm): 7.50–7.47 (m, 3H, H-3', H-4'' & H-5'''), 7.35 (br.d, *J* = 8.7 Hz, 2H, H-2''' & H-6'''), 7.33 (br.d, *J* = 8.7 Hz, 2H, H-3''' & H-5'''), 7.21–7.20 (m, 2H, H-2'' & H-6''), 6.85 (s, 2H, H₂N-2), 5.82 (s, 1H, H-5), 4.30 (s, 2H, CH₂-7'''), 3.76 (s, 2H, CH₂-6); ¹³C-NMR (150 MHz, DMSO-*d*₆, δ, ppm): 168.69 (C-2), 153.97 (C-3'), 149.80 (C-5'), 146.11 (C-4), 136.93 (C-4'''), 133.43 (C-1''), 132.48 (C-1'''), 131.27 (C-3''' & C-5'''), 130.16 (C-4''), 129.92 (C-3'' & C-5''), 128.81 (C-2''' & C-6'''), 127.64 (C-2'' & C-6''), 102.67 (C-5), 35.93 (C-7'''), 28.05 (C-6). Anal. Calc. for C₁₉H₁₆ClN₅S₂ (413.05): C, 55.13; H, 3.90; N, 16.92. Found: C, 55.10; H, 3.82; N, 16.87.

4.6. Designing of ligand 7 in ACD/ChemSketch

The synthesized ligand 7 was sketched in ACD/ChemSketch and accessed in mol format. The biochemical properties and Lipinski's rule of five (RO5) of synthesized ligand 7 was evaluated and justified using online computational tools such as Molinspiration (<http://www.molinspiration.com/>) and Molsoft (<http://www.molsoft.com/>), respectively.

4.7. Cell culture and treatment with compound 7

B16F10 mouse melanoma cells (KCLB no.80008; Korean Cell Line Bank, Seoul, Korea) were grown in Dulbecco's modified Eaglemedium (DMEM; Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen), penicillin (50 U/ml; Sigma-Aldrich) and streptomycin (50 µg/ml; Sigma-Aldrich), in a humid atmosphere, 5% CO₂ and 95% air at 37 °C. The medium was changed twice or three times each week. Cells were detached with 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA) in PBS without trypsin and washed with DMEM [17–20].

4.7.1. Anti-proliferative activity assay

For cell proliferation assessment, cells were seeded into 96-well plates (0.4 × 10⁵ cells/well in 100 µl medium) and cultured for 24 h. Following an overnight incubation, the cell medium was removed, and the cells were treated with compound 7 concentration gradient (0, 5.5, 11, 22 or 44 µM) for 48 h, and then washed with phosphate-buffered saline (PBS). Thereafter, the medium was replaced by fresh medium (200 µl) containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), followed by a 4 h incubation at 37 °C. Finally, 100 µl solubilization buffer (10% SDS, 0.01 N HCl) was added to each well to terminate the MTT reaction and dissolve formazan crystals for the determination of formazan crystal amount which was measured by the absorbance at 595 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). After agitation for 1 h at room temperature, cell proliferation was defined by the optical density as a way to indicate the amount of MTT converted into formazan crystal. Proliferation inhibition rate was calculated as: [1-A595 (experimental well)/A595 (control well)] × 100% [17].

4.7.2. Wound-healing assay

B16F10 cells migration was determined by wound-healing assay. Briefly, B16F10 cells (1.5 × 10⁵/well) were seeded into 35-mm culture dish to 70% confluence followed by forming an injury line with a 10-µl pipette tip. After floating cell debris were washed with medium and incubated with compound 7 for 48 h. The migration distance of cells was then captured using an inverted microscope (IX53; Olympus Corporation, Tokyo, Japan) and photographs were taken at incubation starting time and after 48 h of incubation [21].

4.7.3. Gelatin zymographic assay

Enzymatic activities of MMP-2 and MMP-9 from B16F10 cells were

performed using SDS-PAGE zymography. Briefly, B16F10 cells were split into 35-mm culture dish. After 24 h, cells were serum-starved in DMEM medium (Gibco/Invitrogen) for additional 20 h, and then treated with increasing concentrations of compound 7 (0, 5.5, 11, 22 or 44 µM) for 48 h. Subsequently, 30 µl conditioned medium was mixed with reducing agent-devoid of 4x SDS sample buffer (62.5 mM) and subjected to electrophoresis in 7.5% SDS PAGE gels containing 1 mg/ml gelatin (Sigma-Aldrich). Gels were then washed with 1 X PBS containing 2.5% (v/v) Triton X-100 for 20 min three times at room temperature to remove any remaining sodium dodecyl sulfate (SDS). Subsequently, gels were incubated overnight at 37 °C in a developing buffer containing 10 mM CaCl₂, 150 mM NaCl, and 50 mM Tris–HCl, pH 8.0, for 24 h to facilitate the digestion of gelatin by MMPs. The gels were then stained with 0.5% Coomassie brilliant blue R-250 in 10% acetic acid (v/v) and 50% methanol, for 1 h, and then de-stained in 50% methanol and 10% acetic acid solution at room temperature to clearly visualize the digested bands [21].

4.7.4. Statistical analysis

Values are presented as a mean of three different experiments ± standard deviation (SD). Differences between the calculated means of the each individual group were determined by one-way ANOVA. Any difference was considered statistically significant at *P* < 0.05.

Declaration of Competing Interest

Author's declare no conflict of interest.

Acknowledgments

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