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Increased Gap Junctional Intercellular Communication Is Directly Related to the Anti-Tumor Effect of All-Trans-Retinoic Acid Plus Tamoxifen in a Human Mammary Cancer Cell Line

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Abstract Additive effects against tumor cells might be achieved by combining anti-neoplastic agents directed against one or more altered mechanisms in cancer. We investigated the participation of gap junctional intercellular communication (GJIC), which is commonly dysfunctional in tumor cells as a possible mediating mechanism of the effect of all-trans-retinoic acid (RA) and tamoxifen (Tx) in MCF-7 human breast cancer cell lines. The combination of RA + Tx stimulated GJIC in approximately 53 ± 3% of MCF-7 cells as early as after 6 h of treatment remaining communicated through 144 h of culture. The GJIC enhancement occurred along with immunolocalization of Cx26 and 43 at the membrane of contacting cells and correlated with higher protein levels. Cx40 immunoreactive plaques were detected at cell-to-cell contacts during 48 h of RA + Tx treatment that did not involve higher protein expression, to the contrary, a downregulation occurred after 72 h of treatment. Cell proliferation inhibition upon RA + Tx exposure was observed with optimal effects at 96–120 h of culture with an accumulation of cells primarily in G2/M and G0/G1 cell cycle boundaries. An enhancement of the pre-existing E-cadherin levels was observed after drug exposure along with a downregulation of Bcl-2 and C-myc protein levels and a reduction of telomerase activity, suggesting partial tumor phenotype reversion. Blockage of the RA + Tx-induced GJIC with 18-β-glycyrrhetinic acid (β-Gly) prevented in 34% the inhibition of MCF-7 proliferation and the E-cadherin increment in 30% at 96 h of culture. GJIC blockage did not alter the downregulation of Bcl-2, c-Myc, or telomerase activity induced by RA + Tx. Our results showed the participation of GJIC as a mediator mechanism of the combined action of RA and Tx in MCF-7 cells. The chemopreventive modulation of GJIC might represent an approachable alternative for the improvement of cancer therapy. J. Cell. Biochem. 89: 450–461, 2003.

Key words: gap junctional intercellular communication; chemoprevention; anti-tumor; human breast cancer cells

In most normal tissues, the direct exchange of small molecules between the cytoplasm of contacting cells occurs through gap junctions (GJs) allowing the coordinated homeostatic integration of a group of cells [Yamasaki and Nauss, 1996]. Through the passage of signaling molecules, gap junctional intercellular communication (GJIC) participates in the regulation of cell proliferation, differentiation, cell death, and homeostatic maintenance [Lowenstein, 1979; Yamasaki and Nauss, 1996].

GJ are conglomerates of hexameric structures formed by connexins (Cx) which are coded by a multigene family and are expressed in a species- and cell-specific manner [Bruzzone et al., 1993; Goodenough et al., 1996]. Cxs-forming channels have a unique permeability and electrophysiological properties, which confer selectivity to the exchange of signals through GJ [Lowenstein and Kanno, 1966; Yamasaki, 1990]. The degree of GJIC can be modulated at the transcriptional, translational, and channel gating levels [Sáez et al., 1993]. Cell stimulation with tumor promoter agents, in most cases, inhibits intercellular communication [Trosko

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et al., 1993] and, in agreement with these observations, GJIC capacity of tumor cells are frequently found to be reduced or altered. Transfection of Cxs gene expression vectors into tumor cells restores GJIC and correlates with reduced tumorigenesis in vivo and with inhibition of cell growth in vitro [Eghbali et al., 1991; Mehta et al., 1991]. Additionally, enhanced cell-cell communication in Cx43 overexpressing tumor cells increases drug sensitivity [Huang et al., 2001], probably by inducing the GJIC-dependent bystander effect [Mesnil and Yamasaki, 2000].

Pharmacological stimulation directed to efficiently enhance GJIC in tumor cells might represent an approachable implement for the complementary improvement of anti-neoplastic therapies. Indeed, agents that exhibit tumor-preventing properties or cause cell differentiation, such as retinoids, β-carotene, and cyclic AMP can increase GJIC generally through upregulation of Cx43 [Rogers et al., 1990; Zhang et al., 1992]. In various experimental systems, the enhancement of GJIC due to retinoids has been correlated to cell growth inhibition [Mehta et al., 1989], to neoplastic transformation reversal [Mehta et al., 1986] and to morphogenic transformation suppression induced by some carcinogens [Watanabe et al., 1999], suggesting that cell-to-cell communication mediates, at least in part, the anti-tumor action of these agents

Retinoids exert a variety of biological effects related to cellular differentiation and proliferation, and they have been proven to be useful in the prevention and treatment of cancer [Sporn and Roberts, 1983]. A number of breast cancer cell lines have been demonstrated to be susceptible to proliferation inhibition due to retinoid action, however, cell lines expressing estrogen receptor (ER+) are commonly found to be more sensitive to growth inhibition by retinoids than those lacking the receptor [Fontana et al., 1990]. Growth of ER positive breast cancer cells and tumors depend on estrogen and are therefore sensitive to growth inhibition by ER antagonists, such as tamoxifen (Tx) [Nayfield, 1995]. Tx, as an anti-estrogen, inhibits the growth of breast cancer, primarily through competition for ER binding, although Tx has also been shown to exert an antiproliferative effect in ER-negative cells, suggesting the existence of mechanisms other than ER antagonism [Perry et al., 1995]. Downregulation of c-myc gene expression, in relation to cytostatic effect, was reported to occur after Tx exposure in several breast and cervical cancer cell lines, with the exception of MCF-7 cells, in which c-myc expression was not affected [Kang et al., 1996]. When C-myc, which is overexpressed in ~30% of breast cancer and essential for cell cycling [Koskinen and Alitalo, 1993], and bcl-2, one of the major genes implicated in the regulation of apoptosis [Reed, 1994], are overexpressed, they have been described to confer tumor chemoresistance, resulting in an unfavorable prognosis [Fanidi et al., 1992]. Together, the myc-oncogene and the proto-oncogene bcl-2 have been found to cooperate in the neoplastic transformation of normal rat liver epithelial cells, and this effect was related to GJIC downregulation [DeoCampo et al., 2000]. Retinoids and Tx have been independently studied for their ability to regulate c-myc [Kang et al., 1996; Shang et al., 1998] and bcl-2 expression [Toma et al., 1997; Zhang et al., 1999]. However, the combined effect of retinoic acid (RA) and Tx on c-Myc and Bcl-2 regulation in relation to GJIC, has not yet been studied.

Among the altered mechanisms of tumor cells, increased telomerase activity has been reported for the majority of cancer cells analyzed and its activation seems to be a sign of negative prognosis [Hoos et al., 1998; Hackett and Greider, 2002]. RA and Tx have been independently shown to downregulate telomerase activity in some cell lines [Herbert et al., 2001; Ding et al., 2002]. As part of their anti-tumoral mechanism of action, however, telomerase activity in relation to GJIC antiproliferative effect has not been addressed.

Based on their independent anti-tumor activity, we investigated the effect of RA and Tx in relation to GJIC in MCF-7 human mammary carcinoma cells in an attempt to target additional altered mechanisms in cancer and with the aim of pharmacologically inducing GJIC as a possible modulating mechanism favoring their anti-neoplastic activity.

MATERIALS AND METHODS

Chemicals

All-trans-RA and Tx (Sigma) were dissolved in absolute ethanol (Merck) at a concentration of 10^{-2} M and then diluted in culture medium to the required final concentration. Stock solution of 18-β-glycyrrhetinic acid (β-Gly) (Aldrich

Chemical Co, Inc.) was prepared in DMSO at 50 mM and diluted in cultured medium at a final concentration of 35 μM. All stock solutions were kept at −80°C. Procedures involving RA were performed in subdued yellow light.

Cell Culture

Human mammary-carcinoma MCF-7 (ER+) cell lines were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (Gibco) and antibiotics in a humidified incubator (5% CO_2, 95% air) at 37°C. Cells were synchronized by serum deprivation 24 h before the addition of complete media containing RA, Tx, and/or β-Gly. Cell media and drugs were renewed every 48 h of culture.

Intercellular Coupling

Gap junctional intercellular communication (GJIC) of MCF-7 cells, cultured on glass cover slips, was evaluated based on intercellular transfer of the fluorescent dye, Lucifer yellow (dilithium salt; Sigma, Poole). Single cells were microinjected with 10 mg/ml of the dye dissolved in 150 M LiCl. The dye was microinjected through glass microelectrodes by brief over-compensation of the negative capacitance circuit in the amplifier until the microinjected cell was brightly fluorescent. Cells were observed for 1–3 min to determine whether dye transfer to neighboring cells had occurred. Dye coupling was observed in a Nikon Diaphot microscope with xenon arc lamp illumination (excitation wavelength: 450–490 nm; emission wavelength >520 nm). The cell coupling percentage was scored as the number of microinjected cells that allowed dye transfer to two or more adjacent cells. At least 10 cells per experiment were microinjected of approximately 15 independent experiments.

Immunocytochemistry

Cells grown on glass cover slips were fixed and permeabilized in 70% ethanol at −20°C for 20 min. After the non-specific binding sites were blocked with normal goat serum, cells were incubated with an anti-Cx43,-Cx26 rabbit polyclonal antibody (Zymed), or-Cx40 rabbit polyclonal antibody, generously provided by Dr. Juan C. Sáez; (Department of Physiological Sciences, Pontificia Universidad Católica de Chile). Sub-cellular localization of Cxs was visualized after incubating the cells with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma) and mounted on Gelvatol Airvol 205 plus 100 mg/ml DABCO (Sigma), with a Olympus BX60 microscope and photographed using a T-max 400 film (Kodak).

Western Blotting

Relative protein levels for Cxs 43, 40, 26, E-cadherin (Zymed), c-Myc, or Bcl-2 (Santa Cruz) were analyzed by SDS–PAGE and immunoblot analysis. Cells were scraped-collected in the presence of proteases inhibitors (phenylmethylsulfonyl fluoride 3 mM, soybean trypsin inhibitor 200 μg/ml, EDTA 20 nM, pH 10) and phosphatase inhibitors (pyrophosphate 20 nM, NaF 100 mM, $NaVO_3$ 200 μM), and lysed by sonication with an ultrasonic cell disrupter (Microson, Heat system). Total protein content in cell lysates was determined by the Bradford method (Bradford, Bio-Rad labs, Hercules, CA). Samples containing up to 200 μg of total protein were separated in a SDS–PAGE and electrotransferred to a nitrocellulose membrane. After blocking non-specific binding sites with 5% non-fat milk, the membranes were incubated with the specific antibody at dilutions suggested in accordance with the manufacture's protocol. The membranes were then incubated with a secondary antibody conjugated to alkaline phosphatase. Antigen-antibody complexes were detected with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma). Equal sample loadings were detected by staining the membranes with Ponceau S. Immunostained protein bands were scanned at a resolution of 200 dpi and quantified.

Cell Cycle Parameters

Cells were trypsinized, washed twice with PBS, and resuspended in 0.5 ml of hypotonic fluorochrome solution, 50 μg/ml of propidium iodide (PI; Sigma Aldrich, St. Louis, MO) in 0.1% sodium citrate plus 0.1% Triton X-100. Fluorescence of individual nucleus (10,000 events) was analyzed by flow cytometry (Becton Dickinson FACScan) using LYSIS II Software (BDIS) for instrument control and data acquisition. DNA fluorescent signal pulse processing (pulse area versus pulse width) was used to exclude doublets and aggregates from analysis. The percentage of cells in sub-G_0/G_1 region and the G_0/G_1, S, and G_2-M phases of the cell cycle were determined using Mod FIT software (Verity Software House, Topsham, ME).

Apoptosis Assays

Three approaches were performed to analyze apoptotic cells. Propidium iodide exclusion analyses, TUNEL, and Sub G0/G1 population as part of cell cycle studies, described earlier.

TUNEL. Control and treated cells, grown on cover slips, fixed, permeabilized, and treated with terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling according to manufacture's conditions (Upstate, Lake Placid, NY). Fluorescence cells were observed with an Olympus BX60 microscope under fluorescent light (excitation wavelength: 450–490 nm) and photographed using Kodak T-max 400 film. Experiments were repeated at least twice.

Telomerase Activity

For quantitative analyses of telomerase activity, the PCR-based method was performed using the TRAPeze telomerase detection kit from Intergen according to the manufacturer's protocol (Intergen Company, Purchase, NY). Assays were performed in 25 μl reactions following the manufacturer's protocol for radioisotopic detection. Reactions were incubated for 30 min at 30°C for telomerase-mediated extension of the TS primer, and further subjected to 30 PCR cycles consisting of 94°C, 30 s/58°C, 30 s. PCR products were separated by electrophoresis for 2.5 h at 200V on 10% non-denaturing polyacrylamide gels. Samples were analyzed in at least two independent reactions with reproducible results and equal sample loadings were monitored in a silver stained gel of the same samples used for the telomerase activity assay. Product visualization was done by autoradiography. Densitometric analysis was performed using the ImageQuant 5.0 program (BioRad).

RESULTS

Effect of RA and Tx on GJIC in MCF-7 Cells

According to the majority of the reports analyzing tumor cell lines, MCF-7, human mammary adenocarcinoma cells were poorly communicated through GJ (Fig. 1). Only $15 \pm 5\%$ of the cells transferred Lucifer yellow to neighboring cells without significant differences due to culture time or cell confluence. The working concentration of RA and Tx were chosen after performing time course experiments at doses between 10^{-5} and 10^{-8} M to obtain the maximum increase of GJIC without cell toxicity. Figure 1A,B shows the percentage of MCF-7 cells treated with either 10^{-6} M RA or 10^{-6} M Tx that transferred Lucifer yellow to two or more neighboring cells. An enhancement of GJIC at as early as 2–6 h of treatment was observed after exposure to either agent, reaching a maximum between 24 and 72 h of RA treatment and between 6 and 24 h of Tx and then declining to the GJIC degree of control cells (Fig. 1A,B).

Aiming to pharmacologically induce a higher intercellular communication, we studied the effect of the combination of RA and Tx on GJIC stimulation in MCF-7 cells.

As observed in Figure 1C, RA and Tx together induced a higher GJIC at 6 h of treatment, with an almost additive effect of the agents independently used. Although for the other times of treatment, the combination of RA + Tx did not induce a higher cell-to-cell communication, treated cells remained more communicated than control cells for longer times of culture than with the drugs added separately (Fig. 1C). After the additive effect obtained at 6 h of the combined treatment, approximately 50% of MCF-7 cells were communicated until 72 h of culture and approximately 30% of treated cells stayed more communicated than control MCF-7 cells for the remaining experimental time (Fig. 1C). The effect of RA + Tx on GJIC stimulation was more sustained throughout the culture time than with the drugs independently added, possibly allowing a more durable intercellular signaling exchange.

RA-Tx Effect on MCF-7's Connexin Expression and Immunostaining

In agreement with the low GJIC observed, MCF-7 cells under normal growth conditions showed a diffuse, faint Cx26, Cx40, and Cx43 immunostaining. Cxs were inmuno-visualized predominantly at the cell cytoplasm, however, some Cx26 punctuated labels were detected at cell-to-cell contacts of few control cells, possibly contributing to the low GJIC observed (Fig. 2A). Exposure to RA + Tx induced a sub-cellular re-distribution of the three Cxs studied, to the plasma membrane of a large proportion of contacting cells (Fig. 2A arrows and insets). While Cx26 and 43 remained at the cell-to-cell contact membrane during most of the culture time, Cx40 cell-membrane label diminished after 48 h of treatment, acquiring an immunostaining similar to that of control cells (Fig. 2A).

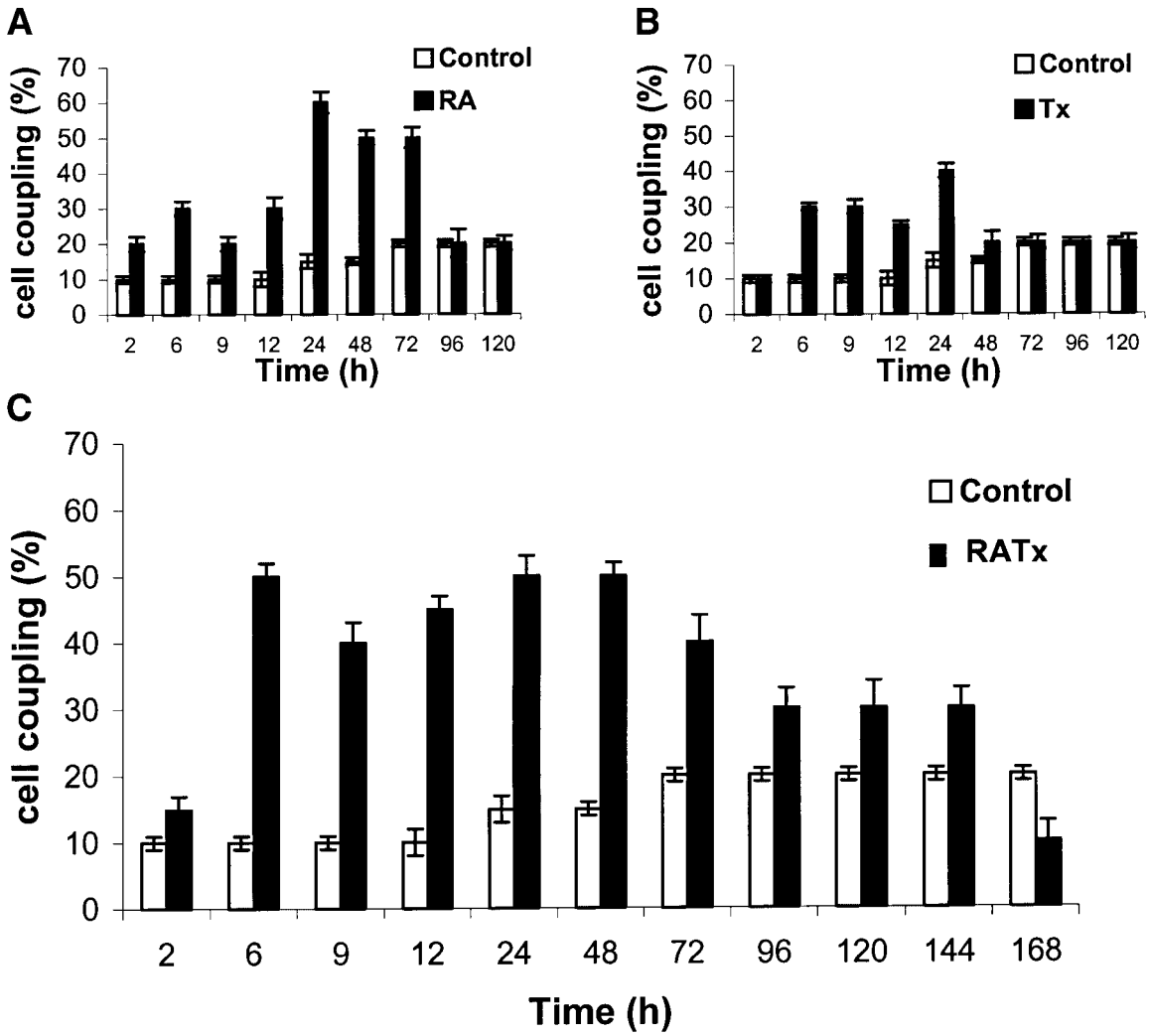

Fig. 1. Effect of RA and Tx on GJIC of MCF-7 cells. Synchronized MCF-7 cells, in the log phase of growth, were treated with (**A**) 10^{-6} M of RA or (**B**) 10^{-6} M of Tx for 120 h, and (**C**) RATx for 168 h as described under Materials and Methods. Graphs represent the mean value of the percentage microinjected of MCF-7 that transfer Lucifer yellow to two or more contacting cells. Open bars: untreated cells; solid bars: treated cells; n = 15.

These observations suggested that the initial increment of GJIC induced by RA + Tx might involve the participation of Cxs 26, 40, and 43, but the maintenance of cell-to-cell communication might preferentially involve Cx26 and Cx43 forming channels.

Western blot analysis indicated that the GJIC enhancement and the Cxs cell-membrane increment stimulated by RA + Tx involved changes in Cxs protein expression (Fig. 2B). An increase of Cx26 and Cx43 protein levels was induced by RA + Tx in MCF-7 cells for 96 h by Cx26 and for 120 h by Cx43 (Fig. 2B). Treatment with the agents separately showed an increase of Cx43 protein levels for 72 h in RA treated cells as well as a slight increase in Cx26 protein during the first 24 h of treatment. Tx alone induced a slight increase of Cx26 protein levels during the first 72 h of treatment, and Cx43 protein variations were not detected (results not shown).

Despite the immuno-visualization of Cx40 at the cell-contacting membrane of some RA + Tx-treated MCF-7 cells for 24–48 h of culture, no significant increase of protein levels was detected, instead a decrease was observed for 96 h of treatment (Fig. 2B). These results suggest that RA + Tx might regulate the expression of Cx40 differently than that of Cx43 or Cx26.

The Western blot results are in agreement with the immunocytochemistry observations,

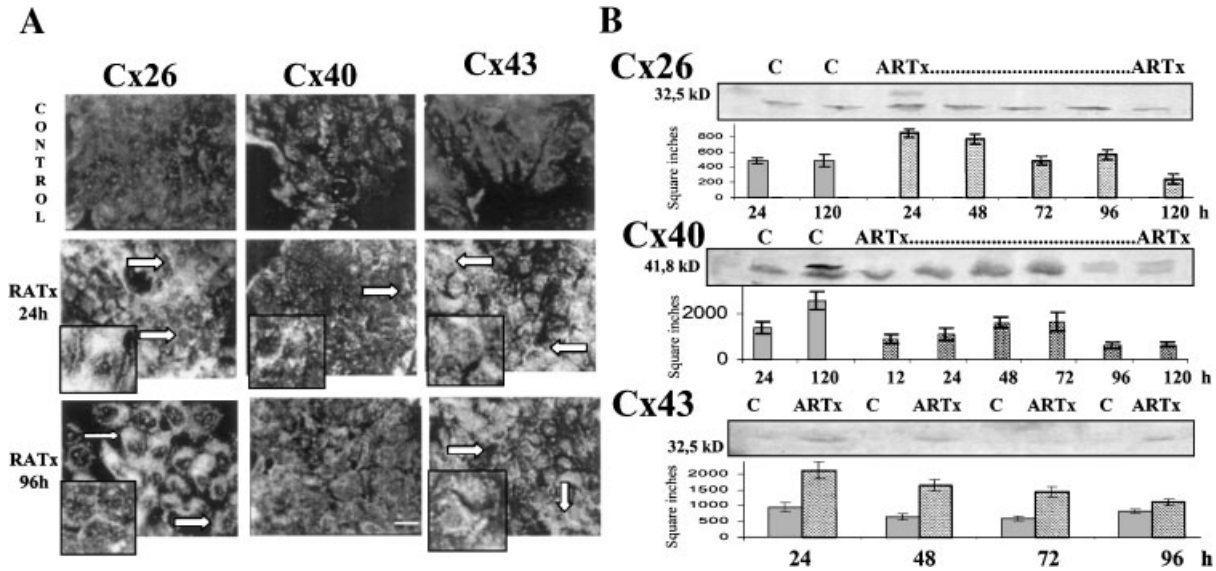

Fig. 2. Effect of RA + Tx on subcellular immunolocalization of connexins and on protein levels. **A**: Immunocytochemistry of MCF-7 control and treated cells for the detection of Cxs 26, 40, and 43 at 24 and 96 h of culture. The arrows show the Cxs immunolabeling at the membrane of contacting cells, and insets show an enlarged view of contacting cell membranes expressing the specified Cxs. Magnification bar = 6×10^{-4} m. **B**: Western blot analysis of Cx26, 40, and 43 in control and RA + Tx treated MCF-7 cells. The graphs for each blot represent the band density quantified with the NIH Image Scion 1.62c program. Results obtained from three independent experiments were used to calculate the standard deviation values.

suggesting that RA + Tx induced an increase of Cx26 and Cx43 protein levels and cell-membrane location for most of the experimental time. However, only a subcellular re-distribution of Cx40 was induced at early times of culture, which did not involve significant changes in its protein levels.

GJIC Participation in Cell Growth and Cell Death

Cell proliferation inhibition of estrogen expressing breast cancer cells after retinoids or Tx treatment has been well documented [Perry et al., 1995; Toma et al., 1997]. We found that chemopreventive doses of RA and Tx, inhibited MCF-7 cell proliferation starting at 48 h of treatment with optimal results at 96 and 120 h of culture where an 84–91% of inhibition was observed (Fig. 3A–C).

In order to determine whether the proliferation inhibition observed might be mediated by GJIC, we blocked the enhancement of cell-to-cell communication induced by RA + Tx with β-Gly at 35 μM final concentration. The working concentration of β-Gly was chosen as optimal after testing several doses for the entire time of culture. We used the concentration of β-Gly that completely abolished cell-to-cell communication for 120 h of culture, assessed by Lucifer yellow microinjections and did not induced cell cytotoxicity or changes related to our studies.

Propidium iodide labeled cell-DNA analysis showed fewer RA + Tx treated MCF-7 cells in the S phase as compared with control cells, starting at 48 h of culture. Compared with control MCF-7 cells, an 84% reduction of treated cells in the S phase was observed by 96 h of culture, with an accumulation of cells mainly at G2-M and some in G1 cell cycle boundaries (Fig. 3A,B). When GJIC was blocked with β-Gly, an inhibition of only 50.3% of cell proliferation at 96 h was observed, suggesting that, at this time of treatment, 34% of the cell growth inhibition observed with RA + Tx was facilitated by GJIC. At 72 and 120 h of treatment, the reversion of cell proliferation inhibition induced by the blockage of GJIC was less than at 96 h, suggesting that the exchange of signaling molecules or metabolites through GJ had an optimal effect by 96 h of treatment (Fig. 3B).

Br-deoxy-Uridine assays and [^{3}H] thymidine incorporation analyses agreed with the results of cell cycle DNA content obtained, showing inhibition of proliferating cells in cultures treated with RA + Tx and a partial prevention of it in non-communicated RA + Tx treated MCF-7 cells. The GJIC mediating activity in cell proliferation inhibition ranged from 30% to 45% at 96 h of culture (not shown).

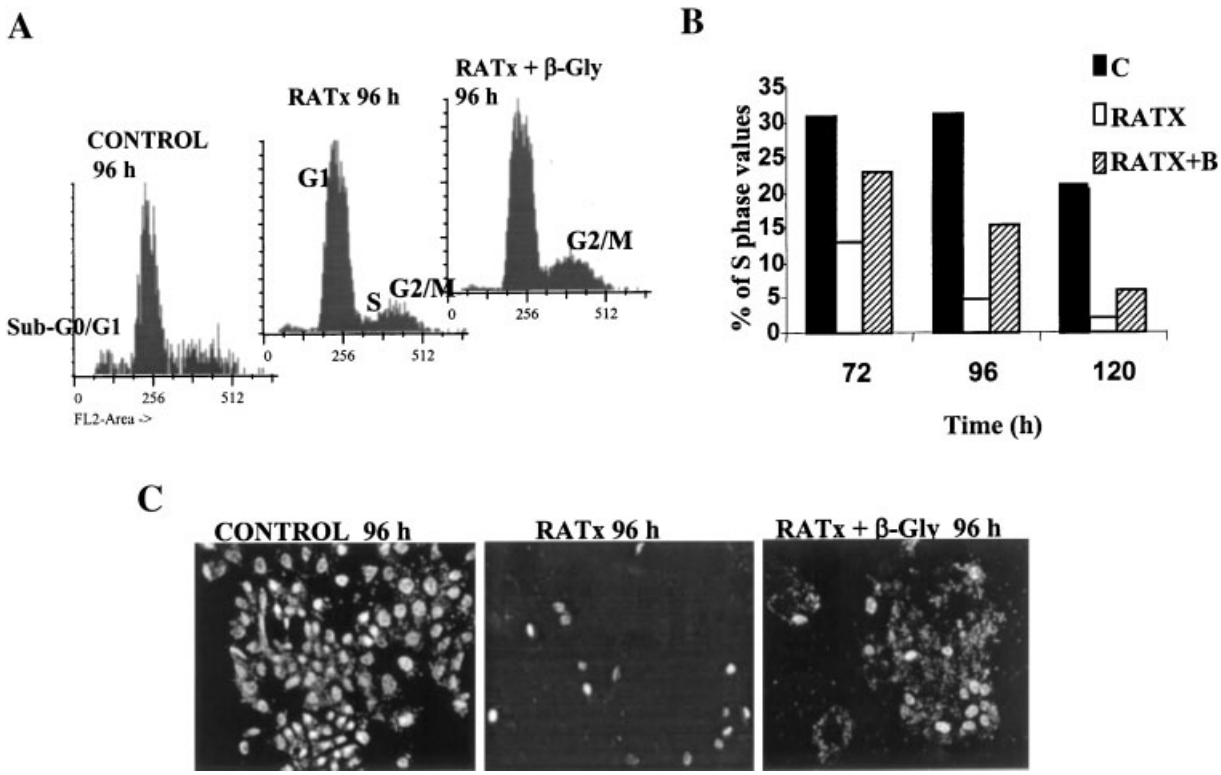

Fig. 3. Cell cycle profile of untreated MCF-7 cells, treated with RA + Tx and with the GJIC blocker β-Gly. **A**: DNA histograms of control and treated MCF-7 cells at 96 h of culture stained with propidium iodide and analyzed by flow cytometry as described in Materials and Methods. Histograms represent one of three independent experiments showing the percentage of cells in G1, G2/M, S, and sub G0/G1 cell cycle phases. **B**: The graph represents the percentage of MCF-7 cells in S phase of the cell cycle under the different culture conditions, at 72, 96, and 120 h of culture. **C**: Analysis of DNA fragmentation by TUNEL assay as described in Materials and Methods in control MCF-7 cells, 10^{-6}M RA + Tx, and 10^{-6}M RA + Tx + β-Gly treated cells. Micro-photographs were taken for the 96 h of treatment and represent one of two experiments.

Analysis of sub-G0/G1 population in MCF-7 cells showed a significant decrease in the number of apoptotic cells in cultures treated with RA + Tx as compared to MCF-7 cells grown under normal conditions, which started to die by 72 h of culture due to cell confluence. These results were corroborated by TUNEL (Fig. 3C), trypan blue, and propidium iodide exclusion assays (not shown). A slight increase in the amount of sub-G0/G1 cells was observed in cultures where GJIC has been blocked, although it was always significantly less than the cell death of control cells, suggesting that in this experimental model, GJIC does not facilitate cell death.

RA+Tx and GJIC Inhibition Effect on E-Cadherin, Bcl-2, and C-myc Protein Levels of Expression

The majority of epithelial tumors show reduced E-cadherin expression, which correlates with loss of tumor differentiation and increased invasiveness [Gamallo et al., 1993]. To determine whether RA + Tx and GJIC affects cell differentiation in addition to cell proliferation inhibition, E-cadherin protein levels were studied at different times of treatment by immunoblotting.

MCF-7 cells expressed E-cadherin when cultured under normal conditions, however, an enhancement over the pre-existing E-cadherin was induced by RA + Tx (Fig. 4) starting at 48 h of treatment. A partial reduction of the E-cadherin increment induced by RA + Tx was observed at 96 and 120 h β-Gly addition, suggesting that GJIC might mediate approximately 30% of the cell signaling exchanges related to cell differentiation.

The myc oncogenes that have been associated with the cellular functions of proliferation, differentiation and apoptosis, and the proto-oncogene bcl-2, mainly with apoptosis inhibition, have been shown to cooperate with neoplastic transformation, and their co-expression in

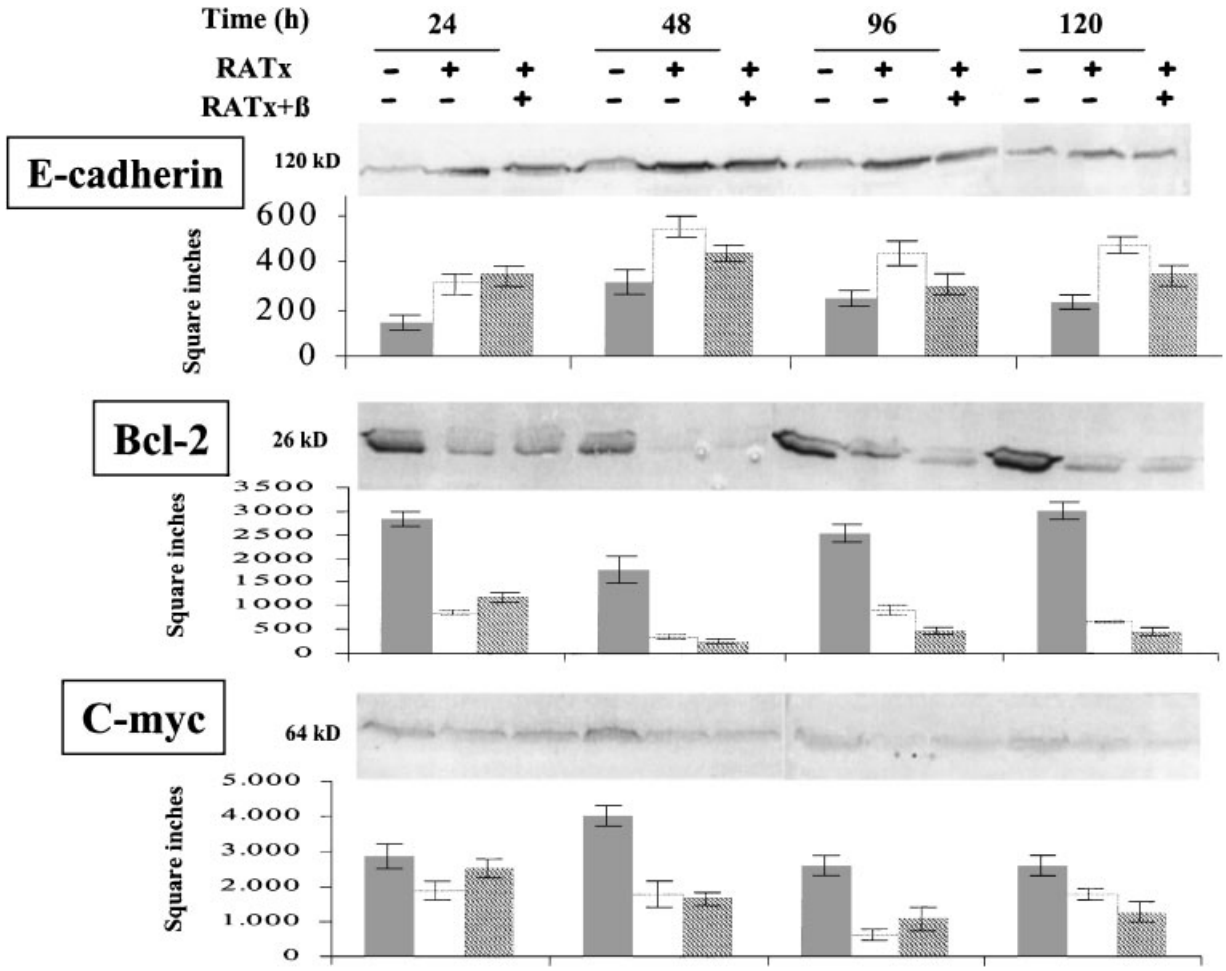

Fig. 4. Expression of E-cadherin, Bcl-2, and C-myc protein levels in MCF-7 cells after treatment with RA + Tx and with the GJIC inhibitor, β-Gly. MCF-7 cells treated with 10^{-6}M RA + Tx and/or 35 μM 18-β-Gly at different times of culture were subjected to Western blot analysis as described in Materials and Methods. The graphs under each immunoblot represent the quantification of each band density using the NIH Image software, Scion 1.62c. The standard deviation values were calculated from the results obtained in three independent experiments.

tumor cells has been related to refractory malignancy. Since MCF-7 cells expressed both c-Myc and Bcl-2 protein, we investigated the effect of RA + Tx on their regulation and a possible relationship with GJIC.

Starting at 24 h, a gradual downregulation of c-Myc occurred throughout the 120 h of treatment with the combination of RA + Tx (Fig. 4) agreeing with the inhibition of cell proliferation observed. In cultures where GJIC has been blocked, the levels of c-Myc remained as low as those obtained with RA + Tx, suggesting that cell-to-cell communication is not related to c-Myc expression.

Despite the low rate of cell death observed in RA + Tx cultures as compared with control cells, Bcl-2 protein levels significantly decreased through culture time starting at 24 h of treatment. GJIC blockage with β-Gly did not prevent the RA + Tx-downregulation of Bcl-2 (Fig. 4) suggesting that other mechanisms are responsible for Bcl-2 downregulation.

The treatment with the combination of RA + Tx, in addition to cell proliferation inhibition mediated by GJIC, downregulated both neoplastic markers, c-Myc and Bcl-2, suggesting a partial neoplastic phenotype reversion.

Telomerase Activity in RA + Tx Treated MCF-7 Cells

Telomerase is activated in the majority of tumors and cancer cell lines analyzed, and its activation is thought to be essential for unlimited cell proliferation [Hackett and Greider, 2002].

We examined the effect of RA + Tx on MCF-7 cell differentiation and cell proliferation inhibition upon telomerase activity and compared it with cultures where RA + Tx-stimulated-GJIC has been blocked with β-Gly. An increase of telomerase activity was observed in untreated MCF-7 cells over culture time as they reach cell confluence (Fig. 5A; lanes 4, 7, 10, 13, and 15). Starting at 72 h of RA + Tx exposure, a progressive decrease of telomerase activity occurred (Fig. 5A; lane 8), with optimal effect at 96 h of treatment (Fig. 5A; lane 11). MCF-7 cells where the induction of GJIC has been prevented with β-Gly, showed similar downregulation of telomerase activity over cultures treated with RA + Tx (Fig. 5; lanes 9, 12, and 17).

Figure 5B shows the values for the relative bands density of telomerase activity in cultures with RA + Tx and with β-Gly, plotted as percentage of inhibition with regard to the respective time control cells. MCF-7 cells treated with RA + Tx for 72 h showed approximately 13% of

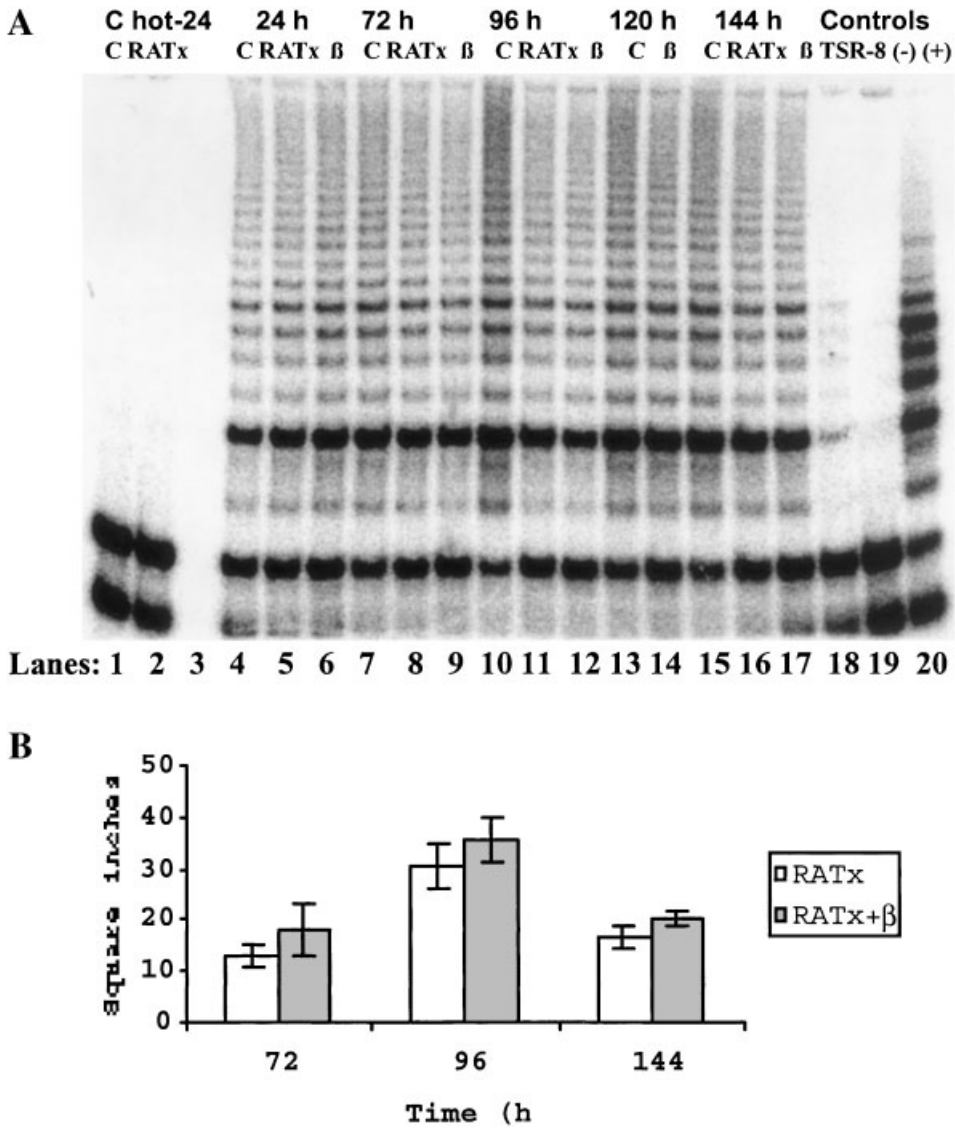

Fig. 5. Telomerase activity in MCF-7 control, RA + Tx, and RATx + β-Gly treated cells as measured by the TRAP method. **A**: Time course of telomerase activity in MCF-7 cells after exposure to 10^{-6} M RA + Tx (RT) or RATx + β-Gly (β), at 24, 72, 96, and 120 h. Internal controls are specified. Sample loading equality was tested by silver staining a 10% SDS–PAGE of the same samples than the telomerase gel. **B**: Values for the total density bands of RA + Tx and RATx + β-Gly treated cells at each time of culture were plotted as a percentage of telomerase activity inhibition compared with the corresponding control at each time of culture. Quantification of the radioactive bands was performed using the ImageQuant 5.0 Bio-Rad program. The results represent one of two independent experiments.

telomerase activity inhibition as compared to control cells at the same time of culture. A maximum of 32% of telomerase activity inhibition is observed by 96 h of RA + Tx treatment, and then by 144 h of culture, a partial recovery of its activity occurred. The presence of the GJIC inhibitor, β-Gly, in RA + Tx treated MCF-7 cells did not prevent the downregulation of telomerase activity observed in cell cultures grown only with RA + Tx (Fig. 5B), suggesting that GJIC participation on cell proliferation inhibition probably involved pathways unrelated to telomerase activity.

DISCUSSION

Even though enhancement of GJIC with antineoplastic agents has not been sufficient to induce tumor reversal, efforts to pharmacologically improve cell-to-cell communication might offer an approachable complementary therapeutic strategy toward the induction of neoplastic phenotype reversion and/or drug sensitivity enhancement.

In an attempt to maximize GJIC in MCF-7 tumor cells, we studied the combined effect of two chemopreventive agents, all-trans-RA, known to modulate GJIC, and Tx, widely used for breast cancer prevention.

Treatment with the combination of chemopreventive doses of RA + Tx enhanced GJIC in MCF-7 cells more efficiently than with the agents separately only at 6 h of treatment. The overall increase in GJIC induced by RA + Tx was similar to that obtained with either RA or Tx. However, the combined treatment induced cell-to-cell communication for longer culture times, which might allow cellular signaling and metabolite exchange for a longer period of time and therefore facilitate a more efficient antitumor effect of RA + Tx.

As in other systems [Mehta et al., 1989; Hirschi et al., 1996], GJIC stimulation was correlated with cell growth inhibition and phenotype changes toward cell differentiation. To study the possible correlation between increased GJIC and the cell proliferation and differentiation changes observed, we blocked the RA + Tx-induced cell-to-cell communication with β-Gly, which completely abolished cell communication through GJ. RA + Tx action, without GJIC as a mediator, had a diminished effect on cell proliferation inhibition and on cell differentiation as assessed by cell proliferation assays and E-cadherin protein expression, respectively. The participation of GJIC in these cellular effects was significant as its blockage prevented approximately 30% of the anti-proliferative effect due to RA + Tx as well as the expression of E-cadherin, a cell differentiation marker.

Increased expression of Cx26 and 43 protein levels was observed after RA + Tx exposure, probably explaining the enhancement of GJIC. Although during the first 48 h of treatment a proportion of cells showed Cx40 immunostaining at the membrane of contacting cells, no significant changes in protein levels were observed, instead a downregulation at later times of treatment occurred. Separate treatment with the agents did not induce significant changes of Cx40 protein levels, with the exception of cells treated with Tx, which showed a slight decrease by 120 h of culture (not shown). Although we cannot currently explain the differential regulation of Cx40 or the appearance of the second immunoreactive band at later periods of culture, it seems that the chemopreventive agents used might negatively regulate the expression of Cx40.

Activation of telomerase has been suggested to be necessary for tumor growth, and its downregulation has been associated with cell proliferation inhibition. Retinoids generally inhibited telomerase activity, suggesting that their anti-tumoral action might be, in part, mediated by their ability to downregulate telomerase activity [Choi et al., 2000]. Estrogens, on the other hand, stimulated telomerase activity [Kyo et al., 1999], and as might be expected, Tx has been shown to inhibit HTERT expression in the presence of estrogen in MCF-7 cells [Wang et al., 2002]. We were interested in studying whether the mediating effect of GJIC on RA + Tx-cell proliferation inhibition was related to telomerase activity. RA + Tx treated MCF-7 cells showed decreased telomerase activity independent of the degree of cell-to-cell communication, suggesting that RA + Tx action towards cell proliferation might involve alternative pathways to achieve similar effects.

Blockage of c-myc expression with c-myc antisense oligonucleotides has demonstrated its crucial participation in cell proliferation. Our results and others [Perry et al., 1995] have shown that c-Myc protein levels did not change when MCF-7 cells were treated with 1 μM of Tx. On the other hand, according to our findings as

well as those of other reports, c-Myc expression was not efficiently downregulated by RA alone [Shang et al., 1998]. Here we show that treatment of MCF-7 cells with the combination of chemopreventive doses of RA + Tx greatly reduced c-Myc protein levels by 48 h of culture, indicating a synergistic effect of RA + Tx on c-Myc downregulation and therefore on MCF-7 cell proliferation inhibition. Blockage of GJIC induced by RA + Tx did not revert the downregulation of c-Myc, suggesting that the mediating effect of GJIC in the inhibition of cell growth uses unrelated mechanisms to those where c-Myc is involved.

Decreased bcl-2 expression has been correlated with the apoptotic activity reported for some retinoids [Toma et al., 1997]. Indeed, some apoptotic cells were observed in MCF-7 cells after 120 h of 1 μM RA exposure, which correlated with a decrease of Bcl-2 protein levels (not shown). Independently, 1 μM Tx has been shown to induce apoptosis in MCF-7 cells after 48 h of treatment [Zhang et al., 1999] along with a decreased expression of bcl-2 but not bax or bcl-X_L. Our results showed that treatment of MCF-7 cells with the combination of 1 μM RA and Tx downregulated Bcl-2 protein by 24 h of treatment, but regardless of the low levels of Bcl-2 during the 120 h of treatment, no significant induction of apoptosis was observed. In fact, treated cultures were followed for 7 days, and while control cells died due to overgrowth, a great proportion of treated cells remained attached. Flow cytometry analysis of cell DNA integrity, TUNEL assays, and dye exclusion tests agreed in the detection of low apoptotic cells in RA + Tx treated MCF-7 cells. Inhibition of RA + Tx-induced GJIC did not have a significant effect on the diminished cell death observed in treated cultures without the GJIC blocker. These results suggested that the enhancement of GJIC due to RA + Tx would mainly mediate neoplastic phenotype reversion in MCF-7 cells and not the processes related to cell death. We have used chemopreventive doses of agents that did not cause cell damage, and therefore GJ participation in the spreading of death signals might have not be expected.

Overall, our results showed that the combined treatment of RA + Tx induced a significant enhancement of GJIC in MCF-7 cells that was responsible for ~34% of the observed cell growth inhibition and for ~30% increase of E-cadherin protein levels, a cell differentiating marker. We believe that the induction of GJIC by RA + Tx along with the downregulation of Bcl-2, c-Myc, and telomerase activity, does not only result in a partial reversion of the malignant phenotype, but may also contribute to a higher sensitivity of tumor cells to the more effective action of traditional anti-neoplastic drugs. Further studies are currently being conducted to determine whether RA + Tx treatment resulted in the sensitization of MCF-7 cells to traditional anti-neoplastic drugs.

The pharmacological restoration of cellular communication through GJ could potentially offer a target mechanism with the possibility of modulation to complementarily improve therapeutic strategies against cancer.

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