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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.

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 Unfortunately, this compound proved later to induce severe teratogenic side effects and for that reason was withdrawn in 1961.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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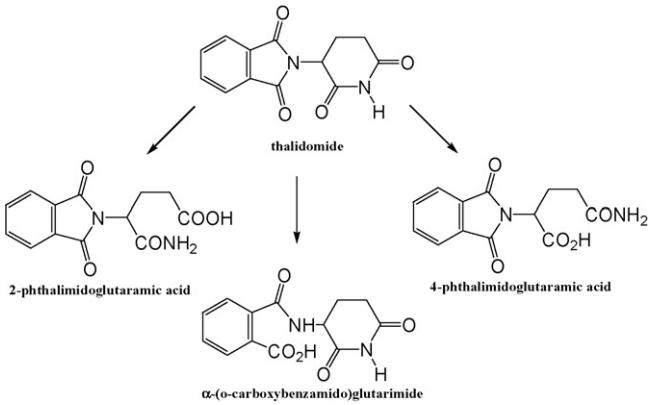


Scheme 1. Opening of thalidomide rings under physiological conditions.

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].

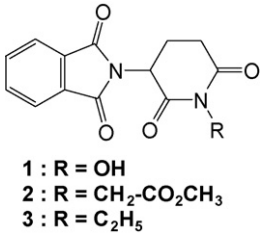
N-hydroxythalidomide (Compound **1**, Scheme 2) had proved to present a teratogenic activity against chicken embryos more efficient than thalidomide [15], and was then a candidate for antiangiogenic activity. The interest of antiangiogenic activity in the area of anti-cancer strategy prompted us to study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

^{1}H 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

borate buffer (pH 10.4; 0.01 M) and phosphate buffer (pH 2.4; 0.067 M).

2.3.2. Lipophilicity of thalidomide and its analogs

The maximum UV absorption of thalidomide and compounds **1**–**3** was 293.5 nm. Standard solution curves were prepared in octanol at concentration ranges of 5–50 mg L^{-1} for each compound. These values were chosen to avoid any precipitation artefacts. The regression line slope equations were obtained by measuring absorbance in UV at 293.5 nm.

Thalidomide : $y = 0.0056 + 0.0008x, \quad r^2 = 0.999$

Compound **1** : $y = 0.0225 + 0.0015x, \quad r^2 = 0.998$

Compound **2** : $y = 0.0033 + 0.0014x, \quad r^2 = 0.998$

Compound **3** : $y = 0.0017 + 0.0017x, \quad r^2 = 0.998$

Hundred milliliter of octan-1-ol were introduced in a flask, 100 mL of phosphate buffer (pH 7.4; 0.067 M) and 5 mg of the product were then added. The flask was shaked during 180 min, and the mixture constituted by the two immiscible solvents was decanted. The organic layer was separated, and the absorbance of this solution was measured in UV at 293.5 nm. The concentration of the compound in this layer (working solution) was determined by reference to standard curves. For each compound, three experiments were realized under the same conditions. The mean of log *P* values was calculated. Results were expressed with the standard deviation from the mean (Eq. (1)).

$$S_n = \sqrt{\frac{Sd^2}{n}} \tag{1}$$

where *d* is the deviation and *n* is the number of determinations.

2.4. Validation data

2.4.1. Calibration curves

Calibration curves were prepared at concentrations ranges of 5×10^{-2}, 3.75×10^{-2}, 2.5×10^{-2}, 1.875×10^{-2} and 1.25×10^{-2} mg mL^{-1} for each compound, and gave the regression line slope equations:

Thalidomide : $y = 0.320 + 2.133x, \quad r^2 = 0.993$

Compound **1** : $y = -0.201 + 0.481x, \quad r^2 = 0.997$

Compound **2** : $y = 0.154 + 4.689x, \quad r^2 = 0.996$

Compound **3** : $y = 0.004 + 4.678x, \quad r^2 = 0.994$

where *y* and *x* represent area and concentration, respectively. For each compound, four concentrations were studied ($n = 3$ for each concentration).

Table 1
Study of repeatability and reproducibility

	Repeatability RSD (%)	Reproducibility RSD (%)
Thalidomide	0.0675	1.35
Compound **1**	0.0489	3.29
Compound **2**	0.0594	0.253
Compound **3**	0.0407	0.698

2.4.2. Repeatability and intermediate precision

The repeatability (intra-day precision) of the method was evaluated by analyzing solutions of thalidomide, compounds **1** to **3** at concentrations of 5×10^{-2} mg mL^{-1} in six replicates.

Reproducibility (inter-day precision) was studied for the same concentrations as those studied for repeatability on three different days.

Results corresponding to these two criteria were expressed by the relative standard deviation (Table 1).

2.5. Data analysis

In the kinetic studies of products degradation, data conformed to a single exponential decay (Eq. (2)).

$$y = a\,e^{-bx} \tag{2}$$

where *y* is the %of hydrolysis and *x* is time.

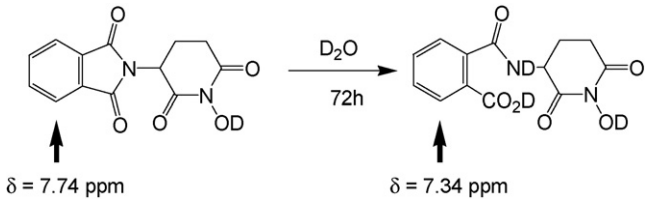
The two parameters *a* and *b* were determined by nonlinear regression of Eq. (2) using Sigma Plot software (version 7.1). For each compound, three experiments were realized under the same conditions, and the half-life was determined from the nonlinear regression curve. The mean of the three experimental half-life values was calculated. Results were expressed with the standard deviation from the mean (Eq. (1), see Section 2.3.2).

3. Results and discussion

3.1. Comparative stability of thalidomide and N-hydroxythalidomide

3.1.1. Hydrolysis regioselectivity

^{1}H NMR spectrometry in D_2O was used to determine where the ring opening mainly occurred. For compound **1**, the signals related to the two methylene groups of glutaramide ring were located at 2–2.8 ppm. Protons in α-position of chiral center were diastereotopic, and each of them had its own signal, respectively at 2.06 and 2.50 ppm. The multiplet at 2.80 ppm corresponded to the methylene in β-position. The peak at 5.25 ppm was the exchangeable methine proton branched to the chiral center. After 24 h, signals of aromatic protons at 7.74 ppm decreased, while a new multiplet appeared at 7.37 ppm. After 48 and 72 h, this phenomenon increased (Scheme 3). Concerning the signals of glutarimide ring, no change was observed. These data proved that the phthalimide ring was highly affected by hydrolysis while the glutarimide ring remained stable. The result of the hydrolysis was then an opening of the phthalimide ring leading to *N*-hydroxy-α-(*o*-carboxybenzamido)glutarimide. These results, compared to the ring opening which occurred for



Scheme 3. Hydrolysis regioselectivity of *N*-hydroxythalidomide.

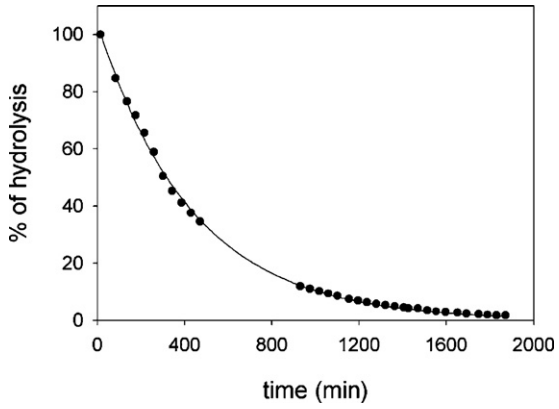


Fig. 1. Hydrolysis curve of thalidomide (pH 7.4, 37 °C).

thalidomide itself, under the same conditions, proved that *N*-substitution by a hydroxy group increased significantly the stability of glutarimide ring. The knowledge of this regioselectivity of hydrolysis could be useful for further studies in order to establish the mechanism of antiangiogenic activity.

3.1.2. *Influence of temperature and pH on N-hydroxythalidomide hydrolysis – comparison of half-lifes for thalidomide and compound **1***

Data for the hydrolysis of thalidomide and compound **1** are respectively presented in Figs. 1 and 2. Because thalidomide and its analogs exhibited very low water solubility, it was necessary to add in the medium an organic solvent and the determinations of half-lifes for both compounds were realized in a solution of phosphate buffer (pH 7.4) and acetonitrile (70/30, v/v) at 37 °C. Half-life values for thalidomide and compound **1** were,

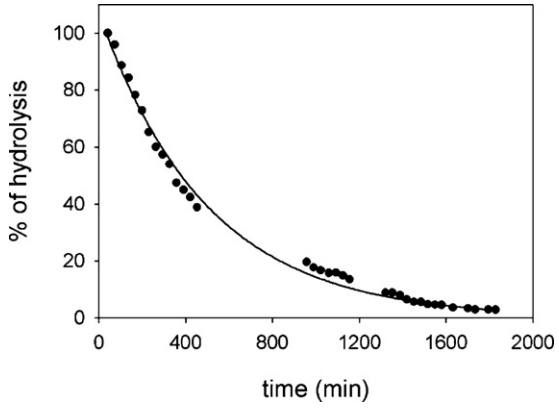


Fig. 2. Hydrolysis curve of compound **1** (pH 7.4, 37 °C).

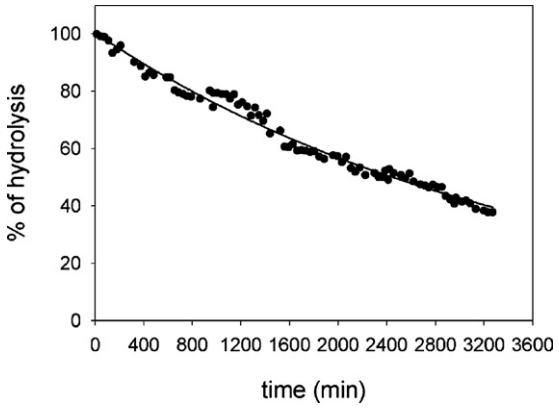


Fig. 3. Hydrolysis curve of compound **1** (pH 7.4, 23 °C).

respectively, 330.28 ± 15.13 min and 350.18 ± 23.29 min. The difference was not significant.

*3.1.2.1. Influence of temperature at pH 7.4 on compound **1** hydrolysis.* For compound **1**, the same experiment was realized at 23 °C (Fig. 3). Its half-life increased by a factor of 6 (2136.56 ± 325.35 min).

*3.1.2.2. Influence of pH at 37 °C on compound **1** hydrolysis.* In the case of additional studies under alkaline conditions (pH 7.4) product **1** was completely hydrolyzed after 30 min. Contrary to that, no more than 20% of the drugs were hydrolyzed after 8500 min in acidic medium (pH 2.4) (Fig. 4).

In conclusion, the half-life of *N*-hydroxythalidomide was mainly influenced by temperature. Actually, when the incubation temperature decreased from 37 to 23 °C, its own half-life increased by a factor of 7.5. Moreover, hydrolysis rate of this compound was pH-dependant: at 37 °C and under alkaline conditions, opening of imide ring was almost spontaneous, while the drug was only slightly hydrolyzed in acidic medium after several days. So, the stability of the pharmacophore depended on the medium but not on physico-chemical intrinsic properties.

Substitution of nitrogen atom by a hydroxy group did not affect significantly the stability of the pharmacophore. The weak difference between thalidomide and *N*-hydroxythalidomide half-lifes was not sufficient to justify important differences in

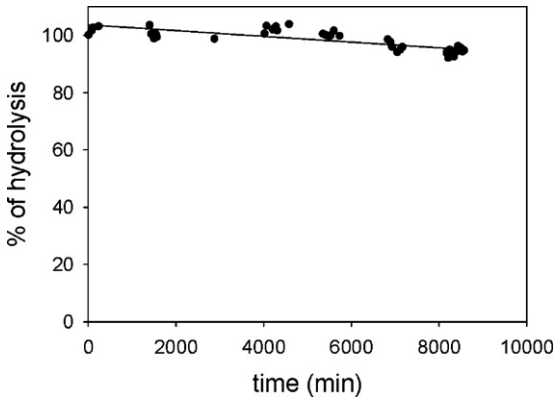


Fig. 4. Hydrolysis curve of compound **1** (pH 2.4, 37 °C).

Table 2
Calculated log *P* values of thalidomide and its derivatives

	log P^a (S.D.)	log P^b (S.D.)	log P^c (S.D.)
Thalidomide	−0.15 (0.47)	0.00 (0.49)	0.49 (0.96)
Compound **1**	0.02 (0.47)	0.49 (0.49)	nd
Compound **2**	−0.38 (0.47)	−0.18 (0.49)	0.35 (1.25)
Compound **3**	0.42 (0.47)	0.59 (0.49)	1.21 (1.03)

[a] Crippen's fragmentation.
[b] Viswanadhan's fragmentation.
[c] Broto's method.

biological activities and that led us to study the stability of other N-substituted derivatives (Scheme 2, compounds **2** and **3**). Differences in biological activities could not be explained by half-lifes in the case of thalidomide and *N*-hydroxythalidomide. That observation led us to study the stability of other N-substituted derivatives.

3.2. Comparison of thalidomide and other N-substituted thalidomide analogs

In order to detect an eventual relation between lipophilicity and stability of different N-substituted analogs of thalidomide, lipophilicity of thalidomide and of the three compounds **1**, **2** and **3** (Scheme 2) were determined under the same conditions and compared.

3.2.1. Determination of log P of thalidomide analogs

So far as calculated log *P* values were concerned, three different methods ((a) Crippen's fragmentation; (b) Viswanadhan's fragmentation; (c) Broto's method; Table 2) [20–22] using the computer program Chem Draw Ultra (version 5), gave results which were very dependent on the calculating method used, and were not very significant considering the standard deviations values.

Log *P* values had then to be experimentally determined for each compound using the method based on the partition coefficients developed by Hansch [23] (Table 3). Compared to thalidomide, an ethyl substituent (compound **3**) caused an increase of lipophilicity ($\Delta \log P = 0.36$). With a methyl acetate group (compound **2**) the increase was slightly lower ($\Delta \log P = 0.24$). Only the hydroxy group (compound **1**) allowed a decrease of lipophilicity ($\Delta \log P = -0.42$).

3.2.2. Hydrolysis of N-substituted analogs of thalidomide

Concerning the two lipophilic analogs **2** and **3**, hydrolysis kinetics performed at physiological pH (7.4) and temperature (37 °C) gave two different profiles described respectively

Table 3
Experimental log *P* values of thalidomide and its derivatives

	log *P* experimental (S.D.)
Thalidomide	1.93 (0.024)
Compound **1**	1.51 (0.111)
Compound **2**	2.17 (6×10^{-3})
Compound **3**	2.29 (8×10^{-3})

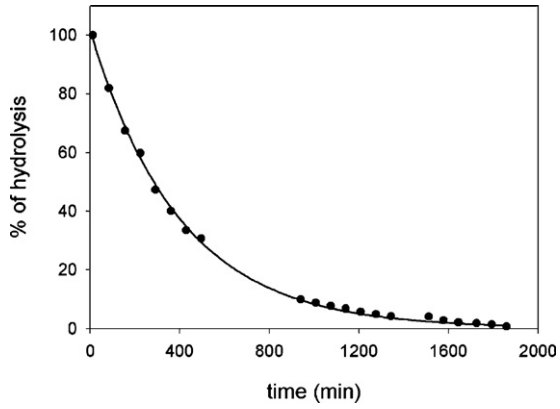


Fig. 5. Hydrolysis curve of compound **2** (pH 7.4, 37 °C).

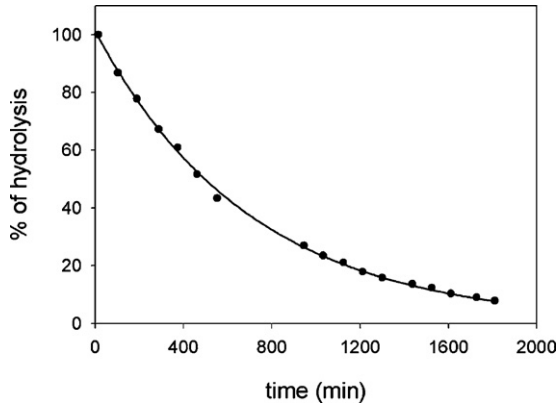


Fig. 6. Hydrolysis curve of compound **3** (pH 7.4, 37 °C).

in Figs. 5 and 6. When the nitrogen atom of the glutarimide ring was substituted by an ester group (compound **2**), the rate of hydrolysis moderately but significantly decreased (half-life = 299.07 ± 11.59 min, (Fig. 5), versus 330.28 ± 15.13 min for thalidomide). Contrary to that, *N*-alkylated thalidomide (compound **3**) was much significantly more stable (half-life = 528.92 ± 18.10 min, Fig. 6) than thalidomide (half-life = 330.28 ± 15.13 min) and *N*-hydroxythalidomide (compound **1**; half-life = 350.18 ± 23.29 min).

All these results showed that substitution by a group which moderately modified the lipophilicity (compound **1** and **2**) of the molecule allowed a slight variation of the half-life of the structure. When the lipophilicity was more considerably increased (compound **3**), the stability of the heterocyclic compound changed significantly to reach an increase of 65% of the half-life of thalidomide.

4. Conclusion

Hydrolysis of thalidomide under biological physico-chemical conditions led to the opening of the phthalimide ring, the glutarimide ring being more stable. The introduction of a hydroxy group on the nitrogen atom of the glutarimide ring reduced the lipophilicity but did not significantly modify the half-life of compound **1** compared to thalidomide. On the other

hand, temperature and pH proved to be important factors acting on the stability of phthalimide ring.

As expected, the introduction of an alkyl group (compound **3**) on the same nitrogen atom provoked an increase of log *P*, which corresponded to an increase of the half-life of the compound. But, when a similar log *P* increase was obtained by introducing an ester group on the nitrogen atom (compound **2**), the half-life was slightly shortened. These behaviours proved that if lipophilicity could increase the stability, other factors than lipophilicity (i.e., steric hindrance, chemical structure and reactivity) could be responsible for the stability of thalidomide-like compounds. These findings could be useful to design new compounds presenting antiangiogenic activities.

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References

[1] P. Jakeman, W.C.S. Smith, Lancet 343 (1994) 432–433.
[2] R. Shimazawa, H. Miyachi, H. Takayama, K. Kuroda, F. Kato, M. Kato, Y. Hashimoto, Biol. Pharm. Bull. 22 (1999) 224–226.
[3] S. Makonkawkeyoons, R.N.R. Limson-Pobre, A.L. Moreira, G. Kaplan, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 5974–5978.
[4] P.H. Chuong, H. Galons, J. Voisin, S. Righenzi, J.M. Warnet, J.R. Claude, D. Huynh-Thien, Int. J. Immunopharmacol. 19 (1997) 289–296.
[5] R.J. D'Amato, M.S. Loughnan, E. Flynn, J. Folkman, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 4082–4085.
[6] W.D. Figg, S. Raje, K.S. Bauer, A. Tompkins, D. Venzon, R. Bergan, A. Chen, M. Hamilton, J. Pluda, E. Reed, J. Pharm. Sci. 88 (1999) 121–125.
[7] S.C. Dixon, E.A. Kruger, K.S. Bauer, W.D. Figg, Cancer Chemother. Pharmacol. 43 (1999) S78–S84.
[8] S. Fabro, R.L. Smith, R.T. Williams, Nature 215 (1967) 296.
[9] H.J. Schmabi, H. Nau, D. Neubert, Arch. Toxicol. 62 (1988) 200–204.
[10] O. Trapp, G. Schoetz, V. Schurig, J. Pharm. Biomed. Anal. 27 (2002) 497–505.
[11] H. Shumacher, R.L. Smith, R.T. Williams, Brit. J. Pharmacol. 25 (1965) 338–351.
[12] H. Shumacher, R.L. Smith, R.T. Williams, Brit. J. Pharmacol. 25 (1965) 324–337.
[13] T. Eriksson, S. Björkmann, A. Fyge, J. Chromatogr. B. 582 (1992) 211–216.
[14] H. Sauer, J. Gunther, J. Hescheler, M. Wartenberg, Am. J. Pathol. 156 (2000) 151–158.
[15] G. Blaschke, H.H. Hess, N.P. Lupke, Arzneimittelforschung 39 (1989) 293–294.
[16] P. Moreau, M. Sancelme, C. Bailly, S. Leonce, A. Pierre, J. Hickman, B. Pfeiffer, M. Prudhomme, Eur. J. Med. Chem. 36 (2001) 887–897.
[17] S. Robin, J. Zhu, H. Galons, C. Pham-Huy, J.R. Claude, A. Tomas, B. Viossat, Tetrahedron Asym. 6 (1995) 1249–1252.
[18] N. Flaih, C. Gadjou, O. Lafont, H. Galons, Synlett 6 (2000) 896–898.
[19] G. Mansard, F. Estour, H. Galons, O. Lafont, Heterocycles 65 (2005) 2957–2964.
[20] A.K. Ghose, G.M. Crippen, J. Chem. Inf. Comput. Sci. 27 (1987) 21–35.
[21] V.N. Viswanadhan, A.K. Ghose, G.R. Revankar, R.K. Robins, J. Chem. Inf. Comput. Sci. 29 (1989) 163–172.
[22] P. Broto, G. Moreau, C. Vandycke, Eur. J. Med. Chem. Chim. Theor. 19 (1984) 71–78.
[23] A. Leo, C. Hansch, D. Elkins, Chem. Rev. 71 (1971) 525–616.