[Chem. Pharm. Bull. 16(11)2289—2292(1968)]

UDC 612.014.46-08 : 615.214.24.03

Negative Effect of Thalidomide and Relative Substances on the Growth of HeLa Cells[1]

Tetsuro Mohri and Haruo Kitagawa

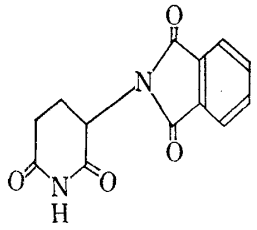
Faculty of Pharmaceutical Sciences, University of Chiba[2]

(Received October 27, 1967)

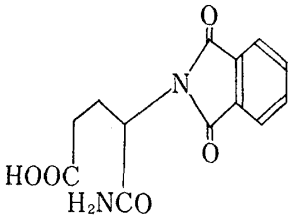
Thalidomide has been suspected as a specific depressant on chondrogenesis or as a general metabolic antagonist such as competitor to vitamins,[3] glutamate[4] or glutamine.[5] On the other hand, there are many reports on a variety of action, sometimes contrary, of thalidomide and related compounds to growth and morphology of isolated cells.[6] In the present paper, thalidomide and its two main metabolites, N-phthaloylisoglutamine and N-(2,6-dioxo-3-piperidinyl)phthalamic acid (Chart 1), were tested for effect on the growth rate of HeLa cells cultured in test tubes, and compared with several drugs in this respect. And the possibility for these thalidomide family to be glutamine competitors was inquired.

Experimental

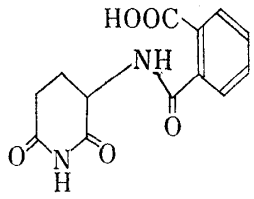
Thalidomide (I in Chart 1) was obtained from Dainippon Pharmaceutical Co., Ltd., and N-phthaloylisoglutamine (II) and N-(2,6-dioxo-3-piperidinyl)phthalamic acid (III) were obtained through the courtesy of Dr. R. Beckmann from Chemie Grünenthal Co., Ltd., West Germany. All of these compounds were mixtures of their D and L forms.



I: thalidomide



II: N-phthaloylisoglutamine



III: N-(2,6-dioxo-3-piperidinyl)-phthalamic acid

Chart 1. Structures of Thalidomide Family Tested

Aminopterine was obtained from Takeda Chemical Industries, Ltd.

HeLa cells were stocked in 250 ml culture bottles as monolayer cultures with 15 ml of medium for each. The medium is composed of Hanks balanced salt solution[7] (HBS, 90% v/v), inactivated bovine serum

1) Partly presented at the 85th Annual Meeting of the Pharmaceutical Society of Japan at Tokushima, Oct. 29, 1965.
2) Location: *1-33 Yayoi-cho, Chiba.*
3) W.F. Robertson, *Brit. Med. J.*, **1**, 792 (1962); F. Kemper, *Z. Ges. Exptl. Med.*, **135**, 454 (1962); H. Tewes, *Münch. Med. Wochschr.*, **104**, 269 (1962); J.M. Leck and E.L.M. Miller, *Brit. Med. J.*, **2**, 16 (1962); O. Frank, H. Baker, H. Ziffer, S. Aaronson, S.H. Hutner and C.M. Leevy, *Science*, **139**, 110 (1963).
4) J.W. Faigle, H. Keberle, W. Riess and K. Schmid, *Experientia*, **18**, 389 (1962); H.M. Rauen, *Arzneimittel-Forsch.*, **14**, 111 (1964).
5) J.B. Boylen, H.H. Horne and W.J. Johnson, *Lancet*, **1**, 552 (1963).
6) *a*) S. Roath, M.W. Elves and M.C.G. Israëls, *Lancet*, **1**, 249 (1963); *b*) L. Villa and S. Eridani, *Lancet*, **1**, 725 (1963); L.L. Gershbein, *Biochem. Pharmacol.*, **14**, 893 (1965); J.A. DiPaolo and C.E. Wenner, *Science*, **144**, 1583 (1964); *c*) K. Lindahl.-Kiessling and J.A. Böök, *Lancet*, **2**, 405 (1963).
7) J.H. Hanks and R.E. Wallace, *Proc. Soc. Exptl. Biol. Med.*, **71**, 196 (1949).

(BS, 10% v/v) and lactalbumin hydrolysate (LH, 0.4% w/v). When the cells attained about 5×10^6 per bottle, they were divided into test tubes with an inoculum of 2×10^4—1×10^5 cells suspended in 1.5 ml of medium for each tube. The medium used throughout the culture for assay was composed of HBS 95% (v/v), BS 5% (v/v) and LH 0.1% (w/v), except for the experiment in which HBS and LH were replaced by 95% of Eagle basal medium[8] (glutamine was reduced to 1/20 of usual). The test tubes with tight stoppers were incubated at an angle against stationary rack at 38°, and the medium was usually all renewed every other day. Any given test compound was added to the medium for a group which consisted of more than 25 of tubes after 48 hr of preincubation. Penicillin was dissolved in a concentration of 25 units/ml. The cell counting was carried out on three tubes for every group just before renewals of medium as follows: cell nuclei were stained and isolated with crystal violet–citric acid solution, suspended uniformly in 2.0 ml of the staining solution by pipetting, and enumerated in a hemocytometer. The cell number in each tube was calculated from the average of replicate counts.

Results and Discussion

Thalidomide and two of its metabolites, (II) and(III) in Chart 1, were all found completely without effect on the growth of HeLa cells at 500 mg/liter as well as 100 mg/liter in the preliminary experiments. As shown in Fig. 1, growth-inhibiting effect was observed at 1000 mg/liter for all. It was noted that the cells became readily detached from the glass and the shapeless nuclei were frequently seen with staining in four days of treatment at this concentration.

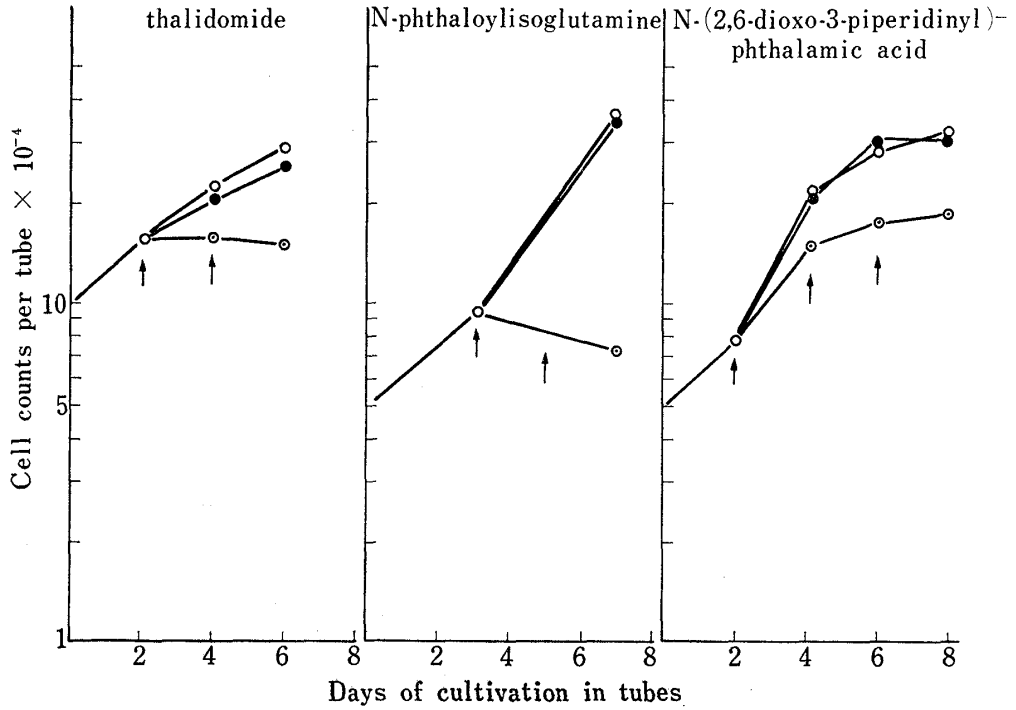


Fig. 1. Influence of Thalidomide and Its Two Metabolites on HeLa Cell Growth

Each compound was added to medium at renewals (arrows) in the concentrations (mg/liter), 0 —○—, 100 —●—, and 1000 —⊙—. Each point is the mean of cell numbers of three tubes.

III was easily soluble in all concentrations. The other two dissolved in 24 hr of culture in 500 mg/liter, but, in 1000 mg/liter, a part of the compounds still remained as sediment in 48 hr of culture.

Fig. 2 shows the ineffectiveness of glutamine for protection against growth inhibition by 1000 mg/liter of II. The ratio of glutamine to II was approximately 1/2 in molar concentration. Growth inhibition by thalidomide (1000 mg/liter) was also not reduced by

8) H. Eagle, *Science*, **130**, 432 (1959).

glutamine supplementation of medium. In Fig. 3, there are shown the results of the experiment using Eagle basal medium which was depleted of glutamine as low as 1/20 of usual concentration. Even in such a growth-depressing condition, 500 mg/liter of thalidomide did not make worse the growth of cells than that of control.

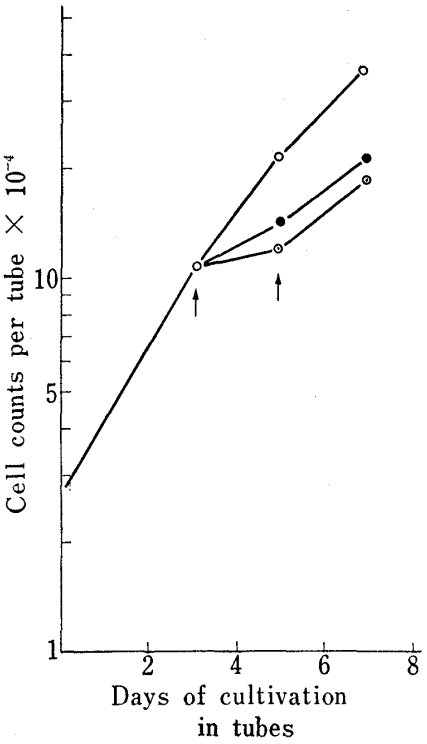


Fig. 2. No Effect of Glutamine Addition Simultaneously with N-Phthaloylisoglutamine (1000 mg/liter) to Medium

—●— N-phthaloylisoglutamine
—⊙— N-phthaloylisoglutamine+glutamine (300 mg/liter)
—○— glutamine (300 mg/liter)

The compounds were added to medium at renewals (arrows). Each point is the mean of cell numbers of three tubes.

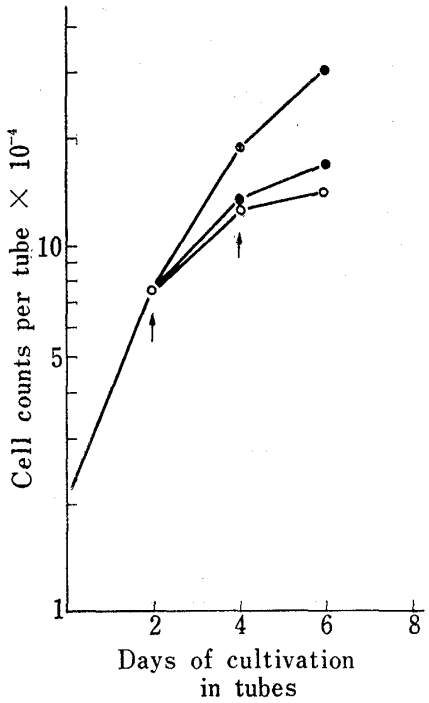


Fig. 3. No Effect of Thalidomide in Glutamine Depletion in Eagle Medium

—○— control (glutamine 15 mg/liter)
—●— thalidomide (500 mg/liter)+ glutamine 15 mg/liter
—⊙— glutamine (300 mg/liter)

Each compound was added to medium at renewals (arrows). Each point is the mean of cell numbers of three tubes.

Roath, *et al.*[6a] have demonstrated that lymphocyte proliferation is inhibited by 3 mg/liter of thalidomide *in vitro*. On the contrary, it is reported that proliferation of human leucocytes cultured has not been affected by thalidomide.[6e] In the present work, it was shown that two of the most abundant metabolites occuring in human urine after oral administration of thalidomide[9] were without effect as well as thalidomide on HeLa cells at 500 mg/liter and below. The growth-inhibiting effect of thalidomide family on HeLa cells at 1000 mg/liter is rather considered to be non-specific, because acetylsalicylate depressed HeLa cell growth in lower concentrations, which were much higher than those found effective with aminopterin, as illustrated in Fig. 4, although thiamine was not found depressing in any concentrations.

From the results of this report and others, it may be concluded that thalidomide and its main metabolites have no definite effect on cellular proliferation, and that no competition between each of thalidomide family and glutamine on HeLa cell growth has eliminated the possibility of their interference in amino acid metabolism.

9) R. Beckmann, *Arzneimittel-Forsch.*, **13**, 185 (1963).

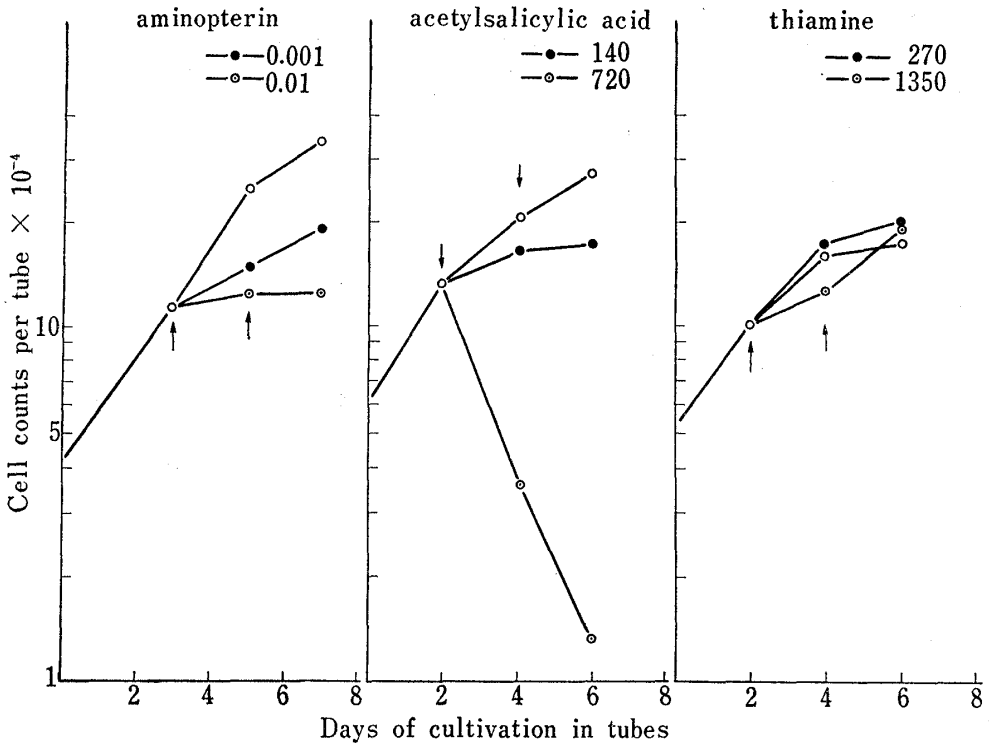


Fig. 4. Some References of Toxicity on HeLa Cell Growth

Concentrations are expressed in mg/liter. Control for every case is shown by —○—. Each compound was added to medium at renewals (arrows). Each point is the mean of cell numbers of three tubes.

Acknowledgement This work was supported in part by Science Research Grant from the Educational Ministry, and the authors express their appreciation to Dr. Y. Ito of the University of Tokyo for his kindness as representative of the Grant. The authors are also indebted to Mrs. S. Saotome, Miss T. Kobayashi and Miss S. Oshida for their technical assistance.

[Chem. Pharm. Bull. 16(11)2292—2296(1968)]

UDC 547.857.4.07

A Convenient Synthesis of 2-Methyl-4-pyrone from Kojic Acid

Kiyomatsu Hasizume, Shosuke Yamamura and Shoji Inoue

Faculty of Pharmacy, Meijo University[1]

(Received July 26, 1968)

In a lot of fungal metabolites, there are many pigments which contain simple or further extended pyrono–quinonoid systems.[2] Recently, we found the simple method to prepare such a pyrono–quinonoid system as shown in Chart 1.[3]

In the pyrono–quinonoid systems of natural pigments, however, they have an alkyl group (or alkenyl group) only at C_2 position. Accordingly, it is necessary to use such a 2-alkyl-,

1) Location: *Yagotourayama, Tenpaku-cho, Showa-ku, Nagoya.*
2) W.B. Whalley, "Chemistry & Biochemistry of Fungi & Yeasts," Butterworths, London, 1963, p. 565.
3) S. Yamamura, K. Kato and Y. Hirata, *Tetrahedron Letters*, **17**, 1637 (1967).