

Formulation and Characterization of a Thalidomide Analogue in Nanoemulsion System

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Abstract—The objectives of the current study were to synthesize a thalidomide (THD) analogue and study its anticancer effect on human breast cancer cell line (MCF-7). Cytotoxic activity on the normal human cell line was assayed to ensure its safety on normal cells. It is quite a surprise to find that the synthesized analogue caused collapsing and shrinking in the shape of (MCF-7) while revealing its safety on the proliferation of normal human cell line. Therefore, it can be a promising compound in the treatment of breast cancer. Moreover, its encapsulation in a nanoemulsion system would enhance its potency. The plain nanoemulsion formula was evaluated by several characteristic methods, which showed that the mean droplet size was (345.6 nm) and mean zeta potential was (-9.98 mV). In addition to their examination under the field emission transmission electron microscope and noticed their spherical shape. Our future work is to study the drug entrapment efficiency and *in vitro* drug release. It is hoped that this study will lead to new insights of THD analogues as a therapeutic agent, particularly in the nanoemulsion system.

Index Terms—thalidomide analogue, anticancer activity, MTT assay, nanoemulsion, TEM

I. INTRODUCTION

Thalidomide (THD) as a derivative of the glutamic amino acid was released by a German pharmaceutical company as a sedative drug [1]. In the late 1950, it was prescribed to pregnant women who suffer from morning sickness [2]. Nevertheless, by the beginning of 1960, it was ultimately withdrawn from most of the world where it resulted in the most prominent medical disaster. It was confirmed to cause children teratogenesis to over 10,000 cases [3]. Surprisingly, the FDA reapproved the drug in 2006 owing to its anti-angiogenesis [4], anti-

inflammation and cell proliferation inhibition [5]. It was recorded to treat solid tumors such as lung cancer, prostate cancer, and primary hepatocellular carcinoma [6]. THD is characterized by its low aqueous solubility, and this continues to be an open problem that affects its therapeutic use [7]. It was assumed that this poor solubility hampered the discovery of its teratogenicity [8]. Therefore, this problem can be addressed by the formulation of THD in a nanodrug delivery system where this formulation is an interesting area of research. Our key idea is to encapsulate the synthesized THD analogue in a submicron emulsion, known as nanoemulsion.

Nanoemulsions are colloidal particles, acting as a drug delivery system. It is worth mentioning that nanoemulsions are characterized by their large surface area with small size ranging 10-1000 nm [9]. In addition to its biocompatibility, relative stability [10], improved drug solubility, controlled delivery profiles and protection of sensitive drug substances [11]. Moreover, several routes of administration were studied regarding their use as a drug delivery system [12]. Therefore, they are more promising over other drug delivery systems. Nanoemulsion consists of a mixture of water and oil, which are immiscible liquids in addition to an emulsifying system (surfactant and co-surfactant) [10]. Mainly, they are divided into two forms; oil in water nanoemulsion (o/w), where oil is dispersed in water and water in oil nanoemulsion (w/o), where water is dispersed in an oily phase [13]. Therefore, they offer a wide range of drugs that can be encapsulated inside them.

As a continuation of our research group endeavors to explore the structural modifications that are postulated to potentiate the antitumor efficacy of some thalidomide analogues [14], a parallel strategy of nanoemulsion formulation was sought to minimize cytotoxicity and to enhance bioavailability. A reasonable approach was followed to validate the presence of phthalimide moiety as a pharmacophoric part essential for the antiangiogenic

activity along with its ring-expanded naphthalimide counterpart (Fig. 1). Various compounds of this direction were synthesized and investigated for their anticancer efficacy against different cell lines (S F Hammad *et al*, unpublished data). Among these synthesized compounds, one of the most potent compounds, (Fig. 2) was studied for its cytotoxic effect on normal human cell line. Furthermore, its anticancer activity was assayed on breast cancer cell line (MCF-7). Our ongoing perspective is to formulate this THD analogue in nanoemulsion system to enhance its potency and this can be a significant step forward in treatment of breast cancer. Moreover, drug entrapment efficiency and *in vitro* drug release will be performed.

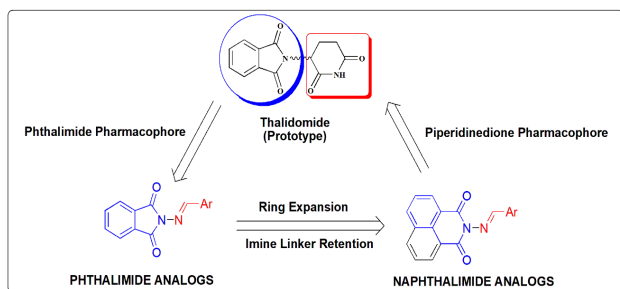


Figure 1. Rational design of synthesized THD analogue.

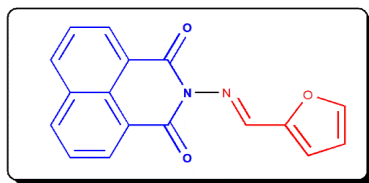


Figure 2. Chemical structure of the synthesized THD analogue.

II. MATERIALS AND METHODS

A. THD Analogue Synthesis

In a continuation with our work, several THD analogues were synthesized (S F Hammad *et al*, unpublished data). In this study, we proposed a method to synthesize one of the promising analogues. Into a 50 mL round bottom flask, 0.212 gm (1 mmol) of N-aminonaphthalimide was charged and 0.12 mL (1.5 mmol) of Furan-2-carbaldehyde were added to 20 mL of ethanol absolute. To the reaction mixture 4 drops of acetic anhydride were added and the mixture was kept under

reflux at 90 °C with stirring for 6 h. where the reaction was monitored by thin-layer chromatography till completion. The reaction mixture was left to cool down at room temperature till a precipitate was formed that was then collected by vacuum filtration and washed with cold diethyl ether and recrystallized from absolute ethanol to yield the desired furfural imine in about 83% yield (Fig. 3). The structure of the synthesized THD analogue was elucidated and characterized by NMR, IR, Mass spectroscopy and elemental analysis.

B. THD Analogue Spectrophotometric Analysis

The proposed method in studying spectrophotometric analysis of the synthesized THD analogue will be used for further studying of drug entrapment efficiency and percentage of *in vitro* drug release. This method involved the preparation of a stock solution of the synthesized THD analogue by dissolving the compound in absolute ethyl alcohol under stirring at room temperature until completely dissolved. Then, it was diluted to get different concentrations, and their absorbance was scanned by UV-visible spectrophotometer (Lambda EZ 201). After that, a calibration curve was plotted between the absorbance and the concentrations of the analyte. Furthermore, the correlation coefficient was calculated from the calibration curve.

C. Cytotoxic Effect of THD Analogue on Normal Human Cell line

Normal human lung fibroblast cell line (Wi-38) was used to detect cytotoxicity of the synthesized compound. Wi-38 cell line was sub-cultured in DMEM medium-contained 10% fetal bovine serum (FBS), seeded as 5×10^3 cells per well in 96-well plates and incubated at 37°C in 5% CO₂ incubator. After 24 h for cell attachment, serial concentrations of THD analogue were incubated with Wi-38 cells for 72 h. Cell viability was assayed by MTT method. 20 µl of 5 mg/mL MTT (Sigma, USA) was added to each well and the plate was incubated at 37°C for 3 h. Then, MTT solution was removed, 100 µl of DMSO was added and the absorbance of each well was measured with a microplate reader (BMG LabTech, Germany) at 570 nm. The effective concentration (IC₅₀) and safe dose (EC₁₀₀) values of the tested compound that cause 50% and 100% cell viability were estimated by the Graphpad Instat software.

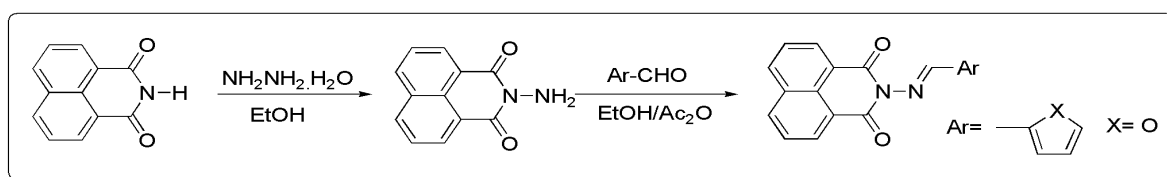


Figure 3. Synthesis scheme of THD analogue.

D. Determination of the Anticancer Activity

Anticancer activity was assayed on human breast cancer cell line (MCF-7). Cells were cultured in RPMI-1640 (Lonza, USA) supplemented with 10% FBS. They were seeded in sterile 96-well plates as a density of 5×10^3 cells/well. After 24h, serial concentrations of the tested compound were incubated with MCF-7 cancer cell line for 72 h at 37°C in 5% CO₂ incubator. MTT method was done as described above. The half maximal inhibitory concentration (IC₅₀) values were calculated using the Graphpad Instat software. Furthermore, cellular morphological changes were investigated using phase contrast inverted microscope with a digital camera (Olympus, Japan).

E. Nanoemulsion Preparation and Characterization

Plain nanoemulsion formula was prepared using oleic acid as oily phase, aqueous phase, and emulsifying system. The preparation process was performed at room temperature at speed stirring of 400 rpm. They were sonicated for about 30 min then degassed. The formula was monitored with respect to pH, particle size, zeta potential as well as indicating their shape with field emission transmission electron microscope.

F. pH Measurements

The pH of the plain nanoemulsion formula and THD-nanoemulsion formula were measured by using a portable pH meter (AD 130 pH/mV/Temperature Portable Meter).

G. Zeta Potential Measurements

The average particle size and zeta potential of the plain nanoemulsion was measured by dynamic light scattering (Malvern Nano ZS Zeta-sizer) at room temperature. It was diluted with distilled water then placed into a specific cuvette. Data analysis was performed using Malvern Zeta-Sizer software version 7.02.

H. Field Emission Transmission Electron Microscope

It was used to capture the images of the plain nanoemulsion formula and THD analogue-nanoemulsion formula. 5 µL of the sample was placed on a copper grid, then 5 µL of phosphotungstic acid was added to provide its negative staining. The model of microscope used was JEOL JEM-2100F.

III. RESULTS AND DISCUSSION

A. THD Analogue Characterization

M.p. 197-199 °C, IR (KBr) 3437, 1653, 1323, 1230, 1184 cm⁻¹; ¹H NMR (DMSO-d₆) 6.78–7.80 (m, 1H, Fur. C₄-H), 7.30-7.31 (m, 1H, Fur. C₃-H), 7.83-7.89 (m, 2H, Naphth. C_{2,6}-H), 8.08 (m, 1H, Fur. C₅-H), 8.44-8.51 (m, 4H, Naphth. C_{1,3,6,8}-H), 8.58 (s, 1H, CH); ¹³C NMR (DMSO-d₆) δ 112.78 (Fur. C₃, C₄), 119.71 (C_{4a}), 122.23 (C₄, C₅), 126.69 (C₂, C₇), 127.25 (C_{8a}), 130.99 (CH), 131.29 (C₁, C₃, C₆, C₈), 134.52 (Fur. C₅), 147.59 (Fur. C₂), 159.87 (C=O), 160.25 (C=O); EIMS m/z = 290 (M⁺).

B. Anticancer and Cytotoxic Effect of THD Analogue

The IC₅₀ and EC₁₀₀ (µmol/mL) of the tested THD analogue against normal human cell line (Wi-38) were (101.01±1.61) and (53.78±1.46) respectively. These values were expressed as mean±SEM. Their high values reveal their safety on the proliferation of normal human cell line. Moreover, the anticancer activity of the synthesized THD analogue were assessed on three human cancer cell lines, liver cancer cell line (HepG-2), breast cancer cell line (MCF-7) and colon cancer cell line (Caco-2) (S F Hammad *et al*, unpublished data). Particularly, our results will focus here on the breast cancer cell line (MCF-7).

It should be noted that when this compound was tested on (MCF-7) breast cancer cell line, the IC₅₀ was of a low value (42.53±2.4). This indicates its high anticancer activity against breast cancer cell line at dose (42.53 µmol/mL) less than its EC₁₀₀ (53.8 µmol/mL) on normal human cells. Moreover, it demonstrates the adequacy of the compound to be safe on normal human cells and their promising use in the treatment of breast cancer. It affects the cancer cell line in different ways including shrinking and collapsing in the normal shape of the cells (Fig. 4). These promising results open an approach to increase its potency and decrease the concentration of the tested dose on cancer cells by formulating the THD analogue in nanoemulsion system which is an important drug delivery system. It is worth mentioning that our preliminary findings of the tested THD-nanoemulsion system on breast cancer cell line reveal more promising results. The anticancer dosage was reduced to nanomolar efficiency instead of micromolar efficiency, which is the most intriguing findings, but these results are still under investigations.

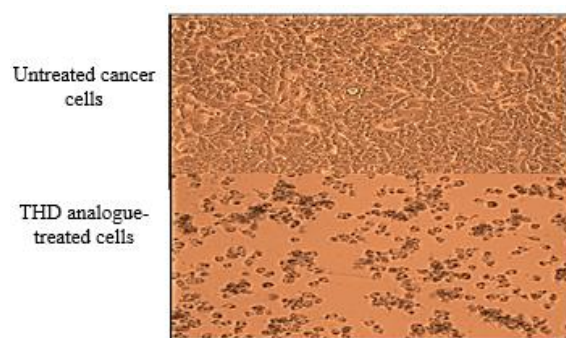


Figure 4. Morphological alterations of the THD analogue-treated MCF-7 breast cancer cell line in comparison with the untreated MCF-7 cancer cell line.

C. Characterization of Plain Nanoemulsion Formula

This section summarizes and discusses the main characterization methods of the plain nanoemulsion formula that will be used as a drug delivery system to enhance THD analogue potency. The pH measurement of the plain nanoemulsion formula was shown to be (4.97±0.01) and was observed to be (4.36±0.1) for THD analogue-nanoemulsion formula. The mean droplet size of the plain formula was (345.6 nm) and this demonstrates that it lies within the most common

size range of nanoemulsion 100-500 nm [9]. This observation agrees with the results reported by others that the normal size ranges 10-1000 nm [15]. The mean zeta potential of the plain nanoemulsion was demonstrated to be (-9.98 mV). Moreover, photomicrographs of the plain formula and THD analogue-nanoemulsion formula were captured showing their small sizes with spherical structures and large surface area (Fig. 5).

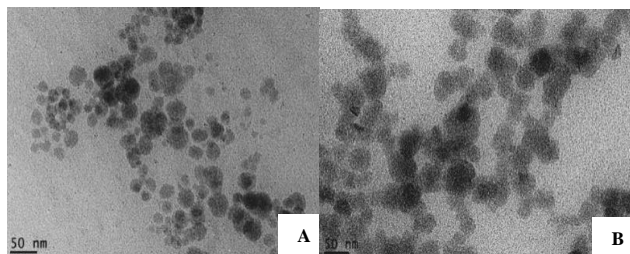


Figure 5. Transmission electron photomicrographs (A) plain nanoemulsion formula, (B) THD analogue-nanoemulsion formula.

IV. CONCLUSION

In the present study, we extended our previous work by synthesizing a promising THD analogue and its characterization to assess its antiangiogenic activity. The anticancer activity showed that the synthesized analogue has a promising effect on three different cancer cell lines (S F Hammad *et al.*, unpublished data), including breast cancer cell line, where it caused cells shrinking and collapsing. An approach was made to increase its potency and decrease its dosage when applied to cancer cells by formulating the compound in nanoemulsion formula. The plain nanoemulsion formula was characterized by different methods regarding their mean droplet size and zeta potential. They were photographed by field emission transmission electron microscope to reveal their spherical structure and small sizes with large surface area which are very important factors in many successful drug delivery systems. Our future investigations are to study the anticancer activity of the formulated THD analogue on breast cancer cell lines and to study the effect of the plain nanoemulsion formula and its safety on normal cell line. This study is a promising step for THD analogue as a therapeutic agent particularly in the nanoemulsion system.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Noran M. Tawfik and Sherif F. Hammad conceived the main idea, writing, editing the manuscript and performed the final submission. Mohammed Teiama, Sameh Samir Iskandar and Ahmed Osman equally contributed to the supervision of the whole work and performed the final revision.

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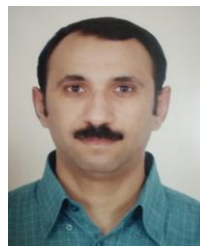
of Science was in Zoology (Biotechnology) in 2016. She got her master's degree under title of "Cytogenetic evaluation and PCR diagnostics of the tumor suppressor gene (P53) in CD34⁺ cord blood stem cells".

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Dr. Sherif F. Hammad is one of the expert Egyptian medicinal chemists especially in the field of API industrial production. Dr. Hammad was graduated in Faculty of pharmacy, Alexandria University in 1997 with an excellent with honor grade after receiving the best ideal student award (first ranked over the university). He started his academic career in Helwan University where he earned a master's degree in pharmaceutical chemistry followed by a Medicinal Chemistry