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Stimulation of gap junctional intercellular communication by thalidomide and thalidomide analogs in human fetal skin fibroblasts (HFFF2) and in rat liver epithelial cells (WB-F344)

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Abstract

Gap junction channels maintain cell–cell communication and are essential for the coordination of tissues, playing a pivotal role in embryonal development. Gap junctional intercellular communication (GJIC), studied here in human fetal skin fibroblasts (HFFF2) and in rat liver epithelial cells (WB-F344), was almost doubled upon exposure to thalidomide (10 μM) in the presence of NADH or NADPH (20 μM). Neither in HFFF2 nor in WB-F344 cells did any detectable alteration in GJIC occur with the thalidomide analog EM 16 (10 μM), known as a non-teratogenic compound. The thalidomide analog EM 364 (10 μM) increased GJIC without prior metabolic activation. It is suggested that GJIC modification may be related to the pharmacological and toxicological properties of thalidomide.

Keywords: Teratogen; Thalidomide; Gap junctions; Intercellular communication; Human fetal skin fibroblasts (HFFF2); Rat liver epithelial cells (WB-F344)

1. Introduction

Gap junctional intercellular communication (GJIC) is a signaling pathway that involves the transfer of messenger compounds between adjacent cells [1]. Signaling is achieved via cell-to-cell channels that connect the cytosol of two neighboring cells, allowing for the exchange of low-molecular-mass compounds. A functional pore is formed from two half-channels, each consisting of a hexamer of proteins that belong to the gene family of connexins.

Intercellular signaling can modulate gene expression at various levels. It has been suggested that untimely or chronic changes in gap junctional communication during embryonal or fetal development may lead to embryonic lethality or teratogenesis [2]. Gap junctional coupling has been demonstrated in embryonic tissues, and it is assumed that GJIC is involved in the process of pattern formation [3], probably providing a pathway for the formation of a putative gradient of morphogens [4,5]. There is some evidence that GJIC plays a part in the patterning of the developing limb [5]. Various types of connexins have been detected within an organism, and there are differences in the expression of connexin genes during embryonal development [6,7]. One of the most abundant connexin proteins is connexin43 (Cx43), which appears to be implicated in limb bud development [5,8,9].

GJIC is affected by various compounds, including retinoic acid [10], retinoid derivatives [11], vitamin D [12,13], and tumor promotors such as phorbol esters [14]. It has been shown that thalidomide induces GJIC in human fibroblasts after metabolic activation [15]. Retinoic acid and thalidomide are known as potent human teratogens. While these compounds induce different patterns of malformation, it is interesting to note that they both modify GJIC at the cellular level.

The present study investigates the stimulation of GJIC by thalidomide in comparison to some of its structural analogs and retinoic acid in human fetal skin fibroblasts (HFFF2) and in rat liver epithelial cells (WB-F344) which show a high level of Cx43 expression [16].

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Abbreviations: GJIC, gap junctional intercellular communication; PGA, 2-phthalimido glutaric acid; Cx43, connexin 43; Cx31, connexin 31; MTT, (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide); and THF, tetrahydrofuran.

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2. Materials and methods

2.1. Chemicals

EM 12, EM 16, EM 20, EM 138, and EM 364 were kindly provided by Grünenthal. Thalidomide, PGA, Lucifer yellow CH, MTT, and *all-trans* retinoic acid were purchased from Aldrich. FAD, NAD^+, $NADP^+$, NADH, and NADPH were purchased from Boehringer. Other chemicals were obtained from Merck.

2.2. Preparation of test compounds

FAD, NAD^+, $NADP^+$ (dissolved in water), NADH, and NADPH (dissolved in 0.1% Na-bicarbonate buffer, pH 9) were present in cell culture at 20-μM final concentration. Thalidomide, EM 12, EM 16, EM 20, EM 138, EM 364, and PGA were dissolved in THF and added to the medium to yield a final concentration of 10 μM. Final concentration of THF in mixture was 0.4%; controls were treated only with solvent.

2.3. Cells and culture conditions

The human fetal skin fibroblasts cell line HFFF2 (ECACC, no. 86031405) and the rat liver epithelial cell line WB-F344, a gift from Dr. I. A. Cotgreave and Dr. L. Wärngard, Institute of Environmental Medicine, Karolinska Institute (Stockholm, Sweden), were cultured in Dulbecco's modification of Eagle's minimal essential medium, supplemented with 2 mM L-glutamine, 0.02 g/L of gentamycin (Aldrich), and 10% (v/v) fetal bovine serum (Greiner). Confluent cells were incubated with test compounds at the indicated concentrations.

2.4. Evaluation of cytotoxicity

The cytotoxicity of compounds was assayed with the MTT reduction assay with minor modifications [17]. Cells were grown in six-well plates to near confluence and treated with test compounds as mentioned. The growth medium was replaced by phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} and supplemented with MTT at a final concentration of 1.2 mM. After incubation for 2 hr at 37°, cells were treated with 10% sodium dodecyl sulfate and 0.1% HCl. The results were expressed as absorbance at 550 nm minus background absorbance at 690 nm of the test sample and compared to the appropriate control. Data were obtained from 3–4 separate incubations for each compound and dose used.

2.5. Preparation of cell lysates

WB-F344 rat liver epithelial cells were grown in tissue culture flasks. Confluent cells were washed twice with cold PBS and detached by trypsinisation. Harvested cells were centrifuged at 500 × *g* for 5 min, resuspended in 10 mL PBS, and sonicated for 5 min. When the solution became opaque, cell lysates were added immediately to the incubation medium at 1:15 dilution. Heat-inactivated cell lysates were prepared by incubating cell lysates at 56° for 30 min and then added to the incubation medium.

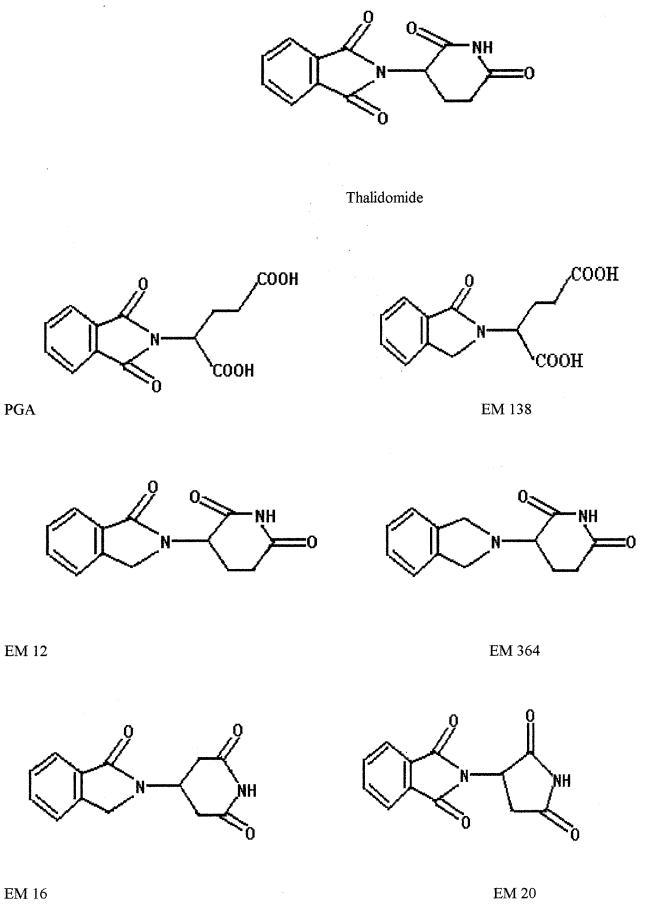

Fig. 1. Thalidomide and related compounds.

2.6. Dye transfer assay

GJIC was assessed by following the transfer of the fluorescent dye Lucifer Yellow CH (10% in 0.33 M LiCl, w/v) from a single microinjected cell into neighbouring cells by means of a microinjector and micromanipulator (Eppendorf) as previously described [15]. The number of fluorescent neighbouring cells was scored 5 min after injection and served as an index of GJIC [18]. Each experiment was repeated at least in triplicate. Differences in comparison to controls were analysed for statistical significance using the Student's *t*-test.

3. Results

The structures of the compounds used are shown in Fig. 1. These include thalidomide, its hydrolysis product, PGA,

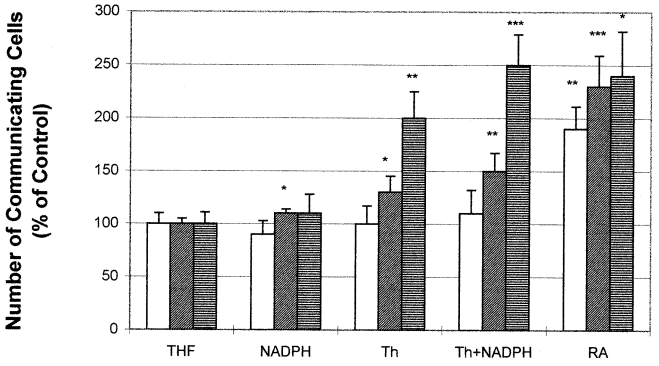

Fig. 2. Time-dependence of gap junctional intercellular communication (GJIC) in WB-F344 cells in the presence of thalidomide. The number of communicating cells (% of control) induced by thalidomide (10 μM) and NADPH (20 μM) were measured on day 1 (□), 3 (▨), and 6 (▤) of incubation. Control was 32.3 ± 2.1 cells. Data represent means ± SD (N = 4). Significantly different from control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

EM 12, EM 20, EM 138, EM 364, and its non-teratogenic analog EM 16.

3.1. Cytotoxicity of compounds

Upon incubation of cells with 10 μM thalidomide or its analogs according to the previous studies [15], none of the compounds was found to be cytotoxic. The lowest value for cell viability was obtained by treating cells with EM 364, which showed 90% of cell viability compared to the control. The coenzymes used in the assay were also non-cytotoxic; only upon incubation with $NADP^+$ was cell viability diminished to 61% of control (data not shown), which is in accordance with previous studies describing cytotoxic effects of $NADP^+$ [19].

3.2. Time-dependence of thalidomide effects on GJIC

The influence of thalidomide in the presence of NADPH on GJIC on day 1, 3, and 6 of incubation was tested in rat liver epithelial cells (WB-F344) and compared to retinoic acid (Fig. 2). Upon incubation of cells with 10 μM of thalidomide alone, no alteration of GJIC was observed until day 3 of incubation. On day 6, GJIC was augmented compared to day 1 and 3 of incubation. When thalidomide was added to the cell culture in the presence of 20 μM NADPH, GJIC was significantly increased on day 3 of incubation, and on day 6, GJIC was more pronounced than with thalidomide alone. NADPH alone had no influence on GJIC, retinoic acid (1 μM) was used as positive control.

3.3. Effects of coenzymes

Since thalidomide requires metabolic activation to affect GJIC [15], induction of GJIC was examined in the presence of coenzymes to investigate their effect on the metabolic

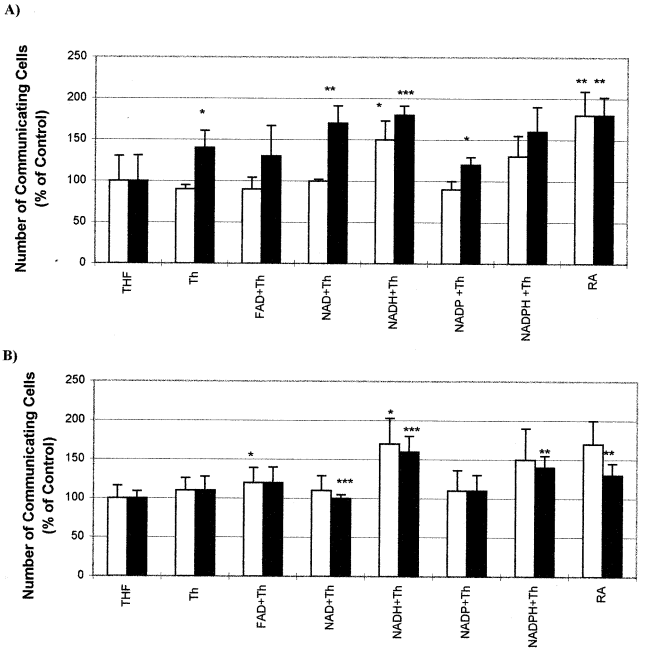

Fig. 3. Induction of gap junctional intercellular communication (GJIC) by thalidomide in the presence of coenzymes. The number of communicating cells (% of control) induced by thalidomide (10 μM) in the presence of coenzymes FAD, NAD^+, $NADP^+$, NADH, NADPH (each 20 μM) or with all-*trans* retinoic acid (1 μM) were measured on day 1 (□) and 3 (■) of incubation. (A) In WB-F344 cells, control was 32.9 ± 7.4 cells. Data represent means ± SD (N = 3). (B) In HFFF2 cells, control was 18.6 ± 3.3 cells. Data represent means ± SD (N = 5). Significantly different from control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

activation in rat liver epithelial cells (WB-F344) and in human fetal skin fibroblasts (HFFF2) (Fig. 3).

In rat liver epithelial cells (WB-F344) in the presence of NADH (20 μM), GJIC increased 1.8-fold on day 3 of incubation with thalidomide, and an increase of 1.6-fold was found using NADPH (Fig. 3A). In human fetal skin fibroblasts (HFFF2) in the presence of NADH (20 μM), GJIC increased 1.7-fold already on day 1 of incubation with thalidomide, and an increase of 1.5-fold was found using NADPH (Fig. 3B). The other coenzymes used in the assay were less active.

3.4. Metabolic activation of thalidomide

Cell lysate freshly prepared from WB-F344 cells was used together with the test compounds. A significant increase in GJIC was observed when cells were incubated with thalidomide in the presence of cell lysate and NADH (Fig. 4). When cell lysates were incubated at 56° for 30 min to inactivate heat-labile enzymes, the effect on GJIC was comparable to controls. NADH alone with cell lysate had no stimulatory effect on GJIC. When thalidomide was used in the absence of NADH and cell lysate, a slight decrease in GJIC was observed. Controls contained cell lysate and tetrahydrofuran (THF) (0.4%).

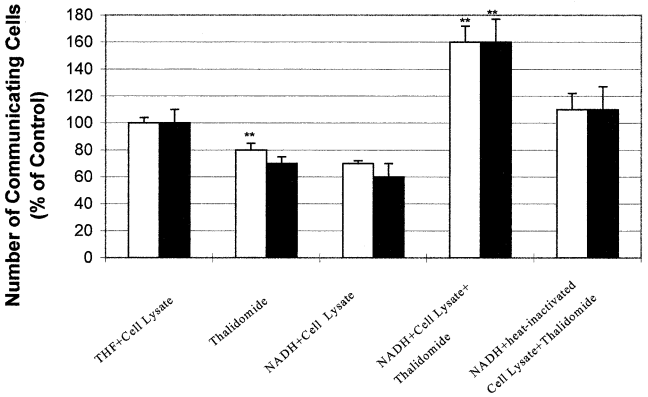

Fig. 4. Induction of gap junctional communication in WB-F344 cells by thalidomide in the presence of NADH and cell lysate. The number of communicating cells (% of control) induced by thalidomide (10 μM) in the presence of NADH (20 μM) and cell lysate (1:15) were measured on day 1 (□) and 3 (■) of incubation. Control was 31.0 ± 3.2 cells. Data represent means ± SD (N = 3). Significantly different from control: $^{**}P < 0.01$.

3.5. Influence of thalidomide analogs on gap junctional intercellular communication

The influence of thalidomide and its analogs on GJIC was tested in the presence or absence of NADH in human fetal skin fibroblasts (HFFF2) and in rat liver epithelial cells (WB-F344) (Table 1). EM 16, a non-teratogenic thalidomide analog [20], did not stimulate GJIC in either cell type, whereas all other thalidomide analogs induced GJIC to some extent in the presence of NADH.

3.5.1. Rat liver epithelial cells (WB-F344)

In the presence of NADH, the potent teratogens in primates, thalidomide, and EM 12 [20,21] and the teratogenic hydrolysis product of thalidomide, PGA [22], were less effective in stimulating GJIC than the other thalidomide analogs (except EM 16) in WB-F344 cells. The metabolite of EM 12, EM 138 [23], was the most effective compound on day 1 of incubation, whereas its effect was similar to that of EM 20 and EM 364 on day 3. NADH alone did not affect GJIC (data not shown).

Table 1
Induction of gap junctional intercellular communication by thalidomide and thalidomide analogs in the presence and absence of NADH

A)

Compounds	Number of Communicating Cells (% of control) in WB-F344 Cells			
	without NADH		with NADH (20 μM)	
	1st Day	3rd Day	1st Day	3rd Day
THF (0.4%)	100 ± 10	100 ± 10	100 ± 20	100 ± 10
Thalidomide (10 μM)	110 ± 25	110 ± 12	150 ± 42	130 ± 20***
PGA (10 μM)	120 ± 25*	110 ± 8	120 ± 24	130 ± 8***
EM 12 (10 μM)	130 ± 14	120 ± 0***	150 ± 52	130 ± 28
EM 16 (10 μM)	80 ± 24	80 ± 12	110 ± 33	110 ± 23
EM 20 (10 μM)	130 ± 30	110 ± 12	150 ± 48	150 ± 7***
EM 138 (10 μM)	140 ± 9**	120 ± 5*	170 ± 36*	150 ± 16**
EM 364 (10 μM)	150 ± 8***	160 ± 8***	150 ± 29*	150 ± 25*
Retinoic acid (1 μM)	140 ± 9**	150 ± 0***	150 ± 25*	160 ± 10*

B)

Compounds	Number of Communicating Cells (% of control) in HFFF2 Cells			
	without NADH		with NADH (20 μM)	
	1st Day	3rd Day	1st Day	3rd Day
THF (0.4%)	100 ± 35	100 ± 59	100 ± 30	100 ± 2
Thalidomide (10 μM)	110 ± 5	130 ± 30	150 ± 28*	150 ± 8***
PGA (10 μM)	150 ± 40	170 ± 40*	150 ± 13**	160 ± 15**
EM 12 (10 μM)	150 ± 40	130 ± 20*	190 ± 5***	190 ± 0***
EM 16 (10 μM)	80 ± 20	110 ± 30	100 ± 8	100 ± 5
EM 20 (10 μM)	120 ± 40*	120 ± 30	150 ± 33	140 ± 10**
EM 138 (10 μM)	170 ± 40*	130 ± 20*	140 ± 14**	130 ± 9**
EM 364 (10 μM)	240 ± 20***	130 ± 30	180 ± 16**	150 ± 10**
Retinoic acid (1 μM)	180 ± 60*	150 ± 20**	180 ± 15***	160 ± 15***

The number of communicating cells (% of control) induced by thalidomide or analogs (each 10 μM) or all-*trans* retinoic acid (1 μM) were measured on day 1 and 3 of incubation. (A) In WB-F344 cells, controls were 31.1 ± 3.7 cells. Data represent means ± SD (N = 4). (B) In HFFF2 cells, controls were 18.3 ± 0.9 cells. Data represent means ± SD (N = 6).

Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

When NADH was absent, only EM 364 produced similar values compared to its effect in the presence of NADH, indicating that no metabolic activation is required.

3.5.2. Human fetal skin fibroblasts (HFFF2)

Thalidomide and EM 12, and PGA, were found to be more effective in stimulating GJIC in HFFF2 cells than in WB-F344 cells in the presence and absence of NADH.

EM 12 was the most effective compound both on day 1 and 3 of incubation in the presence of NADH. PGA and EM 12 also were found to be effective in the absence of NADH, but the effect of EM 12 was increased in the presence of NADH whereas PGA showed similar values, indicating that metabolic activation is not required for this compound. EM 364 and EM 138 increased GJIC in the absence of NADH, although their effects were lower in the presence of NADH. EM 20 had no significant effect on GJIC in the absence of NADH, indicating that metabolic activation is required for this compound. NADH alone did not affect GJIC (data not shown).

4. Discussion

According to previous studies in human skin fibroblasts, stimulation of GJIC by thalidomide requires metabolic activation with liver microsomes and NADPH [15]. Here, we show that in the presence of the coenzymes NADH or NADPH alone, thalidomide was also active and able to stimulate GJIC both in HFFF2 and in WB-F344 cells. Related coenzymes were not capable of activating thalidomide. Upon incubation of WB-F344 cells with thalidomide in the presence of cell lysates and NADH, GJIC was stimulated up to 1.6-fold of control, whereas in the presence of heat-inactivated cell lysate and NADH, GJIC was only 1.1-fold of control. On the other hand, NADH or thalidomide alone showed no effect on GJIC. Thus, heat-labile enzymes in the culture environment are required for the metabolic activation of thalidomide. Which enzymes are involved in the activation process remains to be elucidated. It is also not clear if the activation by liver microsomes and cell lysate is mediated by the same enzymatic system(s).

Using this assay system, we investigated thalidomide analogs for their effects on GJIC. Only EM 16 had no effect on GJIC under any of the conditions applied. EM 16 is a non-teratogenic derivative of thalidomide [20] and differs from all other compounds investigated with respect to the position of the carbonyl groups in relation to the tertiary nitrogen atom; both carbonyl groups in the glutarimide ring are at the γ-position. In all other compounds, one of the carbonyl groups (respectively carboxyl groups) is located in the β-position in relation to the tertiary nitrogen atom.

Thalidomide and its teratogenic analog EM 12 [20,21] both stimulate GJIC, and the effect is more pronounced after activation with NADH. Hydrolysis of the glutarimide ring in both compounds leads to the teratogenic derivatives PGA [22] and EM 138, which both stimulate GJIC in HFFF2 cells without activation. It might be speculated that the hydrolysis of the glutarimide ring to yield a carboxyl group in the β-position to the tertiary nitrogen atom is an important step to obtain an active derivative. A similar product can be formed by the hydrolysis of EM 20 (with one C-atom less) and EM 364, but not with EM 16.

Hydrolytic cleavage of the phthalimide ring may compete with this reaction. Thus, it is interesting to note that EM 364, which can not be cleaved at this position, is the most active compound in the system together with EM 12, where this kind of cleavage is less probable as compared to the parent thalidomide. In all experiments, retinoic acid was included as a test compound and proved to stimulate gap junctional communication as described earlier [10].

The present study shows some interesting correlation of teratogenic compounds and cellular effects on GJIC. It should be noted that there are a number of differences between teratogens such as thalidomide and retinoic acid regarding species specificity or malformation pattern.

Acknowledgments

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