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Investigation of *N*-hydroxythalidomide *in vitro* stability and comparison to other N-substituted derivatives

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Abstract

The stability of N-substituted derivatives of thalidomide was studied and compared to that of thalidomide itself. Nitrogen atom included in glutarimide ring was successively substituted by a hydroxy group, a methyl acetate group, and an ethyl group. Lipophilicities of these compounds were determined using the method based on experimental determinations of partition coefficients developed by Hansch. Hydroxy group led to a decrease of lipophilicity. Substitution of the nitrogen atom by an ethyl group or a methyl acetate group allowed an increase of lipophilicity. Relative stabilities of each compound were determined under physiological conditions: pH (7.4) and temperature (37 °C) using high performance liquid chromatography procedure. The program Sigma Plot was used to fit experimental data in order to obtain the half-lifes of thalidomide and its analogs. In the case of substitution by an ethyl group, the increase of lipophilicity ($\Delta \log P = 0.36$) was in agreement with a higher stability in aqueous medium. In the case of methyl acetate group, hydrolysis of the cycle was chemically favoured despite a higher lipophilicity compared to those of thalidomide. In the case of *N*-hydroxy compound, the decrease in lipophilicity was not sufficient to affect the stability.
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Keywords: Thalidomide; Lipophilicity; Half-life; Hydrolysis kinetics

1. Introduction

Racemic thalidomide (1,3-dioxo-2-[2,6-dioxopiperidin-3-yl]-isoindole) was initially marketed in 1956 as a potent hypnotic and sedative agent and considered at the time as devoided of any side effect. Unfortunately, this compound proved later to induce severe teratogenic side effects and for that reason was withdrawn in 1961. Since 1965, new area of activities appeared for thalidomide. Thalidomide was active against lepra reactions [1,2], in potentially inhibiting the replication of human immunodeficiency virus (HIV-1) [3], and in the therapy of various inflammatory reactions [4]. Furthermore, thalidomide teratogenic effect was partially explained by its antiangiogenic activity [5]. This property appeared to be particularly interesting in the treatment of solid tumors such as brain or prostate cancers, and clinical trials were realized [6,7]. Despite the fact that development of new stereoisomeric drugs requires the submission of unambigous data concerning activity and configurational stability of single enantiomers, thalidomide is still used as a racemate in several countries for the treatment of various diseases. Even if the configuration S of the asymmetric carbon was an essential cause of teratogenicity [8], the racemate could be used because isolated enantiomers undergo very quickly a racemization *in vivo* [9,10]. Another very important fact was the spontaneous degradation of thalidomide in water [11]. Actually, this drug presented two imide rings, both sensible to hydrolysis. Opening of phthalimide and glutarimide rings occurred *in vitro* at several pH [12,13]. Under physiological conditions (pH 7.4

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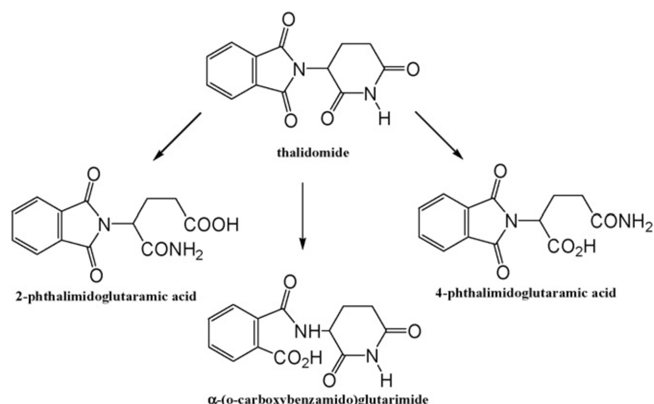


Scheme 1. Opening of thalidomide rings under physiological conditions.

and 37 °C), these degradations led to three derivatives which corresponded to 2- and 4-phtalimido-glutaramic acid, and to α-(*o*-carboxybenzamido) glutarimide (Scheme 1) [12,13].

These metabolites were suspected to be formed *in vivo* and to generate hydroxy radicals which exerted antiangiogenic activity affecting embryos bodies [14].

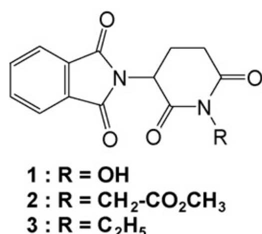
N-hydroxythalidomide (Compound **1**, Scheme 2) had proved to present a teratogenic activity against chicken embryos more efficient than thalidomide [15], and was then a candidate for antiangiogenic activity. The interest of antiangiogenic activity in the area of anti-cancer strategy prompted us to study the stability of the heterocycles of this compound.

In order to check if nitrogen substitution by various groups would modify the hydrolysis profile of the pharmacophore, the stability of other thalidomide derivatives was compared to that of *N*-hydroxythalidomide. At first, the nitrogen atom included in glutarimide ring was then replaced by a methyl glycine ester group (Scheme 2, compound **2**). On the other hand, lipophilicity which is related to the ability of compounds to pass through biological membranes [16], could also influence stabilities of imide rings especially in aqueous media like blood. In that purpose, another compound was then designed with an ethyl group on the nitrogen atom (Scheme 2, compound **3**).

2. Materials and methods

2.1. Chemicals

Thalidomide was a gift of Laphal Industrie (Allauch, France), compound **1** (racemate) [17], compounds **2** and **3** (racemates) were synthezised according to published procedures [18,19].



Scheme 2. Derivatives of thalidomide.

HPLC grade acetonitrile, methanol were obtained from SDS (France). Analytical grade acetic acid, hydrochloric acid, potassium phosphate were purchased from Merck (Fontenay-sous-Bois, France). Deuterium oxyde, Deuterium chloride were purchased from Sigma–Aldrich (St. Quentin Fallavier, France). All other materials were analytical grade and used as received.

2.2. Apparatus

2.2.1. Study of N-hydroxythalidomide in vitro degradation by ^1H NMR spectrometry

^1H NMR Spectra were obtained at a frequency of 300 MHz with a Brücker Spectrospin 300NMR Spectrometer. *N*-hydroxythalidomide solutions were prepared by dissolution of 5.2 mg (19×10^{-3} mmol) of compound **1** in 1 mL of deuterated phosphate buffer (pH 7.4; 0.067 M) and a total of 128 scans per spectrum were acquired for each sample. All samples were maintained at 37 °C during data acquisition.

2.2.2. Kinetics of degradation of thalidomide and its analogs

The HPLC system consisted of an isocratic solvent delivery pump system (Beckman Instruments, Berkeley, USA), equipped with a 20 μL sample loop injector (Rheodyne Cotati, CA, USA), a variable-wavelength UV detector (Varian ProStar Model 340). The chromatography column (150 mm × 4.6 mm i.d.) was packed with Nucleosil 100 C18, 5 μm particle size (Macherey-Nagel, France). The mobile phase was 0.01 M potassium phosphate buffer (pH 7.4; 0.067 M) methanol–acetonitrile (125/30/45) (v/v/v) circulating at a constant flow rate of 0.2 mL/min. Prior to use the mobile phase was degassed in an ultrasonic bath. The detection wavelength was set at 230 nm. All chromatograms were carried out at room temperature. The data recording system consisted of a personal computer with system Star 5.52 Software (Varian).

2.2.3. Determination of log P of thalidomide and its analogs

UV spectra were obtained with a Beckman DU640B spectrophotometer.

2.3. Methods

2.3.1. Hydrolysis kinetics of thalidomide and its analogs

For studies realized at pH 7.4, a solution of 70 ml of phosphate buffer (pH 7.4; 0.067 M) and 30 mL of acetonitrile was prepared. For each compound analyzed, 5 mg were dissolved in this solution. The resulting mixture was sonicated for 3 min, and then stirred at 23 °C or 37 °C. At different intervals of time, an aliquot (20 μL) of this solution was collected and injected into the analytical column. The concentration of the product was determined according to the difference of the amount of the product in the starting solution ($t = 0$) and in the solution at the time of analysis.

For studies realized at pH 10.4 and 2.4, the same protocol was used, but phosphate buffer was respectively replaced by

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