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Stimulation of Gap Junctional Intercellular Communication by Thalidomide and Thalidomide Analogs in Human Skin Fibroblasts

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ABSTRACT. It has been speculated that gap junctional intercellular communication (GJIC), an intercellular signalling pathway, is involved in embryogenesis by coupling compartments of the same developmental potential. We found that thalidomide induces GJIC in human fibroblasts after activation by liver microsomes in concentrations as low as 10^{-7} M. Treatment of cells with the thalidomide analog EM-12 increased GJIC without prior activation. No alteration of GJIC was detected with phthalimide and glutamate, the components of thalidomide. However, 2-phthalimido glutaric acid (PGA), a hydrolysis product of thalidomide, stimulated GJIC without activation at concentrations between 10^{-10} M and 10^{-5} M. We suggest modification of GJIC as a biochemical mechanism responsible for pharmacological and toxicological properties of thalidomide and related compounds. BIOCHEM PHARMACOL 53;10:1553–1557, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. teratogen; thalidomide; gap junctions; intercellular communication; dye transfer; cell culture

Thalidomide is a potent human teratogen which led to limb malformation in newborns after maternal usage [1]. The biochemical mechanisms underlying its teratogenic effect are still under investigation. Various processes have been discussed including interference with biologically relevant molecules, interaction with DNA, or influence on cell-cell interactions [2]. Recently, the down-regulation of adhesion receptors in embryonal cells has been postulated as a mechanism of teratogenic action of thalidomide [3].

Thalidomide is active in the treatment of inflammation in leprosy [4] and currently is in use in the therapy of this disease [5]. Further research revealed additional pharmacological activities of thalidomide, including inhibition of graft-versus-host reactions [6, 7], inhibition of fibroblast growth factor-induced angiogenesis [8], and suppression of the activation of latent human immunodeficiency virus type 1 (HIV1) *in vitro* [9]. It was shown that thalidomide and its derivatives induce changes in the pattern of integrin receptors and other surface markers of lymphocytes [10, 11], properties important with respect to their effects on the immune system. Thalidomide affects cytokine production *in vitro*, enhancing the production of interleukin-4 and interleukin-5 and inhibiting interferon-γ release in stimulated human peripheral blood mononuclear cells [12]. Inhibitory effects on the production of tumor necrosis factor-α by enhancement of mRNA degradation have been discussed in context with its immunosuppressive action [13].

Intercellular communication via gap junctions is an important signalling pathway and involves the transfer of low-molecular-weight compounds such as second messengers or ionic signals between cells [14]. Gap junctions are composed of a system of cell-to-cell channels connecting the cytosol of coupled cells. Gap junctional coupling has been demonstrated in embryonic tissues, and it is assumed that gap junctional intercellular communication (GJIC) is involved in the process of pattern formation [15]. Furthermore, differences in the expression pattern of connexin during embryogenesis suggest that this signalling pathway plays a role in embryonal development [15]. Gap junctional intercellular communication becomes restricted to domains of cells with the same developmental potential [16]. Inhibition of GJIC has been discussed as a mechanistic link between teratogenesis and carcinogenesis [17].

GJIC is stimulated by various compounds, including retinoic acid, retinoid derivatives [18], e.g. 4-oxoretinoic acid [19], and vitamin D [20, 21], whereas tumor promotors such as phorbol ester generally inhibit GJIC [22].

Here we describe the stimulation of this pathway of intercellular communication by thalidomide and some of its analogs, an effect which is potentially related to some of its biological properties.

MATERIALS AND METHODS

Chemicals

Thalidomide and 2-phthalimido glutaric acid (PGA) were kindly provided by Grünenthal (Stolberg, Germany), while

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Abbreviations: GJIC, gap junctional intercellular communication; PGA, 2-phthalimido glutaric acid.

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supidimide and EM-12 were a gift from Dr. Neubert (Free University Berlin, Germany). Lucifer yellow CH, *all-trans* retinoic acid, glutamic acid hydrochloride, and phthalimide were purchased from Aldrich (Steinheim, Germany). Other chemicals were obtained from Merck (Darmstadt, Germany).

Preparation of Microsomes and Preincubation of the Test Compounds

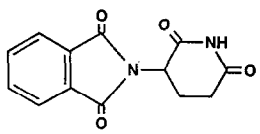
Microsomes were isolated from livers of untreated male Wistar rats as previously described [23]; protein concentration was 0.6 mg/mL microsomal suspension. The mixture was supplemented with NADPH (7×10^{-4} M). Thalidomide, EM-12, and supidimide (dissolved in tetrahydrofuran) were added to the suspension of microsomes to yield a concentration of 4×10^{-4} M. Final concentration of tetrahydrofuran in the mixture was 4%; controls contained only solvent. The microsomal mixtures were incubated for 60 min at 37°C. The incubation mixtures described above were used to test the activated compounds at a final concentration of 10^{-5} M in cell culture; for lower concentrations, less of the parent compound was added to the incubation mixture.

Cells and Culture Conditions

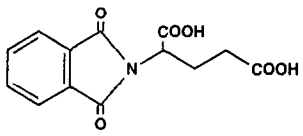
The human skin fibroblast cell line 161 BR (ECACC, no. 90011809) and the human fetal skin fibroblast cell line HFFF2 (ECACC, no. 86031405) were cultured with Dulbecco's modification of minimal essential medium (Gibco BRL, Eggenstein, Germany), supplemented with 10% fetal calf serum (Life Technologies, Eggenstein, Germany) and 2 mM L-glutamine [19, 20]. Confluent cells were incubated with the indicated concentrations of *all-trans* retinoic acid, 2-phthalimido glutaric acid, phthalimide (dissolved in ethanol), thalidomide, supidimide or EM-12 (dissolved in tetrahydrofuran). For treatment with preincubated thalidomide, supidimide or EM-12, 250 µL of the preincubated microsomal mixtures were added to 10 mL of medium which was filtered through 0.22 µm Millex-GV (Millipore S.A., Molsheim, France) prior to addition to cultured cells.

Dye Transfer Assay

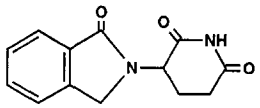
On days 1 and 3, junctional communication was measured by microinjection of the fluorescent dye Lucifer yellow CH (10% in 0.33 M LiCl, w/v) into cells of confluent cultures by means of a microinjector and micromanipulator (Eppendorf, Hamburg, Germany). The number of fluorescent neighbours of the injected cells was scored 5 min after injection and serves as an index of junctional communication [24]. The data plotted in the figures are mean values of 10 individual injections with SEM given as error bars. Differences between means were analyzed for statistical significance by Student's *t*-test. Each experiment was repeated at least in triplicate. Similar significant differences were observed in all experiments. The coefficient of variation between experiments was less than 17%.



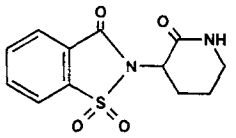
Thalidomide



2-phthalimido glutaric acid



EM-12



Supidimide

FIG. 1. Thalidomide and related compounds. The enantiomers are not specifically indicated.

RESULTS

The structures of the compounds used are given in Fig. 1. These include thalidomide, its hydrolysis product 2-phthalimido glutaric acid as well as the teratogenic analog of thalidomide, EM-12, and its non-teratogenic derivative supidimide.

The influence of thalidomide and its analogs after single treatment of human skin fibroblast cell line 161 BR on gap junctional intercellular communication is summarized in Fig. 2. Upon incubation of cells with 10^{-5} M of thalidomide, no significant alteration of GJIC was observed in the dye transfer assay at days 1 and 3 of cell culture. However, when the compound had been preincubated with liver microsomes, GJIC significantly increased within three days of culturing and approached communication levels comparable to those of the positive control, retinoic acid (10^{-6} M).

Communication was also induced by EM-12 (10^{-5} M). In contrast to thalidomide, no activation was necessary to obtain stimulation of GJIC, but the effects of EM-12 after preincubation with liver microsomes were somewhat more pronounced. Without activation, supidimide (10^{-5} M) did not alter GJIC. After preincubation with microsomes, the compound exhibited stimulation of GJIC after three days in cell culture, but not on day 1.

In addition, 2-phthalimido glutaric acid (10^{-5} M), a hydrolysis product of thalidomide, induced GJIC in fibro-

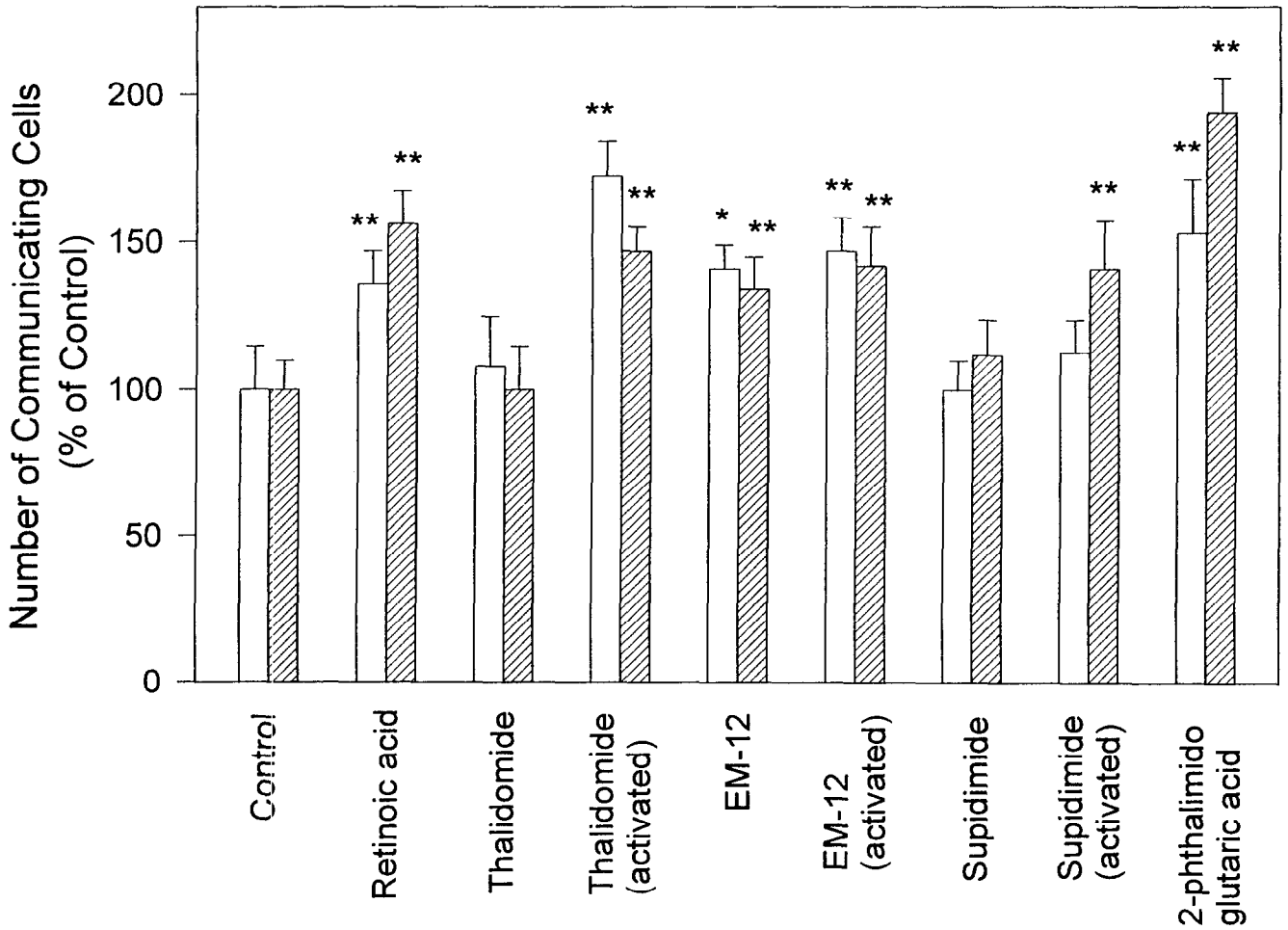


FIG. 2. Induction of gap junctional communication (GJIC) in human skin fibroblasts of the cell line 161 BR. The number of communicating cells (% of control) induced by thalidomide, EM-12, supidimide, and 2-phthalimido glutaric acid was measured at days 1 (□) and 3 (▨). The absolute number of communicating cells in controls was 19.2 ± 2.9 at day 1 and 17.8 ± 1.8 at day 3. Significantly different from control: *$P < 0.01$; **$P < 0.001$.

blasts to about the same extent as activated thalidomide. However, no activation was necessary to observe an increase in GJIC (Fig. 2). No alteration of GJIC was observed when the cells were grown in the presence of phthalimide (10^{-5} M) or glutamic acid (10^{-5} M), both components of thalidomide.

The effects of 2-phthalimido glutaric acid and EM-12 on gap junctional intercellular communication were also detected at lower concentrations. 2-phthalimido glutaric acid and EM-12 at 10^{-7} M stimulated GJIC at 190% and 160% of control, respectively, at day 3 of treatment; 2-phthalimido glutaric acid was active at even 10^{-10} M.

Very similar results were obtained in the human fetal skin fibroblast cell line HFFF2 (Fig. 3). Without activation, no effects of thalidomide (10^{-5} M) were observed, whereas after preincubation with rat liver microsomes GJIC was increased at days 1 and 3. Supidimide was only active after incubation with microsomes. In contrast, GJIC increased after incubation with EM-12 (10^{-5} M) and 2-phthalimido glutaric acid (10^{-5} M) without activation by liver microsomes. The effects of 2-phthalimido glutaric acid were comparable to those of the positive control, retinoic acid (10^{-6} M).

DISCUSSION

The present work is, to our knowledge, the first report on the stimulation of intercellular communication via gap junctions by thalidomide in human cells. No effects of this compound were observed in Hamster V79 cells using the metabolic cooperation assay [25]. Our data show that the stimulation of GJIC is not mediated by the parent compound itself, since activation by a liver microsomal system is required (Fig. 2). The nature of the ultimately active compound is still unknown. However, 2-phthalimido glutaric acid, a product of thalidomide hydrolysis, stimulates GJIC without further activation even at concentrations of 10^{-10} M. The compound was not detectable in incubation mixtures of rat liver microsomes and thalidomide at a detection limit of 10^{-7} M. Similar results were observed with EM-12, a thalidomide analog lacking a keto group in the five-membered ring. This structural property might favor the non-enzymatic hydrolysis of the glutarimide ring preferentially to the isoindolinone system, yielding the respective analog of 2-phthalimido glutaric acid. The hydrolysis of a single imide bond in the

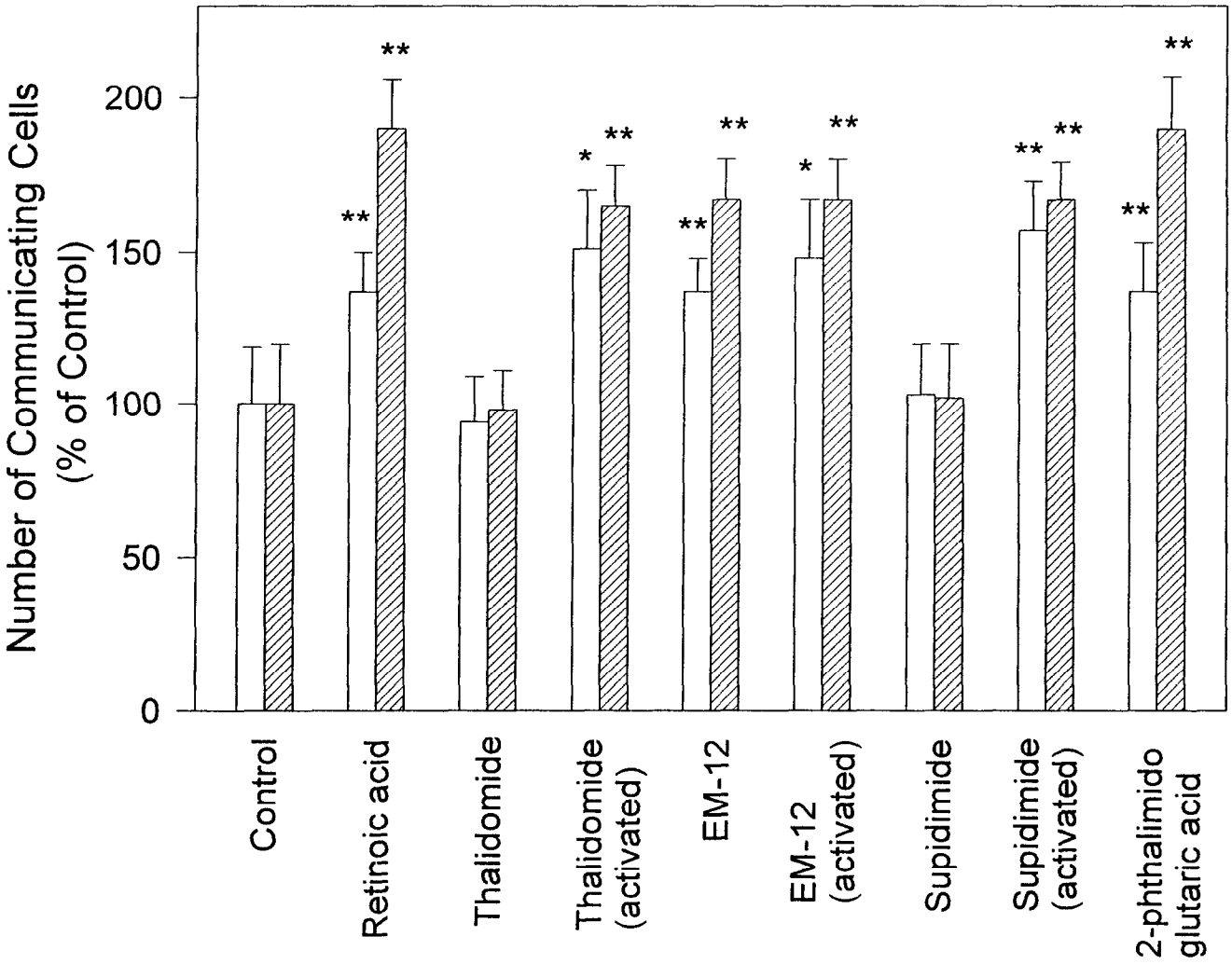


FIG. 3. Induction of gap junctional communication in human fetal skin fibroblasts of the cell line HFFF2. Conditions as in Fig. 2. The absolute number of communicating cells in controls was 16.8 ± 2.9 at day 1 and 18.9 ± 4.2 at day 3.

phthalimide moiety has been associated with an inactivation of the drug [26].

A variety of biochemical pathways are involved in the modulation of GJIC, including the regulation of connexin expression or connexin protein phosphorylation [14]. It might be speculated that ligand-activated receptors such as the retinoic acid receptors or orphan receptors are targets for thalidomide metabolites and analogs.

Both thalidomide and EM-12 are teratogenic in primates, causing typical limb malformations [3]. GJIC plays a role in embryonal development, and the expression of gap junction proteins (connexins) varies during fetal development. Other teratogenic compounds such as retinoic acid and phorbol esters also affect GJIC [18, 22]. In most cases, retinoids induce while phorbol esters inhibit GJIC. It is not known whether these properties are responsible for the teratogenic potential of these compounds. However, it might be speculated that disturbances in GJIC, either by induction or by inhibition, could influence embryonal development. It should be noted that supidimide, which was also found to induce GJIC in fibroblasts after activation (Fig. 3), exhibits little teratogenicity *in vivo*. Thus, further studies are necessary to evaluate the potency of thalidomide and its analogs to induce GJIC in relation to their teratogenic activity.

Thalidomide is used in the therapy of leprosy [4] and shows beneficial effects in the treatment of cutaneous lesions of systemic lupus erythematosus [27]. The latter disorder is also efficiently treated with different retinoids, capable of inducing GJIC. Although it is not known whether GJIC is a relevant biochemical mechanism in the treatment of lupus erythematosus, it appears of interest to test whether similar pathways are operative for retinoids and thalidomide in this disease.

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