Chemical Stabilities and Biological Activities of Thalidomide and Its *N*-Alkyl Analogs

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Purpose. To determine whether the *N*-alkyl analogs of the thalidomide are active and stable, their stabilities in buffer and their abilities to inhibit tumor necrosis factor alpha (TNF-α) *in vitro* in human peripheral blood mononuclear cell cultures were investigated.

Methods. TNF-α concentrations were determined with the aid of ELISA kits. Chemical stabilities of the compounds were determined in three phosphate buffer solutions (pH 6, 6.4, and 7.4) at 25 and 32°C by high-pressure liquid chromatography, and half-lives were calculated.

Results. The addition of *N*-alkyl groups to the glutarimide ring of the thalidomide molecule had little effect on the ability such compounds have to inhibit TNF-α production. There was no statistical difference between the activity of thalidomide and its *N*-alkyl analogs at a 95% confidence level. Like thalidomide, the *N*-alkyl analogs in this series inhibit an average of 60% of the TNF-α synthesis in lipopolysaccharide-stimulated peripheral blood mononuclear cell cultures. Thalidomide and its *N*-alkyl analogs are hydrolyzed at very similar rates, with half-lives ranging from 25 to 35 h at 32°C at pH 6.4 and an average rate constant of 2.35×10^{-2}/h.

Conclusions. Alkylating thalidomide had little effect on its ability to inhibit the production of TNF-α in these cell cultures. All of the compounds tested seem to have some, perhaps comparable, therapeutic potential.

KEY WORDS: thalidomide; *N*-alkyl analogs; biological activity; tumor necrosis factor alpha; chemical stability; half-life.

INTRODUCTION

Thalidomide (α-*N*-phthaloyl-glutamic-acid-imide) was widely prescribed as a sedative in the late 1950s but was withdrawn from the market in 1961 because of its teratogenic effects. Thalidomide has a relatively simple chemical architecture (Fig. 1). In addition to its sedative and teratogenic effects (1), it is now known to possess significant and unique immunomodulatory and anti-inflammatory activities. Numerous reports have appeared on the successful use of thalidomide in the treatment of type 2 lepra (erythema nodosum leprosum) reactions (2,3), chronic graft-versus-host disease (4), discoid lupus erythematosus (5), aphthous ulcers in patients with AIDS (6), and rheumatoid arthritis (7). Its effectiveness has been attributed to its ability to inhibit the synthesis of tumor necrosis factor-alpha (TNF-α). Studies have shown that thalidomide is a powerful inhibitor of TNF-α produced by monocytes *in vitro* (8,9).

Oral therapy of thalidomide has proven to be effective in rheumatoid arthritis but is associated with unacceptable side effects (7). There is good reason to believe that thalidomide acts on TNF-α expression at the local tissue level (8,10–12). Therefore, if the delivery of the drug can be targeted, via percutaneous delivery, it should be feasible to treat localized inflammation selectively. However, thalidomide itself is too polar and too high melting to achieve this end. Therefore, several *N*-alkyl analogs of thalidomide that do not possess its marked polarity and high melting properties were prepared. We have shown that these *N*-alkyl analogs of thalidomide are delivered percutaneously far easier than thalidomide itself (13). However, these improvements would be little avail if the analogs were not themselves active compounds. Therefore, in this study, the TNF-α inhibitory effects of thalidomide and its *N*-alkyl analogs were assessed in cell culture. Peripheral blood mononuclear cells (PBMCs) were stimulated with lipopolysaccharide (LPS), which induces TNF-α production. Thalidomide was used as a control.

MATERIALS AND METHODS

Synthesis Method

The racemic form of thalidomide and its *N*-alkyl analogs were synthesized according to literature methods but with some modifications in the purification procedures. Detailed synthetic procedures have been presented in our previous study (14). Briefly, thalidomide was prepared by condensing *N*-phthaloyl-DL-glutamic anhydride with urea. Similarly, *N*-methyl, *N*-propyl, and *N*-pentyl thalidomide were synthesized by condensing *N*-phthaloyl-DL-glutamic anhydride with methyl amide, propyl amine, and amyl amine, respectively. Identification and levels of purity >96% were assured through element analysis, electron impact mass spectrometry, nuclear magnetic resonance spectroscopy, high-pressure liquid chromatography (HPLC), and by the sharpness of melting points.

Stability Determination

The chemical stabilities of thalidomide and its *N*-alkyl analogs were investigated. Methanolic stock solutions of each compound (160 μg/mL) were prepared. A series of dilutions of each compound in study into phosphate buffer solutions of pH 6, 6.4, and 7.4 was made to give final drug concentrations of 2 μg/mL containing less than 1% methanol. To obtain the two lower pH values, the 0.1 M phosphate buffer (pH 7.4) was adjusted to pH 6 and 6.4 with ortho-phosphoric acid. The various solutions were incubated at 25 and 32°C for 24 h, with samples being taken at 1, 2, 4, 6, 8, and 24 h. The chemical

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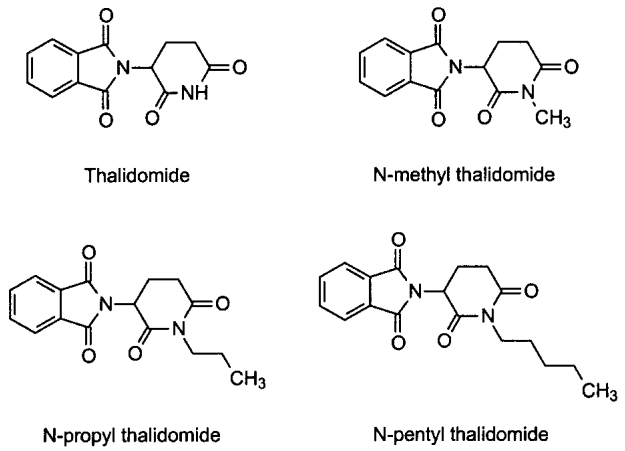

Fig. 1. Structural formula of thalidomide and its *N*-alkyl analogs.

stability studies for thalidomide and its analogs at pH 6.4 at 32°C were performed in duplicate. The standard deviations were not more than 5% and therefore the other chemical stability studies were not repeated. The hydrolysis reaction was immediately quenched by diluting the samples with the appropriate mobile phase (pH 2.0) for each compound. These were then stored at –20°C until analyzed by HPLC. Normal log [concentration]-time profiles of thalidomide and its analogs were prepared and the first linear part of the curve was used to determine the rate constants. Half-lives were calculated by dividing 0.693 by the individual rate constants.

HPLC Procedure

Thalidomide and its *N*-alkyl analogs were assayed by HPLC as described previously (14). Briefly, the HPLC consisted of a Beckman 114M solvent delivery system and a Spectraflow 783 variable wavelength UV detector set at 220 nm. A C_8 Spheri-5 micron cartidge (220 × 4.6 mm) with a guard column was used. The mobile phase consisted of 25% acetonitrile/water for thalidomide and *N*-methyl thalidomide and 35 and 45% acetonitrile for the *N*-propyl and *N*-pentyl analogs, respectively. The flow rate was set at 1.2 mL/min. Calibration curves showed excellent linearity over the entire concentration range from 0.01 μg/mL to 10 μg/mL.

Isolation of PBMCs

PBMCs were isolated from the blood of six healthy human donors by Ficoll Hypaque (Sigma Chemical Co., St. Louis, MO, USA) density centrifugation. The cells were washed three times in cell culture medium (RPMI 1640; Bio Whittacker, Walkersville, MD, USA) and centrifuged for 10 min at 1,200 × g at 4°C. The supernatant was discarded and the pellet (PBMCs) was resuspended in R-10 (89% RPMI 1640, 10% sterile human serum and 1% 100 U/mL PCN-penicillin). The cells were counted on a hemacytometer, using Trypan Blue (Sigma), and the final volume of cell suspension was adjusted with R-10 to obtain 2×10^6 cells/mL. One hundred microliters of the cell suspension (2×10^6 cells/mL R-10) were added to wells of a 96-well flat-bottom microtiter plate (15).

TNF-α Induction

PBMCs were stimulated with *Salmonella minnesota* R592 (LPS; Sigma) to produce TNF-α. A stock solution of LPS (2 mg/mL sterile water) was stored at –20°C. LPS was diluted in R-10 and used at 2 μg/mL for the assay. After the plate was incubated for 1 h, 50 μL of the LPS solution (2 μg/mL) was added to each well (with exception of the negative control). The negative controls received 50 μL of regular R-10. The final volume of the cultures was 200 μL. The plate was covered and incubated for 16-18 h in a humidified 5% CO_2 incubator at 37°C (15).

TNF-α Inhibition

The compounds in the study were dissolved in DMSO (Sigma) and further dilutions were performed with 0.06% acidified R-10 (0.1 M HCl + R-10). Final solutions (50 μg/mL) contained 0.5% DMSO. Fifty microliters of each drug solution was added separately to wells containing 100 μL of cell solution and incubated for 1 h at 37°C in a humidified 5% CO_2 incubator before the addition of LPS. Each drug was tested in triplicate from six donors, thus, 18 assays for each compound. The positive and negative controls were also run in triplicate, in which case 50 μL of 0.5% DMSO in R-10 were added instead of the drug solution (15).

TNF-α Determination

After incubating the plate for 16–18 h, it was centrifuged for 2 min at 2 × g at 4°C (Sigma Laboratory Centrifuge, Model 3-15). One hundred μL of the supernatants were harvested and TNF-α concentrations were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer's directions. The absorbance (492 nm) of each well was determined with an automated plate reader. The optical density was read in triplicate samples. The values for the standards were plotted vs. the concentration of the same standards, and the best curve was obtained. The data were linearized using regression analysis and presented as pg/mL. A regression analysis equation was used to determine the TNF-α concentration for each well. Percentage TNF-α inhibition was calculated as: 100 × [1 – (TNF-α experimental/TNF-α control)]; where TNF-α experimental represents TNF-α secretion by stimulated PBMCs that were cultured in the presence of the drug, and TNF-α control represents TNF-α secretion by stimulated PBMCs that were cultured in the absence of the drug. PBMCs cultured in medium containing equivalent amounts of DMSO in the presence or absence of the stimulating agent were used as controls for drug treated cells.

STATISTICAL ANALYSIS

The concentration of TNF-α (pg/mL) in the supernatant of three replicate cultures of human mononuclear cells stimulated with LPS and containing drug were compared to the TNF-α concentration (pg/mL) in the supernatants of three replicate cultures stimulated with LPS and not containing drug (positive control). Student's *t* test was used to determine statistical significance, and p values of < 0.05 were considered significant.

RESULTS

It is known that thalidomide hydrolyzes spontaneously in aqueous media. The rate of its hydrolysis accelerates with

increasing pH and, of course, temperature (16). To roughly determine whether the *N*-alkyl analogs hydrolyze to a comparable extent, the compounds were placed in solution in aqueous buffers at pH values of 6, 6.4, and 7.4 and the stabilities of each were assessed at 25 and 32°C. Because the reactions are pseudo-first order, the natural logarithms of concentration of thalidomide and its odd chain *N*-alkyl analogs were plotted as a function of time and the slopes of the linear curves were taken as the rate constants. The half-lives are listed in Table I.

The TNF-α inhibitory effects of thalidomide and its *N*-alkyl analogs were measured in the supernatant of human PBMCs stimulated with LPS. The addition of *N*-alkyl groups to the glutarimide ring of the thalidomide molecule (Fig. 1) had little effect on the respective abilities of the compounds to inhibit TNF-α production as shown in Fig. 2. The data in Fig. 2 represent mean percent TNF-α inhibition ± SD of three replicate cultures from six donors. There were no statistical significant differences in the abilities of thalidomide and its *N*-alkyl analogs to inhibit TNF-α at a 95% confidence level ($p < 0.05$). Like thalidomide, the *N*-alkyl analogs in this series inhibit an average of 60% of the TNF-α synthesis in LPS-stimulated PBMCs.

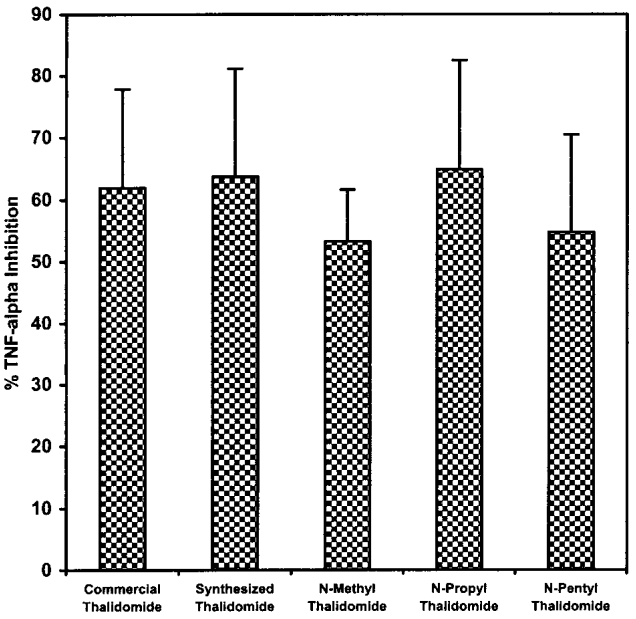

Fig. 2. Percentage tumor necrosis factor alpha inhibition of thalidomide and its *N*-alkyl analogs.

DISCUSSION

Thalidomide is sparingly soluble in aqueous solution (approximately 50 μg/mL at 25°C) and is broken down in the body mainly by hydrolytic mechanisms. Schumacher *et al.* (16) studied the spontaneous hydrolysis of thalidomide in solution and found that, at pH values above 6, thalidomide undergoes spontaneous hydrolysis, the rate of which accelerates with increasing pH. Similar trends have been observed for the *N*-alkyl analogs. Although all the substituted amide bonds of the molecule are sensitive to hydrolysis, from pH 6 to 7 only the phthalimide ring undergoes cleavage. At pH 7 and above, the glutarimide moiety also suffers measurable hydrolytic splitting. Theoretically, 12 different products can be formed from thalidomide by hydrolysis of one or more amide bonds. The spontaneous rates of hydrolysis of thalidomide and its *N*-alkyl analogs have been compared in this study. Thalidomide and its *N*-alkyl analogs are hydrolyzed at very similar rates with half-lives ranging from 25 to 35 h at 32°C at pH 6.4. The average rate constant was 2.35×10^{-2}/h and was dependent on temperature and pH.

We have studied the biological activity (TNF-α inhibition) of thalidomide and its *N*-alkyl analogs by stimulating PBMCs with LPS. First, we showed that our synthesized thalidomide inhibits the production of TNF-α to the same degree as the thalidomide purchased from ICN, giving us confidence in the synthetic outcome and also the TNF-α test. Like thalidomide, its *N*-alkyl analogs also suppressed the synthesis of TNF-α in PBMCs stimulated with LPS at a concentration of 50 μg/mL. The activities of all four compounds were, for all practical purposes, identical. This strongly suggests that the structural features of thalidomide responsible for its activity were conserved upon making the analogs. ED_{50} values were not determined here, and one could not compare potencies of these compounds relative to one another. The *N*-alkyl fragments added to the thalidomide molecule spanned a range from one carbon to five. One might expect a five-carbon chain to sterically inhibit receptor association at the imide position if this position was somehow involved in the occupation of the receptor. It appears, therefore, that the imide nitrogen is not an essential "lock-and-key" position for activity. Moreover, the *N*-alkyl analogs are incrementally, exponentially more hydrophobic than thalidomide. The similar inhibitions of the compounds indicate that they are not driven out of solution and onto the receptor sites by their hydrophobicities. This suggests comparable structuring of water takes place around the molecules when bound as when "free" (or there is a cancellation of two opposite effects). These cultures contained 10^6 human mononuclear cells. Thalidomide's ability to suppress TNF-α in cultures of PBMCs is also in agreement with the finding of Shannon and Sandoval (17). They demonstrated a significant reduction in TNF-α produced by PBMCs stimulated with LPS and treated with 50 μg/mL thalidomide.

Table I. Half-lives of Thalidomide and Its *N*-Alkyl Analogs

	Half-lives, $t_{1/2}$ (h)					
	25°C			32°C		
Compound	pH 6	pH 6.4	pH 7.4	pH 6	pH 6.4	pH 7.4
Thalidomide	140.5	72.6	12.6	45.3	25.4	5.7
N-Methyl Thalidomide	137.2	102.8	13.9	49.7	29.0	5.5
N-Propyl Thalidomide	169.4	88.7	12.6	49.6	30.3	6.2
N-Pentyl Thalidomide	318.3	96.6	16.7	74.9	35.1	6.2

CONCLUSION

It was shown that alkylating thalidomide had little effect on the ability of the compounds to inhibit the production of TNF-α in these cell cultures. Based on this observation, all of the compounds tested seem to have some, and perhaps comparable, therapeutic potential.

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