STIMULATION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION BY THE HUMAN TERATOGENS RETINOIC ACID AND THALIDOMIDE

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To My Family

“Two roads diverged in a wood, and I took the one less travelled by.

And that has made all the difference.”

Robert Frost

ZUSAMMENFASSUNG

Gap Junctions sind Zell-Zell Kanäle, die an der zellulären Signalgebung teilnehmen und eine wichtige Rolle bei der embryonalen Entwicklung spielen. Die Stimulation der interzellulären Kommunikation über Gap Junctions (GJIC) wurde durch Teratogene wie Thalidomid, Retinsäure und einige ihrer Analoga in verschiedenen Zellen von Mensch, Maus und Ratte untersucht.

All-*trans*-Retinsäure und verwandte Verbindungen, die eine Steigerung der GJIC verursachten, stabilisierten Connexin 43 (Cx 43) mRNA; Cx 43 ist ein weit verbreitetes Gap Junction Protein. Das starke Teratogen 13-*cis*-Retinsäure stimulierte die GJIC und stabilisierte Cx 43 mRNA ähnlich wie die all-*trans*-Retinsäure. Dagegen transaktivierte 4-oxo-13-*cis*-Retinsäure, ein schwaches Teratogen, zwar den RARß2-Promotor, stabilisierte aber weder Cx 43 mRNA, noch induzierte es die GJIC. Alle Retinoide transaktivierten den RARß2-Promotor in F9 Maus-Teratokarzinom Zellen; dennoch scheinen ihre Wirkungen auf die GJIC durch eine posttranskriptionale Regulierung von Cx 43 mRNA zu erfolgen.

Die starken Teratogene, Thalidomid, sein Analog EM 12 und der Thalidomidmetabolit 2-Phthalimido-Glutarsäure (PGA), stimulierten die GJIC signifikant in humanen fötalen Hautfibroblasten. EM 12 und PGA waren weniger effektiv bei der Stimulierung der GJIC in Rattenleber Epithelzellen (WB-F344). Das PGA Analog, EM 138, stimulierte die GJIC effektiver in WB-F344 Zellen, während das EM 12 Analog EM 364 in HFFF2 Zellen effektiver war. Dies deutet auf eine speziesspezifische Wirkung von Thalidomid und seiner Analoga auf die GJIC hin, und könnte in Beziehung zu deren speziesspezifischer teratogenen Wirkung stehen. Weder in HFFF2 noch in WB-F344 Zellen, ergab sich eine Wirkung auf die GJIC bei Behandlung mit EM 16, einem nicht-teratogenen Thalidomidanalogon. All-*trans*-Retinsäure, die ein starkes Teratogen bei Mensch und Ratte ist, stimulierte die GJIC signifikant in Zellen beider Spezies.

Der Phorbolester, 12-O-Tetradekanoylphorbol-13-Azetat (TPA) aktiviert die Protein Kinase C (PKC) und bewirkt dadurch eine Phosphorylierung und Translokation von Cx 43 Protein von der Membran in das Cytosol, was die GJIC vermindert. EM 364, das die GJIC in Zellen beider Spezies stimuliert, verhinderte effektiv die TPA-verursachte Translokation von Cx 43; EM 16, das die GJIC nicht in Zellen beider Spezies stimulieren konnte, war nicht effektiv. Ob Thalidomid und seine Analoga die GJIC über einen PKC-abhängigen Weg stimulieren, muß noch weiter untersucht werden.

Die hier vorgeschlagene Modifikation der GJIC könnte mit den pharmakologischen und toxikologischen Wirkungen der all-*trans*-Retinsäure, des Thalidomids und deren Analoga in Beziehung stehen.

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ABBREVIATIONS

AF	Transcription activation function
AP-1	Activator protein-1
AP-1 RE	Activator protein-1 response element
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CREB	Cyclic adenosine monophosphate response element binding protein
Cx	Connexin
DAG	Diacylgylcerol
db-cAMP	Dibutyryl-adenosine-3´, 5´-cyclic monophosphate
DMEM	Dulbecco´s minimal essential medium
DMSO	Dimethly sulfoxide
DNA	Deoxyribonucleic acid
DOTAP	N-[1-(-2,3-Dioleoyloxy) propyl]-N, N, N-trimethylammonium-methylsulfate
EDTA	Ethylene glycol-O, O'-bis-(2-aminoethyl)-N, N, N'-tetraacetic acid
EGF	Epidermal growth factor
EtBr	Ethidium bromide
FAD	Flavin adenine nucleotide
FCS	Fetal calf serum
FGF	Fibroblast growth factor
fos	Protooncogen coding partially for AP-1
GJIC	Gap junctional intercellular communication
HPLC	High performance liquid chromatography
HRE	Hormone response element
IGF	Insulin, insulin-like growth factor
IP_3	Inositol-1, 4, 5-triphosphate
jun	Protooncogen coding partially for AP-1
kDa	10^3 Dalton

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
min	Minute
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethly-thiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NGS	Normal goat serum
ONPG	Ortho-nitrophenyl-ß-D-galactopyranoside
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PGA	2-Phthalimido glutaric acid
PIP_2	Phosphatidylinositol-4, 5-diphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator-activated receptor
RA	Retinoic acid
raf	Protein kinase activated by ras (member of monomeric G-proteins)
RAR	Retinoic acid receptor
RARE	Retinoic acid receptor responsive element
RBP	Retinoid binding protein
rpm	Revolutions per minute
RXR	Retinoid-X-receptor
RXRE	Retinoid-X-receptor responsive element
TATA-Box	AT rich sequence at the start point of transcription

TGF-ß	Transforming growth factor-β
Th	Thalidomide
THF	Tetrahydrofuran
TNF-α	Tumor necrosis factor-α
TPA	12-O-Tetradecanoylphorbol-13-acetate
TR	Thyroid hormone receptor
UTR	Untranslated region
VDR	Vitamin D receptor
v-src	Rous sarcoma virus

1. INTRODUCTION

1.1. Intercellular Communication

Intercellular communication is necessary in complex and differentiated organisms to coordinate the cellular activities of differentiation and growth. Two modes of cell-cell communication contribute to this coordination: one form involves the secretion of signal substances like neurotransmitters, hormones and growth factors into the extracellular fluid. Target cells expressing receptors to these substances respond appropriately upon encountering the respective chemical modulators. The other form of communication operates within the limiting plasma membrane of groups of cells which are coupled into integrated structures (gap junctions) that permit free diffusion of molecules of low molecular weight.

1.1.1. Intercellular Communication via Gap Junctions

1.1.1.1.Structure and Formation of Gap Junctions

Gap junctions are membrane-spanning channels that allow the passage of small molecules (<1 kDa) such as second messengers or ionic signals from one cell to another. The name "gap" derives from the 2-4 nm gap between the two apposing membranes of neighbouring cells connected by channels. Each gap junction is comprised of two hexamers termed connexons that, in turn, are comprised of six subunits termed connexins (Caspar et al., 1977). Transmembrane topology of a generic connexin polypeptide creates four conserved membrane domains (M1-M4), one variable cytoplasmic (C), two conserved extracellular domains (E1-E2) and the N and C-termini face the cytoplasm (Simon and Goodenough, 1998) (Figure 1).

At least 14 mammalian connexins have been identified according to their molecular weights, among which connexin 43 (43 kDa) and connexin 32 (32 kDa) are widely expressed (Donahue, 2000). The oligomerisation of connexin to connexon occurs

in the Golgi apparatus, after their synthesis in the endoplasmic reticulum (Musil and Goodenough, 1993). With the help of adhesion molecules on the cell surface, connexons bind to the other connexons on the surface of neighbouring cells. Due to their short half-life (1-3 hours) they are degraded and leave the cell surface (Laird 1996; Laing et al., 1997).

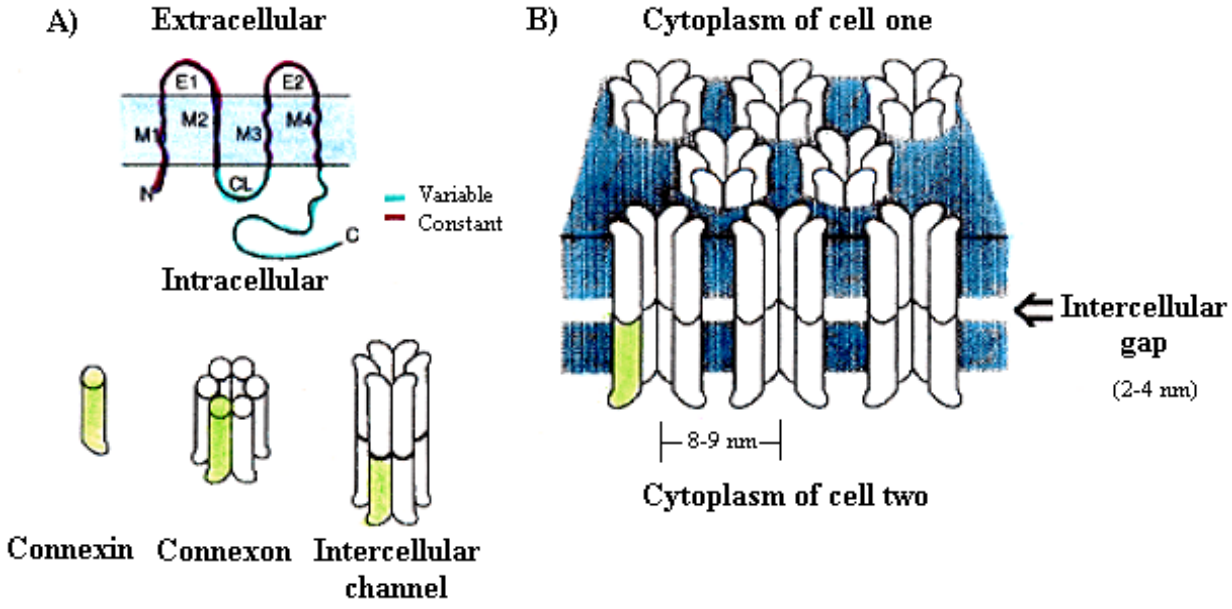


Figure 1. Structure of Gap Junctions. A) Transmembrane topology of a generic connexin polypeptide with membrane (M1-M4), cytoplasmic (C) and extracellular domains (E1-E2). **B)** Gap junctions are intercellular channels clustered at close appositions of the plasma membrane of two cells. Modified from (Simon and Goodenough, 1998)

Connexin 43 (Cx 43) which is examined in this study, is widely expressed in a variety of organs including skin, the lens and cornea of the eye, heart muscles, smooth muscles, brain, kidney, intestine, inner ear and developing limb of the embryo (Dealy et al., 1994; Forge et al., 1999; Vaney, 1999; Nadarajah et al., 1999; Severs, 1999; Kistler et al., 1999). The majority of connexin genes have a simple structure, in which the entire coding region

is present within a single exon, although a new class has emerged that contains an intron within the coding region (Simon and Goodenough, 1998).

1.1.2. Regulation of Gap Junctional Intercellular Communication

Communication through the gap junctions is commonly measured by three ways: electrophysiologically (Loewenstein, 1979), by fluorescent dye transfer (usually lucifer yellow) (Steward, 1978), or by metabolic cooperation (Loewenstein, 1979). Because gap junctions are unique in their ability to transfer molecules as large as lucifer yellow intercellularly, dye transfer is the most widely used screening method.

The permeability of gap junctions is rapid (within seconds) and reversibly decreased by experimental manipulations that decrease cytosolic pH or increase the cytosolic concentrations of free Ca^{2+} . These observations indicate that gap junction channels can undergo a reversible conformational change that closes the channel in response to changes in the cell. Tumor promotors (phorbol esters) via PKC (Doble et al., 2000), growth factors [e.g. insulin, insulin like growth factor (IGF), fibroblast growth factors (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF)], and oncogens (ras, raf, scr) cause a decrease in gap junctional intercellular communication (GJIC), whereas cAMP via protein kinase A (PKA) (Spray et al., 1985), carotenoids (Zhang et al., 1991), vitamin D (Stahl et al., 1994), thyroid hormones (Stock et al., 1998), retinoids (Hossain et al., 1989) and thalidomide (Nicolai et al., 1997) cause an increase in GJIC. Adhesion molecules can also interact with the formation of the gap junction channels within the membrane and either increase or decrease GJIC according to the cell type (Wang and Rose, 1997). Mostly these effects fall into one of the two categories of regulation: either due to the amount of connexin proteins or due to the phosphorylation of connexin proteins.

1.1.2.1. Regulation due to the Amount of Connexin Proteins

Previous research has shown that in the promoter region of Cx 43, there resides the TATA-Box and the AP-1 and AP-2 binding sites (De Leon et al., 1994; Chen et al., 1995) which indicates a transcriptional regulation. As a result, the binding of AP-1 to the promoter region causes, upregulation of transcription, thereby increasing the amount of Cx 43 protein that can be obtained (Geimonen et al., 1996; 1998). The effect of retinoids on GJIC seem to be due to the increased levels of Cx 43 protein (Clairmont et al., 1996). This can be performed by either increasing the transcription of Cx 43 or by stabilization of Cx 43 mRNA which normally undergoes a rapid degradation due to its UA rich 3′ untranslated region (3′UTR) (Ross, 1995; 1996). The latter shows presence of a possible post-transcriptional regulation of Cx 43 gene.

1.1.2.2. Regulation due to the Phosphorylation of Connexin Proteins

Phosphorylation of the connexin proteins causes alteration in GJIC possibly due to a structural change of the protein which often results in a translocation of the protein to the cytoplasm instead of forming gap junctions within the cell membrane. As a result, phosphorylation of connexins initiates a decrease in GJIC (as in the case of Cx 43) , although counteracting increased levels of cAMP causes phosphorylation of Cx 32 via cAMP dependent-PKA pathway which leads to an increase in GJIC (Musil and Goodenough et al., 1990).

Tumor promotors (phorbol esters) are known to decrease GJIC via increased protein kinase C (PKC) activation which in turn phosphorylates Cx 43 protein and decreases the amount of gap junctions formed within the cell membrane (Doble et al., 2000). Phorbol esters mimic the diacylgylcerol (DAG) which is formed by the phospholipase-C (PLC) mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2), and bind to PKC to activate it instead of DAG (Nishizuka, 1986). Cleavage of PIP_2 forms also inositol-1, 4, 5-triphosphate (IP_3) which in turn increases free Ca^{2+} levels

in the cytosol and then Ca^{2+} increases PKC activity (Nishizuka, 1986) and causes a decrease in GJIC not only due to rapid conformational change of gap junctions but also due to the phosphorylation of connexin proteins by PKC in long term.

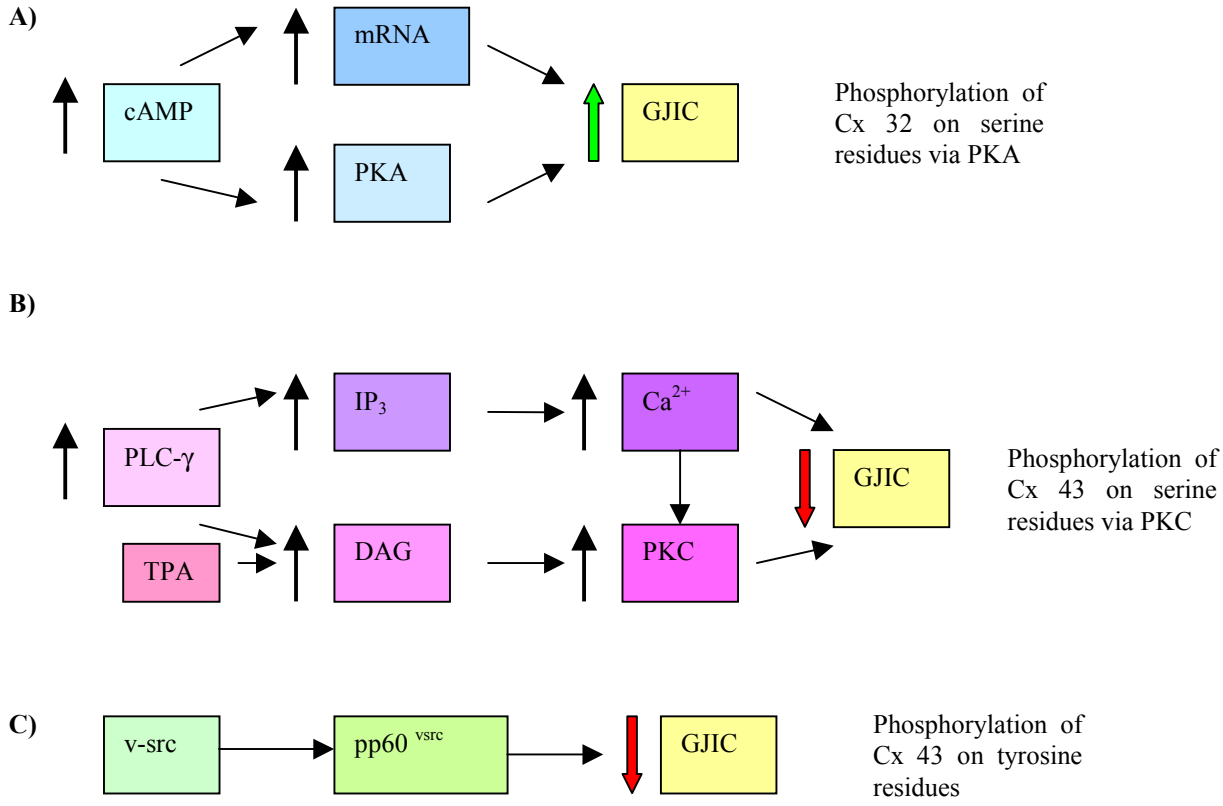


Figure 2. Regulation of Gap Junctional Intercellular Communication via Phosphorylation of Connexin Proteins.

Sequence analysis of the Cx 43 protein has shown that there various functionally important residues on it which can be phosphorylated by PKC, PKA and mitogen-activated protein kinase (MAPK) (Kanemitsu and Lau, 1993; Yamasaki, 1997). It is known that MAPK can be activated by PKC (Yamagutchi et al., 1995). Furthermore, the growth factors IGF, FGF and PDGF are the ligands for receptor tyrosine kinases which in turn activate a PKC dependent downstream pathway and decrease GJIC as mentioned

before. On the other hand, neurotransmitters can cause alterations on GJIC which may be related to the possible involvement of protein kinases (Saez et al., 1993).

Tyrosine kinases ($pp60^{v\text{-}src}$ and $p130^{gag\text{-}fos}$) also cause phosphorylation of Cx 43 which causes a decrease in GJIC (Crow et al., 1990) (Figure 2).

1.1.3. Functions of Gap Junctional Intercellular Communication

Gap junctions provide a mechanism for coordinating the activities of cell groups. Previous studies have shown that these mechanisms fall into five general classes: speed, synchrony, switching, symbiosis and stimulus/suppression. In the heart, gap junctions permit the rapid cell-cell transfer of action potentials, ensuring coordinated contraction of the cardiomocytes (Severs, 1999). Gap junctions are also present in the nervous system, where electrical synapses are used in neuronal pathways requiring high speed, synchronous neuronal firing and switching between neuronal pathways which occur in areas such as the eye, inner ear and brain (Nadarajah et al., 1999; Forge et al., 1999; Vaney, 1999). In nonexitable cells, gap junctions allow for symbiotic interactions between highly differentiated, functionally compromised cells and more active, renewable cells, which perform cellular functions for both cell types, as in the lens of the eye (Kistler et al., 1999). Junctions can also act as suppressors of somatic cell mutations in key metabolic and signalling enzymes: normal cells deliver junction-permeable intermediary, permitting the survival of mutant cells with blocked enzymatic pathways. In this last case, gap junctions might also serve as tumor suppressors (Holder et al., 1993), since tumor cells tend to decrease GJIC which in turn promotes lack of growth control and differentiation of tumor cells.

On the other hand, the effect of gap junctions in vertebrate development is well studied and will be discussed further. Furthermore, there appears to be a link between teratogenicity and carcinogenesis, that most carcinogens are also compounds possessing

teratogenic properties whereas teratogenic compounds are not necessarily implied to be carcinogenic (Vainio, 1989). The compounds which are stimulating GJIC like retinoids, vitamin D and thalidomide can also suppress tumor formation.

1.1.4. The Relation Between Gap Junctional Intercellular Communication and Teratogenicity-The Effect on Embryogenesis

Gap junctional coupling has been demonstrated in embryonic tissues, and it is assumed that GJIC is involved in the process of pattern formation (Goodenough et al., 1996). The most abundant connexin protein, connexin 43 (Cx 43) is differentially expressed during the development of mouse (Ruangvoravat and Lo, 1992; Dahl et al., 1995; Sullivan et al., 1993) and human embryos (Hardy et al., 1996). Cx 43 transcripts appear to be distributed as a gradient in regions spanning the midbrain/hindbrain junction, in the telencephalon, and within the mouse embryo limb mesenchyma (Dahl et al., 1995; Sullivan et al., 1993). Furthermore, Cx 43 proteins have been implicated in the morphogenesis of the chick limb bud during the early development of chick embryo (Dealy et al., 1994; Green et al., 1994; Makarenkova and Patel, 1999). It has been shown that congenital heart defects could be related to GJIC (Dasgupta et al., 1999). Furthermore, gap junctions can also regulate bone cell differentiation (Donahue, 2000).

GJIC is stimulated by teratogenic compounds, retinoic acid (Mehta et al., 1989; Stahl et al., 1998) and thalidomide (Nicolai et al., 1997) showing similar malformations due to their teratogenicity which include limb malformations, craniofacial structures, congenital heart defects, ear malformations or deafness, facial palsy, absent or shrunken eyes, cataract, ocular movement abnormalities, malformed intestines, kidney malformations, central nervous system defects and mental retardation and which will be discussed later. As it is previously mentioned, gap junctions are important for the central nervous system, ear, brain, eye and heart not only in performing their normal functions in a

normal adult but also for their formation during embryonal development. Furthermore, as mentioned above, Cx 43 plays a very important role in the developing limb, where malformations also occur upon application of both retinoic acid and thalidomide. As a result, GJIC could be a possible key mechanism explaining the mode of action of retinoic acid, thalidomide and their derivatives.

1.2. Teratogenicity

Teratogenesis is defined as the dysgenesis of fetal organs detected either structurally or functionally (e.g. brain functions). The typical manifestations of teratogenesis are restricted growth or death of the fetus, carcinogenesis and malformations defined as defects in organ structure and function (Koren et al., 1998). It has been shown that fetuses could be deformed by external influences, including poisoning and therapeutic drugs, infection, X-rays and malnutrition (Dally, 1998).

1.2.1. The Retinoids

1.2.1.1. The Metabolism of Retinoic Acid

The International Union of Pure and Applied Chemistry-International Union of Biochemistry (IU-PAC-UB) has stated that vitamin A (retinol) and related compounds of natural and synthetic origin will be classified as *retinoids*. Vitamin A is the transcriptionally inactive precursor of retinoic acid (RA) (Gerster et al., 1997). ß-carotene in plants and retinyl esters in animal tissues are the two major sources of vitamin A (Goodman et al., 1984). The first step of conversion of ß-carotene to vitamin A is in the intestinal mucosa which is an oxidative cleavage producing all-*trans*-retinal, which is then reduced to retinol (Ong et al., 1994).

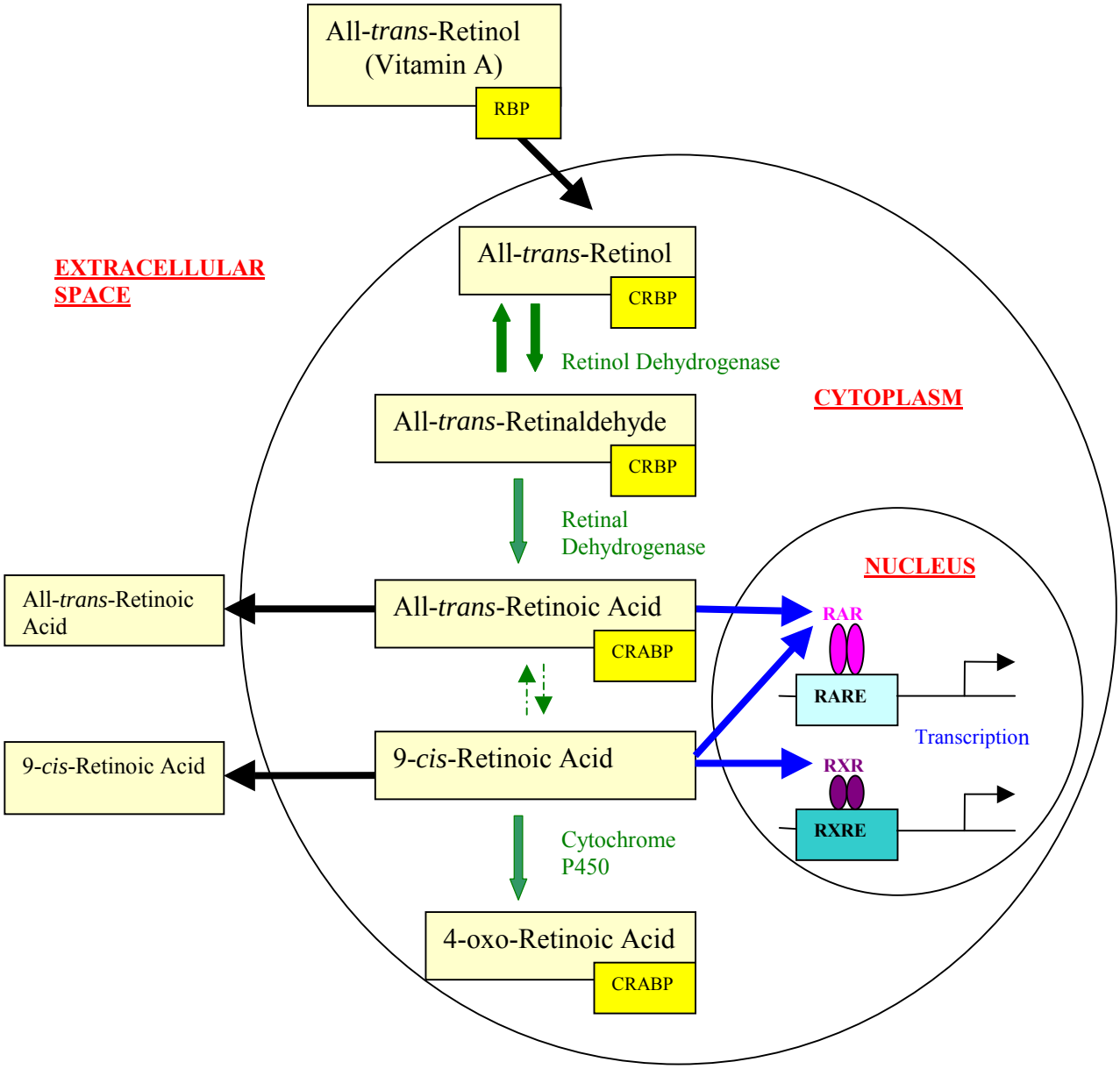


Figure 3. Summary of Retinoic Acid Metabolism and Signaling Pathway. Retinoic acid synthesised from retinol in a two step pathway involving oxidation first to retinaldehyde, then to retinoic acid. Retinoic acid then enters the nucleus, binds to specific nuclear receptors and activates transcription via an autocrine activation route. Alternatively, retinoic acid can be released from the cell to induce transcription in a neighbouring cell via a paracrine pathway. Modified from (McCaffery and Dräger, 2000).

Retinol in the mucosal cell is re-esterified (Goodman et al., 1984) and retinyl esters are then transport by means of the lymphatic system into the general circulation. After being taken up by liver, retinol is secreted into the circulation bound to retinol-binding protein (RBP) which is synthesized by the liver (Ong et al., 1994). The presence of a specific RBP receptor has been postulated for the uptake of retinol by cells (Napoli, 1996). After delivery to the target tissue, retinol is either esterified into retinyl esters for storage or further metabolized into the biologically active all-*trans*-RA. Alternatively, all-*trans*-RA can be taken from the all-*trans*-RA pool present in the plasma where it circulates bound to albumin. The enzymes that catalyze the oxidation of retinol to retinaldehyde are generally considered to be dehydrogenases which are relatively nonspecific (Malik et al., 2000). Aldehyde dehydrogenase can convert retinaldehyde into all-*trans*-RA (Duester et al., 1998). The first reaction is reversible, whereas the second is irreversible (Napoli, 1999). All-*trans*-RA can then enter the nucleus, bind to specific nuclear receptors and acitivate transcription, alternatively it can be released from the cell to induce transcription in a neighbouring cell (Figure 3).

Cytochrome *P*-450 appears to catalyze all-*trans*-RA metabolism. Although non-specific members of the superfamily can catalyze all-*trans*-RA metabolism *in vitro*, major activity derives from retinoid-induced, possibly species specific, cytochrome *P*-450 enzymes *in vivo*. As a result, all-*trans*-RA also undergoes further metabolism into 4-hydroxy-RA, 4-oxo-RA and 18-hydroxy-RA and isomerizes into 9-*cis*-RA and 13-*cis*-RA (Napoli, 1999) (Figure 3 and 4). These conversions seem to send all-*trans*-RA down paths of degradation and elimination, and thereby help to control retinoid signaling. All-*trans*-RA has a short half-life *in vivo* and in cultured cells (approximately 6-7 hours) (Napoli, 1986; Randolph et al., 1997). All-*trans*-RA metabolism in mammals and mammalian cells reduces its biopotency, establishing an autoregulatory loop by altering the rates of retinol esterification vs. conversion into all-*trans*-RA (Haq et al., 1988). At least five

naturally occurring biologically active retinoids have been identified. These are all-*trans*-RA, 9-*cis*-RA, all-*trans*-4-oxo-RA, 3,4-didehydro-RA and 14-hydroxy-4,14-retroretinol (Malik et al., 2000).

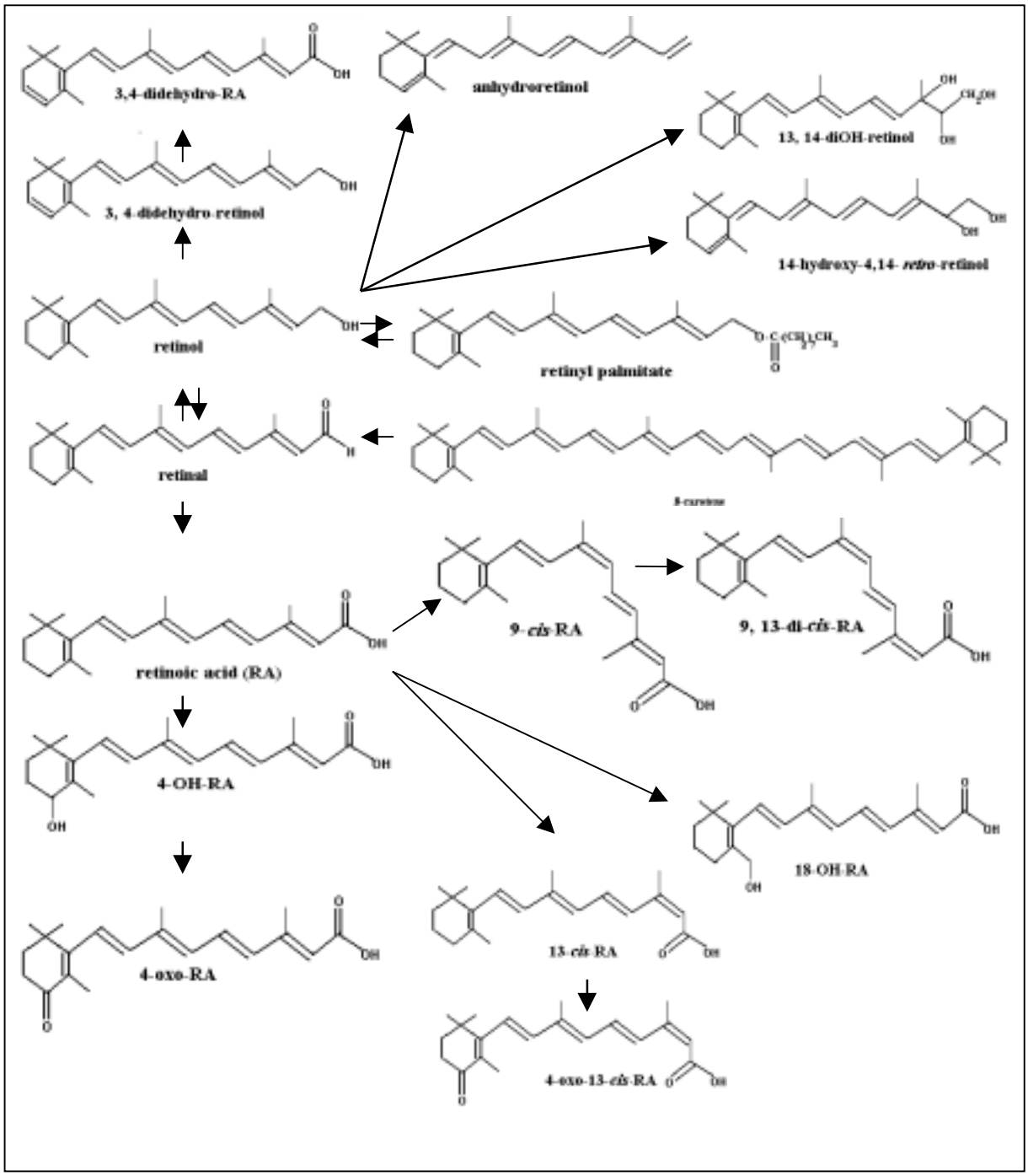


Figure 4. Metabolites and Derivatives of Retinol (vitamin A) and Retinoic Acid. Modified from (Napoli, 1999).

1.2.1.2. Retinoid Binding Proteins, Receptors and Responsive Elements

Retinoid Binding Proteins. The hydrophobic nature of the retinoids is circumvented by the presence of specific carrier proteins. At least four intracellular binding proteins: cellular retinol binding proteins (CRBP); CRBPI and CRBPII, cellular all-*trans*-RA binding proteins (CRABP); CRABPI and CRABPII, contribute to retinoid hormone-signaling physiology, as indicated by their widespread distribution throughout the embryo and adult tissues (Napoli, 1999).

CRBPI and CRBPII can bind both retinol and retinal and discriminate against all-*trans*-RA, whereas CRABPI and CRABPII bind all-*trans*-RA and some of its metabolites and discriminate against retinol and retinal (Figure 3). Both types of binding proteins; CRBP and CRABP, appear to play a critical part in facilitating the interaction of retinoids with binding sites on nuclei and consequently play an active role in the intracellular distribution of the retinoids (Napoli, 1996). Retinol bound to CRBPII can be oxidized to retinaldehyde by a retinol dehydrogenase (Napoli, 1993). The retinaldehyde, remained bound to CRBPII, is then available for oxidation to all-*trans*-RA by retinaldehyde dehydrogenase (Ottonello, 1993). In contrast to CRBP, CRABP is thought to promote the breakdown of all-*trans*-RA (Boylan et al., 1992) while CRABPII, which has been reported to be present in both the cytoplasm and nucleus, may have some part to play in the movement of all-*trans*-RA into the nucleus (Gustafson et al., 1996). Since CRABPII can also associate with the *all-trans*-RA receptors, it can act as a transcriptional regulator (McCaffery and Dräger, 2000). Thus rather than being essential for retinoid metabolism, the binding proteins seem to increase the effiency of retinoid signaling. On the other hand, the concentrations of both types of cellular binding proteins exceed those of the ligands. One reason could be to protect cellular membrane from retinoids whilst at the same time protecting retinoids from nonenzymatic chemical transformation. Furthermore, each binding protein exhibits affinities for its ligands similar to the affinities of receptors for

their hormones and to a much higher specificity than typical enzymes for their substrates (Napoli, 1996). It has been shown that CRAB can bind also some metabolites of all-*trans*-RA such as 4-hydroxy-RA and 4-oxo-RA (Napoli et al., 1995) whereas it remains still unclear that CRAB or CRBP can bind to 9-*cis*-RA which is the isomerization product of all-*trans*-RA (Napoli, 1999).

Retinoid Receptors. Retinoid receptors belong to the nuclear receptor superfamily which represent an evolutionary conserved class of transcription factors being present from flies to mammals (Mangelsdorf et al., 1995). In contrast to membrane bound hormone receptors, they bind to small lipophilic hormones such as steroids like androgens and estrogens, non-steroids like thyroid hormons and retinoids and finally steroid derivatives like vitamin D. They function in the cell nucleus as ligand modulated transcription factors (Mangelsdorf and Evans, 1995). The relatively small and simple RA molecule can regulate the expression of multiple genes in a time and locus specific manner to produce complex results such as limb morphogenesis (Maden et al., 1994). Such events rely on three ligand-activated transcription factors, retinoic acid receptors (RARα, RARβ and RAR γ) each encoded by distinct genes and each with multiple isoforms produced by alternative splicing or differential promoter use (Chambon, 1996). RARs bind to RA responsive elements (RARE) within the promoters of target genes (Figure 3), but function in a heterodimeric partnership with a second group of receptors, retinoid x receptors (RXR α, RXR β and RXR γ) (Figure 5). Three distinct genes encode the three distinct RXRs, and each RXR also has multiple isoforms. In all, 48 potential combinations of the RXR-RAR heterodimer could occur and RAR and RXR isoforms can have distinctive expression patterns in adults and distinctive spatial-temporal expression patterns during embryogenesis (Napoli, 1999). Apparently only the RAR partner requires ligand binding to affect transcription. Additionally, RXRs function as homodimers with distinct retinoid x receptor responsive elements, RXRE, and heterodimerize with several

other receptors, including those for thyroid hormone (TR), vitamin D (VDR), and peroxisome proliferator activated receptors (PPAR) (Figure 5) (Rowe, 1997). RXR seem not to require ligand to influence co-receptor, but liganded RXR may enhance action of a heterodimer pair (McCaffery and Dräger, 2000; Lu et al., 1997). However, it still remains unknown whether or not 9-*cis*-RA is a major or the only ligand of RXR (Napoli, 1999).

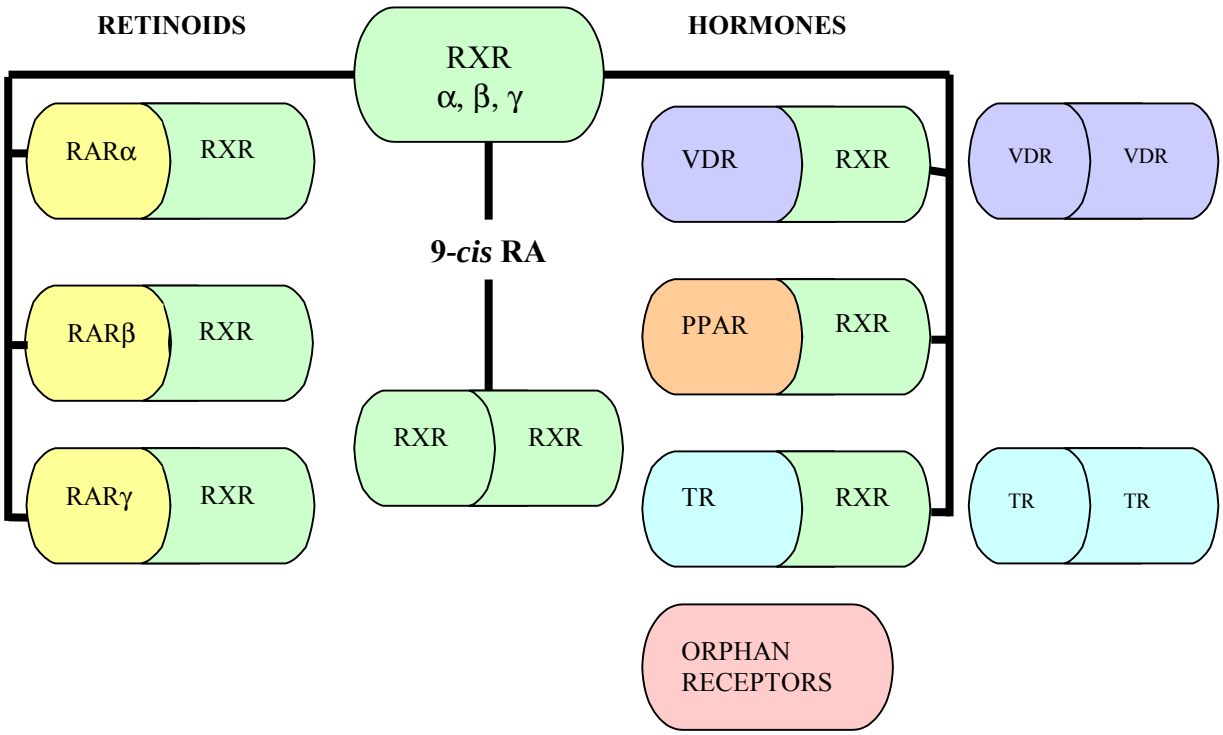


Figure 5. Interactions Between the Nuclear Receptors. RXR is a cofactor required for transcriptional activation by several members of the steroid/thyroid hormone nuclear receptor superfamily and also RAR family. Modified from (Rowe, 1997).

Nuclear receptor superfamily members also differ from each other by their mode of binding to their DNA response elements. According to this, receptors for steroids are associated with heat shock factors which dissociate upon ligand binding and then there receptors bind to DNA sequences as homodimers. Receptors for which no ligand has been

found (like orphan receptors), can bind DNA as monomeric forms or as dimers to direct repeats, whereas receptors for retinoids, thyroid hormone and vitamin D are bound to DNA in the absence of a ligand. Binding of a ligand leads to a conformational change of the ligand binding domain and to transcriptional activation (Tenbaum and Baniahmad, 1997). Retinoid receptors like other nuclear receptors possess a common conserved structure with regions designated A through F. The amino terminal A and B regions have transcription activation function (AF-1) due to a promoter and contex specific activation which is ligand-independent function. The C region, contains two zinc fingers involved in DNA recognition and binding. The D region is responsible from corepressor binding. The highly conserved region E, amongst the subtypes of RARs and RXRs, contains the ligand binding domain, the ligand-dependent transcriptional activation (transactivation) function (AF-2) and a dimerization surface. The F region is derepression and CBP [cAMP response element binding protein (CREB)-binding protein] binding domain. Ligand binding causes a conformational change in the receptor heterodimer, causing a corepressor dissociation and coactivator binding that enzymatically disrups local chromatin structure and increases transcription (Chambon, 1996; Rowe, 1997; Tenbaum and Baniahmad, 1997; Lawson and Berliner, 1999; Collins and Mao, 1999) (Figure 6).

Figure 6. Structure of Retinoid Receptors. Regions are as follows: A/B, promoter and contex specific activation (AF-1); C, zinc fingers involved in DNA recognition and binding; D, corepressor binding; E, transactivation (AF-2), dimerization and ligand binding; and F, derepression domain and CBP [cAMP response element binding protein (CREB)-binding protein] binding. Modified from (Lawson and Berliner, 1999).

Retinoid Receptor Responsive Elements. RAR and RXR bind as heterodimers to specific DNA sequences called retinoic acid response elements (RARE) in regulatory regions of target genes and stimulate target gene transcription upon ligand binding by a process known as transactivation. RARE most offen consists of two copies of directly repeated motives or closely related sequences that can be spaced one, two or five basepairs apart; in addition inverted or everted repeats have been identified (Giguère, 1994). However, the complex formed due to interaction of retinoid receptors with transcription factor AP-1, which is composed of jun and fos proteins, is unable to transactivate either RARE or AP-1 response element (AP-1 RE), thereby not stimulating target gene transcription (transrepression). On the other hand, interaction of RXRs with different nuclear receptors such as vitamin D, thyroid hormone and peroxisome proliferator-activated receptor, limits the ability of RXRs to bind to RARs. Each heterodimeric complex binds to specific hormone response elements (HRE) in target genes. Binding of ligands (L) by these receptors stimulates transactivation for the set of genes other than the ones for retinoid signaling patways (Fisher and Voorhees, 1996) (Figure 7).

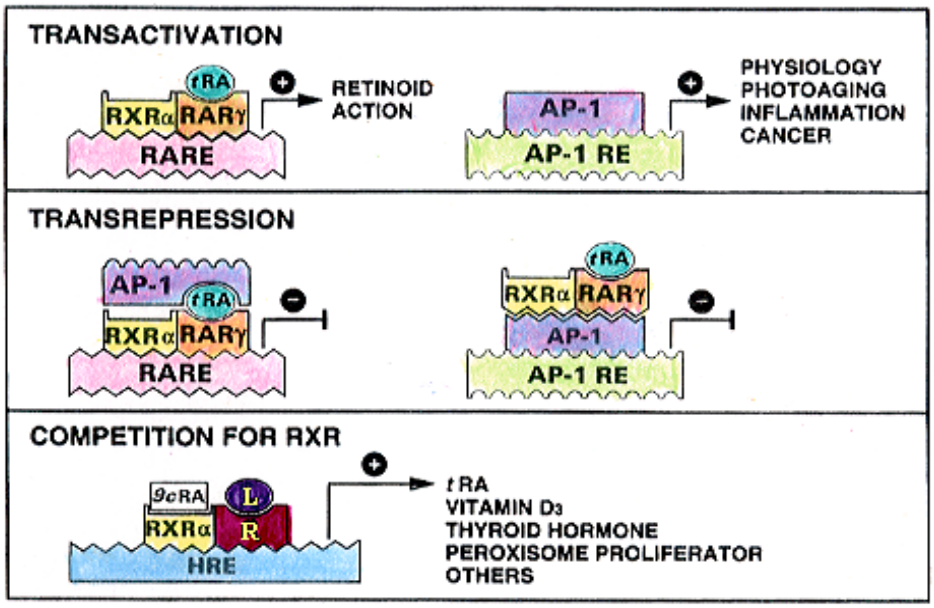


Figure 7. Functional Interactions of Retinoid Receptors. Modified from (Fisher and Voorhees, 1996).

The genes having RARE upstream of their promoters are well known for their effect on embryogenesis, growth and differentiation processes (De Luca, 1991). A family of nuclear transcription factors known as the homeotic genes, which are important in the positional identity of embryonic tissues, also have RARE sequences in their promoter regions (Marshall et al., 1996).

1.2.1.3. Different Functions and Usage of Retinoic Acid

An important set of genes are under direct control of RA itself: several transcription factors (e.g. Hox), signaling molecules (e.g. sonic hedgehog), RA signalling components (RARα2, RARβ2, CRBP and CRAB), adhesion proteins (e.g. intercellular adhesion molecule-1) and enzymes (e.g. PKC) each of which can activate multiple pathways of development and also that of the adult signaling pathway (McCaffery and Dräger, 2000). Further more, effects of retinoids on growth factors and their receptors [transforming growth factor-ß (TGF-ß), EGF, IGF, nerve growth factors (NGF) etc.], effects on activity and synthesis of hormones (growth hormone etc.), on the activity and synthesis of cellular enzymes and effectors (alcohol dehydrogenase, PKC, PKA, components of extracellular matrix etc.), effects on gap junctional communication, on viral replication, on the cells of immune system, effects on visual process, effects on differentiation, on embryogenesis, on reproduction and on cancer are well studied to date (Gudas, 1994; McCaffery and Dräger, 2000). As a result, vitamin A deficiency alone can cause blindness and is also likely to increase vulnerability to severe infections and iron decificiency anemia. Furthermore, it may be an important factor contributing to diarrheal, respiratory and measles morbidity, growth retardation in childhood and congenital malformations in pregnancy (Underwood and Arthur, 1996). However excess usage of retinoids, especially RA, is also hardly handled by the organism and can cause mainly severe malformations of the embryo during pregnancy which will be discussed later.

Due to their wide range of effects on organisms, both naturally occuring and synthetic retinoids have already been used in the therapy of various skin diseases, especially acne, for augmenting the treatment of diabetes and as cancer chemopreventive agents (Napoli, 1999). Tazaroten (Zorac®), for the treatment of Psoriasis, Tretinoin (*all-trans* Ra) as Epi-Aberel®, Isotretinoin (13-cis Ra) as Roaccutan® or Isotrex® for the treatment of acne and Vesanoid® for the treatment of leukemia are some of the drugs already found on the German pharmaceutical market.

The recommended daily intake of vitamin A levels ranges between 8,000 and 10,000 IU/day (1 IU of vitamin A is equivalent to 0.3 μg of retinol) (Underwood and Arthur, 1996; Azaïs-Braesco and Pascal, 2000).

1.2.1.4. Teratogenicity of Retinoic Acid

Vitamin A is transferred from mother to the embryo across the placenta; vitamin A concentrations in fetal blood are approximately half of those in the mother. RBP is involved with this transfer (Azaïs-Braesco and Pascal, 2000). Later on vitamin A is metabolised to form RA in embryonic tissues. Although little information is available about the biogenesis of RA in pregnancy or in the embryo and about the teratogenic threshold of circulating RA, an intake exceeding 10,000 IU of vitamin A significantly increases the risk of malformations (Underwood and Arthur, 1996; Azaïs-Braesco and Pascal, 2000).

Teratogenesis of high vitamin A intakes have been reported in several animal species (Hathcock et al., 1990) with more than 70 types of malformation including exencephaly, spina bifida, craniofacial, ear and ocular malformations as well as limb malformations (Shenefeld, 1972). Similar abnormalities occur during the therapeutic treatment with RA and 13-*cis*-RA (Figure 8), the latter forms RA again in the human placenta and embryo 72 hours after the treatment whereas maternal serum concentrations

of both isomers remain low. However, animal studies showed that a vitamin A deficient diet also gave birth to malformed offspring with abnormalities of the cardiac, lung, and urogenital system which shows that retinoids participate in the development of diverse embryonic structures including face, heart, eye, limb and nervous system (Smith et al., 1998; Collins and Mao, 1999; Azaïs-Braesco and Pascal, 2000). The impact of retinoids on vertebrate limb development have been illustrated by experiments in salamanders as serial duplications during the limb formation and pointed out the ability of retinoids to specify limb formation (Maden, 1982). A family of nuclear transcription factors known as the homeotic genes, which are important in the positional identity of embryonic tissues, are differentially induced by RA due to RARE sequences in their promoter regions and alteration of the established Hox code may be responsible for some abnormalities seen both in vitamin A deficiency and as well as in excess RA consumption (Marshall et al., 1996). In humans, RA has been found to be equal in its teratogenicity to thalidomide which will be discussed later (McCaffery and Dräger, 2000).

Three strategies have been used in developing embryos to study the role of retinoids in embryonic development: elimination of RAREs, chemical inhibition of endogenous RA synthesis, and transfection with constructs that inhibit RAR activities *in vivo* or establish a reporter cell line *in vitro*. The expression of the RARß at particular locations in the embryo is believed to indicate the local presence of RA. Furthermore, limb malformations produced from excess retinoid administration have been correlated with induction of RARß2 expression (Jiang et al., 1994). Thus, several laboratories generated transgenic mice with constructs containing one or multiple copies of this RARE, varying stretches of the RARß-promoter region and the ß-galactosidase gene to further examine the role of RA in various processes of RA, especially on development (Mendelsohn et al., 1991). By the same approach, F9 teratocarcinoma cells which are known to express high levels of RA receptors, were transfected with a construct containing

the RARE from the RARß driving ß-galactosidase and called F9-RARß-lacZ reporter cell lines (Wagner et al., 1992). By using these reporter cell lines, several studies have been performed hypothesising the role of gap junctional intercellular communication (GJIC) as the possible mechanism of action of retinoid teratogenesis (Clairmont et al., 1996; Clairmont and Sies, 1997; Teicher et al., 1999).

Components of retinoid signaling pathways such as binding proteins or receptors were also hypothesized to be imported during embryogenesis. While CRABII is expressed widely throughout embryonic tissues, in the adult organism it is mainly restricted to the skin. However, CRABI expression sites have been correlated with sites most vulnerable to the teratogenic effect of RA (Dencker et al., 1990). Expression of CRABs could be a mechanism of the organism to protect itself from excess RA. On the other hand, besides the interspecies conservations for a given RAR or RXR type, the transcripts of each RAR and RXR type has also distinct expression patterns in the developing embryo and in various adult tissues (Chambon, 1996).

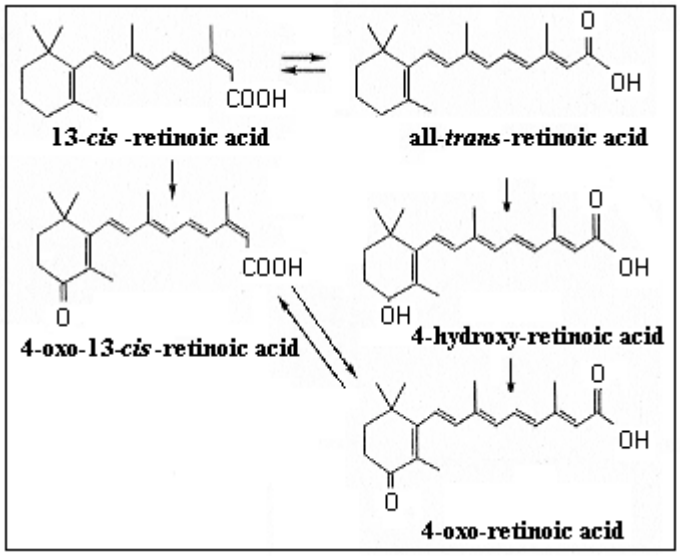


Figure 8. The Main Metabolic Pathways of Retinoic Acid Leading to 13-*cis*-Retinoic Acid and 4-oxo Metabolites.

1.2.2. Thalidomide

Thalidomide (α–N–phthalimidoglutarimide) was synthesized as a sedative drug first in 1953 in Germany by Chemie Grünenthal and created one of the most dramatic disasters in the history of medicine due to its teratogenic properties. Thalidomide (Th) was first marketed in Germany under the name Contergan® in 1956 and then was subsequently used in the rest of Europe, New Zealand, Australia and Canada, although it was never approved in USA. The drug was taken by many pregnant women to treat morning sickness, since it was mistaken to be 'safe'. Early in 1960s, it was suggested that Th was associated with neuropathies and a little later, associated birth defects were reported. Consequently, Th was taken off the market in 1961. Since it was the third best selling drug in Europe and sold in 48 countries, by the time the drug was withdrawn from sale, 12,000 babies with Th-associated birth defects had been born, of which about two-thirds survived (Dally, 1998; Miller and Strömland, 1999; Marriott et al., 1999).

However, thalidomide has actions that have led to its re-evolution in a large variety of medical conditions and it has once again become available in some countries. In the early 1990s, Th was being imported illegally for distrubution amongst AIDS patients in the USA and, was finally granted FDA approval in the USA in 1998 under strict conditions, and it is also available in Canada, Brazil, Mexico and England. Thalidomide is marketed by the brand name Thalomide® by Celgene Corporation located in Warren, N.J., USA.

1.2.2.1. The Metabolism of Thalidomide

Thalidomide (Th) is a synthetic derivative of glutamic acid with two rings; phthalimide and glutarimide (Figure 9) and has two optically active forms, (+) R- and (-) S- forms (enantiomers) that interconvert rapidly under physiologic conditions. However, only (-)-S-thalidomide is teratogenic enantiomer, whereas (+)-R-thalidomide is non-teratogenic and

thalidomide has always been used clinically as a racemic mixture [an equal mixture of S-(-) and R-(+) enantiomers].

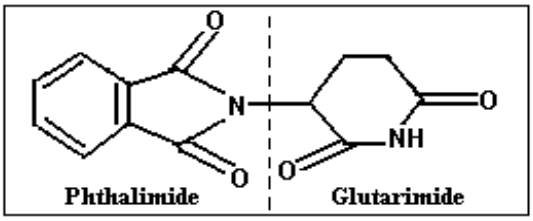
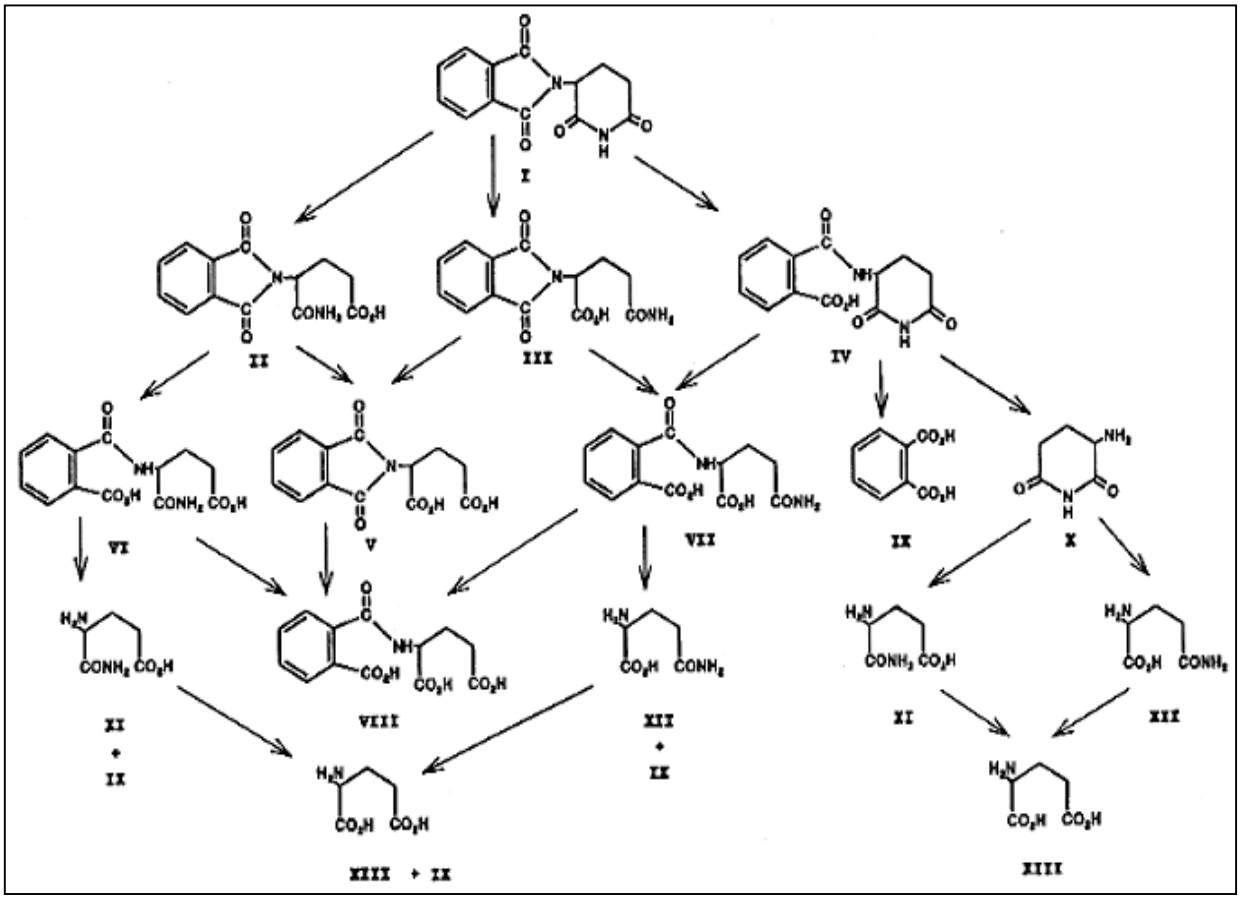


Figure 9. The Structure of Thalidomide.

Th is normally given to patients in an oral capsule at doses of 50-400 mg/day, although up to 1200 mg/day can be taken (Marriott et al., 1999). However, the absolute bioavailibility of Th from capsules has not yet been characterised in human subjects due to its poor aqueous solubility (Stirrling et al., 1997). At the present time, the exact metabolic route and fate of Th is not known in humans. Although, Th appears to undergo non-enzymatic spontaneous hydrolysis in aqueous solution on a pH greater than 6.0 (Schumacher et al., 1965; Tseng, 1996) (Figure 10), the cytochrome *P* 450 system is also implicated in the metabolism of Th (Reepmeyer and Cox, 1997). Furthermore, it has been shown that active Th metabolites can be generated by using liver microsomes which demonstrate metabolic cytochrome *P* 450 activity (Nicolai et al., 1997). On the other hand, this effect is species-specific, i.e. microsomes from human and rabbit were able to activate Th metabolically whereas microsomes from rat were not (Bauer et al., 1998).

In studies of healthy volunteers, after a 200 mg oral dose, the mean peak plasma concentrations ranged from 2.9 to 5.7 hours indicating Th is slowly absorbed from the gastrointestinal tract (Tseng, 1996). The mean half-life is about 8-9 hours and total body clearance around 11 hours (Tseng, 1996). However, the extent of plasma protein binding is unknown.



I Thalidomide

II 4-Phthalimidoglutaramic acid

III 2-Phthalimidoglutaramic acid

IV α-(*o*-Carboxybenzamido)glutarimide

V 2-Phthalimidoglutaric acid (PGA)

VI 4-(*o*-Carboxybenzamido)glutarimic acid

VII 2-(*o*-Carboxybenzamido)glutarimic acid

VIII 2-(*o*-Carboxybenzamido)glutaric acid

IX Phthalic acid

X α-Aminoglutarimide

XI Isoglutamine

XII Glutamine

XIII Glutamate

Figure 10. Hydrolysis Products of Thalidomide. Modified from (Bauer et al., 1998)

1.2.2.2. Different Functions of Thalidomide

Since its formulation in 1956, possibly as many as 5000 papers have been published about thalidomide (Th), but its complex mechanism of action is still be poorly understood (Stephens et al., 2000). Mostly, biological activities of thalidomide are carried out by its teratogen derivatives, whereas non-teratogen thalidomide derivatives failed to show the same effect with Th (Stephens et al., 2000). Here some of its most important functions on organisms will be discussed (Figure 11).

Tumor Necrosis Factor (TNF)-α. TNF-α is a key cytokine involved in the host immune response and also contributes to the pathogenesis of both infectious and autoimmune dieseases. Thalidomide (Th) can selectively inhibit the production of TNF-α in human monocytes. Although the mechanism by which Th reduces TNF-α production is still unclear, the drug seems to reduce the half-life of TNF-α. It has been shown that in addition to its inhibitory effect on the production of monocytes cytokines, Th exerts a costimulatory effect on T cell responses (Corral and Kaplan, 1999). The immune modulating and anti-inflammatory effects of Th in patients may thus be attributable to a balance between the inhibition of production of monocytes cytokines, including TNF-α and the costimulation of T cell activity.

Nuclear Factor-κ B (NFκB). Th also inhibits the activation of the nuclear factor-κ B (NF-κB), a promoter for the transcription of TNF-α (Marriot et al., 1998).

Fibroblast Growth Factor (FGF)-2. Fibroblast growth factor-2 is a ligand for its receptor tyrosine kinase on the cell membrane which in turn can activate a PLC-γ/PKC/raf-1/MAPKK/MAPK pathway or activate NF-κB which binds to DNA and causes the transcription of further proteins (Alberts et al., 1994; Gaubert et al., 2000). FGF-2 has been shown to play an important role in angiogenesis (the formation of new blood vessels from previously existing microvessels) (D´Amato et al., 1994) and also in limb

development (Li et al., 1996). Th has been shown to inhibit FGF-2 induced angiognenesis (D´Amato et al., 1994).

Insulin, insulin-like Growth factor (IGF)-1. Insulin, insulin-like growth factor (IGF)-1 also belongs to the receptor tyrosine kinase family and can activate the same pathways with FGF-2 (Alberts et al., 1994). Furthermore, it has been shown IGF-1 is also important in limb formation (Stephens et al., 1998) and can activate angiogenesis together with FGF-2 (Punglia et al., 1997). Thalidomide can inhibit angiogenesis stimulated by IGF-1 (Stephens et al., 2000).

Anti-cancer. Thalidomide has also recently been reported to exhibit anti-cancer activity through the inhibition of angiogenesis *in-vivo* which does not seem to be mediated by inhibition of TNF-α (Long et al., 1998; Figg et al., 1998; Fine et al., 1998; Eisen et al., 1998).

Adhesion Molecule Receptors. Extracellular matrix adhesion is also required in FGF-2 stimulated angiogenesis (Ingber and Folkman, 1989). On the other hand, it has already been shown that increased protein kinase C activity can further cause TNF-α–induced NF-κB binding to I-CAM-1 promoter and increase I-CAM-1 transcription (Rahman et al., 2000; Chen et al., 2000). Th can selectively modulate (mostly down regulate) the density of cell surface molecules involved in the adhesion cascade; I-CAM-1, V-CAM-1, E-selectin and L-selectin (Geitz et al., 1996), the same effect has also been obtained with EM 12, a potent teratogen derivative of Th (Neubert, 1996) but not non-teratogenic compounds (Thiel et al., 2000).

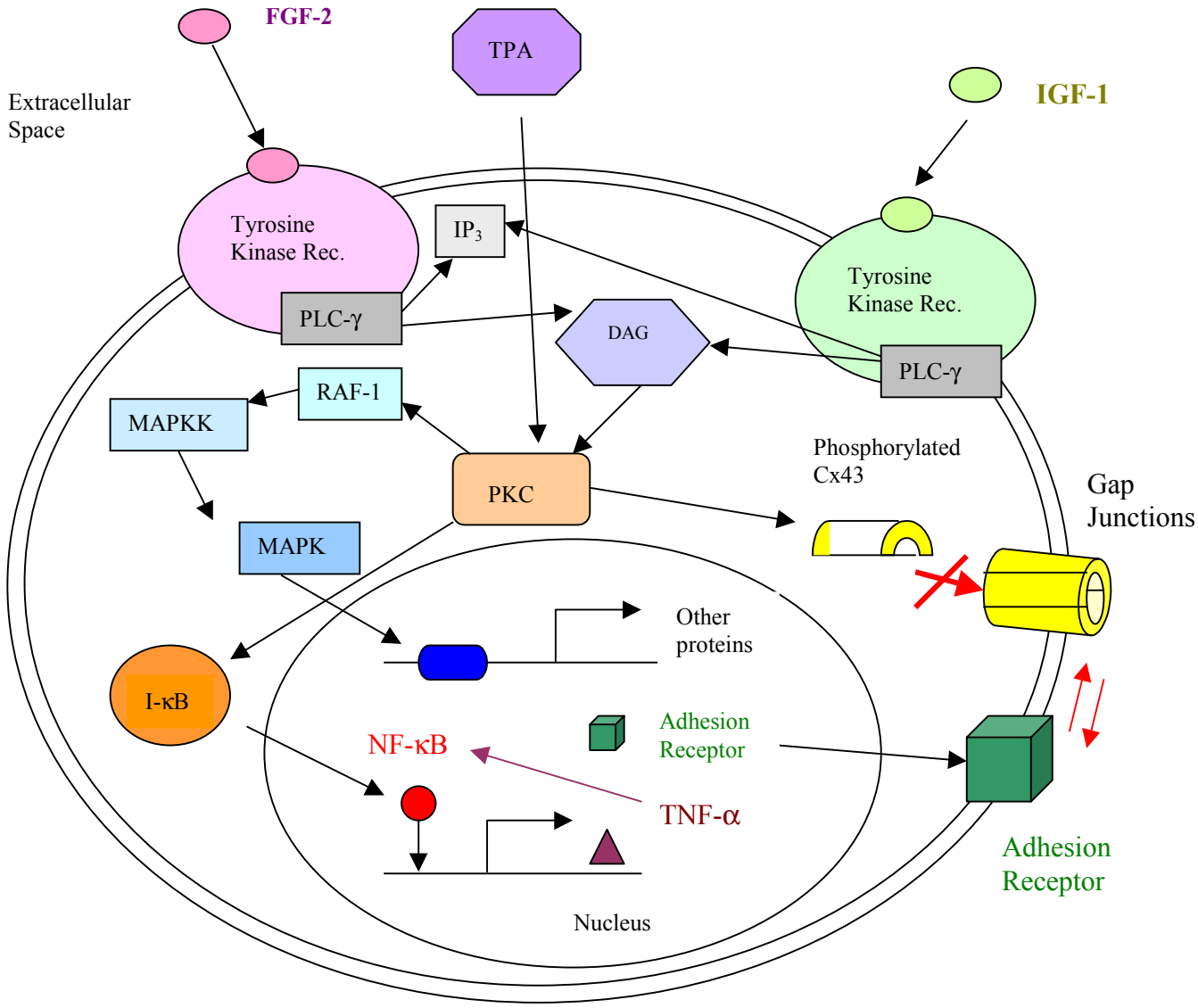


Figure 11. Hypothetical Signal Transduction Pathway Related to Thalidomide. Ligands (FGF-2, IGF-1) bind and activate receptor tyrosine kinases which in turn activate a wide range of pathways (some shown here). As a key protein kinase, activation of PKC can further cause inhibition of GJIC (Doble et al., 2000), translocation of NF-κB to the nucleus which produces subsequent transcription of TNF-α, adhesion receptors and some other proteins. Adhesion molecules can further interact with gap junctions on the membrane (Wang and Rose, 1997). The effects of thalidomide performed via TNF-α, FGF-2 and IGF-1 could be due to activation or inhibition of further protein cascades at any points which are still unclear.

Gap Junctional Intercellular Communication (GJIC). Recently, it has been shown that thalidomide can stimulate GJIC in the presence of liver microsomes *in-vitro*. The effect could not be performed by non-teratogen derivatives of compound (Nicolai et al., 1997). On the other hand, down regulation of GJIC has been reported due to increased levels of TNF-α in rat heart during inflammation (Fernandez-Cobo, 1999) and due to increased level of cadherins which are cell-cell adhesion molecules (Wang and Rose, 1997).

1.2.1.3. Clinical Applications of Thalidomide

Due to its anti-inflammatory, immunomodulatory and anti-angiogenic properties, thalidomide is currently being used clinically for more than a hundred indications, including mycobacterial diseases, dermatologic disorders, HIV/AIDS and related disorders, autoimmune diseases, cancer and associated disorders some of which are summarized in (Table 1).

Due to its anti-inflammatory and immunomodulatory effects mediated by degrading TNF-α mRNA (Corral and Kaplan, 1999) and reducing phagocytosis by leukocytes (Calabrase and Fleischer, 2000), thalidomide is very effective in the treatment of diseases, especially the ones associated with increased levels of TNF-α, such as leprosy with acute inflammatory lesions, graft-versus-host disease which is seen after bone-marrow transplations, rheumatoid arthritis, HIV-associated wasting syndrome (weight loss) or diarrhea. However, due to its anti-angiogenic properties, by inhibiting IGF-1 and FGF-2 (Stephens et al., 2000), thalidomide is shown to be effective for the treatment of several solid-tumor types, including breast, renal, ovarien, prostate cancer and gliomas (Table 1).

Table 1. **The Use of Thalidomide in the Treatment of Disorders**.

DISORDERS	Authors
DERMATOLOGIC DISORDERS	
Leprosy	(Iyer et al., 1971)
Leprosy associated erythema nodosum leprosum	(Iyer et al., 1971)
Chronic cuteneous lupus erythematosus	(Knop et al., 1983)
Behçet's disease	(Saylan and Saltik, 1982)
Aphthous stomatitis	(Mascaro et al., 1979)
Prurigo nodularis	(van den Broek, 1980)
CANCER	
Breast	(Long et al., 1998)
Ovarian	(Eisen et al., 1998)
Prostate	(Figg et al., 1998)
Gliomas	(Fine et al., 1998)
Renal	(Eisen et al., 1998)
HIV-RELATED CONDITIONS	
Primary HIV infection	(Makonkawkeyoon et al., 1993)
HIV-diarrhea	(Sharpstone et al., 1995)
HIV-tuberculosis	(Marriot et al., 1999)
HIV-associated aphthous ulcers	(Jacobson et al., 1997)
HIV-associated wasting syndrome	(Reyes-Terán et al., 1996)
HIV-associated Kaposi´s sarcoma	(Solér et al., 1996)
MISCELLANEOUS	
Tuberculosis	(Tramontana et al., 1995)
Rheumatoid arthritis	(Huizinga et al., 1996)
Graft-versus-host disease	(Saurat et al., 1988)

However, there are also severe adverse effects of Th such as its teratogenicity which will be discussed later and peripheral neuropathy. Peripheral neuropathy is a neurologic disorder associated with Th and characterized as symetrical, painful paresthesias of hands and feet, often accompanied by sensory loss in the lower limbs and

can be irreversible (Ochonisky, 1994). Dizziness due to its sedative effect, headache, weight gain, edema and nausea may also occur (Calabrase and Fleischer, 2000).

1.2.1.4. Teratogenicity of Thalidomide

For decades it was believed that the placenta served as a barrier that protected the fetus from adverse effects of drug. The thalidomide disaster drastically changed this idea, since even a single dose can cause severe birth defects, including phocomelia, a condition in which the long bones of limbs are absent (amelia) or severly deficient (peromelia), congenital heart defects, absence or small external ears or deafness, facial palsy, absent or shrunken eyes, cataract, ocular movement abnormalities, malformed intestines, kidney malformations, central nervous system defects and mental retardation. Among these malformations, limb malformations, craniofacial structures and ear malformations were the most frequently reported anomalies (Koren et al., 1998; Miller and Strömland, 1999; Stephens et al., 2000). The critical period of thalidomide-induced teratogenicity occurs between the 34^{th} and 50^{th} day after the last menstrual period (26-36 days postfertilization). Malformations of upper limbs are seen before those of the lower limbs, since the arms form slightly before the legs during development (Miller and Strömland, 1999).

To date, no definitive mechanism of action has been determined for the teratogenic activity. However, at least 30 hypothesis concerning the mechanism of action of this drug have been advanced. 13 of which have been found incorrect whilst some of the others are supported by data which are summarized in Table 2 (Stephens et al., 2000).

Thalidomide and its metabolites have been shown not to be genotoxic and do not have carcinogenic potentials (Zhu et al, 1999). Furthermore, none of the postulated mechanisms could explain alone the species specific teratogenicity of thalidomide, i.e. teratogenic to primates but not to rats or mice. However, as discussed in the previous section, the last five of the proposed mechanisms are of main current research interest and

it may be that many of them are correct and can fit into a unified model incorporating several mechanisms.

Table 2. Thalidomide Proposed Mechanisms of Action.

1.	B vitamin antagonism
2.	Interference with glutamic acid metabolism
3.	Complexes formed by polar thalidomide metabolites with essential cations
4.	Pteroylglutamic acid antagonism
5.	Nucleic acid synthesis
6.	Axial limb artery degeneration
7.	Cell death
8.	Direct effect on limb bud
9.	IGF-1 antagonist
10.	Intercalation into DNA
11.	Down regulation of TNF-α
12.	Inhibition of angiogenesis
13.	Regulation of adhesion molecules
14.	Cell-cell interactions

Modified from (Stephens, 1988; Stephens et al., 2000).

1.3. Aim of the Study

The present study investigates the stimulation of gap junctional intercellular communication (GJIC) by the potent human teratogens thalidomide and retinoic acid, and some of their analogs in different cell types originated from human, mouse and rat. Since thalidomide and retinoic acid share important characteristics, mainly concerning the similar malformations in embryo due to their teratogenicity and since GJIC is shown to be important in embryonic development, their effects on the regulation and stimulation of GJIC has been examined to clarify the following situations:

1. Effects of retinoic acid and its derivatives on GJIC, on transactivation of RARß2 promoters and on connexin 43 gene regulation.
2. Effects of thalidomide and its analogs on GJIC and a comparison of differences between human and rat cells to check species specificity of the effect.
3. The relationship between teratogenicity of compounds and their effects on GJIC.
4. A comparison of retinoic acid and thalidomide for their effects on GJIC.

2. Materials and Methods

2.1. Equipment and Materials

Balance	Sartorius, Göttingen
Cell culture materials	Greiner, Solingen
Centrifuges: Universal 30 RF Bench centrifuge 5415C RC5B Plus (GSA and SM24 Rotors)	 Hettich,Tuttlingen Eppendorf, Hamburg, Sorvall, Bad Homburg
Electrophoresis chamber	Biorad, München
ELISA-Reader Victor 1420 multilabel counter	Wallac Oy, Turku, Finnland
Femtotips	Eppendorf, Hamburg
Fluorescence microscopy (Axiovert)	Zeiss, Köln
Fluorescence microscopy (Typ IM 35)	Zeiss, Köln
HPLC-Pump	Merck, Darmstadt
Incubator	Heraeus, Osterode
Integrator	Merck, Darmstadt
Microinjector (Typ 5242)	Eppendorf, Hamburg
Micromanipulator (Typ S 170)	Eppendorf, Hamburg
Spectrophotometer	Perkin Elmer, Überlingen
Sterile filter	Sartorius, Göttingen
UV/VIS Detector	Merck, Darmstadt
Voltage supplier	Biorad, München

2.2 Chemicals

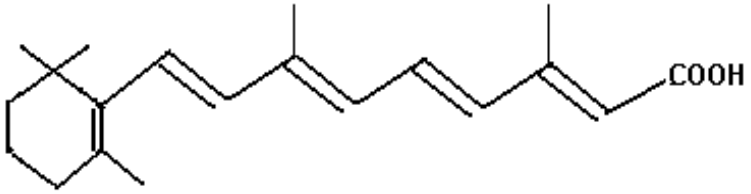
(-)-S-Thalidomide	Sigma, Deisenhofen
(+)-R-Thalidomide	Sigma, Deisenhofen
(+/-) Thalidomide	Sigma, Deisenhofen
2-Phthalimidoglutaric acid	Sigma, Deisenhofen
13-*cis*-retinoic acid	Sigma, Deisenhofen
2-Nitrophenyl-ß-D-galactopyranoside	Fluka, Neu-Ulm
All-*trans*-retinoic acid	Sigma, Deisenhofen

Antibodies: Alexa 546 goat anti rabbit Ig G (H+L) Rabbit anti-connexin 43	 Molecular Probes, Göttingen Zymed, California, USA
db-cAMP	Boehringer, Mannheim
DMEM-Medium	Sigma, Deisenhofen
Dotap	Promega, Mannheim
E.coli K12-HP101	Promega, Mannheim
F9 cells (No.85061803)	EACC, Salisbury, GB
FAD	Boehringer, Mannheim
Fetal Calf Serum	Greiner, Solingen
Gentamicin solution	Sigma, Deisenhofen
HFFF2 cells (No.86031405)	ECACC, Salisbury, GB
Lucifer Yellow CH dilithium	Sigma, Deisenhofen
Molecular weight marker III and VI	Boehringer, Mannheim
MTT	Sigma, Deisenhofen
NAD^+	Boehringer, Mannheim
NADH	Boehringer, Mannheim
$NADP^+$	Boehringer, Mannheim
NADPH	Boehringer, Mannheim
Normal goat serum	Vector, California, USA
Plasmid pCH110	Pharmacia Biotech, Freiburg
Plasmid pCH110	Pharmacia Biotech, Freiburg
Reporter lysis buffer	Promega, Mannheim
Restriction enyzme buffers (B, H and M)	Promega, Mannheim
Restriction enyzmes (Eco RI, PST I, Hind III and PVU II)	Promega, Mannheim

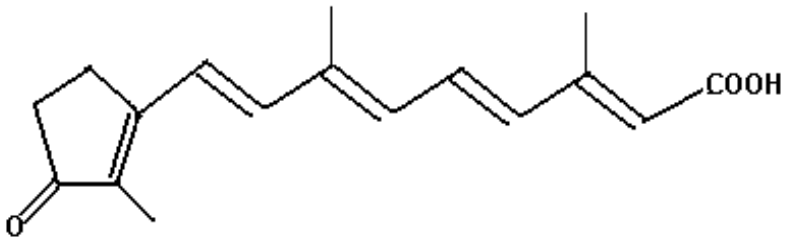
1,1-dimethyl-2-nor-4 oxo-retinoic acid, 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid, 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid, 4-oxo-13-*cis*-retinoic acid, 4-oxo-11, 13-*cis*-retinoic acid, 3-hydroxy-retinoic acid were kindly provided by Dr. Paust (BASF). EM 12, EM 16, EM 20, EM 138 and EM 364 were kindly provided by Grünenthal, Stolberg. F9RARβlacZ cells were a kind gift from Dr. P. van der Saag. All other chemicals were purchased from Merck, Darmstadt.

2.3. Structures of the Test Substances

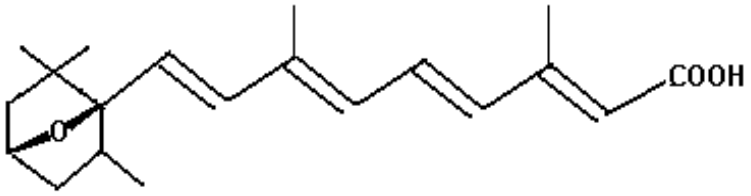
2.3.1. Retinoic Acid and its Derivatives



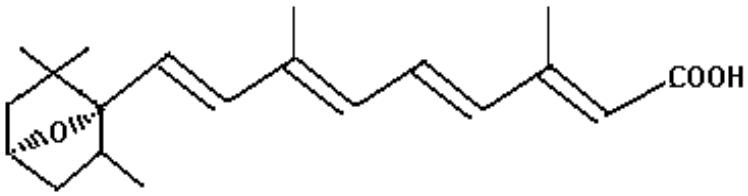
All-*trans*-retinoic Acid



1,1-didemethyl-2-nor-4-oxo-retinoic acid

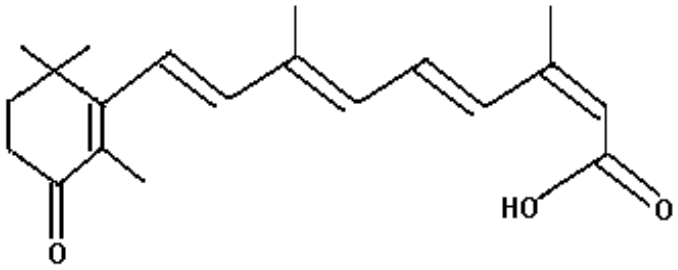


3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid

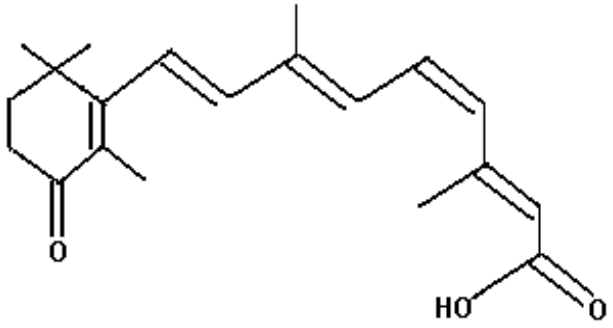


3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid

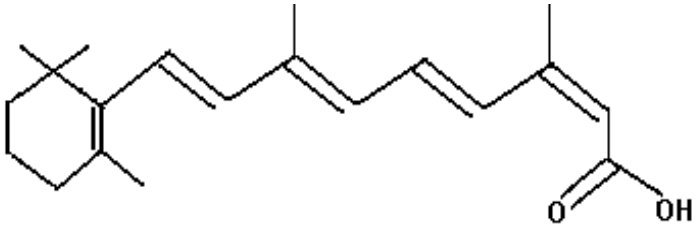
Figure 12. Retinoic Acid and its Derivatives



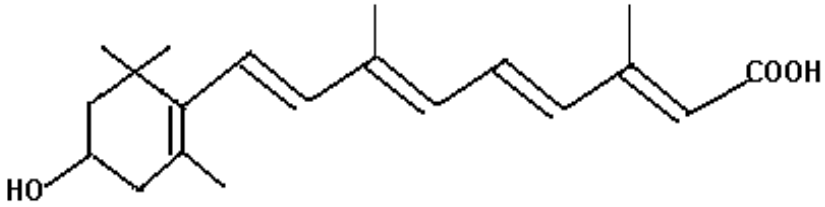
4-oxo-13-*cis*-retinoic acid



4-oxo-11-*cis*, 13-*cis*-retinoic acid



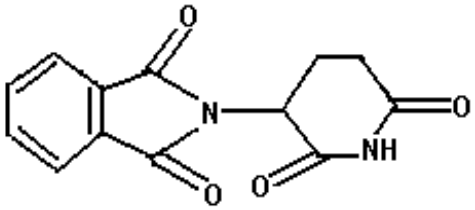
13-*cis*-retinoic acid



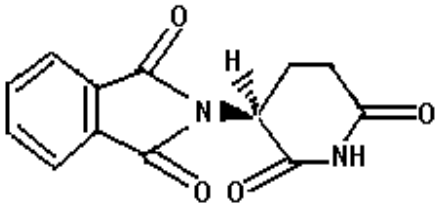
3-hydroxy-retinoic acid

Figure 13. Retinoic Acid and its Derivatives

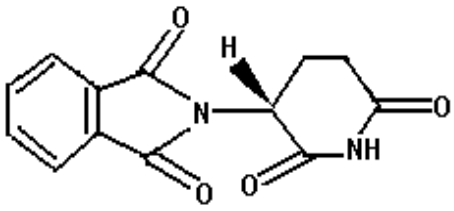
2.3.2. Thalidomide and its Analogs



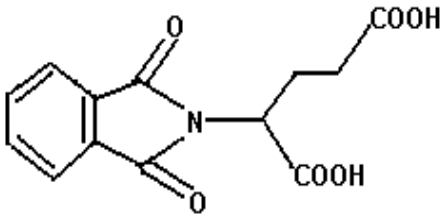
(+/-) Thalidomide



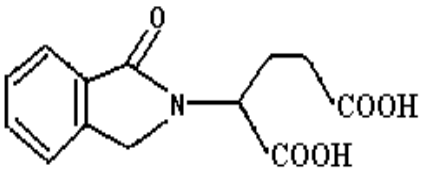
(-)-S-Thalidomide



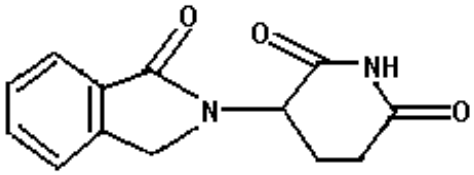
(+)-R-Thalidomide



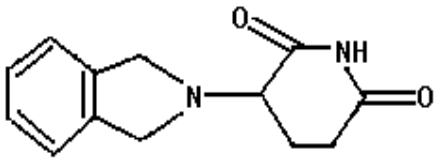
2-Phthalimidoglutaric acid



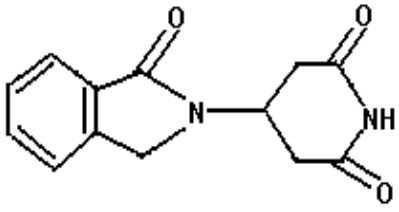
EM 138



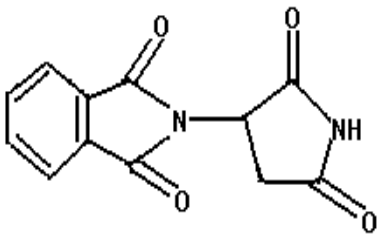
EM 12



EM 364



EM 16



EM 20

Figure 14. Thalidomide and its Analogs

2.4. Stock Solutions

Substances	Concentration	Solvent
2-Nitrophenyl-ß-D-galactopyranoside	0,01 M	0.1 M Na_2HPO_4 Buffer
BSA	0.5 mg/ml	0.9% NaCl
db-cAMP	10 mM	H_2O
FAD	10 mM	H_2O
MTT	5mg/ml	PBS
NAD^+	10 mM	H_2O
NADH	10 mM	0.1% $NaHCO_3$
$NADP^+$	10 mM	H_2O
NADPH	10 mM	0.1% $NaHCO_3$
Retinoic acid and related compounds	1 mM	Ethanol or THF
Thalidomide and related compounds	10 mM	THF

FAD, NAD^+, NADH, $NADP^+$ and NADPH are kept at 4 °C for a maximum of 2 weeks, whereas others were stored at -80 °C.

2.5. Cell Culture

2.5.1 Characterisation of Cells used in Assays

HFFF2

These cells are human skin fibroblasts (HFFF2) obtained from 14-18 week old fetuses. These cells were used in the assay until the 15th passage.

WB-F344

Rat liver epithelial cells (WB-F344) are a diploid cell line from phenotypically normal liver of an adult rat (Tsao et al., 1984). They show a high level of connexin 43 expression (Zhang and Thorgeirsson, 1994). These cells were used in the assay until the 15th passage.

F9

The mouse teratocarinoma cell line was obtained from 4 day old embryoes (Guenet et al., 1974). These cells were used in the assay until the 20th passage.

F9RARβlacZ

This reporter cell line arose from stable transfection of F9 cells with a gene construct of RARβ2-promoter of mouse and lacZ (β-galactosidase) reporter gene (Shen et al., 1992). These cells were used in the assay until the 20th passage.

2.5.2. Subcultivation of the Cells

Cells were grown in Dulbecco´s modification of Eagle´s minimal essential medium (DMEM) supplemented with 2 mM L-glutamine, 0.02 g/l gentamicyn and 10% (v/v) foetal calf serum (FCS). FCS was heat-inactivated before supplementation for 30 minutes at 56 °C aliquoted and stored at -20 °C. All throughout the experiments, standard, sterile cell culture techniques were performed. After cells were grown as a single monolayer in a humidified atmosphere of 5% CO_2 at 37°C, they were washed first with PBS to remove excess medium from the environment. Then cells were detached by trypsinisation with Trypsin-EDTA solution (30% in PBS). Trypsin was removed from the environment and inactivated by adding DMEM containing 10% (v/v) FCS. Cells were centrifuged at 500 x g for 5 min and seeded in new culture flasks of 75 cm ². Confluent cells were passaged 1:3 in the case of HFFF2 cells, 1:5 in the case of WB-F344 cells and 1:7 for F9 and F9RARβlacZ cells. Teratocarcinoma cells were grown on a film of 1% gelatine solution.

2.5.3. Freezing and Thawing of the Cells

After trypsinisation, cells were resuspended in complete medium containing 10% FCS (v/v) and 10% DMSO. The resulting suspensions were dropped into cryovials in a way to

create three vials from each flask and frozen at -70 °C for 24 hours to make cells ready for further freezing and then frozen cells were stored in liquid nitrogen to have cell stocks.

To obtain new cultures from frozen cell stocks, cells were thawed quickly at 37°C and immediately transferred to a 75 cm² culture flask containing complete medium supplemented with 10% (v/v) FCS. To remove DMSO, the medium was changed with fresh medium after 24 hours.

2.5.4. Incubation with Test Compounds

All cell types were incubated with test compounds in a humidified atmosphere of 5% CO_2 at 37 °C.

2.5.4.1. Incubation Conditions for Gap Junctional Intercellular Communication

For the cell communication assay, cells were grown on petri dishes with a diameter of 35 mm until they reached 70-90% confluence. They were made quiescent by keeping them in complete medium containing no FCS for 24 hours (serum deprivation) to decrease the basal level of cell communication. Compounds to be tested were added to the complete medium containing no FCS at the indicated concentrations for WB-F344 cells and added to the medium containing 3% (v/v) FCS in the case of HFFF2 cells. Cell were incubated with this incubation medium for 24 and 72 hours at 37 °C. For each experiment a control group was carried out either in the presence of 0.2% ethanol or 0.4% of THF according to the stock solutions of test compounds.

2.5.4.2. Incubation Conditions for Examination of Connexin 43 Gene and Affinity to RARß2-Promoter

F9 and F9RARβlacZ cells were seeded on the petridishes one day before the incubation and throughout the whole experiment incubated in complete medium containing 10% (v/v)

FCS. Test compounds were added to the medium and cells were treated for 24 hours. For the measurement of β-galactosidase activity in F9RARβlacZ cells, 50 μM db-cAMP was added to the incubation medium together with test compounds (Kruyt et al., 1992, Martin et al., 1990). Light sensitive compounds were used and kept away from day light during the experiments (Murayama et al., 1997). For each experiment a control group was carried out either in the presence of 0.2% ethanol or in 0.4% THF due to the stock solutions of test compounds used to treat these cells.

2.5.4.3. Incubation Conditions for Immunohistochemistry Assays

For immunohistochemistry assays, WB-F344 cells were grown on coverslips in petri dishes with a diameter of 35 mm until they reached 70-90% confluence. They were made quiescent by keeping them in complete medium containing no FCS for 24 hours (serum deprivation) to decrease the basal level of cell communication. After treatment of cells for 24 hours with EM 364 (10 μM) or EM 16 (10 μM) in the complete medium containing no FCS, TPA or staurosporine (each 100 nM in DMSO) were added to the indicated dishes prior 30 and 60 minutes to the assay. Staurosporine was used as positive control. For each experiment a control group was carried out in the presence of DMSO as negative control.

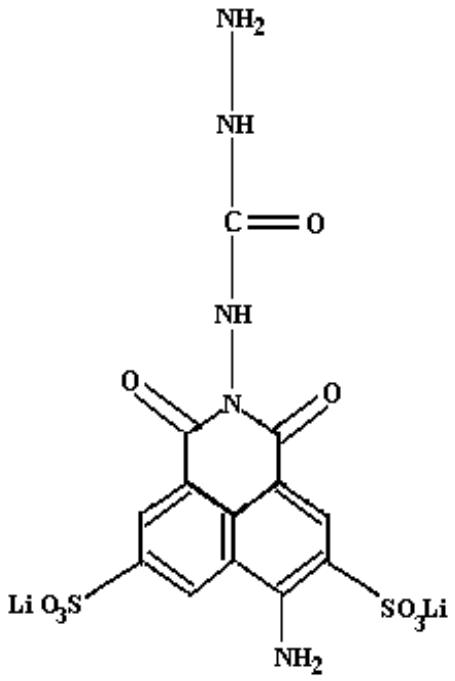
2.6. Gap Junctional Communication Assay

Gap junctional intracellular communication (GJIC) was performed by the efficacy of fluorescent dye, Lucifer Yellow CH, from a single microinjected cell to its neighbouring cells. The movement of dye from cell to cell via the gap junctions, termed "dye-coupling" occurred between the cells known to be electrically coupled. Intact cell membranes are impermeable to this water soluble dye due to its sulphanate groups (Figure 15 a) which have very low pK values and fully ionized at physiological pHs. The excitation

wavelength of Lucifer Yellow CH is at 426 nm whereas emission maximum is at 540 nm (Figure 15 b). Unlike other dyes, it has a constant quantum yield (the ratio of photons emitted to photons absorbed) between pH 1 and 10 (Steward, 1978; Nicolai et al., 1997).

The measurement of GJIC was performed with a combination of equipment consisting of a fluorescence microscopy, micromanipulator and microinjector. 50-200 femtoliter of Lucifer Yellow CH (10% in 0.33 M LiCL w/v) were loaded to the femtotips and dye is injected to a single cell for a duration of 0.5 seconds under 50 hPa of pressure. Dye-coupled cells were counted 5 minutes after the injection and the mean values of 10 individual injections were determined (Figure 16).

A)



B)

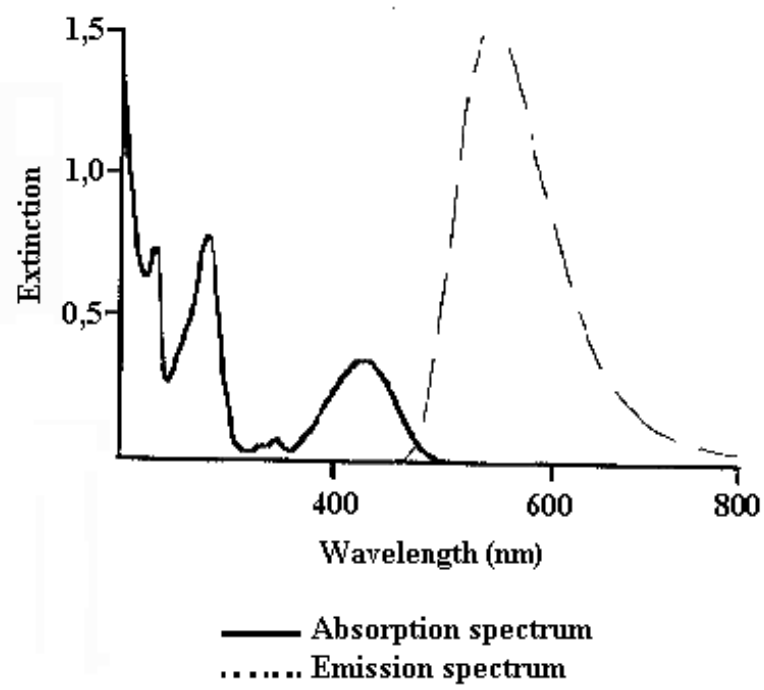
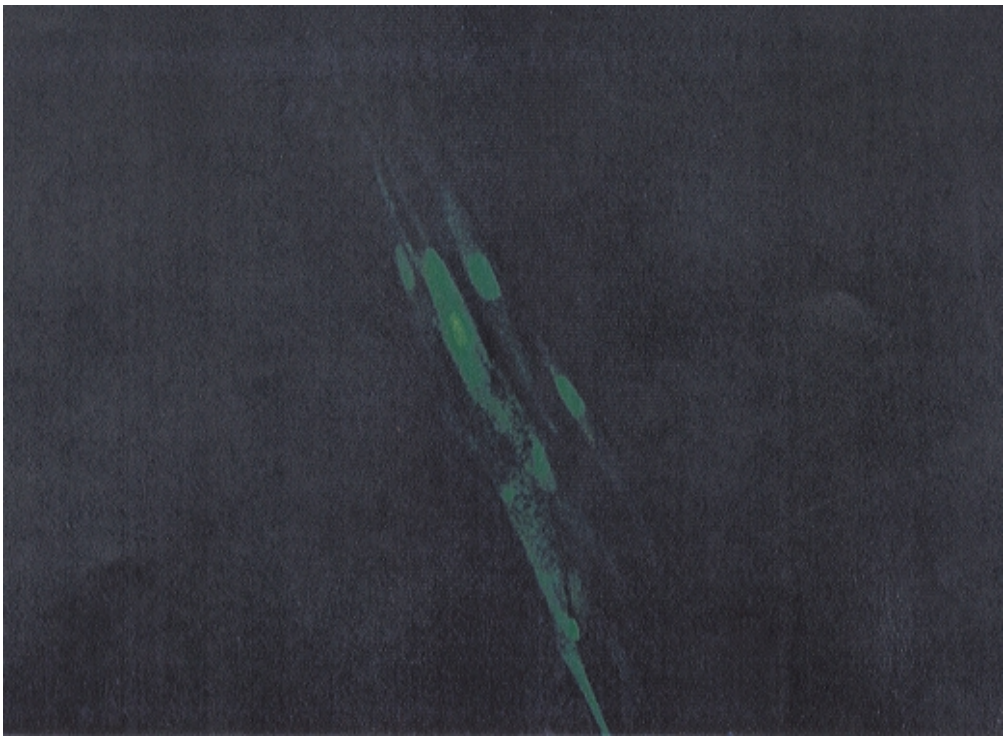


Figure 15. Structure, Absorption and Emission Spectrum of Fluorescence Dye Lucifer Yellow CH. Structure (A), absorption and emissions spectrum (B) of Lucifer Yellow CH (Steward, 1978).

A)



B)

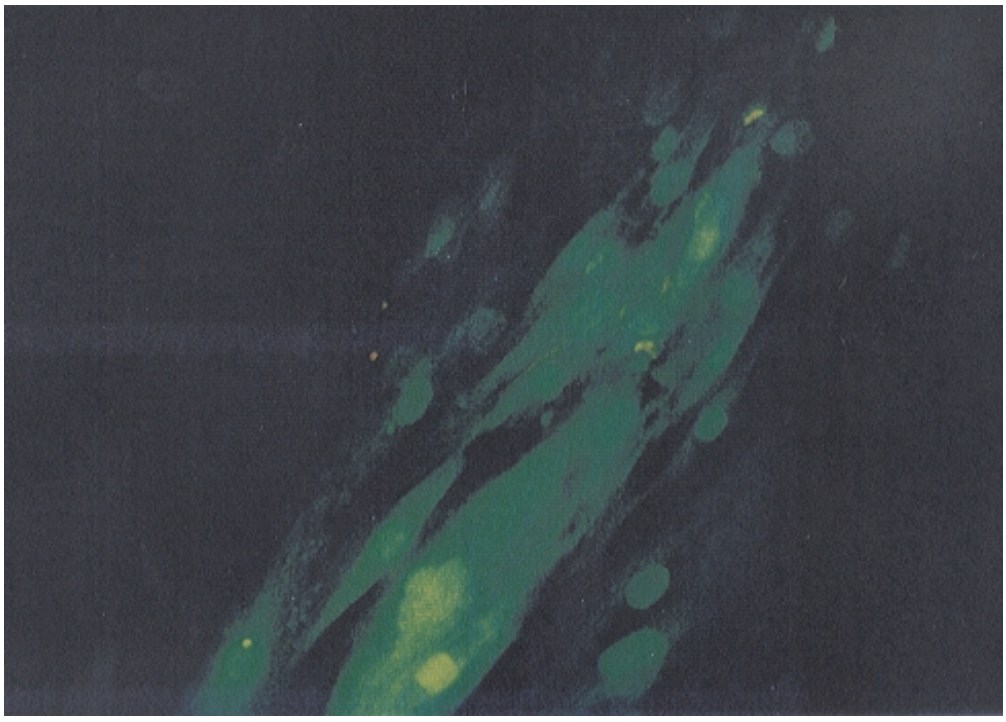


Figure 16. Demonstration of Gap Junctional Intercellular Communication in Human Skin Fibroblasts (HFFF2) In controls (**A**), cells are communicating less, whereas cells treated with appropriate compounds show an increased gap junctional intercellular communication (**B**).

2.7. Examination of the Connexin 43 Gene

2.7.1. Transformation of *E Coli* by Plasmids and Storage of Plasmids

Bacteria cells were grown in LB medium (1% trypton, 0.5% yeast extract, 0.5% NaCl) overnight and after incubation on ice for 15 minutes, they were centrifuged for 13 minutes at 2500 rpm at 4 °C. The pellet was resuspended in $CaCl_2$ 50 mM. After incubation on ice for 15 minutes, they were centrifuged for another 13 minutes at 2500 rpm at 4 °C. Pellets were placed on ice for 15 minutes and immediately transferred to –70 °C to store as compotent cells. For transformation, compotent cells were thawed on ice and 1% (v/v) of plasmid were added to bacteria cells. Then cells were incubated for 1 hour on ice and 60 seconds at 42 °C. Then they were replaced on ice again for 2 minutes. After addition of 700 µl of LB medium, cells were incubated at 37 °C for 1 hour. Then cells were distributed on petri dishes containing 1.8% agar in LB medium supplemented by 100 µg/ml ampiciline. After overnight incubation, one of the surviving colonies was selected and replaced in LB medium containing 1% ampiciline and incubated overnight. Next day, plasmids were isolated and checked by a restriction enzyme cut assay as described above to see the efficacy of transformation.

This procedure was repeated many times for different colonies from petri dishes until the best result obtained for each single plasmid. Then bacteria producing the best results were stored in 10% gylcerol.

2.7.2. Plasmid Isolation Assay

On the first day of the experiment, 0.05% (v/v) of bacteria stocks containing different plasmids were added to LB bacteria medium containing 10% (v/v) ampiciline solution. After overnight incubation at 37 °C, plasmids (CxlucCx, CxlucSV40, PT81 and CMVlucSV40) were separately isolated according to the instructions of the Qiagen Plasmid Purification Assay system. Bacteria grown overnight in LB medium were

centrifuged at 4 °C for 10 minutes at 4000 rpm. The pellet was resuspended at a solution of 50 mM Tris HCl pH 8.0; 10 mM EDTA and 100 µg/ml RNAse A. After 5 minutes, the solution was neutralised with potasiumacetate buffer pH 5.5 to remove free chromosomal DNA. Plasmid-DNA remained in the solution. After centrifugation at 4 °C for 30 minutes at 3500 rpm, particle free supernatant containing plasmid was poured through a specific column which had been already equilibrated. Only plasmid-DNA was bound to the column while the artefacts consisting of RNA, protein etc. are washed out through the column with the wash buffer (1 M NaCl, 50 mM MOPS pH 7.0 and 15% Isopropanol). Plasmid-DNA was eluted from the column by the help of buffer solution of 1.25 M NaCl, 50 mM Tris pH 8.5 15% Isopropanol. After incubation plasmid-DNA at room temperature by 70% isopropanol, it was centrifuged at 4 °C for 30 minutes at 15000 rpm and resulting pellet was dried out by air. Pellet was resuspended by $_dH_2O$ and examined for purity at 260 nm and 280 nm spectrophotometrically.

2.7.3. Treatment with Restriction Enzymes and Gel Electrophoresis

Isolated plasmids were treated by proper restriction enzymes to check whether they are reliable for further assays or not. For this aim, plasmids were cut by related restriction enzymes; Eco RI, PST I, Hind III and PVU II in the presence of related buffers by incubating for 1 hour at 37 °C. Then gel electrophoresis was performed by using 1% (w/v) agarose in TBE buffer (89 mM Tris, 89 mM boric acid. 2 mM EDTA). After 2 hours of running at 110 volt, the gel was washed in an EtBr bath and photographed. The plasmid providing the relevant bands were chosen for the further experiments.

2.7.4. Transient Transfection and Reporter Gene Assay with Luciferase Gene

The insertion of foreign DNA to cells of higher organisms is called transfection. Since the inserted DNA will not be integrated in the DNA of the host cell, it can not be replicated extra-chromosomally and will be lost upon further multiplication of the host cell. As a result it is called transient transfection. By the help of liposomal transfection reagent, DOTAP, foreign DNA forms a stabile complex which can be given directly to the incubation medium.

For the assay, F9 cells were grown by cell culture procedures and seeded on 60 mm petri dishes and after 24 hour, cells were transfected by adjusting DOTAP-reagent mixed with 2.5 µg of the plasmids in the presence of HBS buffer (20 mM Hepes, 5 M NaCl, pH 7.4). After 24 hours, test compounds were added to the fresh culture medium containing 10% FCS. After 24 hours incubation with the compounds, cells were harvested and luciferase activity was determined from cell extracts using luciferase assay system (Promega, Madison).

To prove the efficiency of transfection, cells were co-transfected by plasmid pCH110 which has SV40 promoter region coupled with lacZ gene, which encodes for ß-galactosidase. On the other hand, the SV40 promoter is not activated by the test compounds. Since efficiently cells contains both luciferase and ß-galactosidase genes, it is possible to check luciferase and ß-galactosidase activities at the same time. For this aim, collected samples were treated separately with luciferase test reagent, luciferin (Figure 14) and ONPG solution containing 10 mM MgCl, 100 mM NaCl and 50 mM Tris, pH 7.9. According to the calculated efficiency of transfection, luciferase activity obtained for each sample was determined.

To analyse the effect of retinoic acid and its different derivatives on the 5´-and 3´-regulatory elements of connexin 43 gene, plasmids having the coding sequence of luciferase gene, which was 5´-flanked by different promoter sequences and 3´-flanked with

either connexin 43 3′- untranslated region (UTR) or SV40 sequences, were used according to the previous studies (Clairmont et al., 1997) (Figure 17). The plasmid pT81luc is 5′-flanked by the thymidine kinase (TK) promoter and 3′-flanked by SV40 sequences. It was used as negative control. The plasmid pCMVlucSV40, which contains the cytomegalievirus (CMV) promoter holding the retinoic acid responsive element sequence and which is 3′-flanked by SV40 sequences, was used as positive control. On the other hand, pCxlucCx and pCxlucSV40 have connexin 43 promoter from rat. To examine the effect of test compounds on the 3′-UTR of connexin 43 gene, pCxlucCx was 3′-flanked by mouse connexin 43 3′-UTR whereas pCxlucSV40 was 3′-flanked by SV40 sequences. The luciferase activity obtained for these last two plasmids was compared to clarify the effect of test compounds on 5′-and 3′-regulatory elements of connexin 43 gene in the presence of positive and negative controls.

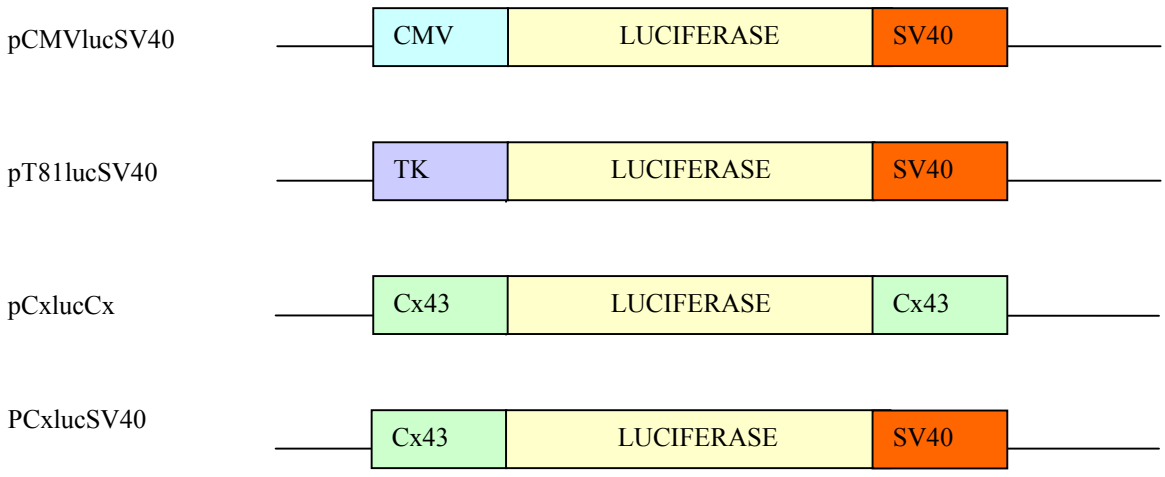


Figure 17. The Plasmids used for the Transient Transfection Assays.

2.8. Measurement of the Effects of Retinoic Acid and its Analogs on the RARβ2-Promoter

The F9-RAR-β2-LacZ reporter cell line contains the RAR-β2-promoter sequence fused with lacZ reporter gene (Nikawa et al., 1995). The retinoids bind to the retinoic acid receptors (RAR or RXR) which further bind as heterodimers to the retinoic acid responsive element (RARE) in the RARß2-promoter region. This will lead to the transcription of lacZ reporter gene. The resultant reporter gene product, ß-galactosidase, was isolated and measured according to previous studies (Rosenthal, 1987, Wagner, 1997) (Figure 18).

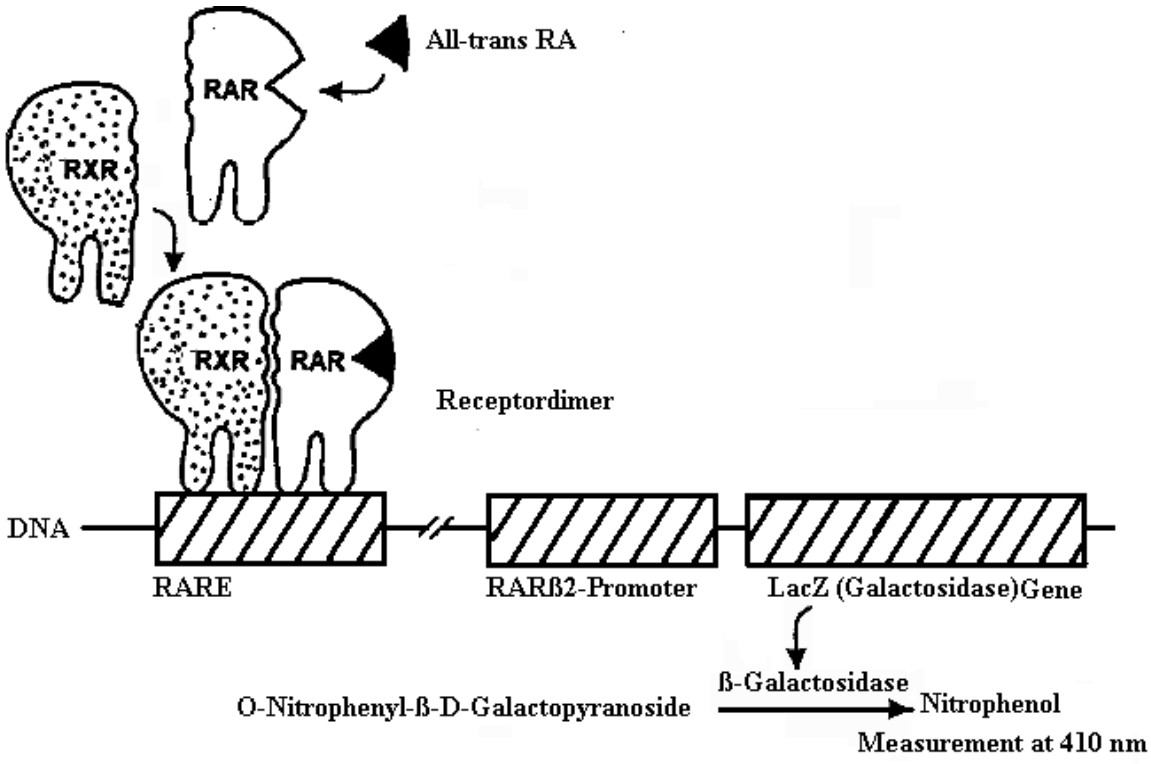


Figure 18. Mechanism of the F9-RARß2-LacZ Test

2.8.1. Harvesting the Cell Extracts for β-Galactosidase Activity Assay

After 24 hours of treatment with the test compounds, F9-RARß2-lacZ cells were washed three times with cold PBS, removed from the dishes and transferred into reagent tubes. After centrifugation for 2 min at 1000 rpm at 4 °C, the pellet was resuspended in 250 mM Tris-HCl, pH 8.0 and the suspension was stored at –80 °C until analysis. For the analysis the suspension was thawed and sonificated for 30 s. After centrifugation for 3 min at 4000 rpm at 4 °C, the supernatant was used for protein measurement and determination of ß-galactosidase activity.

2.8.2. β-Galactosidase Activity Assay

To measure the enzyme activity in the supernatant, 20 µl of supernatant was added to the reaction mixture of 3 µl magnesium-mercaptoethanol-buffer (0.1 M MgCl, 5 M mercaptoethanol), 66 µl of ONPG solution (0.01 M ortho-nitrophenyl-ß-D-galactopyranoside in 0.1 M Na_2HPO_4 -buffer, pH 7.3) which was finally added up to 300 µl with Na_2HPO_4 -buffer, (0.1 M Na_2HPO_4 -buffer, pH 7.3). After the incubation of the reaction mixture at 37 °C for 1 hour, the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 solution. The amount of o-nitrophenol formed in the enzymatic reaction was determined spectrophotometrically at 410 nm. The blank contained 20 µl of 250 mM Tris-HCl, pH 8.0 instead of supernatant from cell extracts (Rosenthal, 1987).

2.8.3. Protein Measurement

Protein concentration was measured in accordance with the method of Bradford (Bradford, 1976). Bovine-serum-albumin (BSA) solution (50 mg/100ml of 0.9% NaCl) at 0.1; 0.2 and 0.3 mg/ml dilutions were used to create a standard curve of the protein concentration. To measure the protein concentration different dilutions of each sample in 0.9% NaCl was added to the 1 ml of Coomassie Brilliant Blue G 250 solution (0.005% in 8.4% phosphoric

acid and 4.75% ethanol). After incubation at room temperature for 10 min, absorbance at 595 nm was measured for each sample and compared to the standard curve of BSA.

2.9. HPLC and Spectrophotometry to check the Purity of Compounds

Test compounds were checked by HPLC for the purity. At the same time, these compounds were also checked by spectrophometry between 220-520 nm to check absorbance patterns of compounds between these wavelengths.

On the other hand, isolated plasmids were checked by spectrophotometry at 260 nm and 280 nm to see the purity of DNA.

2.9.1. Retinoic Acid

Column: Suplex pKb 100 (250x4.6 mm) 5µm

Precolumn: Suplex pKb 100 (20x4.6 mm) 5µm, Supelco, PA, USA

Running solution: acetonitrile/methanol/water/acetic acid (64.5:20:15:0.5)

Flow: 1ml/min

Detection: 350 nm

Injection volume: 50 µl

HPLC was performed according to previous studies (Sundquist et al., 1993).

2.9.2. Thalidomide

Column: Supelcogel TPR-100 (150x4.6 mm) 5µm

Precolumn: Supelcogel TPR-100 (20x4.6 mm) 5µm

Running solution: acetonitrile/water/ acetic acid (20:79.5:0.5)

Flow: 1ml/min

Detection: 220 nm

Injection volume: 50 µl

Stock solutions were prepared in methanol as 10 mM concentrations.

2.10. Immunohistochemistry

Treated cells were washed with PBS containing 0.3% Triton X-100 three times, they were fixed by 2 ml of absolute methanol for 10 min at –20 °C. After washing with PBS containing 0.3% Triton X-100 three times, cells were blocked with 3% normal goat serum (NGS) in PBS containing 0.3% Triton X-100 for 30 min at room temperature. They were incubated by primary antibody for connexin 43 (diluted to 1:1500 in PBS with 1 % NGS) overnight at 4 °C. After washing with PBS containing 0.3% Triton X-100 three times, cells were incubated with appropriate fluorescent secondary antibody (diluted to 1:800 in PBS with 1 % NGS) for 1 hour at 37 °C. After three times washing, they were embedded with a drop of Immunomount (Shandon). Images were obtained with a Zeiss Axiovert coupled to a camera (ORCA II, Hamamatsu).

2.11. Evaluation of Cytotoxicity

The cytotoxicity of compounds were assayed with the MTT reduction assay with minor modifications (Carmichael et al., 1987). Cells were grown in six-well plates to near confluent, then the growth medium was replaced by phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and supplemented with the test compounds. After incubation for 2 h at 37 °C, cells were washed with PBS and incubated for 24 h in growth medium before the MTT test. MTT at a final concentration of 1.2 mM was added to each well; after incubation for 2 h at 37 °C, 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, compared with the appropriate control. Data were obtained from 3-4 separate incubations for each compound and dose used.

2.12. Statistical Analysis

Student *t-test* (one-tailed) was used to determine the statistical differences between the treated and the control groups by Microsoft Excel (version 7.0).

* $P<0,05$

** $P<0,01$

*** $P<0,001$

3. RESULTS

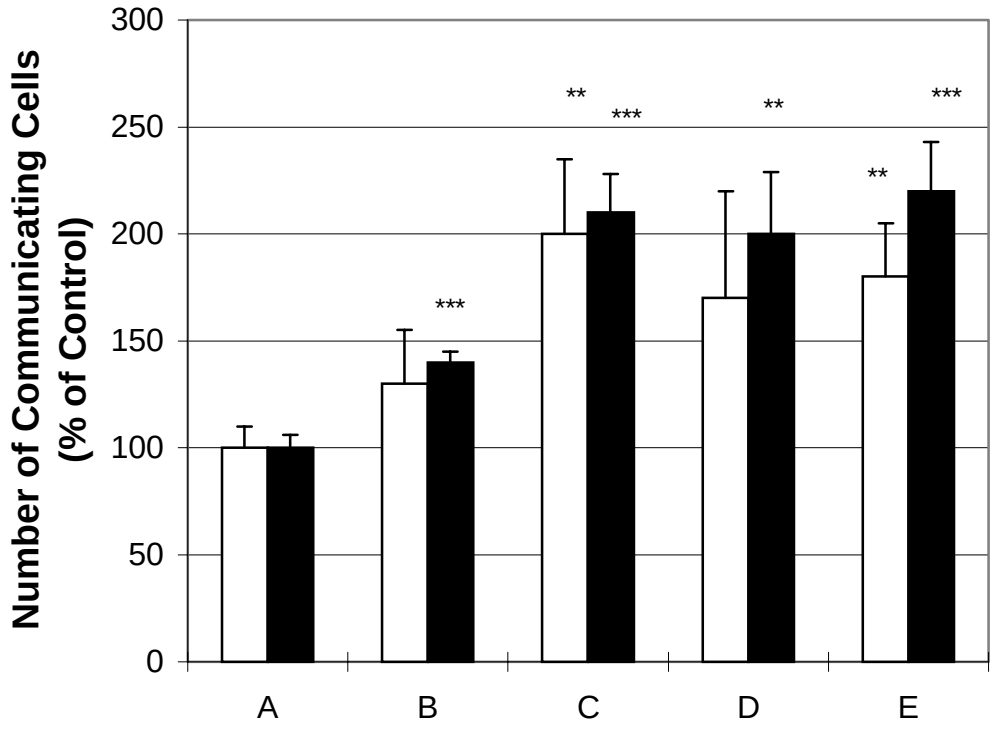
The structures of the compounds investigated in the present study are shown in Figures 12-14.

3.1 Retinoic Acid

3.1.1. Induction of Gap Junctional Intercellular Communication by Retinoic Acid and its Derivatives

The influence of retinoic acid and its derivatives on GJIC were tested in human fetal skin fibroblasts (HFFF2) on day 1 and 3 of incubation. Enantiomers, 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid and 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid significantly stimulated gap junctional intercellular communication (GJIC) up to 2.0-fold on day 1 and up to 2.1-fold on day 3 of incubation in comparison to the control. 1,1-didemethyl-2-nor-4-oxo-retinoic acid, which is a five-membered ring derivative of retinoic acid, was able to induce GJIC only up to 1.4-fold over the control on day three of incubation. On the other hand, 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid was more effective enantiomer to stimulate GJIC. All-*trans*-retinoic acid itself induced GJIC up to 2.2-fold compared to the control on day 3 of incubation (Figure 19).

A potent human teratogen 13-*cis*-retinoic acid and its 4-oxo metabolites; 4-oxo-13-*cis*-retinoic acid and 4-oxo-11, 13-*cis*-retinoic acid were tested together with 3-hydroxy-retinoic acid and all-*trans*-retinoic acid for their effect to stimulate GJIC in HFFF2 cells. 4-oxo-11,13-*cis*-retinoic acid was the most effective derivative stimulating GJIC up to 1.7-fold over the control on day 1 of incubation, which is followed by 13-*cis*-retinoic acid (1.6-fold) and 3-hydroxy-retinoic acid (1.5-fold). However, on day 3 of incubation, 3-hydroxy-retinoic acid was the most effective derivative and stimulated GJIC up to 1.6-fold in comparison to the control. On the other hand, 4-oxo-13-*cis*-retinoic acid could only stimulate GJIC up to 1.3-fold on day 3 and was not effective on day 1 of incubation (Figure 20).



A) Ethanol (0.2%)

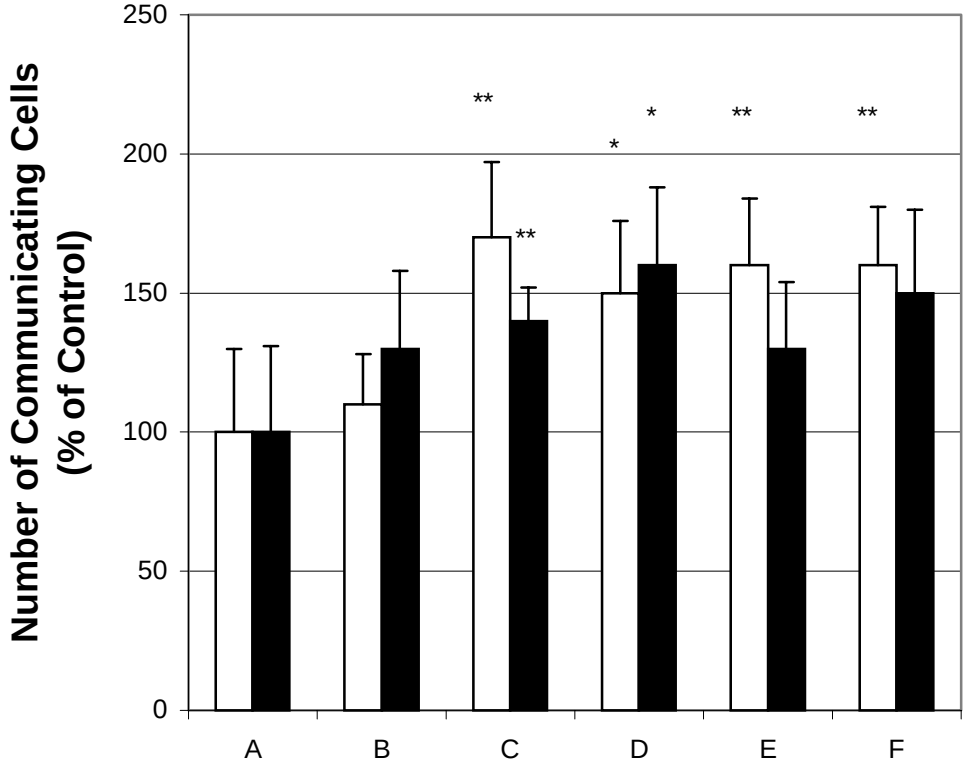
B) 1,1-didemethyl-2-nor-4-oxo-retinoic acid (1μM)

C) 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1μM)

D) 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1μM)

E) All-*trans* retinoic acid (1μM)

Figure 19. Induction of Gap Junctional Communication in HFFF2 Cells by Retinoic Acid and its Derivatives. The number of communicating cells incubated with test compounds were determined and indicated as induction of gap junctional communication measured on day 1 (□) and 3 (■) of incubation.. The absolute number of communicating cells in controls (30.5 ± 5.5) was set at 100%. Data represent means ± SD (n=4) (**P<0.01,*** P<0.001).



A) Ethanol (0.2%)

B) 4-oxo-13-*cis*-retinoic acid (1µM)

C) 4-oxo-11, 13-*cis*-retinoic acid (1µM)

D) 3-hydroxy -retinoic acid (1µM)

E) 13-*cis*-retinoic acid (1µM)

F) All-*trans* retinoic acid (1µM)

Figure 20. Induction of Gap Junctional Communication in HFFF2 Cells by Retinoic Acid and its Derivatives. The number of communicating cells incubated with test compounds were determined and indicated as induction of gap junctional communication measured on day 1 (□) and 3 (■) of incubation.. The absolute number of communicating cells in controls (18.0 ± 1.8) was set at 100%. Data represent means ± SD (n=4) (* $P<0.05$,** $P<0.01$,***$P<0.001$).

3.1.2. Transactivation of RARß2-Promoter

The F9-RAR-ß2-LacZ reporter cell line which contains the RAR-ß2-promoter sequence fused with lacZ reporter gene was used in these assays. The retinoids bind to the retinoic acid receptors (RAR or RXR) which further bind as heterodimers to the retinoic acid responsive element (RARE) in the RARß2-promoter region. This will lead to the transcription of lacZ reporter gene and the isolated reporter gene product, ß-galactosidase is measured to quantify the transactivation activity of the test compound

After 24 hours of incubation, the effects of 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid, 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid and 1,1-didemethyl-2-nor-4-oxo-retinoic acid together with all-*trans*-retinoic acid were tested on the transactivation of RARß2-promoter (Table 3). These compounds produced similar effects on the transactivation of RARß2-promoter as their effects on GJIC. According to this 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid was the most effective derivative of all-*trans*-retinoic acid whereas 1,1-didemethly-2-nor-4-oxo-retinoic acid did not show a pronounced effect on the transactivation of RARß2-promoter (Table 3A).

On the other hand, 13-*cis*-retinoic acid and its 4-oxo metabolites; 4-oxo-13-*cis*-retinoic acid and 4-oxo-11, 13-*cis*-retinoic acid and 3-hydroxy-retinoic acid significantly increased transactivation to the values that are very close to the effect of all-*trans*-retinoic acid. 3-hydroxy retinoic acid was the most effective derivative followed by 4-oxo-11, 13-*cis*-retinoic acid, 13-*cis*-retinoic acid and 4-oxo-13-*cis*-retinoic acid respectively. Although their effects on transactivation were also parallel to that in the stimulation of GJIC, 4-oxo-13-*cis*-retinoic acid was very effective to perform transactivation but it did not stimulate GJIC to a pronounced extent (Table 3B).

Table 3. Induction of ß-Galactosidase Activity in F9-RAR-ß2 Cells Following Incubation with Retinoic Acid and its Derivatives

A)

Test Compounds	Induction of ß-Galactosidase Activity
Ethanol (0.2%)	1.0 ± 0.3 *
1,1-didemethyl-2-nor-4-oxo-retinoic acid (1µM	1.7 ± 0.3 **
3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1µM)	3.9 ± 1.8 **
3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1µM)	2.9 ± 2.0
All-*trans*-retinoic acid (1µM)	4.2 ± 1.1 **

B)

Test Compounds	Induction of ß-Galactosidase Activity
Ethanol (0.2%)	1.0 ± 0.1 **
4-oxo-13-*cis*-retinoic acid (1µM)	3.1 ± 1.7 *
4-oxo-11, 13-*cis*-retinoic acid (1µM)	3.6 ± 2.6 *
3-hydroxy retinoic acid (1µM)	3.9 ± 1.0***
13-*cis*-retinoic acid (1µM)	3.5 ± 1.2***
All-*trans*-retinoic acid (1µM)	4.1 ± 1.2 ***

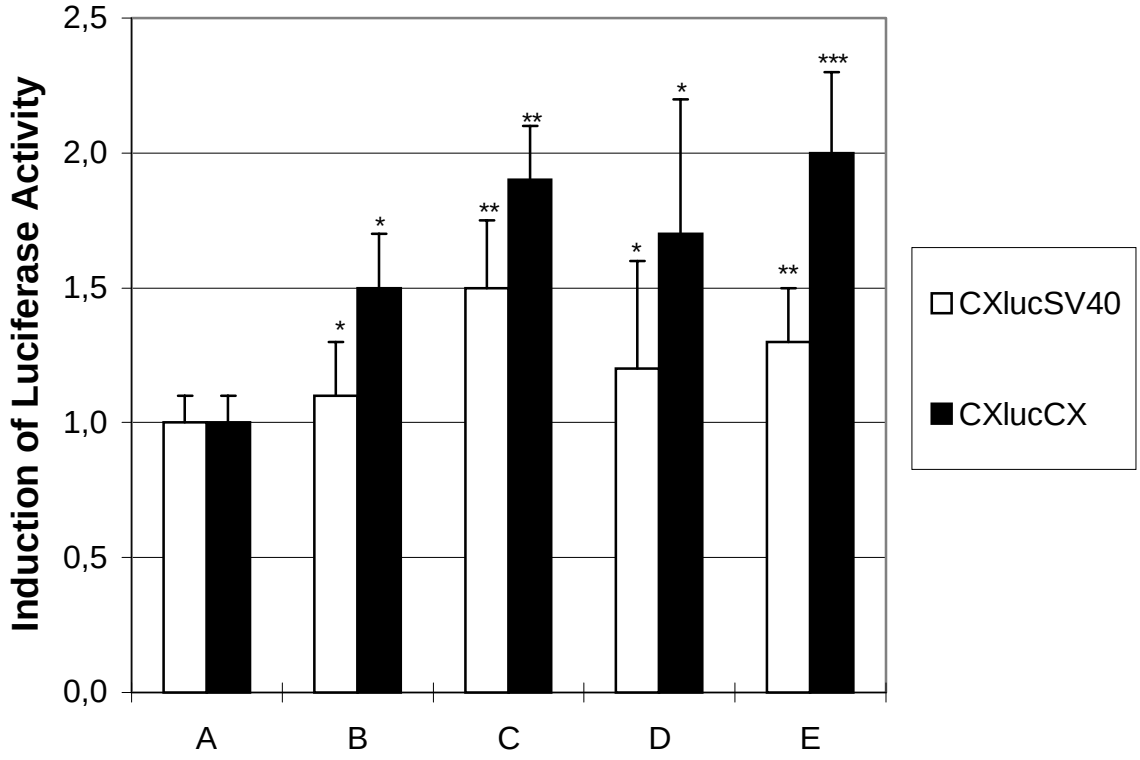
After 24 hours of incubation with indicated compounds, ß-galactosidase activity in F9-RAR-ß2 cells was measured. Control group (0.2% ethanol) was set to 1. Data represent means ± SD (n=5) (*P<0.05 ,** P<0.01 ,*** P<0.001).

3.1.3. The Effect of Retinoic Acid and its Derivatives on Connexin 43 Gene Regulation via Transient Transfection

To analyse the regulation of connexin 43 gene expression by retinoic acid and its different derivatives, F9 mouse teratocarcinoma cells were transiently transfected with different plasmids having variable constructs in their 5′and 3′regions which are summarised in the material and methods section. Plasmids; pCxlucCx and pCxluc SV40 contain the connexin 43 promoter from rat and are 3′-flanked by mouse connexin 43 3′-UTR or by SV40 sequences respectively which were of main interest while the others were used either to quantify the assay or as control plasmids which will be discussed further. The luciferase activity obtained for these two plasmids was compared to claim the effect of test compounds on the 5′-and 3′-regulatory elements of connexin 43 gene in the presence of positive and negative controls.

After 24 hours of incubation, the effects of 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid, 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid and 1,1-didemethyl-2-nor-4-oxo-retinoic acid together with all-*trans*-retinoic acid were tested by means of reporter gene assay. The relationship between the structure of the compound and its effect on GJIC and RARß2-promoter was also clearly seen here, i.e. the compounds which were previously effective, were further able to regulate post-transcriptional modifications of connexin 43 gene to the same extent. According to this 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro retinoic acid was the most effective derivative of all-*trans*-retinoic acid whereas 1,1-didemethly-2-nor-4-oxo-retinoic acid was only effective to some extent. Furthermore, as expected, experiments done with the plasmid pCxlucSV40 which lacks connexin 43 3′UTR, showed lower values than that performed with plasmid pCxlucCx which indicates that retinoids can promote their effects due to stabilisation of Cx 43 m RNA on 3′UTR which otherwise undergo rapid degradation (Figure 21).

On the other hand, similar results observed by 13-*cis*-retinoic acid, 4-oxo-13-*cis*-retinoic acid, 4-oxo-11, 13-*cis*-retinoic acid and 3-hydroxy-retinoic acid. The only one exception was observed with 4-oxo-13-*cis*-retinoic acid which was very effective to perform RARß2-promoter mediated transactivation, but has no regulatory effect on Cx 43 mRNA post-transcriptional modifications. However, this could explain why it was also not stimulating GJIC (Figure 22).



A) Ethanol (0.2%)

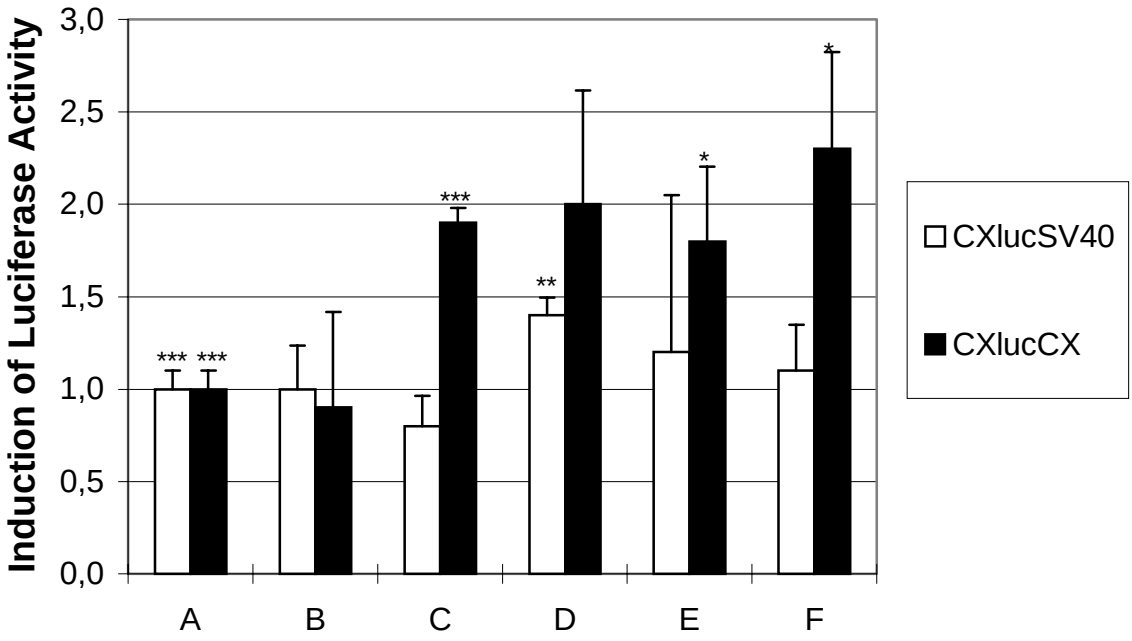
B) 1,1-didemethyl-2-nor-4-oxo-retinoic acid (1µM)

C) 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1µM)

D) 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1µM)

E) All-*trans*-retinoic acid (1µM)

Figure 21. Induction of Luciferase Activity by Retinoic Acid and its Derivatives. F9 cells were transiently transfected with the plasmids; pCxlucSV40 and pCxlucCx and incubated with test compounds for 24 hours. Control group (0.2% ethanol) was set to 1. Data represent means ± SD (n=5) (**P*<0.05 ,** *P*<0.01 ,*** *P*<0.001).



A) Ethanol (0.2%)

B) 4-oxo-13-*cis*-retinoic acid (1μM)

C) 4-oxo-11, 13-*cis*-retinoic acid (1μM)

D) 3-hydroxy-retinoic acid (1μM)

E) 13-*cis*-retinoic acid (1μM)

F) All-*trans*-retinoic acid (1μM)

Figure 22. Induction of Luciferase Activity by Retinoic Acid and its Derivatives. F9 cells were transiently transfected with the plasmids; pCxlucSV40 and pCxlucCx and incubated with test compounds for 24 hours. Control group (0.2% ethanol) was set to 1. Data represent means ± SD (n=3) (* $P<0.05$,** $P<0.01$,*** $P<0.001$).

3.1.3.1. Quantification of Luciferase Activity via Co-transfection and Transient Transfection of F9 Cells with other Plasmids

To prove the efficiency of transfection, cells were co-transfected by plasmid pCH110 which has SV40 promoter region coupled with lacZ gene, which in turn codes for ß-galactosidase and can not be activated by the test compounds. Since 90% of the cells contain both luciferase and ß-galactosidase genes, it is possible to check efficiency of transfection for each single experiment (Teicher et al., 1999). According to the calculated efficiency of transfection, luciferase activity obtained for each sample was determined

Moreover, for each reporter gene assay performed with luciferase, the plasmid pT81lucSV40 was used as the negative control and the plasmid pCMVlucSV40 was used as positive control as mentioned in material and methods. Results are summarised in Table 4

Upon transient transfection with plasmid pT81lucSV40, none of the compounds showed an increase in the luciferase activity as expected and upon transient transfection with plasmid pCMVlucSV40, all compounds showed increased luciferase activities to different extents, probably due to their different ability to regulate connexin 43 gene expression.

Table 4. Induction of Luciferase Activity by Retinoic Acid Derivatives Following Transient Transfections

A)

Test Compounds	**pT81**	**PCXlucSV40**	**PCMVlucSV40**
Ethanol (0.2%)	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.3
1,1-didemethyl-2-nor-4-oxo-retinoic acid (1μM	1.1 ± 0.2	1.1 ± 0.2 *	1.4 ± 0.6
3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1μM)	1.1 ± 0.7	1.5 ± 0.2 **	2.8 ± 1.2 *
3-R-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid (1μM)	1.1 ± 0.6	1.2 ± 0.5 *	1.6 ± 0.2 **
All-*trans*-retinoic acid (1μM)	1.0 ± 0.3	1.3 ± 0.3 **	2.5 ± 0.8 ***

B)

Test Compounds	**pT81**	**PCXlucSV40**	**PCMVlucSV40**
Ethanol (0.2%)	1.0 ± 0.1	1.0 ± 0.1 ***	1.0 ± 0.2
4-oxo-13-*cis*-retinoic acid (1μM)	1.1 ± 0.6	1.0 ± 0.2	1.3 ± 0.4
4-oxo-11,13-*cis*-retinoic acid (1μM)	1.1 ± 0.3	0.8 ± 0.2	2.3 ± 0.6***
3-hydroxy-retinoic acid (1μM)	1.0 ± 0.3	1.4 ± 0.1 **	2.5 ± 0.9**
13-*cis*-retinoic acid (1μM)	1.1 ± 0.2	1.2 ± 0.8	2.2 ± 0.5 *
All-*trans*-retinoic acid (1μM)	1.0 ± 0.5	1.1 ± 0.2	2.7 ± 0.4 ***

F9 cells were transiently transfected by indicated plasmids; and incubated with test compounds for 24 hours. Control group (0.2% ethanol) was set to 1. In (**A**) data represent means ± SD (n=5) and in (**B**) means ± SD (n=3) (*P<0.05 ,** P<0.01 ,*** P<0.001).

3.2. Thalidomide

3.2.1. Cytotoxicity of Compounds

Upon incubation of cells with 10 μM thalidomide or its analogs according to previous studies (Nicolai et al., 1997), none of the compounds were 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 $NADP^+$did cell viability diminish to 61% of the control (data not shown).

3.2.2. Time Dependence of Thalidomide Effects on Gap Junctional Intercellular Communication

The influence of thalidomide in the presence of NADPH on gap junctional communication on day 1, 3 and 6 of incubation was tested in rat liver epithelial cells (WB-F344) and was compared to retinoic acid (Figure 23). 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 of NADPH, GJIC was significantly increased from day 3 of incubation, and on day 6, was more pronounced than with thalidomide alone. NADPH alone had no influence on GJIC. Retinoic acid (1μM) was used as positive control.

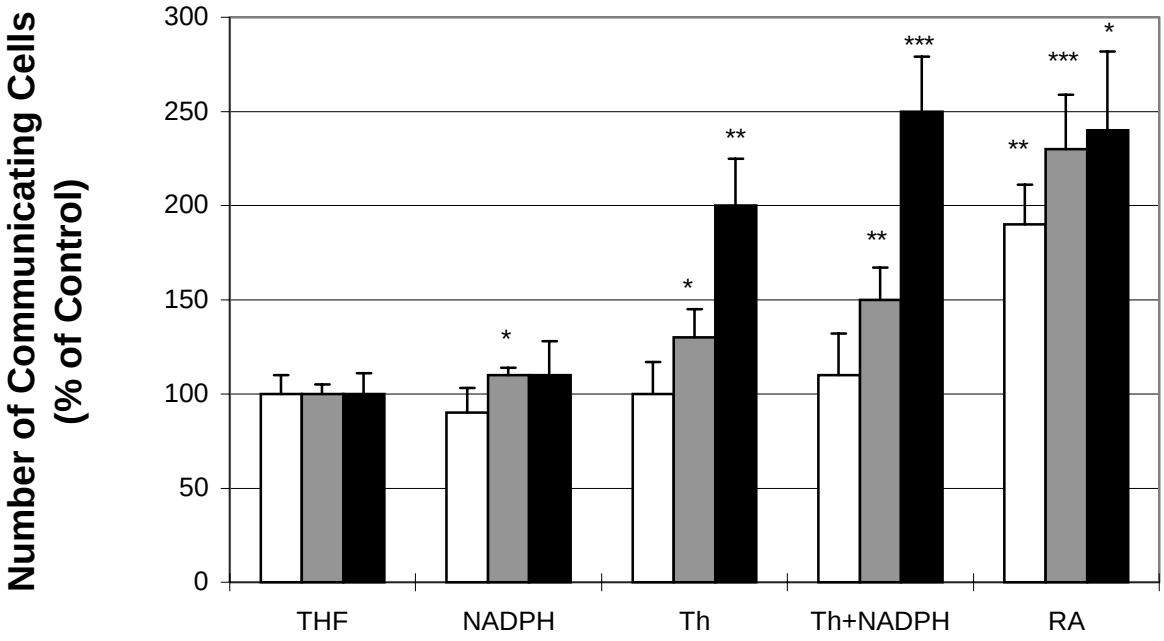


Figure 23. . **Time Dependence of Gap Junctional Intercellular Communication (GJIC) in WB-F344 Cells in the Presence of Thalidomide.** The number of communicating cells (% of control) induced by thalidomide (10 μM) and NADPH (20 μM) were measured on day 1(□), 3 (▒) and 6 (■) of incubation. The absolute number of communicating cells in controls (32.3 ± 2.1) was set at 100%. Data represent means ± SD (n=4) (*P<0.05, ** P <0.01, *** P <0.001).

3.2.3. Effects of Coenzymes

Since thalidomide requires metabolic activation for its effect on GJIC (Nicolai et al., 1997), induction of GJIC was examined in the presence of coenzymes to investigate their effect on the metabolic activation in rat liver epithelial cells (WB-F344) and in human fetal skin fibroblasts (HFFF2).

3.2.3.1. In Rat Liver Epithelial Cells (WB-F344)

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, an increase of 1.6-fold was found using NADPH (20 μM) and NAD^+ (20 μM) was only effective on day 3 of incubation. The other coenzymes used in the assay did not show a pronounced effect on GJIC (Figure 24).

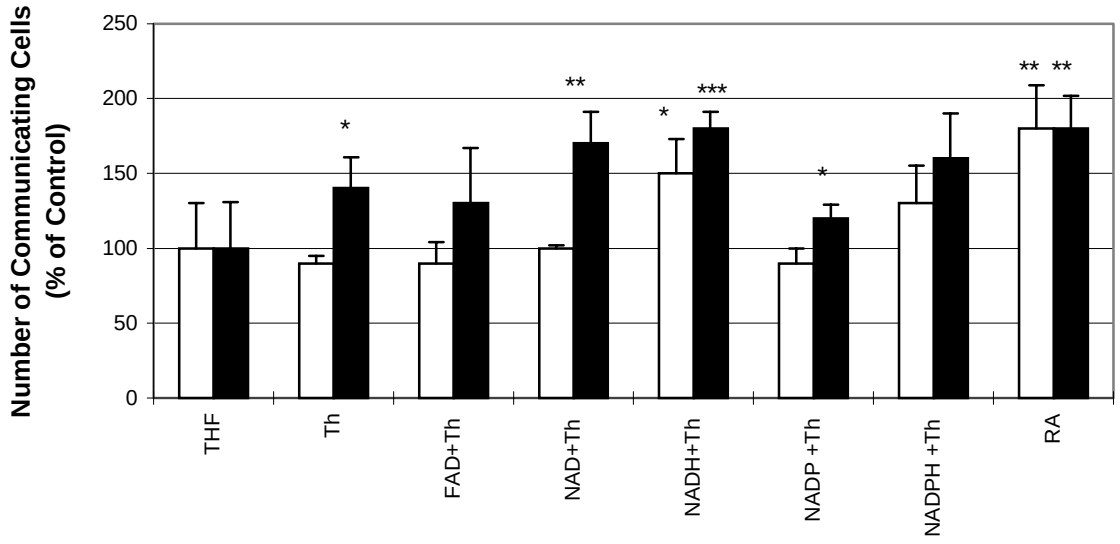


Figure 24. Induction of Gap Junctional Intercellular Communication (GJIC) by Thalidomide in the Presence of Coenzymes in WB-F344 Cells. The number of communicating cells (% of control) induced by thalidomide (10 μM) in the presence of coenzymes: FAD, NAD^+, $NADP^+$, NADH, NADPH (each 20 μM) or with all-*trans* retinoic acid (1 μM) were measured on day 1 (□) and 3 (■) of incubation. The absolute number of communicating cells in controls (32.9 ± 7.4) was set at 100%. Data represent means ± SD (n=3) (**P*<0.05, ** *P* <0.01, *** *P* <0.001).

3.2.3.2. In Human Fetal Skin Fibroblasts (HFFF2)

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. The other coenzymes used in the assay did not show a pronounced effect on GJIC (Figure 25).

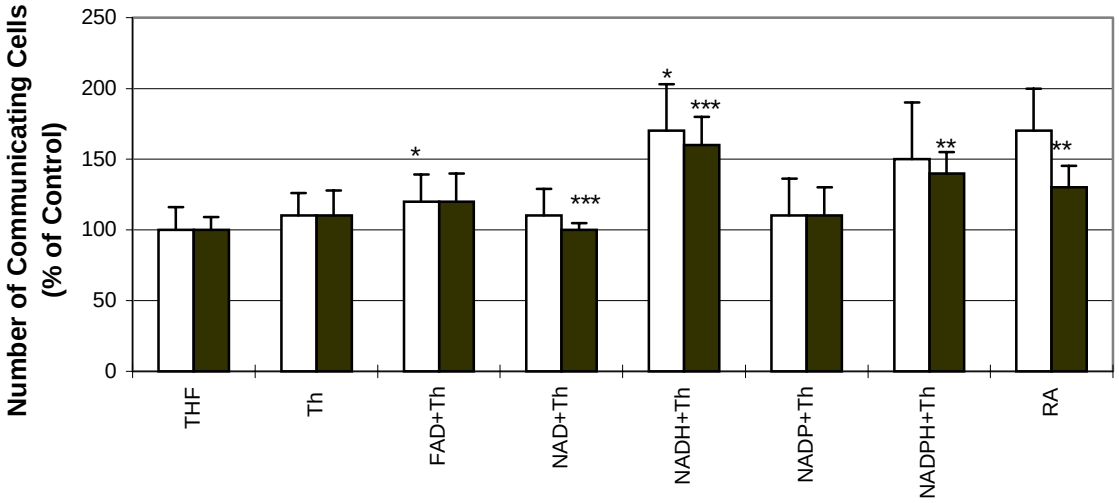


Figure 25. Induction of Gap Junctional Intercellular Communication (GJIC) by Thalidomide in the Presence of Coenzymes in HFFF2 Cells. The number of communicating cells (% of control) induced by thalidomide (10 μM) in the presence of coenzymes; FAD, NAD^+, $NADP^+$, NADH, NADPH (each 20 μM) or with all-*trans* retinoic acid (1 μM) were measured on day 1 (□) and 3 (■) of incubation. The absolute number of communicating cells in controls (18.6 ± 3.3) was set at 100%. Data represent means ± SD (n=5) (*P<0.05, ** P <0.01, *** P <0.001).

3.2.4. Metabolic Activation of Thalidomide-Involvement of Enzymes

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 (Figure 26). When cell lysates were incubated at 56 °C for 30 min. to inactivate heat-labile factors, the effect on GJIC was comparable to controls. NADH alone together 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 solvent.

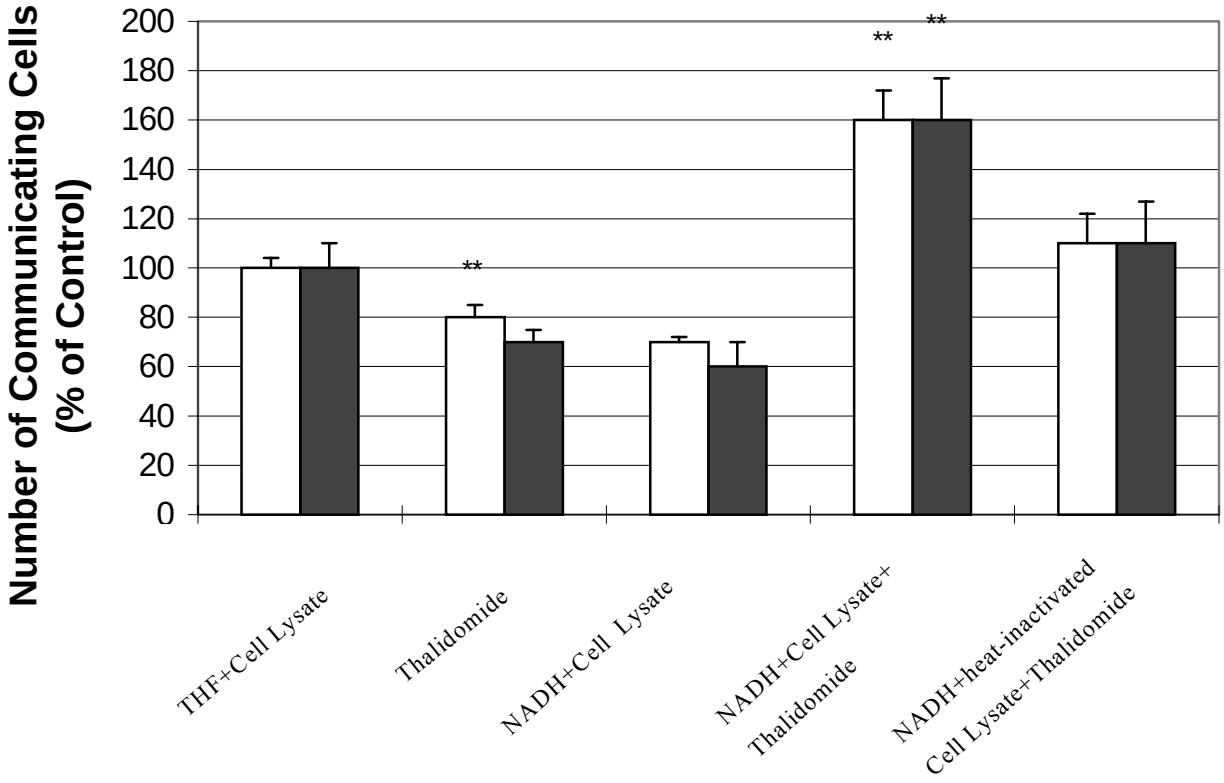


Figure 26. **Induction of Gap Junctional Communication in WB-F344 Cells by Thalidomide in the Presence of NADH and Cell Lysate.** The number of communicating cells (% of control) induced by thalidomide (10 μM) in the presence of NADH (20 μM) and cell lysate (1:15) were measured on day 1 (□) and 3 (■) of incubation. The absolute number of communicating cells in controls (31.0 ± 3.2) was set at 100%. Data represent means ± SD (n=3) (** $P<0.01$).

3.2.5. Influence of Thalidomide Analogs on Gap Junctional Intercellular Communication

The influence of thalidomide and its analogs on GJIC were tested in the presence or absence of NADH in human fetal skin fibroblasts (HFFF2) and in rat liver epithelial cells (WB-F344). EM 16, a non-teratogenic thalidomide analog (Nogueira et al., 1996), did not stimulate GJIC in both cell types, whereas all other thalidomide analogs induced GJIC to some extent in the presence of NADH.

3.2.5.1. Rat Liver Epithelial Cells (WB-F344)

In the presence of NADH, the potent teratogens in primates, thalidomide and EM 12 (Nogueira et al., 1996; Neubert et al., 1996), and the teratogen hydrolysis product of thalidomide, PGA (Meise et al., 1973), were less effective stimulating GJIC than the other thalidomide analogs (except EM 16) in WB-F344 cells. EM 138 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).

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. EM 16 again did not show an effect on GJIC, also thalidomide did not. Likewise, EM 12 and PGA did not affect GJIC in the absence of NADH. EM 20 had no significant affect on GJIC, whereas EM 138 stimulated GJIC on day 1 of the incubation; however, its effect decreased on day 3 (Table 5).

Table 5. **Induction of Gap Junctional Intercellular Communication by Thalidomide and Thalidomide Analogs in the Presence and the Absence of NADH in Rat liver Epithelial Cells (WB-F344).**

Compounds	Number of Communicating Cells (% of control) in WB-F344 Cells			
	Without NADH		with NADH (20μM)	
	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 *

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. The absolute number of communicating cells in controls (31.1 ± 3.7) was set to 100%. Data represent means ± SD (n=4) (**P*<0.05, ** *P* <0.01, *** *P* <0.001).

3.2.5.2. Human Fetal Skin Fibroblasts (HFFF2).

Thalidomide, EM 12 and PGA, were found to be more effective stimulating GJIC in HFFF2 cells than in WB-F344 cells in the presence and also the absence of NADH. The non-teratogenic compound, EM 16, did not show an effect on GJIC also in HFFF2 cells, both in the presence and the 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 were found to be also effective in the absence of

NADH, but the effect of EM 12 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, however their effects were decreased in the presence of NADH from 2.4-fold to 1.8-fold and from 1.7-fold to 1.4-fold respectively. EM 20 had no significant affect 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) (Table 6).

Table 6. **Induction of Gap Junctional Intercellular Communication by Thalidomide and Thalidomide Analogs in the Presence and the Absence of NADH in Human Fetal Skin Fibroblasts (HFFF2).**

Compounds	Number of Communicating Cells (% of control) in HFFF2 Cells			
	Without NADH		with NADH (20μM)	
	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. The absolute number of communicating cells in controls (18.3 ± 0.9) was set to 100%. Data represent means ± SD (n=6) (*P<0.05, ** P <0.01, *** P <0.001).

3.2.6. Influence of Thalidomide Enantiomers on Gap Junctional Intercellular Communication in WB-F344 Cells

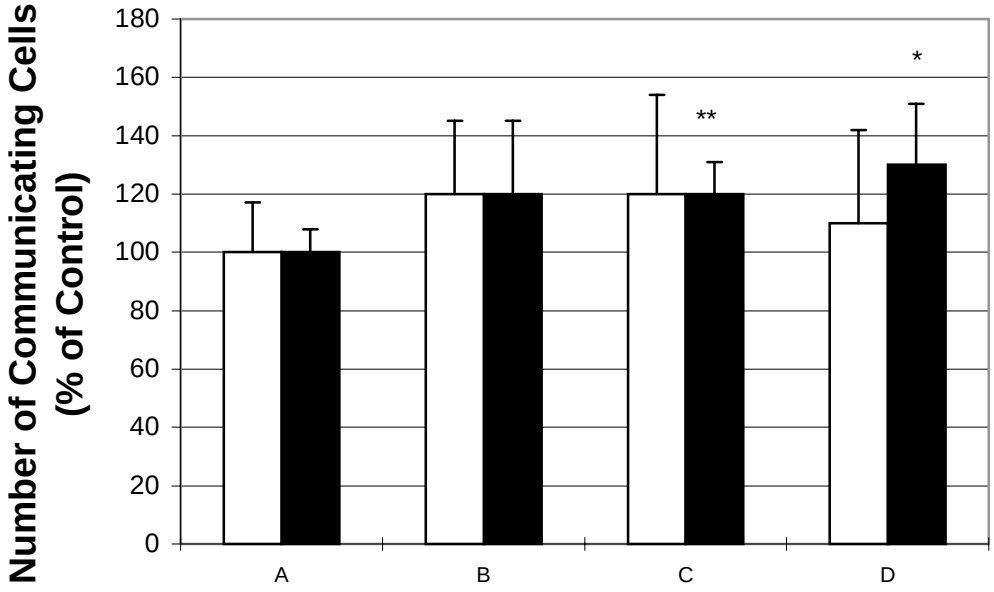
It has been shown that teratogenic potential of thalidomide is carried out only by one enantiomer of compound: (-)-S-thalidomide (Blaschke et al., 1979). Furthermore, it has been found that another potent teratogen, EM 12 exhibits a marked species difference and stereoselective teratogenicity, i.e. the (-)-S-enantiomer were found to be more effective. On the other hand, it is also known that thalidomide and EM 12 are not as teratogenic as to rat and mouse embryos as they are to human. As a result, the effect of enantiomers on GJIC has been examined both in the presence and the absence of NADH in WB cells.

3.2.6.1. Effect of Enantiomers in the Absence of NADH

In the absence of NADH, there was no significant induction of GJIC or no important differences due to stereoselectivity of the enantiomers. Both (-)-S-enantiomer and racemic thalidomide stimulated GJIC only up to 1.2-fold in comparison to the control which was normal for racemic thalidomide, since it needs metabolic activation to show its effect (Nicolai et al., 1997) (Figure 27).

3.2.6.2. Effect of Enantiomers in the Presence of NADH

In the presence of NADH, GJIC was induced by racemic thalidomide up to 1.4-fold in comparison to the control whereas both enantiomers could induce GJIC only up to 1.3-fold and again there was no important differences due to stereoselectivity of the enantiomers which could be a result of rapid chiral inversion of enantiomers (Reist et al., 1998) (Figure 28).



A) THF (0,4 %)

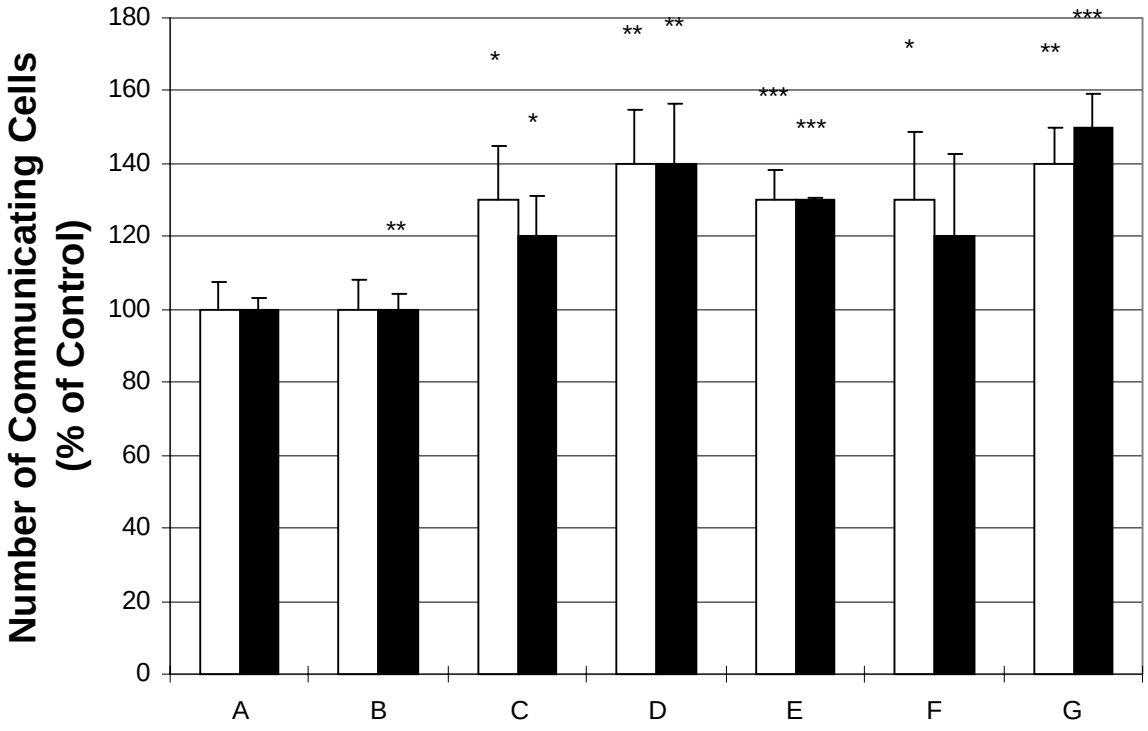
B) (+/-) Thalidomide (10 µM)

C) (-)-S-Thalidomide (10 µM)

D) (+)-R-Thalidomide (10 µM)

Figure 27. Induction of Gap Junctional Communication in Rat Liver Epithelial Cells (WB-F344) by Thalidomide and Thalidomide Enantiomers without Metabolic Activation.

The number of communicating cells (% of control) induced by (+/-) thalidomide, (-)-S-thalidomide and (+)-R-thalidomide were measured on day 1 (□) and 3 (■) of incubation. The number of communicating cells in controls (43,4 ± 1,1) was set at 100 %. Data represent means ± SD (n=6) (* $P<0,05$,** $P<0,01$,*** $P<0,001$).



A) THF (0,4 %)

B) NADH (20 µM)

C) (+/-) Thalidomide (20 µM)

D) (+/-) Thalidomide (10 µM)

E) (-)-S-Thalidomide (10 µM)

F) (+)-R-Thalidomide (10 µM)

G) All-*trans* Retinoic acid (1µM)

Figure 28. Induction of Gap Junctional Communication in Rat Liver Epithelial Cells (WB-F344) by Thalidomide and Thalidomide Enantiomers in the Presence of NADH. The number of communicating cells (% of control) induced by (+/-) thalidomide, (-)-S-thalidomide, (+)-R-thalidomide in the presence of NADH and alone by all-*trans* retinoic acid were measured on day 1 (□) and 3 (■) of incubation. The number of communicating cells in controls (45,4 ± 6,1) was set at 100 %. Data represent means ± SD (n=4) (* $P<0,05$,** $P<0,01$,*** $P<0,001$).

3.2.7. Effect of Thalidomide Analogs, EM 364 and EM 16 on the Translocation of Connexin 43 Protein upon Treatment with TPA in WB-F344 Cells

By using fluorescent antibodies for Cx 43 protein, immunohistochemistry assays were performed in WB cells in the presence of the most active thalidomide analog EM 364 and well-known non-teratogen analog EM 16 up to 10-fold higher concentrations of both compounds.

As described before, TPA is a PKC activator which can mimic DAG and activate PKC and its downstream events and can cause phosphorylation of Cx 43 protein which in turn translocate to the cytosol from the membrane and thereby decreases GJIC.

Upon treatment with TPA, a down regulation of gap junctions, via activation of PKC and thereby translocation of Cx 43 from membrane to cytosol, were performed. The ability of EM 364, which stimulated GJIC significantly both in WB-F344 cells and in HFFF2 cells and the ability of EM16, a non teratogen analog which did not stimulate GJIC in both cell types, were examined to prevent this effect of TPA. Staurosporine, a potent inhibitor of PKC was used as positive control whereas DMSO was negative control.

EM 364 was effective in preventing down regulation of gap junctions upon treatment with TPA for 30 and 60 minutes, both at 10 μM and 100 μM concentrations (Figures 29-32). However, EM 16 failed to prevent TPA-induced down regulation of gap junctions, even at 100 μM concentration (Figures 33-34). Although there are no reports showing the exact relationship between thalidomide and PKC inhibition, thalidomide seems to be involved in the PKC pathway either upstream or downstream as mentioned before.

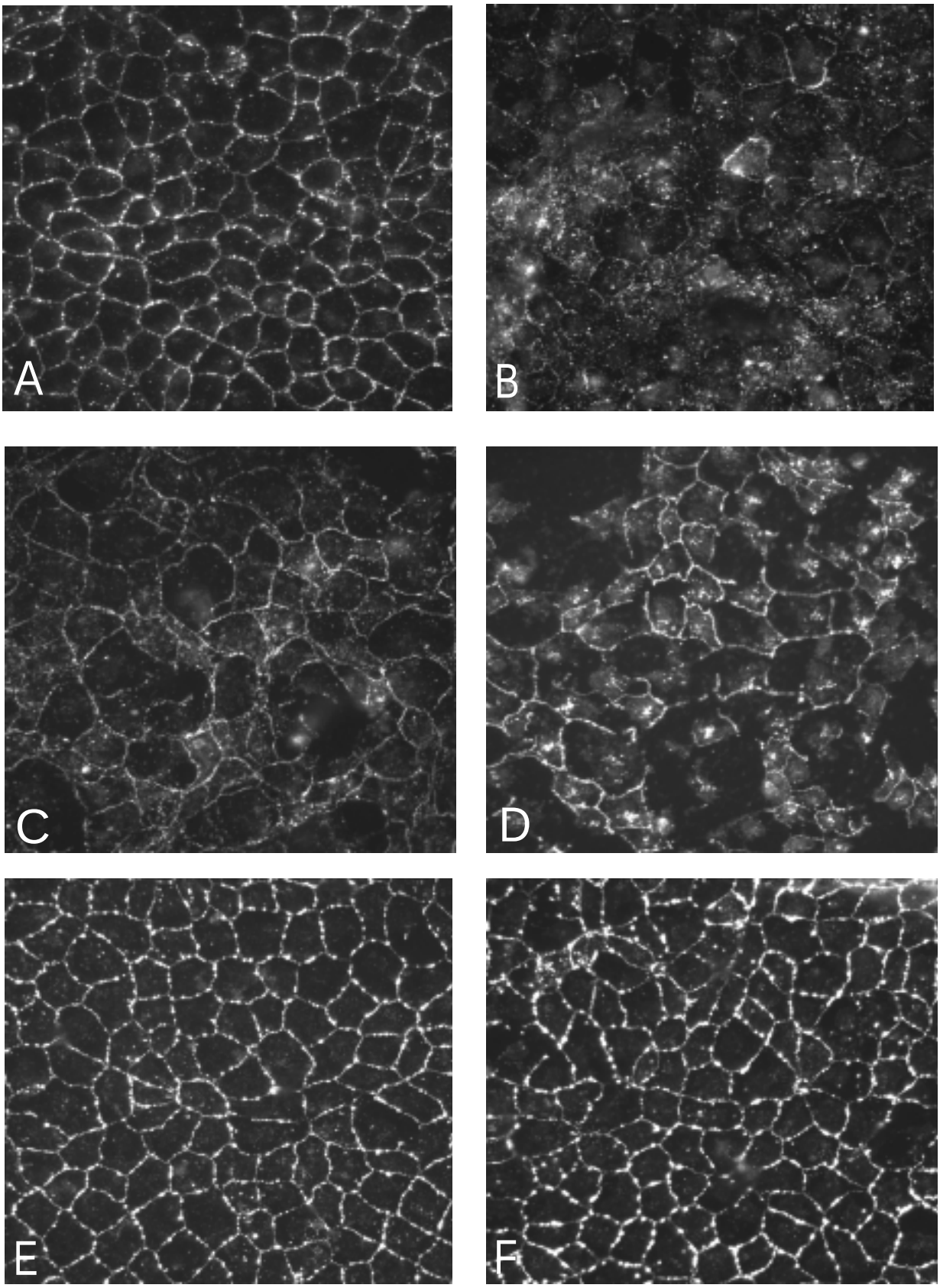


Figure 29. Subcellular Distribution of Connexin 43 upon 30 Minutes Treatment with TPA and EM 364 (100 μM) in WB-F344 Cells. In untreated cells **(A)**, connexin 43 is located in the cell membrane. Upon 30 minutes treatment with TPA (100 nM) alone **(B)**, phosphorylated connexin 43 translocated from membrane to cytosol. Upon treatment 30 minutes with staurosporine (100 nM) together with TPA (100 nM) **(C)** or with EM 364 (100 μM)together with TPA (100 nM) **(D)**, translocation of connexin 43 to cytosol due to phosphorylation was prevented. Upon treatment with staurosporine (100 nM) alone **(E)** or with EM 364 (100 μM) alone **(F)** connexin 43 is located in the cell membrane as more pronounced. Staurosporine was used as positive control.

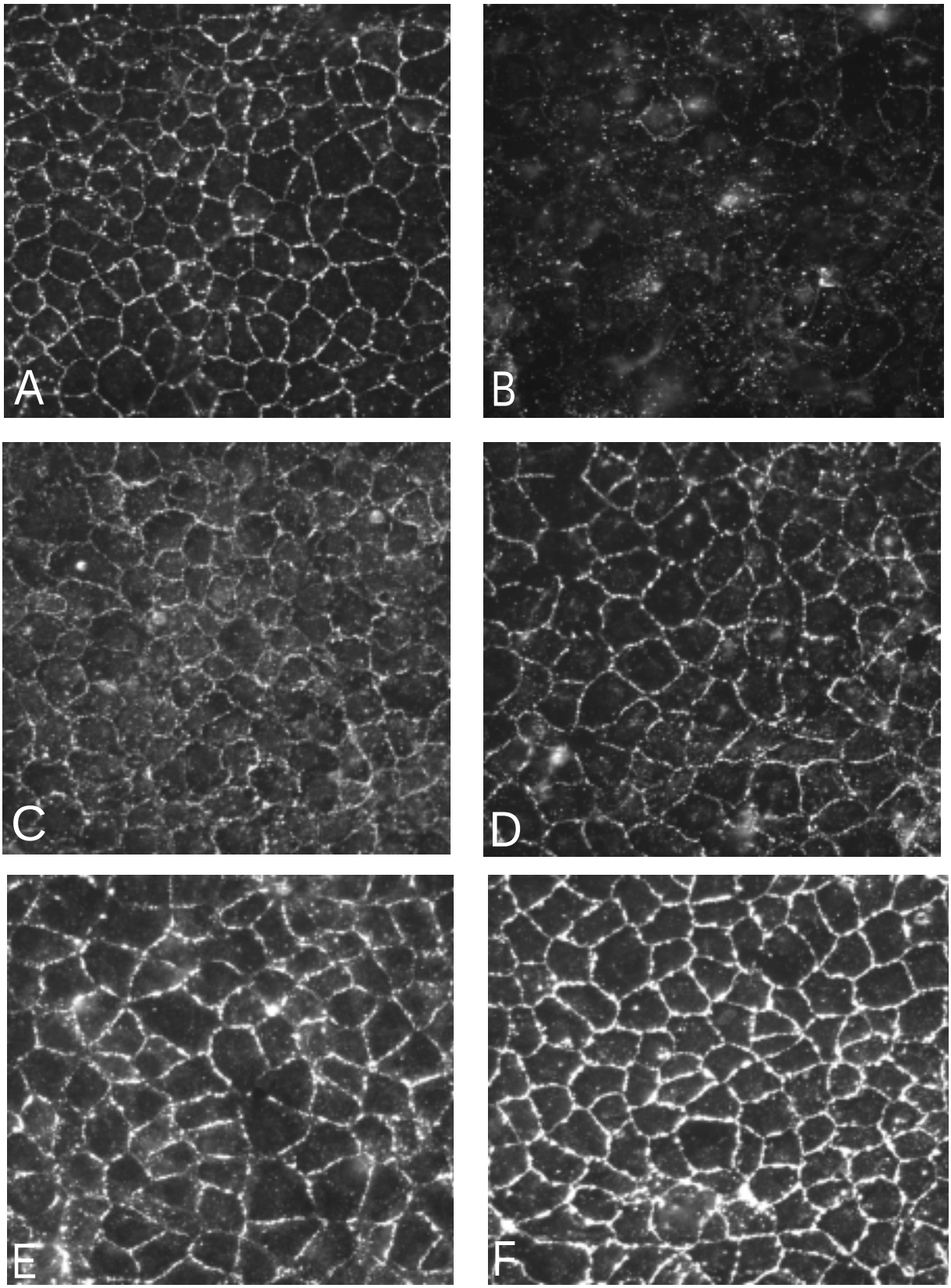


Figure 30. Subcellular Distribution of Connexin 43 upon 60 Minutes Treatment with TPA and EM 364 (100 µM) in WB-F344 Cells. In untreated cells **(A)**, connexin 43 is located in the cell membrane. Upon 60 minutes treatment with TPA (100 nM) alone **(B)**, phosphorylated connexin 43 translocated from membrane to cytosol. Upon treatment 60 minutes with staurosporine (100 nM) together with TPA (100 nM) **(C)** or with EM 364 (100 µM) together with TPA (100 nM) **(D)**, translocation of connexin 43 to cytosol due to phosphorylation was prevented. Upon treatment with staurosporine (100 nM) alone **(E)** or with EM 364 (100 µM) alone **(F)** connexin 43 is located in the cell membrane as more pronounced. Staurosporine was used as positive control.

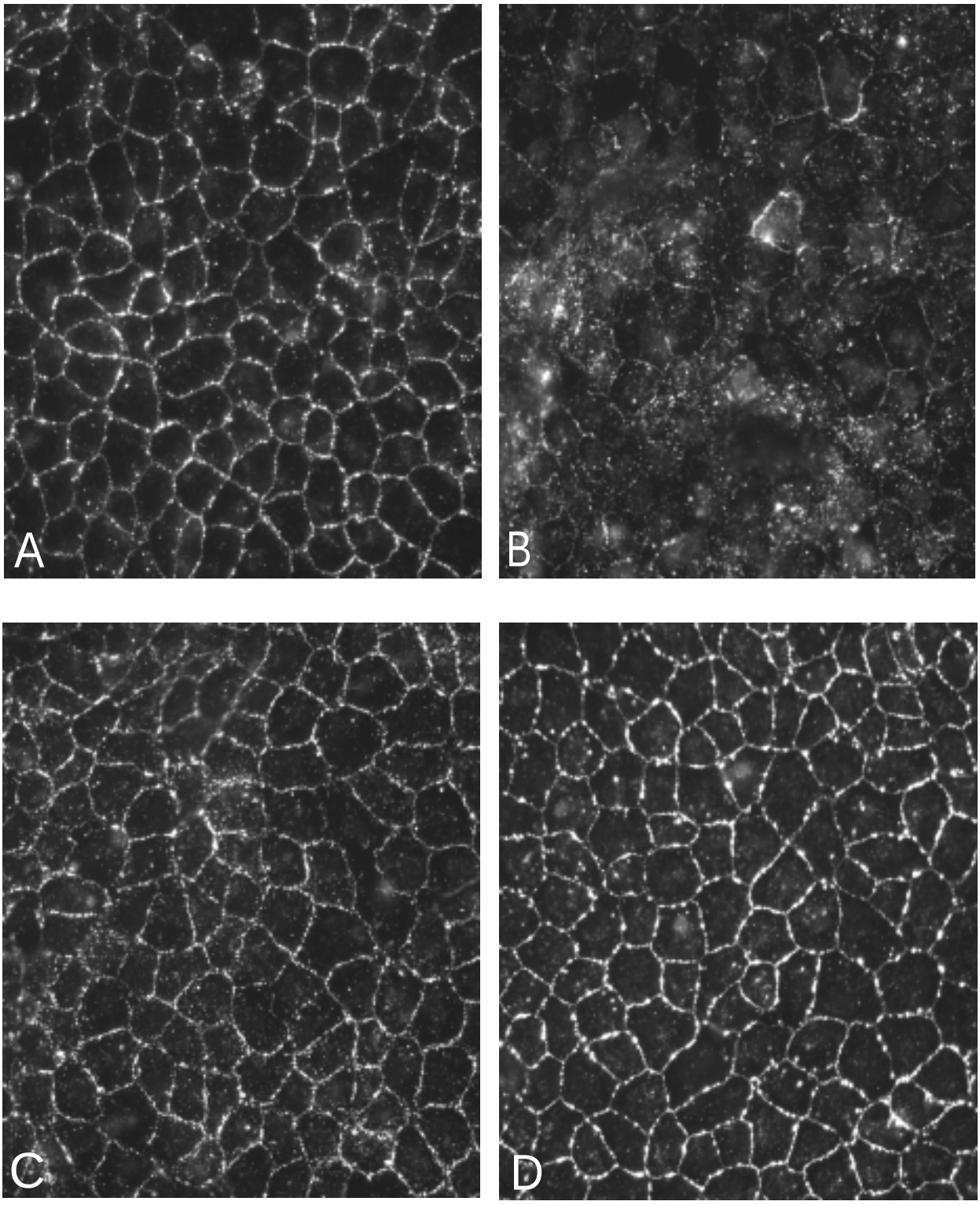


Figure 31. Subcellular Distribution of Connexin 43 upon 30 Minutes Treatment with TPA and EM 364 (10 μM) in WB-F344 Cells. In untreated cells **(A)**, connexin 43 is located in the cell membrane. Upon 30 minutes treatment with TPA (100 nM) **(B)**, phosphorylated connexin 43 translocated from membrane to cytosol. Upon treatment 30 minutes with TPA (100 nM) and EM 364 (10 μM) **(C)**, translocation of connexin 43 to cytosol due to phosphorylation was prevented. Upon treatment with EM 364 (10 μM) alone **(D)**, connexin 43 is located in the cell membrane.

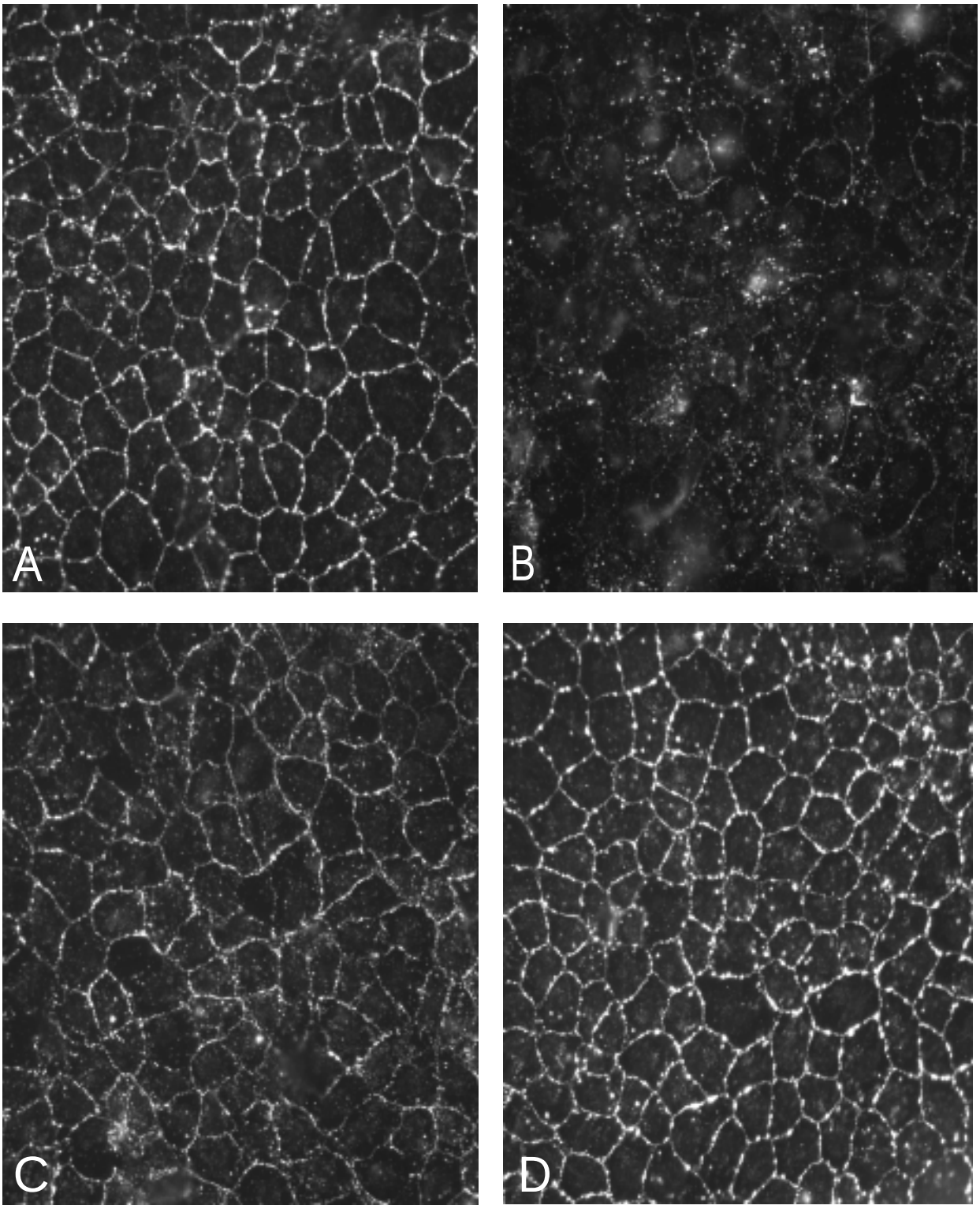


Figure 32. Subcellular Distribution of Connexin 43 upon 60 Minutes Treatment with TPA and EM 364 (10 μM) in WB-F344 Cells. In untreated cells **(A)**, connexin 43 is located in the cell membrane. Upon 60 minutes treatment with TPA (100 nM) **(B)**, phosphorylated connexin 43 translocated from membrane to cytosol. Upon treatment 60 minutes with TPA (100 nM) and EM 364 (10 μM) **(C)**, translocation of connexin 43 to cytosol due to phosphorylation was prevented. Upon treatment with EM 364 (10 μM) alone **(D)**, connexin 43 is located in the cell membrane.

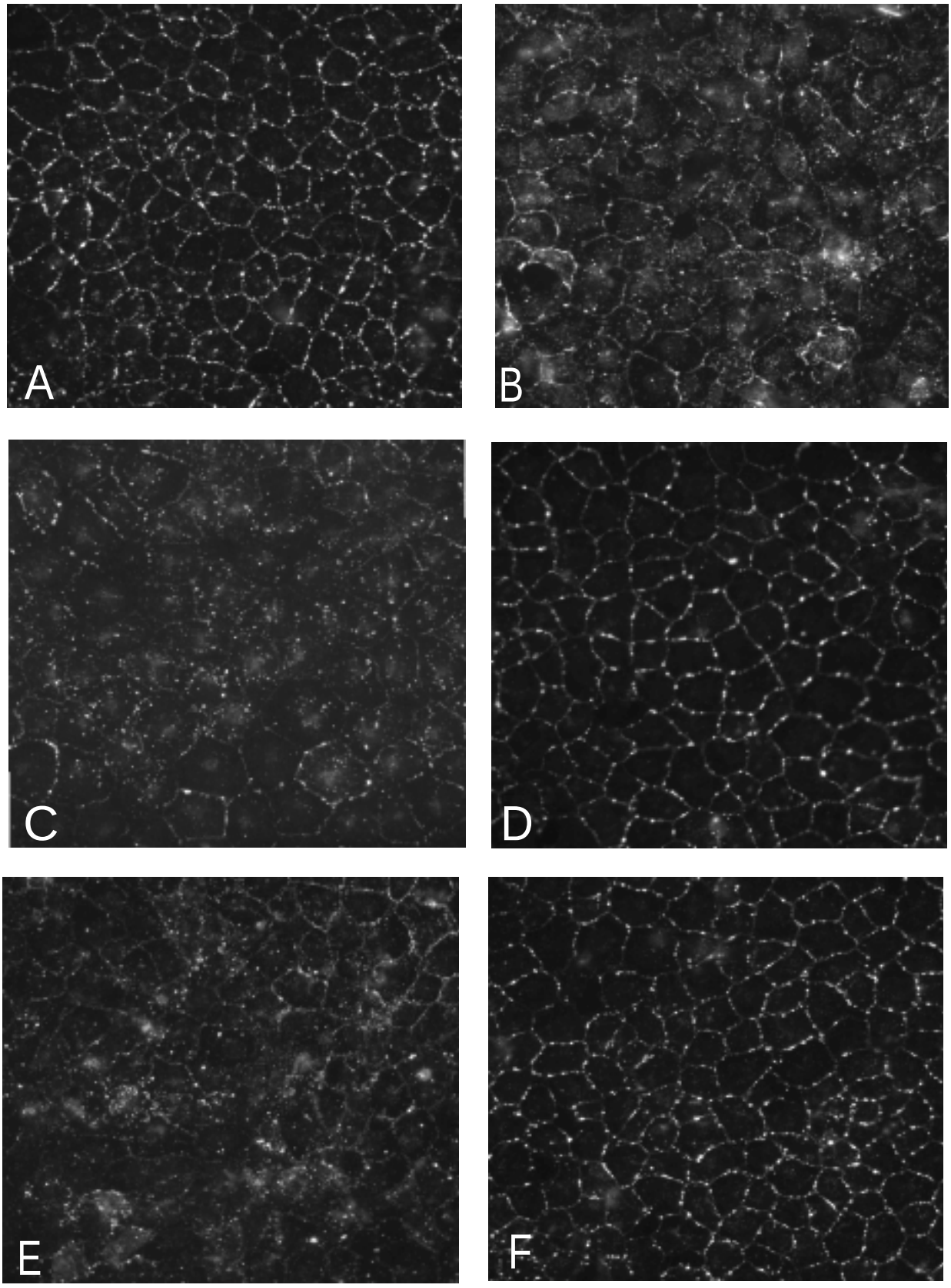


Figure 33. Subcellular Distribution of Connexin 43 upon 30 Minutes Treatment with TPA and EM 16 in WB-F344 Cells. In untreated cells **(A)**, connexin 43 is located in the cell membrane. Upon 30 minutes treatment with TPA (100 nM) **(B)**, phosphorylated connexin 43 translocated from membrane to cytosol. Upon treatment 30 minutes with TPA (100 nM) and EM 16 (10 μM) **(C)**, translocation of connexin 43 to cytosol due to phosphorylation could not be prevented. Upon treatment with EM 16 (10 μM) alone **(D)**, connexin 43 is located in the cell membrane. Due to treatment 30 minutes with TPA (100 nM) and 10 times higher concentrations of EM 16 (100 μM) **(E)**, translocation of connexin 43 to cytosol still could not be prevented. Upon treatment with EM 16 (100 μM) alone **(F)**, connexin 43 is located in the cell membrane.

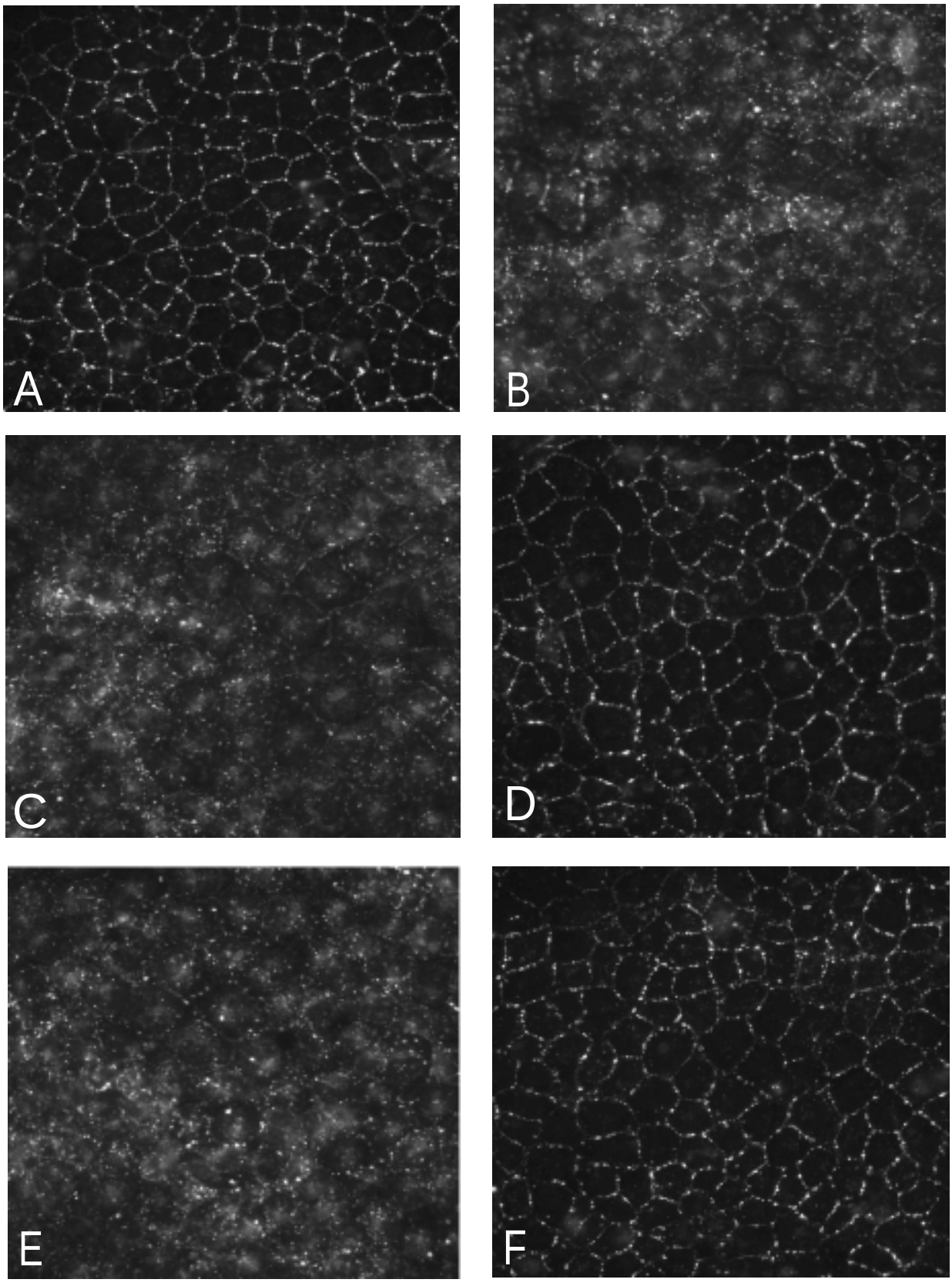


Figure 34. Subcellular Distribution of Connexin 43 upon 60 Minutes Treatment with TPA and EM 16 in WB-F344 Cells. In untreated cells **(A)**, connexin 43 is located in the cell membrane. Upon 60 minutes treatment with TPA (100 nM) **(B)**, phosphorylated connexin 43 translocated from membrane to cytosol. Upon treatment 60 minutes with TPA (100 nM) and EM 16 (10 μM) **(C)**, translocation of connexin 43 to cytosol due to phosphorylation could not be prevented. Upon treatment with EM 16 (10 μM) alone **(D)**, connexin 43 is located in the cell membrane. Due to treatment 60 minutes with TPA (100 nM) and 10 times higher concentrations of EM 16 (100 μM) **(E)**, translocation of connexin 43 to cytosol still could not be prevented. Upon treatment with EM 16 (100 μM) alone **(F)**, connexin 43 is located in the cell membrane.

4. DISCUSSION

4.1. Retinoic Acid

The influence of retinoic acid and its derivatives on GJIC, on the stabilisation of Cx 43 mRNA and on the transactivation of the RARß2-promoter were tested in human fetal skin fibroblasts (HFFF2), in a mouse teratocarcinoma cell line (F9) and a mouse teratocarcinoma reporter cell line (F9lacZ), respectively.

During the metabolism of vitamin A, 4-oxo-retinoic acid is produced as the main metabolite of retinoic acid through 4-hydroxy-retinoic acid or from 13-*cis*-retinoic acid through 4-oxo-13-*cis*-retinoic acid (Napoli, 1999) (Figures 4 and 8). In previous studies, it has been shown that 4-oxo-retinoic acid has effects similar to that of retinoic acid on the stimulation of GJIC, on the stabilisation of Cx 43 mRNA via the 3´UTR and also on the transactivation of the RARß2-promoter (Teicher et al., 1999). Retinoic acid and 13-*cis*-retinoic acid are human teratogens and research has shown that 4-oxo-retinoic acid exhibits a teratogenic potential similar to retinoic acid (Creech et al., 1989). However, 4-oxo-13-*cis*-retinoic acid was shown to be 10 times less teratogenic than retinoic acid (Kraft et al., 1992). To examine a possible relationship between GJIC and the teratogenicity of retinoids, the effects of 13-*cis*-retinoic acid, 4-oxo-13-*cis*-retinoic acid, 4-oxo-11, 13-*cis*-retinoic acid and 3-hydroxy-retinoic acid were investigated in the above mentioned assay systems compared to retinoic acid. 4-oxo-11, 13-*cis*-retinoic acid was the most effective derivative in stimulating GJIC followed by 13-*cis*-retinoic acid and 3-hydroxy retinoic acid on day 1 of incubation. However, on day 3 of incubation, 3-hydroxy-retinoic acid stimulated GJIC most effectively. 4-hydroxy-retinoic acid is a biologically active metabolite of all-*trans*-retinoic acid and is a precursor of 4-oxo-retinoic acid (Figures 4 and 8) (Napoli, 1999). 3-Hydroxy-retinoic acid has a similar structure as 4-hydroxy-retinoic acid except the position of hydroxy group in the ring. It was found to be very effective in the above mentioned assay systems. Therefore, it could be also a

biologically active compound and a hydroxy group in the ring structure could be important for biological activities. On the other hand, 4-oxo-13-*cis*-retinoic acid which is less teratogenic than retinoic acid, was interestingly, less effective also in stimulating GJIC compared to retinoic acid. The teratogenic potential and biological relevance of 4-oxo-11, 13-*cis*-retinoic acid is unknown; it was more effective than its isomer 4-oxo-13-*cis*-retinoic acid in stimulating GJIC. 9-cis-Retinoic acid is the isomerization product of all-*trans*-retinoic acid (Figure 4) (Napoli, 1999) and the only known ligand for the RXR (Figure 5) (Rowe, 1997; Napoli, 1999). Since 4-oxo-11, 13-*cis*-retinoic acid is much more effective then its isomer, the isomerization, giving rise a bending on the chain structure like in the case of 9-cis-retinoic acid from retinoic acid, it could have a biological importance by binding to different retinoic acid receptors. In other words, 4-oxo-11, 13-*cis*-retinoic acid could be also a ligand for RXR, although in this study its affinity to RAR was tested only and its affinity to RXR should be tested alone further. Furthermore, 4-oxo-11, 13-*cis*-retinoic acid, 13-*cis*-retinoic acid and 3-hydroxy-retinoic acid stimulated GJIC almost to a similar extent. 4-oxo-13-*cis*-retinoic acid is a metabolite of 13-*cis*-retinoic acid and can isomerize to 4-oxo-11, 13-*cis*-retinoic acid. 4-Hydroxy-retinoic acid is a metabolite of all-*trans*-retinoic acid which can be formed from 13-*cis*-retinoic acid (Figure 8). This could explain why 4-oxo-11, 13-*cis*-retinoic acid, 13-*cis*-retinoic acid and 3-hydroxy-retinoic acid had similar activities in all assays.

Although 4-oxo-13-*cis*-retinoic acid was not very effective in stimulating GJIC, it was very effective in the transactivation of the RARß2-promoter. On the other hand, other compounds showed even higher affinities to the RARß2 receptor, especially 4-oxo-11, 13-*cis*-retinoic acid and 13-*cis*-retinoic acid which produced almost identically effects whereas the effect of 3-hydroxy-retinoic acid was very close to that of retinoic acid.

The same set of test compounds was examined for their effect to regulate Cx 43 gene expression. The most effective compound was retinoic acid itself, which was

followed by 3-hydroxy retinoic acid, 4-oxo-11, 13-*cis*-retinoic acid and 13-*cis*-retinoic acid. They showed a Cx 43 mRNA stabilisation via the 3´UTR but they showed no significant effect on the Cx 43 promoter located on the 5´ region of the plasmid construct with the exception of 3-hydroxy retinoic acid. However, 4-oxo-13-*cis*-retinoic acid was ineffective both on the 5´ promoter and 3´ untranslated regions of Cx 43 gene which could explain why it did not stimulate GJIC.

Enantiomers with an oxygen atom centered in the ring structure, 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid and 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid and a five-membered ring derivative of retinoic acid; 1,1-didemethyl-2-nor-4-oxo-retinoic acid were also tested in the same assay systems. 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid and 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid significantly stimulated GJIC up to 2.0-fold on day 1 and up to 2.1-fold on day 3 of incubation in comparison to the control, whereas 1,1-didemethyl-2-nor-4-oxo-retinoic acid was only able to induce a 1.4-fold increase in GJIC on day 3 of incubation. Interestingly, 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid was more effective in stimulating GJIC on day 1 of incubation, but on day 3 of incubation there was no difference. All-*trans*-retinoic acid itself induced GJIC up to 2.2-fold of control on day 3 of incubation. According to this, both enantiomers with an oxygen atom in the center of the ring structure were as effective as retinoic acid itself in stimulating GJIC whereas five-membered ring analog was less effective which could be due to the smaller size of ring structure and is also in accordance with the previous studies done with five-membered ring analogs of retinoic acid (Teicher, 1999).

3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid stimulated transactivation of the RARß2-promoter comparable to that of retinoic acid whereas 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid showed a lower level of the transactivation by a factor of 1.4 in comparison to retinoic acid. 1,1-Didemethly-2-nor-4-oxo-retinoic acid was not very

effective in the transactivation of the RARß2-promoter. On the other hand, the difference in the activities of the two enantiomers here was obvious and 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid was significantly favoured with respect to 3-S-6-R-all-*trans*-3,6-epoxy-6-hydro-retinoic acid to transactivate the RARß2-promoter. This could be due to differences of two enantiomers in 3-dimensional structure, i.e. oxygen atom facing front and facing back out of the planar ring structure. According to this, 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid could be favoured with its oxygen atom facing front due to a key-lock model of the receptors and their ligands.

The same compounds were tested for their effects on Cx 43 gene regulation. 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid was the most effective derivative, whereas 1,1-didemethly-2-nor-4-oxo-retinoic acid was only effective to some extent as in accordance to their effects on the stimulation of GJIC. In this assay, the difference between the efficiency of two enantiomers was relatively lower compared to their efficiency to transactivate the RARß2-promoter. According to this experiment, compounds promote their effects due to the stabilisation of Cx 43 mRNA via 3´UTR, since none of the compounds was able to regulate the Cx 43 promoter on 5´region except 3-R-6-S-all-*trans*-3,6-epoxy-6-hydro-retinoic acid which was effective to some extent.

Many of the mRNAs coding for regulatory proteins in eucaryotic cells are unstable because of an evolutionary conserved 50-nucleotide AU-rich sequences on their 3´UTR regions which promotes the removal of poly-A tail and thereby stimulating mRNA degradation. Other unstable mRNAs contain specific recognition sites in their 3´UTR for specific endonucleases that cleave the mRNA (Alberts et al, 1994). Retinoic acid and all of its derivatives, which cause an increase in GJIC, also stabilised Cx 43 mRNA via 3´UTR region. The experiments performed by using the CxlucSV40 plasmid showed that the influence of the test compounds on the Cx 43 promoter, which is located on the 5´ region of the plasmid, is alone not enough to show an increase in the reporter gene

expression. On the other hand, these compounds have similar effects on the transactivation of the RARß2-promoter as their effects on the other two assay systems with one exception; 4-oxo-13-*cis*-retinoic acid was able to perform transactivation but neither increased GJIC nor the mRNA stability. Both enantiomers with oxygen atom on the ring stimulated GJIC and were effective in the stabilisation of Cx 43 mRNA comparable to each other but they showed a big difference in their ability to transactivate RARß-promoter. Furthermore, previous studies have shown that retinoic acid causes an increase in GJIC and an increase in the amount of Cx 43 protein but there was no increase in the level of mRNA (Clairmont et al., 1996). As a result, the effect of retinoids on GJIC seems to probably occur due to the stabilisation of Cx 43 mRNA via posttranscriptional regulation rather than leading an increase in transcription rate of Cx 43 gene.

On the other hand, all well known teratogenic retinoids: retinoic acid, 13-*cis*-retinoic acid and 4-oxo-retinoic acid stimulated GJIC whereas 4-oxo-13-*cis*-retinoic acid, which is approximately 10 times less teratogenic than retinoic acid, was also less effective in stimulating GJIC. Since it has been established that GJIC has an effect on embryogenesis, there could be a link between teratogenicity of retinoids and their effects on GJIC.

4.2. Thalidomide

According to previous studies, in human skin fibroblasts stimulation of GJIC by thalidomide requires metabolic activation, which was performed via liver microsomes in the presence of NADPH (Nicolai et al., 1997) and also by NADPH alone (Nicolai, 1997). Although it is still unclear how NADPH-dependent mechanism can biologically activate thalidomide, it is speculated that some enzymes released to the culture environment could be involved (Nicolai, 1997). Here, it has been shown that in the presence of NADH alone, thalidomide was activated and able to stimulate GJIC both in HFFF2 and in WB-F344

cells. The other coenzymes used in the assay were not capable of activating thalidomide in both cell types. Upon incubation of cells with thalidomide in the presence of cell lysates and NADH in WB-F344 cells, GJIC was stimulated up to 1.6-fold of control, whereas in the presence of heat-treated cell lysate and NADH, GJIC was only 1.1-fold of control. NADH or thalidomide, when used alone, showed no effect on GJIC. Consequently, there are enzymes required to mediate the effect of NADH on the metabolic activation of thalidomide.

Using this assay system, the effects of thalidomide and its analogs on GJIC have been further examined. Thalidomide and EM 12, a thalidomide analog lacking a keto group in the five-membered ring, are potent teratogens in primates (Nogueira et al., 1996; Neubert et al., 1996) and both stimulated GJIC to some extend in WB-F344 cells in the presence of NADH. However, in the absence of NADH, neither compound show a pronounced stimulation of GJIC, although EM 12 seemed more effective than thalidomide. EM 12 affected GJIC in HFFF2 cells also in the absence of NADH and both compounds were relatively more effective in HFFF2 cells than in WB-F344 cells. However, EM 12 seems to be more effective than thalidomide to stimulate GJIC in general, which is in accordance with their teratogenic potential, i.e. EM 12 is also a more potent teratogen than thalidomide (Nogueira et al., 1996). EM 16, a non-teratogenic thalidomide analog (Nogueira et al., 1996), did not stimulate GJIC.

On the other hand, EM 364, which is the only analog carrying no keto groups in the five-membered ring, stimulated GJIC in both cell types in the presence and also in the absence of NADH. In other words, EM 364 did not require metabolic activation and was more effective than thalidomide and EM 12 in WB-F344 cells but similar to EM 12 in HFFF2 cells.

PGA, a teratogenic product of thalidomide hydrolysis (Meise et al., 1973), did not stimulate GJIC in the presence and absence of NADH in WB-F344 cells, although it was

effective in HFFF2 cells. However, EM 138, a stable metabolite of EM 12 (Kenyon et al., 1997) and also a PGA analog lacking a keto group in the five-membered ring, stimulated GJIC effectively up to 1.7-and 1.4-fold of control in the presence and absence of NADH respectively. These values were reversed when this compound treated HFFF2 cells.

EM 20, which has 2 keto groups in the phthalimide ring and a succinimide ring instead of a glutarimide ring, stimulated GJIC in both cell types upon incubation with NADH at very similar values and exhibited no effect in the absence of NADH. As a result, having a succinimide ring instead of a glutarimide ring did not cause a lost in the activity of the compound. Although having no keto group in phthalimide ring could be important in the efficiency of the other analogs, the effect of EM 20 on GJIC showed that there should be some other factors that could be important in the activity of compound.

Aside the possible effect of decreasing keto groups in the phthalimide ring, the open ring pattern of metabolism may be also important for the efficiency of compounds to stimulate GJIC. In other word, the hydrolysis of the glutarimide ring causes opening of the ring structure and give rise structures which have asymmetrical mirror images in all anologs except EM 16 which is not active and also non-teratogen. EM 12 and thalidomide have both a glutarimide ring but EM 12 has less keto group in the phthalimide ring and is both more effective in stimulating GJIC and more teratogen than thalidomide. Furthermore, EM 364 has also a glutarimide ring like EM 12 and thalidomide, but it has no keto group in the phthalimide ring, was the only compound effective without addition of NADH in both cell types and was more active than EM 12 and thalidomide in WB-F344 cell. EM 138 has the same hydrolysis product of glutarimide ring with PGA, but it has one less keto group in the phthalimide ring than PGA has. EM 138 was more effective in WB-F344 in stimulating GJIC compared to PGA, whereas PGA was more effective in HFFF2 cells. Therefore, the number of keto groups found in the ring could be important for stimulating GJIC in the case of having a glutarimide ring or having the same hydrolysis

product of glutarimide ring and this effect change also according to the cell type, namely less keto group is favoured in the presence of NADH in WB-F344 cells.

Enantiomers of thalidomide were tested for their effects on GJIC in WB-F344 cells in the presence and in the absence of NADH. In the absence of NADH both enantiomers did not show any effect on GJIC whereas GJIC was increased in the presence of NADH. However, even this increase was only up to 1.3-fold of control. There was almost no difference between the two enantiomers, although only S-enantiomer is teratogenic (Blaschke et al., 1979) which could be due to rapid chiral inversion in the aqueous solutions (Reist et al., 1998). Since compounds have the same ring structures concerning keto groups and asymmetrical hydrolysis products of the glutarimide ring, the difference in their teratogenic potential needs some further explanations. Although there is no study showing existence of a receptor for thalidomide, which could explain differences between enantiomers by a key-lock model, there are some studies showing that thalidomide could intercalate into the major groove of DNA (Stephens et al, 2000). According to this, it counts to have a 3-D structure compatibility fitting to the DNA groove and non-teratogen (+)-R-thalidomide can not fit to the major groove of DNA according to this hypothesis whereas teratogen (-)-S-thalidomide can. Therefore, differences in the activities of two enantiomers could be explained with their 3-D structures rather than having the different analogs of the phthalimide and glutarimide rings that should be further examined. However, PGA neither increased the transcription nor the translation of Cx 43 protein according to the previous studies (Nicolai, 1997), which excludes the possibility of binding of thalidomide and related compounds to the promoter region of Cx 43 gene on DNA.

Although thalidomide and EM 12 are very potent teratogens in primates (Nogueira et al., 1996; Neubert et al., 1996), they are found to be not teratogenic to rat embryos, i.e. thalidomide and its related compounds are species-specific teratogens. Thalidomide

stimulated GJIC in both cell types but it was slightly more effective in HFFF2 cells, whereas the difference in the efficiency of more potent teratogen, EM 12, in stimulating GJIC was more obvious and it was much more effective in HFFF2 cells. Enantiomers of thalidomide were also not very effective to stimulate GJIC in WB-F344 rat cells. Furthermore, the metabolic activation process may also be species specific, similar to the teratogenic properties associated with thalidomide, since thalidomide was metabolically active in the presence of human or rabbit microsomes, but not in the presence of rat microsomes (Bauer et al., 1998). Teratogen hydrolysis product of thalidomide, PGA, did not stimulated GJIC in WB-F344 cells, when it was very effective both in the presence and also absence of NADH in HFFF2 cells. According to this, the effect of thalidomide and analogs on GJIC may be species-specific and seems to be in accordance with their teratogen potential.

On the other hand, there are numerous reports supporting the importance of GJIC on embryonic development. However, connexin proteins, which form gap junctions, show differences in their types and importance among species. During the early stages of the rat embryo development, expression of another connexin protein, Cx 31, was found to be abundant whereas expression of Cx 43, which is most abundant in human embryo, remained relatively constant (Reuss et al., 1997). Different effects of thalidomide and its analogs on GJIC in human fetal skin fibroblasts (HFFF2) and in rat liver epithelial cells (WB-F344) could be related to the alterations in the expression of connexin proteins. If alterations of GJIC is the key mechanism explaining the teratogenic effect of thalidomide, then it would be also able to explain species-specific effect of the compound, which is always a problem to be explained for most of the hypotheses about the mechanism of thalidomide teratogenicity.

In previous studies performed with PGA, it has been shown that the effect of thalidomide on GJIC is not mediated by transactivation of RARß2-promoter. After

treatment with PGA alone or with PGA and retinoic acid, there was no transactivation observed (Nicolai, 1997). Moreover, no activation of VDR, which forms heterodimers with RXR, was observed due to treatment with PGA (Nicolai, 1997). Therefore, thalidomide does not bind to the retinoic acid and vitamin D receptors. PGA neither increased the transcription nor the translation of Cx 43 protein (Nicolai, 1997) which means that other effects of the compound rather than the regulation of connexin gene or mRNA as in the case of retinoic acid play a role. As a possible mechanism for regulation of GJIC, the effect of PGA on the phosphorylation of connexin proteins was tested, but PGA showed no effect on the phosphorylation of Cx protein on tyrosine residues (Nicolai, 1997).

On the other hand, PKC activated by phorbolesters; TPA or phorbol 12-myristate 13-acetate (PMA), phosphorylates connexin proteins on serine residues. Phosphorylated connexin proteins translocate from the membrane into the cytosol and thereby decreasing GJIC. However, 30 minutes simultaneous treatment of cells with PGA (10 μM) and PMA showed recovered values of GJIC compared to the treatment with PMA alone (Nicolai, 1997). On the other hand, after 30 and 60 minutes of treatment with TPA, it is possible to observe the translocation of phosphorylated Cx 43 proteins from membrane into the cytosol by immunohistochemistry assays using fluorescent Cx 43 antibody. Here it is for the first time shown that EM 364 was able to prevent translocation of Cx 43 proteins. After the treatment with EM 364 (10 μM) in the presence of TPA (100 nM), Cx 43 proteins mainly located in the membrane and the effect of TPA was prevented. This effect was comparable to that of PKC inhibitor staurosporine which is shown to decrease PKC activity and increase GJIC (Granot and Dekel, 1994; Tenbroek et al., 1997). However, the non-teratogen analog of thalidomide, EM 16, was unable to perform the same results even at 10 times higher concentrations. This effect could be performed either by preventing the

phosphorylation of connexin proteins or inhibiting PKC activity, which should be further examined with Western Blot analysis and PKC activity assays relatively.

Furthermore, thalidomide decreases angiogenesis mediated by IGF-1 and FGF-2. IGF-1 and FGF-2 are ligands for receptor tyrosine kinases on the cell membrane which in turn can activate a PLC-γ/PKC/raf-1/MAPKK/MAPK pathway or activate NF-κB which binds to DNA and causes the transcription of further proteins like TNF-α (Alberts et al., 1994; Gaubert et al., 2000) (Figure 11). On the other hand, thalidomide also inhibits the activation of the nuclear factor-κ B (NF-κB) (Marriot et al., 1999) and can selectively inhibit the production of TNF-α (Corral and Kaplan, 1999). Therefore, anti-angiogenesis effect, inhibition of NF-κB and TNF-α performed by thalidomide seems to be mediated due to alterations in PLC-γ/PKC/I-κB/NF-κB/TNF-α pathway probably at downstream of PKC (Figure 11). This patway further involve in the regulation of adhesion receptors (Rahman et al., 2000; Chen et al., 2000) and also gap junction channels (Figure 11). Furthermore, both IGF-1 (Stephens et al., 1998) and FGF-2 (Li et al., 1996) has been shown to play an important role in limb development which is also important concerning the malformed limbs due to thalidomide treatment during pregnancy. On the other hand, increasement of FGF-2 (Doble et al., 2000) and TNF-α (Hu and Cotgreave, 1997; Fernandez-Cobo et al., 1999) causes the decrease of GJIC.

On the other hand, thalidomide (Geitz et al., 1995) and EM 12 (Neubert et al., 1996) but not non-teratogenic compounds (Thiel et al., 2000) can selectively modulate (mostly down regulate) I-CAM-1, V-CAM-1, E-selectin and L-selectin which are cell surface molecules involved in the adhesion cascade. This further supports involment of thalidomide in the above mentioned PKC-related pathway. On the other hand, Ca^{+2}-dependent cell adhesion molecules (cadherins) shown to decrease GJIC (Wang and Rose, 1997). All these thalidomide effects can fit into a unified model incorporating several mechanisms which finally lead to the alteration on GJIC (Figure 35) and therefore, a

possible involvement of thalidomide in the PKC-related pathways, thereby stimulating GJIC could be a possible mechanism of its teratogenic action.

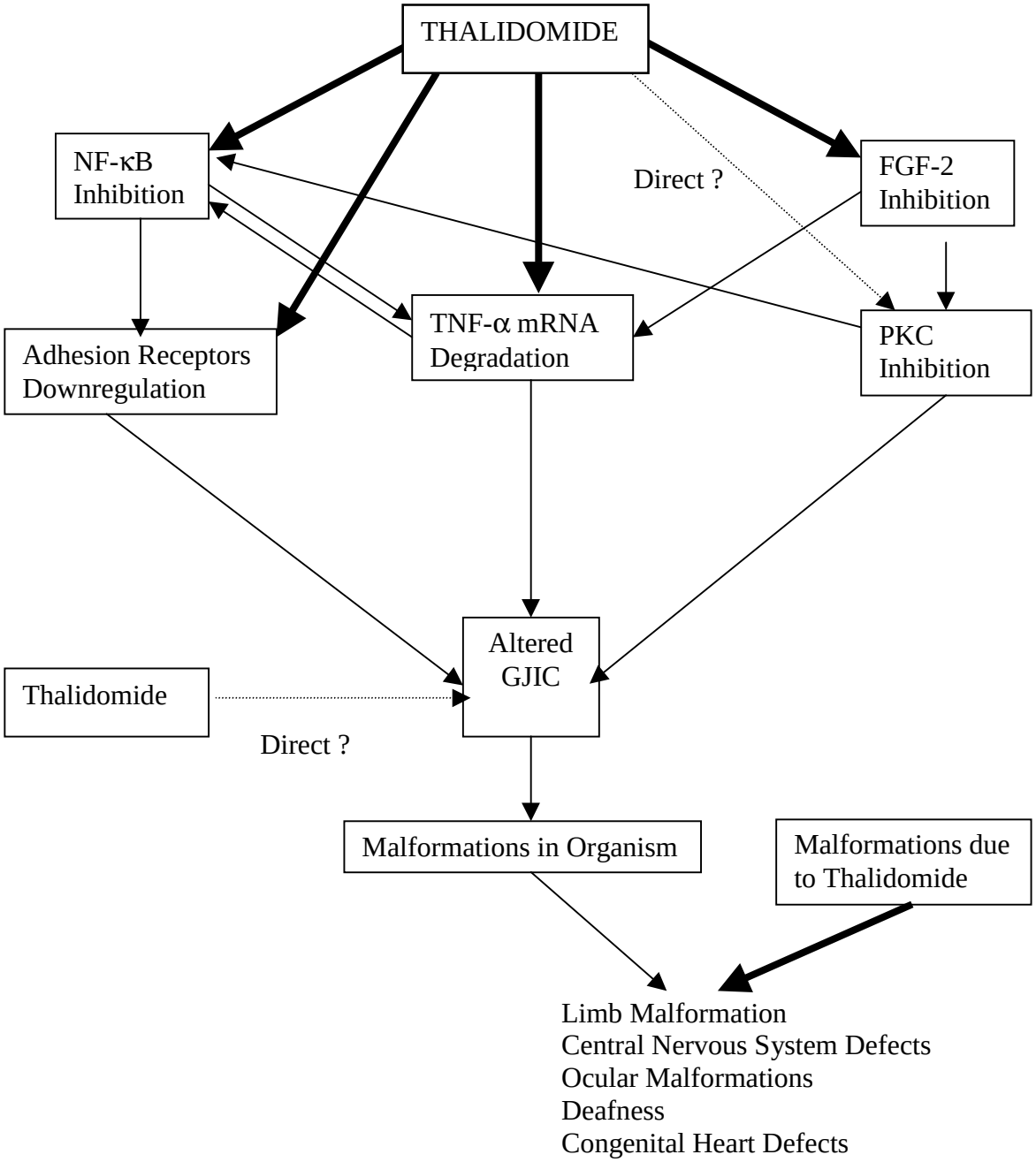


Figure 35. A Model Summarising Several Mechanisms That Might Lead to Alterations on Gap Junctional Intercellular Communication by Thalidomide. Bold arrows indicate already known effects of thalidomide whereas others indicate already studied interactions without involvement of thalidomide. The dotted arrows show suggested interactions of thalidomide.

4.3. Relation Between Teratogenicity and Gap Junctional Intercellular Communication

Gap junctional coupling has been demonstrated in embryonic tissues, and shown to be involved in the process of pattern formation (Goodenough et al., 1996). The most abundant connexin protein, connexin 43 (Cx 43) is differentially expressed during the development of mouse (Ruangvoravat and Lo, 1992; Dahl et al., 1995; Sullivan et al., 1993) and human embryos (Hardy et al., 1996). Cx 43 transcripts appear to be distributed as a gradient in regions spanning brain and embryo limb mesenchyma (Dahl et al., 1995; Sullivan et al., 1993). Furthermore, Cx 43 proteins have been implicated in the morphogenesis of the chick limb bud during the early development of chick embryo (Dealy et al., 1994; Green et al., 1994; Makarenkova and Patel, 1999). Moreover, gap junctions can also regulate bone cell differentiation (Donahue, 2000) and cartilage differentiation (Coelhe and Kosher, 1991). Relationship between the occurrence of congenital heart defects and alterations of GJIC has already been documented (Dasgupta et al., 1999).

Pattern in the developing limb depends on signaling by polarizing region mesenchyme cells which are located at posterior margin of the limb bud (Makarenkova et al., 1999). According to this polarization, cell-to-cell interactions in early limb development are considered within the framework of the signals; STOP, GO, STAY, and POSITION (Wolpert, 1990). Studies examining gap junctions and patterning in the developing limb showed that Cx 43 is asymmetrically distributed in the limb bud (Warner, 1999). During early limb development, Cx 43 was expressed higher at apical ectoderm ridge where outgrowth of limb occurs, whereas at non-ridge limb ectoderm Cx 43 expression was quite low. Furthermore, Cx 43 expression was high only on posterior subridge mesoderm, whereas it was low at anterior mesoderm (Dealy et al., 1994; Makarenkova et al., 1999). Therefore gradient expression of Cx 43 gives the necessary

direction (posterior) for the limb outgrowth which could be confused by increasing/decreasing GJIC in everywhere, thereby could cause a STOP signal for the limb elongation and could start formation of digits.

Adhesion molecules can interact with the formation of gap junctions and either increase or decrease GJIC according to the cell types and type of adhesion molecule (Wang and Rose, 1997). On the other hand, the importance of adhesion molecules in embryonic development has been also well established. According to this, they play a role not only in the formation of the gap junctions and the aggregation of cells in specific locations (Lehtonen et al., 1984, Frenzel and Johnson, 1996) but also are involved in the migration of the embryonic cells by decreasing the contact of cells and forming new adherent and gap junctions in new locations (Aoyama et al., 1985). GJIC is shown to modulate cell proliferation, cell differentiation, apoptosis (Trosko et al., 2000) and cell migration (McDonough et al., 1999). Therefore, alterations in the levels of growth factors and adhesion molecules or GJIC can further alter cell migration and aggregation of cells during embryonal development. Formation of cell aggregations or cell migration in developmentally wrong locations could then give rise to malformed structures. On the other hand, thalidomide has been shown to inhibit cell proliferation by inhibiting NF-κB activation (Moreira et al., 1999), which could further increase GJIC, and migration process during morphogenesis probably by binding adhesion molecule N-cadherin (Thiele et al., 2000). Retinoic acid is supposed to be itself a positional morphogen causing cell polarisation (Wolpert, 1990) and shown to regulate cell migration (Helige et al., 1993) and cell proliferation (Gibson et al., 1989). Furthermore, it is known that retinoic acid and related compounds affect adhesion molecules and growth factors (McCaffery and Dräger, 2000) and here also further proved that they can regulate Cx 43 gene post-transcriptionally, whereas thalidomide can downregulate adhesion molecule receptors and

inhibit angiogenesis induced by growth factors, IGF and FGF (D´Amato et al., 1994; Stephens et al., 2000).

GJIC is stimulated by all the well known teratogen compounds studied up to date: valproic acid (DiCarlo, 1990; Nicolai, 1997), 4-oxo-retinoic acid (Teicher et al., 1999), retinoic acid (Mehta et al., 1989; Stahl et al., 1998), 13-*cis*-retinoic acid, thalidomide, EM 12 and PGA (Nicolai et al., 1997) stimulated GJIC whereas non-teratogen EM 16 and weak teratogen, 4-oxo-13-*cis*-retinoic acid, were either ineffective or less effective in stimulating GJIC, respectively. As mentioned above, gap junctions are important for the central nervous system, ear, brain, eye and heart not only in performing their normal functions in normal adult but also for their formation during the development of embryo. Retinoic acid and thalidomide cause similar malformations including, limb malformations, craniofacial structures (ear malformations, absent or shrunken eyes), cataract, ocular movement abnormalities, deafness, congenital heart defects, malformed intestines, kidney malformations, central nervous system defects and mental retardation. Since both compounds are involved in a wide range of pathways interfering with GJIC and thereby alter GJIC and since both alteration of GJIC and treatment with these compounds give rise to similar defects on embryo, GJIC could be a possible key mechanism in the center of several incorporating mechanisms and thereby causing possibly malformed embryos.

5. SUMMARY

Gap junctions are cell-to-cell channels involved in cellular signaling and play a pivotal role in embryonal development. Stimulation of gap junctional intercellular communication (GJIC) by the potent human teratogens thalidomide and retinoic acid, and some of their analogs were examined in different cell types originating from human, mouse and rat tissues.

All-*trans*-retinoic acid and all of its derivatives that caused an increase in GJIC, also increased the stability of connexin 43 (Cx 43) mRNA which codes for the most abundant gap junction protein. The potent human teratogen, 13-*cis*-retinoic acid stimulated GJIC and increased the stability of Cx 43 mRNA to a similar extent as all-*trans*-retinoic acid. On the other hand, 4-oxo-13-*cis*-retinoic acid, which is weaker teratogen than all-*trans*-retinoic acid, was able to transactivate the RARß2-promoter but did not increase Cx 43 mRNA stability or GJIC. Although all retinoids transactivated the RARß2-promoter in F9 mouse teratocarcinoma cells, it seems that they affect GJIC via the post-transcriptional regulation of Cx 43 gene expression, rather than leading to an increase in the transcription rate.

The potent teratogens, thalidomide, its analog, EM 12, and the thalidomide hydrolysis product, 2-phthalimidoglutaric acid (PGA), stimulated GJIC significantly in human fetal skin fibroblasts (HFFF2). On the other hand, EM 12 which is known to be non-teratogenic in rats and PGA were less effective to stimulate GJIC in rat liver epithelial cells (WB-F344). The PGA analog, EM 138, stimulated GJIC more effectively in WB-F344 cells, whereas EM 12 analog, EM 364, was relatively more effective in HFFF2 cells. This might be related to a species-specific effect of thalidomide and its derivatives on GJIC which might be linked to their species-specific teratogenicity. Neither in HFFF2 nor in WB-F344 cells, there was any detectable alteration of GJIC with the thalidomide analog

EM 16, known as a non-teratogenic compound. All-*trans*-retinoic acid, which is a potent teratogen in humans and rats, stimulated GJIC significantly in cells from both species.

The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) activates protein kinase C (PKC) and thereby causes phosphorylation and translocation of Cx 43 protein from the membrane into the cytosol which results in a decrease of GJIC. EM 364 stimulating GJIC in both cell types, was effective to prevent TPA-induced translocation of Cx 43, whereas EM 16, which did not stimulate GJIC, was found to be not effective. Thus, thalidomide and its derivatives may stimulate GJIC by interfering with one of the PKC related pathways or by inhibiting PKC activity directly, thereby regulating phosphorylation of channel proteins which should be further examined.

It is suggested that modification of GJIC is related to the pharmacological and toxicological properties of all-*trans*-retinoic acid, thalidomide and their derivatives.

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July 98 - Sept . 98	Federation of European Biochemical Societies (FEBS), full fellowship, for living expenses during stay in Bern, Switzerland.
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May 26 - June 6 /97	Boğaziçi University Foundation Fellowship, supplement for expenses for NATO Advanced Study Meeting held in Antalya, Turkey.
May 26 - June 6 /97	UNESCO Fellowship, supplement for the expenses during NATO Advanced Study Meeting held in Antalya, Turkey.
Feb . 97 - July . 97	UNESCO, full fellowship, for the living expenses during stay in Bern, Switzerland

List of Abstracts and Publications:

Onat, D., Stahl, W. and Sies, H., ″Stimulation of Gap Junctional Intercellular Communication by Thalidomide and Thalidomide Analogs in Human Skin Fibroblasts (HFFF2) and in Rat Liver Epithelial Cells (WB-F344)″ Biochem. Pharmacol. 2001, in process.

Onat, D., Stahl, W. and Sies, H., ″Stimulation of Gap Junctional Communication by Thalidomide, 2-Phthalimido Glutaric Acid (PGA) and Retinoic Acid in Rat Liver Epithelial Cells (WB-F344) ″, Annual Fall Meeting of the German Society for Biochemistry and Molecular Biology (GBM), held in Munich, Germany during Oct. 10 - 13 /00.

Onat, D., Stahl, W. and Sies, H., ″ Stimulation of Gap Junctional Communication by Thalidomide, 2-Phthalimido Glutaric Acid (PGA) and Retinoic Acid in Rat Liver

Epithelial Cells (WB-F344) ″, 18th International Congress of Biochemistry held in Birmingham, England during July 16 - 20 /00.

Onat, D., Boscoboinik, D., Azzi A. and Başağa, H., " Effect of α-tocopherol and SDH on the proliferation of human skin fibroblasts" Biotechnol Appl Biochem. 1999 Jun; 29 (Pt 3): 213-5.

Onat, D., Boscoboinic, D., Ricciarelli, R., Başağa, H., Azzi, A., ″Activity of Protein Kinase C as a Function of In vivo and In vitro Ageing in Human Skin Fibroblasts and The Role of Antioxidants″, The First Reginal Meeting on Medical Sciences:The Roles of Free Radicals in Health and Disease, held in Jerusalem and Amman during March 22 – 27/98.

Onat, D., Boscoboinik, D., Azzi A. and Başağa, H., " Activity and Expression of Protein Kinase C as a Function of Age in Human Skin Fibroblasts Obtained from Different Age Donors″ NATO Advanced Study Meeting held in Antalya, Turkey during May . 26 - June .6/97.