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New Thalidomide Derivative with An Anti-Migrative and Anti-Proliferative Effects on Lewis Lung Carcinoma Cell

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Abstract

Due to the crucial demand for novel chemotherapeutic agents, for the first time it was reported in the current study the potential anticancer effects of a new thalidomide derivative against Lewis lung carcinoma (LLC) cell line. The potential anticancer activities of the analog were detected through determination the anti-proliferation, anti-migration, and anti-invasive activities using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), wound healing, and Matrigel invasion assays, respectively. In addition, the effect on the gene status of the pathway of PI3K/Akt/mTOR in LLC was determined. Moreover, the mRNA expression levels of transforming growth factor beta 1 (TGF-β1), Snail, Slug, and matrix metalloproteinase-2 (MMP-2) were measured through Quantitative Real-time RT-PCR. Results revealed significant inhibition of thalidomide analog on the proliferation, migration, and invasion of LLC cells was observed, compared to thalidomide drug. Furthermore, the analog inhibited the gene expression of PI3K, Akt, and mTOR. This chemical also decreased extensively the TGF-β1 mRNA expression, Snail, Slug, and MMP-2 than thalidomide. Taken together, our findings showed that the new thalidomide analog might be a potential anticancer candidate more than thalidomide drug against LLC cells through inhibition of cell proliferation, migration, and invasion.

Keywords: Thalidomide; Lung cancer; Proliferation; Migration; Anticancer

1. Introduction

Thalidomide remains a potent drug with several clinical and therapeutic applications, regardless of its known teratogenic effects [1]. This chemical exhibits several anticancer properties, and it is used in the treatment of a variety of tumors [2,3]. Dithiocarbamates are chelating ligands and possess numerous applications in medicine and materials science [4]. Thalidomide analogs, resulting from the merging of thalidomide with dithiocarbamate group, prior to being administrated to Ehrlich Ascites Carcinoma cells, displayed an evident regulation of the levels of nitric oxide and intracellular adhesion molecules [5] and possessed in vitro antiangiogenic effects against cancer cell lines [6]. Moreover, thalidomide analogs exhibited potential anticancer activity through decreasing the expression of the vascular endothelial growth factor and interleukins in A459 cells [7].

Lung cancer is the prominent cause of cancer death according to Global Cancer Statistics 2021 [8]. Despite the numerous investigations established against lung cancer, this disease is still the leading cause of cancer-related mortality worldwide [9]. Therefore, the development of effective chemotherapeutics agents to reduce mortality in patients with lung cancer are required. The mammalian target of rapamycin (mTOR) signaling regulates several cellular events including cell

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cycle, proliferation, growth, and survival. Also, it plays a central role in cancer initiation and progression [10]. Constitutive activation of PI3K/Akt/mTOR signaling cascades exhibits a crucial role in the survival and metastasis of tumor cells [11] as, when activated, the signal can be spread through Akt, a downstream effector of PI3K to mTOR, causing the phosphorylation of p70S6K and leading to rapid proliferation of tumor cells [12].

Transforming growth factor beta (TGF-β1) exhibited a crucial role in the regulation of cell proliferation [13] and perform a significant role in cancer as a tumor suppressor inhibits epithelial and hematopoietic cell proliferation [14,15]. Additionally, TGF-β1 regulates the expression of transcription factors Slug and Snail through TGF-β1/smad signaling pathway [16]. Also, Snail activates the expression of matrix metalloproteinase (MMP) that plays an essential role in cancer metastasis and survival [17,18].

Based on our interest in searching for new compounds with antitumor activities [19–25], the present study was carried out to assess the in vitro anticancer activity of thalidomide dithiocarbamate analog in Lewis Lung Cancer cells and to determine the mechanisms underlying its anticancer effect through the regulation of the PI3K/Akt/mTOR signal pathway. Moreover, the expression levels of transforming growth factor-β1 (TGF-β1), Snail, Slug, and MMP-2 were measured through qRT-PCR and the anti-proliferation, anti-migration, and anti-invasive activities were determined through MTT assay, wound healing migration assay, and Matrigel invasion assay, respectively.

2. Materials and Methods

2.1. Thalidomide derivative production

The analog, Fig. 1, was synthesized according to the published article [26]. The dissolve of Thalidomide and its new analog in dimethylsulfoxide (DMSO) was performed in order to prepare stock solutions (1 mM), that was kept in vials for further storage at 4 ˚C, and possible future analysis. Solvents and chemicals were ordered from E. Merck (Darmstadt, Germany) and Sigma–Aldrich.

2.2. Lewis Lung Cancer cells and Culture

Lewis Lung Cancer (LLC) cells (from RIKEN BRC, Japan) and culture were performed as previously described [7]. Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) and 100 µg/mL streptomycin and 100 units/mL penicillin (Nacalai Tesque, Kyoto, Japan) was used for cells' growth and culture. The cell culture medium was replaced every other day and kept in a 5% CO2-humidified incubator at 37 ˚C.

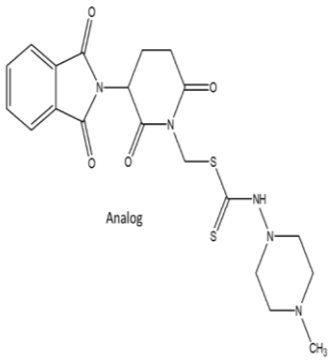

Figure 1. Chemical structure of thalidomide dithiocarbamate analog.

2.3. MTT Assay

As described previously by [7], the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the effect of thalidomide and its analog (6.25–100 µM) against LLC cells viability.

2.4. Wound Healing Migration Assay

As published previously [6], the anti-migrative ability of both thalidomide and its analog (12.5–100 µM) was detected against LLC cells. Using the imaging program DP2-BSW (Olympus), the cell-free areas distance was measured.

2.5. Matrigel Invasion Assay

The anti-invasive activity of thalidomide and its analog (25 and 100 µM) against LLC cells (5×10^4) was assayed in 24-well plates with 8 µm pore cell culture inserts (BD, Franklin Lakes, NJ, USA), pre-coated with BD Matrigel Matrix (Corning, New York, USA), according to the manufacturer's protocol as previously mentioned [27].

2.6. RNA extraction

Trizol reagent was used to isolate total RNA from untreated and treated LLC cells with vehicle, thalidomide, and its analog (25 and 100 µM) in accordance to the manufacturer´s protocol.

2.7. cDNA synthesis

Using an oligo-dT primer and GoScript Reverse Transcription System (Promega), and in accordance to the supplier´s protocol, first-strand complementary DNA (cDNA) synthesis was carried out.

2.8. Primer designing

Primers were designed for mTOR, ALK-1, PI3K ca, TGF-β1, Snail, Slug, MMP-2 and GAPDH genes. The primers used for each gene are given in Table 1.

2.9. Quantitative Real-time RT-PCR

Quantitative Real-time RT-PCR was performed using the SYBR green method with a real-time PCR detection system (Roche, Basel, Switzerland). The relative gene expression levels were calculated by comparative Ct(ΔΔCt) method, where Ct represented the threshold cycle and the relative expression was calculated as $2^{-\Delta\Delta Ct}$ using three independent experiments were completed where GAPDH as the reference gene.

2.10. Statistical Analysis

The results were expressed as means ± standard deviation (SD). The statistical significance for two groups comparisons was estimated by the student's t-test, and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). A p value of < 0.05 was viewed statistically significant.

3. Results

3.1. Effect of the analog on LLC Cell viability

In comparison with vehicle control (0.3% DMSO), LLC cell viability was reduced when applying the thalidomide analog in a concentration-dependent manner as shown in Fig. 1. Within the concentration of 6.25−100 µM of thalidomide, LLC cell viability was not evidently affected. However, the thalidomide analog has significantly reduced LLC cell viability at 25 and 100 µM by 33.7% and 45.9%, respectively (Fig. 2).

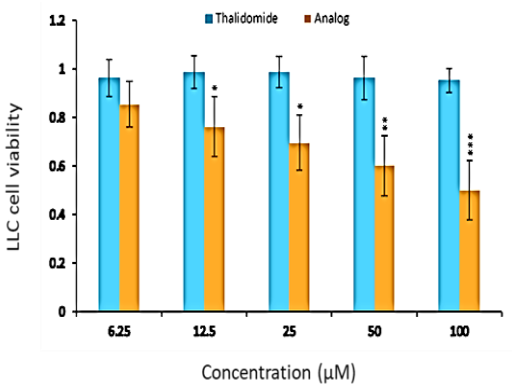

Figure 2. The inhibition of LLC cell viability and growth after the 48h administration of thalidomide and its analog. Using the MTT assay, the cell numbers in each well were calculated. The absorbance at 570 nm corresponding to the initial number of the cells was defined as 1.

Table 1. qRT-PCR primer sequences.

Gene	Primer Sequences (5'-3')	
	Forward primer	Reverse primer
mTOR	TTCCTGAACAGCGAGCACAA	GTAGCGGATATCAGGGTCAGG
ALK-1	CCGCCTGATCAAGTTCTCCT	TTCAGATGATCCATGCGGGG
PI3Kca	GCCACAGACACTACTGCGT	CACCGAACAGCAAAACTCCG
TGF-β1	TGATACGCCTGAGTGGCTGTCT	CACAAGAGCAGTGAGCGCTGAA
Snail	CACGTCCGCACCCACACTGG	GCGAGGGCCTCCGGAGCA
Slug	CACATTCGAACCCACACATTGCC	TGTGCCCTCAGGTTTGATCTGTC
MMP-2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA

3.2. Effect of the analog on LLC cell migration

The wound healing migration assay of LLC was accomplished to inform about the impact of thalidomide and its analog. The highest concentration of thalidomide (100 µM), as illustrated in Fig. 3A, LLC migration within 24 h was declined significantly. The thalidomide analog, conversely, exhibited potent inhibitory activity toward LLC migration as it inhibited the LLC cell migration at 25 µM by 39%. At a concentration of 100 µM 0.3% DMSO vehicle control, thalidomide, or thalidomide analog, the wounds healed as shown in Fig. 3B.

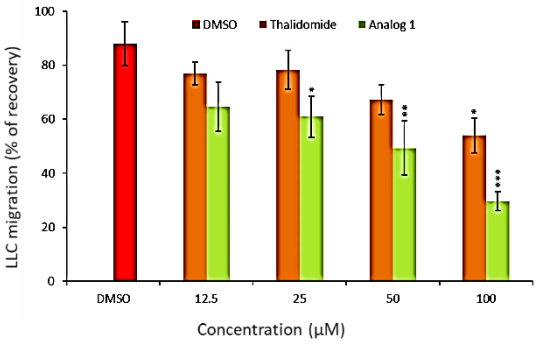

Figure 3A. Data of the scratch-wound assay of LLC migratory ability after thalidomide and its analog treatment. Data are presented as mean ± standard deviation, and (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) are considered significantly different.

The thalidomide analog, conversely, exhibited potent inhibitory activity toward LLC migration as it inhibited the LLC cell migration at 25 µM by 39%. At a concentration of 100 µM 0.3% DMSO vehicle control, thalidomide, or thalidomide analog, the wounds healed as shown in Fig. 3B.

3.3. Effect of the analog on LLC cell invasion

Matrigel migration assay was used to test the invasive ability of LLC cells treated with thalidomide analog. LLC cells migration was significantly reduced by thalidomide only at 100 µM within 48 h, compared with the vehicle control (Fig. 4). Thalidomide, conversely, analog significantly diminished LLC migration at 25 and 100 µM by 40% and 59%, respectively.

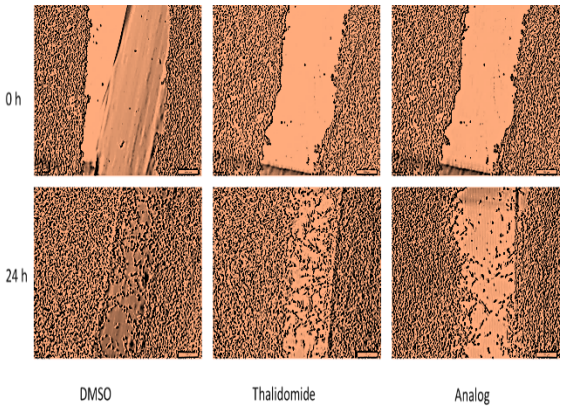

Figure 3B. Illustrative images of the wounds healing ability of 100 µM vehicle control (0.3% DMSO), thalidomide, or thalidomide analog.

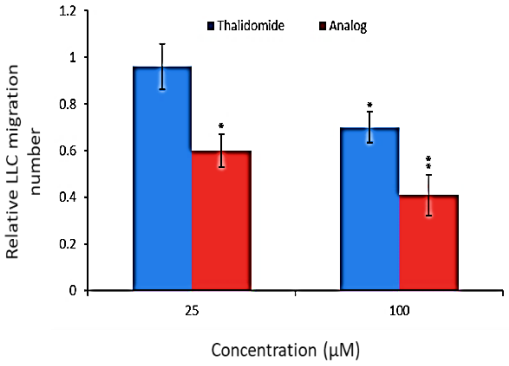

Figure 4. The trans-well migration assay and the invasive ability of LLC cells, under the effect of thalidomide and its analog, was tested. Invasive cell numbers were normalized against those in the vehicle control. Data are introduced as mean ± standard deviation, and (*$p < 0.05$, **$p < 0.01$) are considered significantly different.

3.4. Effect of the analog on the Gene expression status of the PI3K/Akt/mTOR pathway

As shown in Fig. 5, thalidomide (100 µM) reduced the expression levels of mTOR, ALK-1, and PI3Kca by 28%, 31%, and 34%, respectively,

relative to the vehicle control. Differently, the expressions were extensively reduced after treatment with the thalidomide analog by 63%, 65% and 62.6%, respectively. Contrasting to the vehicle control, the lowest concentration of thalidomide (25 µM) had no evident influence on the genes expressions.

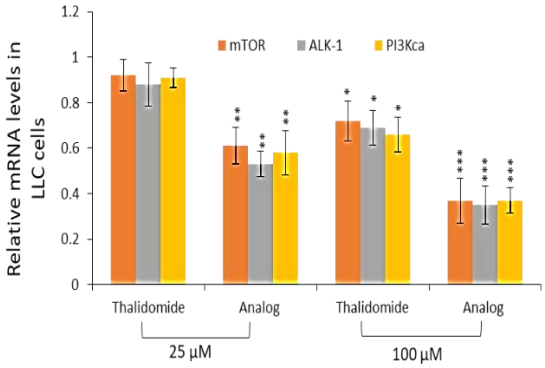

Figure 5. mTOR, ALK-1, and PI3Kca mRNA as expressed in LLC cells under the effect of thalidomide and its analogs. Data are introduced as mean ± standard deviation, and (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) are considered significantly different.

3.5. Effect of the analog on the mRNAs expression levels of TGF-β1, Snail, Slug, and MMP-2

Thalidomide (25 µM) has not able to inhibit the expression levels of TGF-β1, Snail, Slug, and MMP-2, compared with the vehicle control (Fig. 6). On the other hand, thalidomide analog reduced the expression of the same genes by 39%, 47% and 42%, respectively.

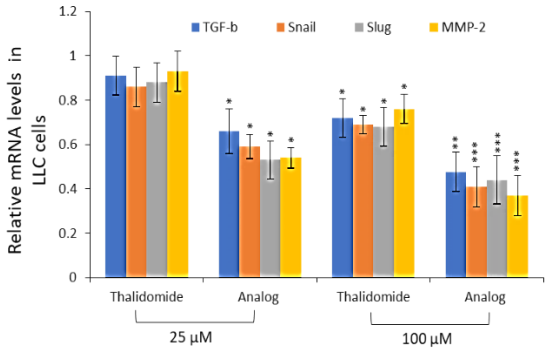

Figure 6. Effect of thalidomide and its analog on expression of TGF-β1, Snail, Slug, MMP-2 mRNA LLC cells. Data are presented as mean ± standard deviation. Significantly (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) different from the vehicle control.

4. Discussion

For the first time, as far as we know, the effect in vitro of the thalidomide analog on cell proliferation, migration, and invasion on LLC cells was demonstrated. Additionally, its activity on the expression status of PI3K/Akt/mTOR signal pathway as well as on TGF-β1, Snail, Slug, and MMP-2 were investigated.

The MTT experiment results, in comparison to the vehicle control, the LLC cells viability when receiving thalidomide analog significantly decreased in a concentration-dependent manner. This may be due to the presence of the added dithiocarbamate group. On the other hand, thalidomide was not able to show any effect in the studied concentration range, 6.25−100 µM. The presence of piperazine moiety and electron-donating properties of the methyl group participate in enhancing the biological activity of the compound [28,29]. Therefore, the outstanding inhibitory activity of thalidomide analog more than thalidomide drug may be associated to the occurrence of these groups in its chemical structure. Our result was in agreement line with previous reports showing that the N-substitution of the imido hydrogen in the glutarimide ring of thalidomide, with moieties containing sulfur atoms, caused greater anticancer activity when compared to thalidomide [26].

Cancer cell migration and invasion was revealed to be correlated to Akt activation. Akt has a key role in cell motility through the stability of microtubules increasing, subsequently, the invasiveness of cells [30,31]. Therefore, the effect of thalidomide and its analog on the LLC cells migration and invasion was investigated by Matrigel invasion assay and cell scratch assay. In the current study, LLC cells migration and invasion were significantly reduced only by the highest concentration of thalidomide (100 µM). However, the cell scratch healing assay in addition to the number of invasive cells were significantly reduced after treatment with the thalidomide analog at a concentration of 25 µM. The potent activity of the thalidomide analog, contrarily to thalidomide, inhibited the LLC cells migration and invasion owned to the activity of the PI3K/Akt/mTOR signaling pathway.

PI3K/Akt/mTOR pathway is a milestone in the cascade of survival, proliferation, and migration of various tumor cells [32,33]. Therapeutic agents able to suppress this signaling pathway can decrease the cancer cell growth [34]. In the current study, compared to vehicle control, thalidomide analog inhibited the PI3K/Akt/mTOR pathway by

downregulating the mRNA expression levels of PI3K, Akt, and consequently mTOR. The results agree with previous studies that explained how PI3K activates Akt, leading to phosphorylation followed by a cascade of events and finally mTOR activation [35]. These expression levels were reduced after the treatment with the lowest dose of the analog thalidomide (25 μM). Conversely, thalidomide could inhibit the gene expressions of this pathway but only at 100 μM, indicating that the thalidomide analog possessed higher ability to block the activation of the PI3K/Akt/mTOR pathway more than thalidomide itself. In a previous report, thymoquinone targeted the PI3K/Akt/mTOR pathway and induce cytotoxic effects on gastric cancer cells [36]. Also, ginkgolic acid blocked the PI3K/Akt/mTOR pathway, leading to the inhibition of migration and invasion of lung cancer cells [37].

TGF-β1 is a multifunctional cytokine which promotes lung adenocarcinoma progression and metastasis [38-40]. The mechanism of action of TGF-β1 initiates with binding to TβRII, leading to the activation of TβRI [41]. After, the activated TβRI induces the phosphorylation of the Smad2 and Smad3, forming heterodimeric complexes with Smad4. Subsequently, these complexes translocate into the nucleus and interact with transcription factors; for example Slug and Snail [42]. Gelatinases including MMPs can alter the cell adhesion by changing the contacts of cell-cell and cell-extracellular matrix [43]. Snail expression facilitates the cancer cells invasion by activating MMPs [44]. Therefore, to study the underlying molecular mechanism by which thalidomide analog affects the function of LLC cells, the potential effect of thalidomide analog on the mRNA encoding TGF-β1, Snail, Slug, and MMP-2 in LLCs expression was investigated.

Our qRT-PCR results showed that the thalidomide analog had a more significant inhibitory activity than thalidomide in reducing the expression of TGF-β1, leading to the decrease of expression of Snail, Slug, and MMP-2 mRNA. It has been reported that TGF-β1 can activate cell metastasis via PI3K/Akt/mTOR signaling pathway [45,46]. Also, isoviolanthin inhibited TGF-β1 in hepatocellular carcinoma cells via the deactivation of the TGF-β1/Smad and PI3K/Akt/mTOR signaling pathways [47].

In summary, the potent anti-proliferative, anti-migration, and anti-invasive ability of thalidomide analog toward LLC cells could be due to the inhibition of the PI3K/Akt/mTOR and TGF-β1/Snail signaling pathways. Although, this is the first time to document that the new synthesized thalidomide analog exhibited anticancer effects against LLC cells. Further investigations are now under consideration focusing on the in vivo model, using of LLC cells to induce LLC-solid tumor in mice, to validate the obtained results in experimental animals.

5. Conclusion

The current study provided important evidence related to the thalidomide analog inhibitory activity on the proliferation, LLC cells invasion and migration. These findings were explained by the interference in the PI3K/Akt/mTOR pathway, reducing the mRNA expression levels of TGF-β1, Snail, Slug, and MMP-2 in LLC cells. Consequently, thalidomide analog can be considered as a promising therapeutic candidate for lung cancer.

6. Conflict of Interest

The authors declare no conflict of interest.

7. Authors contributions

B.E.-A. conceived and designed the study. B.E.-A. and M.Z. conducted research and provided research materials. B.E.-A and E. E.-T. collected, organized, analyzed, and interpreted data. B.E.-A. and E. E.-T. wrote initial and final draft of article. B.E.-A., E. E.-T., and M.Z. have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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