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ORIGINAL ARTICLE

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Upregulation of gap junctional intercellular communication and connexin 43 expression by cyclic-AMP and all-*trans*-retinoic acid is associated with glutathione depletion and chemosensitivity in neuroblastoma cells

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Abstract *Purpose*: Downregulation of gap junctional intercellular communication (GJIC) has been implicated in carcinogenesis. This is a result of altered expression of connexins, the proteins that mediate GJIC, including connexin 43 (Cx43). Our aim was to evaluate the effect of known inducers of Cx43 on the chemosensitivity of the human neuroblastoma cell line IMR-32 to chemotherapeutic agents. *Methods*: We examined the effect of dibutyryl-cyclic AMP (db-cAMP) and all-*trans*-retinoic acid (tRA) on Cx43 and GJIC, glutathione (GSH) and γ-glutamyl-cysteine-synthetase (γ-GCS) levels, and glutathione S-transferase (GST) activity. Finally, we performed cell survival assays to measure the response of IMR-32 cells to the chemotherapeutic drugs doxorubicin, melphalan and bis-chloronitrosourea (BCNU), after treatment with db-cAMP and/or tRA. *Results*: Exposure to db-cAMP led to the upregulation of GJIC and Cx43 expression and phosphorylation. On the other hand, exposure to tRA led to the upregulation of GJIC but Cx43 expression and phosphorylation were not greatly affected. The combination of both agents was more potent in inducing GJIC in comparison to treatment with db-cAMP or tRA alone. Treatment with db-cAMP, but not with tRA, was associated with a significant increase in the cytotoxic effects of the anticancer drugs doxorubicin, melphalan and BCNU as shown by a decrease in their IC_{50} values. Concomitant exposure to db-cAMP and tRA, however, had a more pronounced effect on cell sensitization to chemotherapy drugs (particularly doxorubicin) than exposure to db-cAMP or tRA alone. Under the db-cAMP and tRA treatment conditions (which upregulate GJIC and modulate drug response), GSH levels were significantly reduced while the levels of GST and γ-GCS activities remained unchanged. *Conclusions*: This study suggests that GJIC plays a role in cellular drug resistance, and highlights the potential use of GJIC modulators in combination with chemotherapy. Also, this is the first study exploring the ability of both db-cAMP and tRA to enhance cell chemosensitivity.

Key words Connexin 43 · Intercellular communication · Glutathione · Chemosensitivity

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Introduction

The benefits of currently used chemotherapy drugs are limited in part by the development of tumor cells with a multidrug resistance phenotype. The cellular mechanisms of drug resistance have been studied extensively, and some of the molecules involved have been characterized, e.g. P-glycoprotein [33], multidrug resistance-associated protein [14], topoisomerase enzymes [7], and glutathione (GSH) and associated enzymes (reviewed in reference 6). The resistance patterns associated with these mechanisms generally result in cross-resistance to structurally and functionally different drugs.

Cell-cell interaction via gap junctions (GJs) is considered to be of fundamental importance in the maintenance of tissue homeostasis [27, 28], and their alteration has been observed in cancer cells. GJs are composed of multiple hemi-channels (connexons) in the plasma membrane of one cell joined in mirror symmetry with the same number of hemi-channels in the apposing cell membrane. Connexons are formed from members of a multigene family of distinct but functionally related proteins called connexins [10, 19]. Among various con-

nexins, connexin 43 (Cx43) is consistently reduced in chemically transformed and cancer cells [47, 49]. Upregulation of Cx43 can lead to the reversal of features of the transformed phenotype, induction of differentiation, and a reduction in the rate of cell growth [30, 37].

Connexins are tightly regulated by growth factors, a number of secondary messengers including cyclic AMP [3] (cAMP), and phosphorylation by various protein kinases [19, 25]. Numerous studies have demonstrated that cAMP is a potent inducer of differentiation and has an antiproliferative effect [13]. Treatment with cAMP leads to decreased proliferation of neoplastic cells and the expression of antigens characteristically observed in maturing nontransformed cells of the same lineage. In the case of solid tumors, differentiation may also include the restoration of intercellular communication with the mature surrounding cells [20]. Indeed, it has been shown that tumor cell differentiation is associated with restoration of a diminished gap junctional intercellular communication (GJIC) that is often observed in transformed cells [29]. Although cAMP-dependent protein kinase has been associated with resistance to chemotherapeutic agents [16], cAMP has not been examined to date.

Retinoids have been studied as antiproliferative therapeutic agents since clinical trials with all-*trans*-retinoic acid (tRA) demonstrated dramatic effects in the treatment of acute promyelocytic leukemia (APL), even in patients who relapsed after previous treatment with chemotherapy [45]. It has also become evident that resistance to tRA occurs in a large number of cases [34] and current data suggest that a combination of tRA treatment followed by chemotherapy is optimal for APL [41]. Recent work in the area has demonstrated that tRA can alter cell chemoresistance, but its mechanism of action is as yet unclear [44]. In addition to their interaction with retinoic acid receptors, retinoids as well as their precursor carotenoids have been shown to upregulate GJIC and Cx43 in some cancer cells [5].

In this study we investigated the role of tRA and/or dibutyryl-cAMP (db-cAMP) in the context of chemoresistance to the chemotherapeutic agents doxorubicin, melphalan and bis-chloronitrosourea (BCNU). Our results strongly support a link between GJIC and drug resistance.

Material and methods

Chemicals and reagents

tRA, db-cAMP, L-phenylalanine mustard (melphalan), and doxorubicin were obtained from Sigma Chemical Co. (St. Louis, Mo.). BCNU was obtained from Bristol Laboratories (Canada). Reagents for the GSH assay were from Boehringer Mannheim (Laval, Quebec). Radioisotopes were from ICN Biochemicals (St. Laurent, Quebec). Calcein acetoxymethyl ester (calcein AM) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were obtained from Molecular Probes (Eugene, Ore.). The Cx43 antibody was a kind gift from Dr. Laird (University of Western Ontario, ON). The antibody to GAPDH was purchased from Santa Cruz Biotechnology, Calif.

Cell culture

The human neuroblastoma IMR-32 cells (obtained from the American Type Cell Culture Depository, Rockville, Md.) were grown in basic medium, also referred to as proliferating medium, which contained Eagle's minimal essential medium, 1% sodium pyruvate, 1% non-essential amino acids, 1% nucleosides, 1% glutamine, 10% heat-inactivated fetal bovine serum, and 0.6% penicillin-streptomycin. Cells were maintained at 37°C under an atmosphere of air containing 5% CO_2. When indicated, db-cAMP and tRA were added at concentrations of 0.5 m*M* and 10 μ*M*, respectively. These concentrations, which have no toxic effect on IMR-32 cells, were established based on the results of preliminary studies. Cytotoxicity to db-cAMP treatment was examined at concentrations 0.5, 1.0, and 2.0 m*M*, while tRA toxicity was examined at concentrations of 1, 10, and 100 μ*M* (not shown).

Western blotting for Cx43

A rabbit polyclonal antibody against Cx43 was used at a dilution of 1:1000. Treated cells were washed with PBS, collected by trypsinization, and centrifuged for 30 s at 12,000 *g*. Each pellet was resuspended in 0.4 ml 0.01 *M* Tris-Cl buffer (pH 8.0), containing 0.5 m*M* phenylmethylsulfonyl fluoride, 60 m*M* KCl, 1 m*M* EDTA, 1 m*M* DTT, 0.5% NP-40, 1 m*M* sodium orthovanadate, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, and 0.01 mg/ml aprotinin. The sample was then incubated on ice for 30 min, and centrifuged for 10 min at 12,000 *g*. The soluble fraction was collected and assayed for protein content. Equal amounts of protein were then electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. The immunoblots were processed as described previously [9] and immune complexes were detected by horseradish peroxidase conjugates. A mouse antibody against GAPDH was used to control for protein loading.

Functional assay of GJIC

GJIC was assessed by fluorescence-activated cell sorting (FACS) analysis as described by Kiang et al. [22] with minor changes. Treated cells were collected by trypsinization, washed in Dulbecco's PBS, and stained with either 4 μ*M* DiI (recipient cells) for 10 min or 0.5 μ*M* calcein AM (donor cells) for 15 min. Cells were subsequently washed with PBS and incubated for 2 h in 12-well plates at a concentration of 500,000 cells/well and a donor/recipient ratio of 1:9. After the incubation, cells were washed with PBS, collected by trypsinization, resuspended in PBS and analyzed by flow cytometry. Two-color sorting in a Coulter Epics XL-MCL flow cytometer was used to count 20,000 cells. The excitation wavelength was set at 488 nm, and the emission was at 525 nm for calcein AM and 575 nm for DiI. Percentage GJIC is defined as the proportion of recipient cells that received calcein AM from donor cells, corrected for background using a no-incubation control. Four replicates per condition were used.

Cell survival assay

The cytotoxic effects of various drugs on IMR-32 cells were determined using the MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were plated in 100 μl medium at a concentration of 1×10^3 cells per well in 96-well microtiter plates. Plates were then incubated for 18 h at 37°C under an atmosphere of air containing 5% CO_2. Medium (100 μl) containing the tested drug dissolved in appropriate solvent was added to quadruplicate wells and incubated for an additional 96 h. The medium was then removed from the wells and 200 μl MTT (1 μg/ml in complete medium) was added to each well followed by a 3-h incubation. The formazan crystals were dissolved in 100 μl dimethyl sulfoxide buffered with 25 μl glycine-NaCl solution (0.1 *M* glycine, 0.1 *M* NaCl, pH 10.5). The absorbance was measured in an

enzyme-linked immunoabsorbent assay plate reader (Bio-Rad) at a wavelength of 570 nm. Concentrations inhibiting 50% cell growth (IC_{50}) were determined for the various drugs tested.

Biochemical and Northern blot hybridization studies of resistance-related markers

Intracellular GSH concentration and total glutathione S-transferase (GST) activity were assayed using methods we have described previously [4, 40]. To measure the level of γ-glutamyl-cysteine-synthetase (γ-GCS) RNA, Northern hybridization was used. Total RNA was isolated from exponentially growing cells using the RNAzol B kit (TEL-TEST "B", Tex.). RNA was electrophoresed through a 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane by capillary action for 18 h in 20× SSC. Filters were prehybridized for 2 h at 42 °C in prehybridization buffer (50% v/v formamide, 5× SSC, 5× Denhardt's buffer, 250 mg/ml sonicated calf thymus DNA, 0.5% SDS). Probe was labeled to a high specific activity with ^{32}P-dCTP using an oligo labeling kit (Pharmacia Biotech, Montreal), and added to the blots at a concentration of 10^6 cpm/ml in hybridization buffer.

Hybridization was carried out for 20 h at 42°C in hybridization buffer (dextran sulfate/prehybridization buffer 1:4 v/v). Membranes were washed three times for 10 min at room temperature in 1× SSC containing 0.1% SDS, three times at 60°C for 10 min in 0.1× SSC containing 0.1% SDS, and subjected to autoradiography. The resulting X-ray films were scanned with a spectrophotometer equipped with an LKB Ultrascan laser densitometer. After each probing, filters were stripped of probes by four washes in 0.1× SSC, 0.1% SDS at 100 °C for 15 min, and exposed to X-ray film to ensure that the probe was completely removed. The stripped nitrocellulose membranes were reprobed with a mouse β-actin cDNA. The γ-GCS probe (kindly provided by T. Mulcahey, University of Wisconsin, Wisconsin) was isolated from a plasmid, gel-purified, and eluted prior to labeling.

Statistical analysis

Student's *t*-test was performed to compare IC_{50} values, and GSH and GST levels for different treatment conditions.

Results

Effect of db-cAMP and tRA on Cx43 expression and GJIC

The human neuroblastoma cell line IMR-32 used in this study expressed a moderate level of Cx43 protein (Fig. 1, lane 1). Exposure of cells to a nontoxic dose of db-cAMP (0.5 m*M*) led to an increase in Cx43 protein as well as its phosphorylated forms (Fig. 1, lane 2) which are important for GJ activity. This effect was observed following a 24-h exposure to db-cAMP, remained stable for at least another 24 h, and was associated with morphological changes reflecting induction of differentiation of IMR-32 cells (data not shown). tRA alone at 10 μ*M* slightly enhanced the Cx43 level (Fig. 1, lane 4). Combined treatment with 0.5 m*M* db-cAMP and 10 μ*M* tRA further enhanced Cx43 expression, as compared to the effects of db-cAMP or tRA alone (Fig. 1, lane 3). The level of GJIC was determined by FACS analysis. As shown in Fig. 2, there was a significant induction of GJIC from 11.4 ± 1.03% in control cells to 28.2 ± 0.6%, 26 ± 1.2%, and 42.8 ± 2.8% in cells treated for 48 h with 0.5 m*M* db-cAMP, 10 μ*M* tRA, and 0.5 m*M* db-cAMP plus 10 μ*M* tRA, respectively.

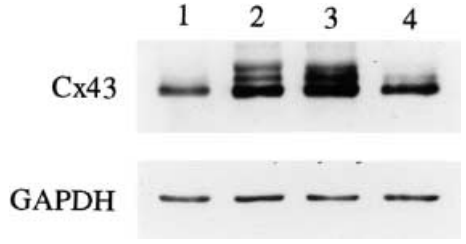

Fig. 1 Effect of db-cAMP and tRA on Cx43 protein expression. Protein extracts (20 μg/lane) from cells treated for 48 h were analyzed by Western blot using a rabbit polyclonal antibody to amino acids 346–360 of the rat Cx43. IMR-32 cells were untreated (*lane 1*) or treated for 48 h with 0.5 m*M* db-cAMP (*lane 2*), a combination of 0.5 m*M* db-cAMP and 10 μ*M* tRA (*lane 3*), or 10 μ*M* tRA (*lane 4*). The migration of Cx43 is related to its phosphorylation as revealed by immunoreactive bands at Mr 43 kDa (unphosphorylated form), 44 kDa and 46 kDa (phosphorylated forms). A mouse anti-GAPDH antibody was used as a control for protein loading

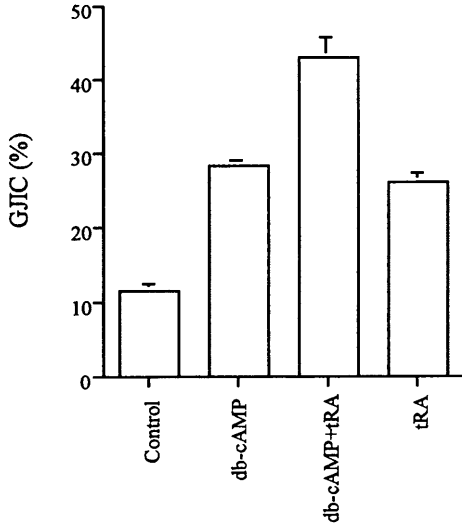

Fig. 2 Effect of db-cAMP on GJIC. Cells were treated for 48 h, as described in Fig. 1 and Materials and methods. FACS analysis was performed and the percent GJIC was estimated for different treatments. Each value is to the average from quadruplicate experiments

Effect of db-cAMP and tRA on drug cytotoxicity

Table 1 shows the IC_{50} values of each of the tested cytotoxic drugs in IMR-32 cells. The IC_{50} values (which are also a measure of drug cytotoxicity) of doxorubicin, melphalan, and BCNU were significantly decreased in cells exposed to 0.5 m*M* db-cAMP. As compared to control cells, db-cAMP increased the cytotoxicity of doxorubicin by approximately fourfold, and that of melphalan and BCNU by twofold. While 10 μ*M* tRA

only had a slight effect on drug modulation, it was found to significantly potentiate the drug cytotoxic effect when combined with db-cAMP. This modulation was more pronounced with doxorubicin than with melphalan or BCNU. Cell survival was not affected by exposure to db-cAMP and tRA in the absence of cytotoxic drugs (data not shown).

Effect of db-cAMP and tRA on GSH, γ-GCS and GST

Table 2 shows the intracellular GSH and GST activities. Exposure to 0.5 mM db-cAMP resulted in an approximately 40% reduction in intracellular GSH concentration, and further addition of 10 μM tRA resulted in an even more significant depletion to less than 20% of the level in control cells. tRA alone had no significant effect on GSH levels. The level of total GST activity was not affected by db-cAMP or tRA. The level of γ-GCS, which is the rate-limiting enzyme for GSH synthesis, was not significantly different in the absence and in the presence of db-cAMP alone or in combination with tRA, based on mRNA (based on densitometric analysis of the ratio of γ-GCS to β-actin levels; Fig. 3).

Discussion

Several drug resistance markers have been associated with clinical relapses in neuroblastoma patients, including overexpression of the ATP-cassette transporter P-glycoprotein [33], and the multidrug resistance-associated protein [14]. Accumulating evidence indicates that the tumor microenvironment can affect the outcome of anticancer drug therapy. In particular, cell-cell interactions have been shown to play a role in metabolic cooperation and drug resistance [24]. Enhanced cell sensitivity to drugs in resistant cells has been suggested to occur via cell-cell communication [43]. Moreover, this mechanism has been found to be important in the ''bystander effect'' observed in gene therapy using retrovirus/adenovirus gene delivery systems [17, 32]. In this study, we focused on the role of GJIC in the sensitivity of the neuroblastoma cell line IMR-32 to chemotherapy.

GJIC occurs between adjacent cells and mediates the exchange of various metabolites, including secondary messengers cAMP and inositol-triphosphate (IP3), as well as a variety of small peptides such as GSH [26]. GJIC has been implicated in many biological functions including the regulation of cell growth, differentiation, and the maintenance of tissue homeostasis [26, 46]. During tumorigenesis, the interruption of the physiological interaction of normal cells with their neighboring cells, and the loss of features of differentiation are a

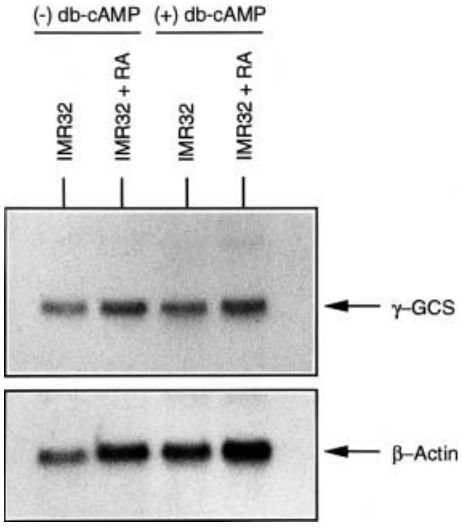

Fig. 3 Effect of db-cAMP and tRA on γ-GCS mRNA expression. Total RNA was extracted from IMR-32 cells treated with db-cAMP and/or tRA (*RA*) for 48 h. The level of γ-GCS mRNA was determined by Northern blot analysis. The first two lanes represent γ-GCS mRNA from IMR-32 cells that were exposed to saline (control) or 10 μM tRA. The following two lanes represent γ-GCS mRNA from IMR-32 cells that were exposed to 0.5 mM db-cAMP alone or in combination with 10 μM tRA. A mouse β-actin probe was used as a control for RNA quality and gel loading

Table 1 Effect of db-cAMP and tRA on the cytotoxic effect of chemicals on IMR-32 cells estimated using the MTT cell survival assay. Values are mean ± SD IC_{50} values (μM) from at least three independent assays

Chemical	Control	db-cAMP	tRA	db-cAMP + tRA
Doxorubicin	2.7 ± 0.3	0.7 ± 0.1*	1.9 ± 0.5	0.07 ± 0.004*,**
Melphalan	40.5 ± 4.8	19.8 ± 1.8*	43.6 ± 5.5	13.7 ± 0.3*,**
BCNU	75.8 ± 9.7	45.0 ± 7.7*	61.2 ± 9.6	32.5 ± 5.7*,**

*$P < 0.01$ vs control cells, **$P < 0.05$ vs db-cAMP-treated cells, Student's t-test

Table 2 Effect of db-cAMP and tRA on total GSH and GST activity in IMR32 cells assayed as described in Materials and methods

	Control	tRA	db-cAMP	db-cAMP + tRA
GSH (nmol/mg protein)	12 ± 2.8	10.4 ± 1.3	7.7 ± 0.2*	2.1 ± 0.06*,**
GST (nmol/min/mg protein)	27.5 ± 3.8	22.8 ± 0.5	26.6 ± 1.0	26.0 + 1.04

*$P < 0.01$ vs control cells, **$P < 0.01$ vs db-cAMP-treated cells, Student's t-test

common denominator in tumor progression [46]. Alteration in GJIC is believed to be among the earliest changes during cell tumorigenesis [11, 23]. A variety of activated proto-oncogenes, chemical carcinogens, and tumor promoters are known to downregulate GJIC [46, 48]. In general, transformed and cancer cell lines lack functional GJIC [20, 31], while there is evidence that loss of GJIC in tumors is associated with high metastatic potential [20, 23]. There is also evidence suggesting that some connexins, such as Cx43, act as tumor suppressors [38].

Our study in the human neuroblastoma cell line IMR-32 indicates that db-cAMP and tRA upregulate GJIC and Cx43 expression and phosphorylation, in agreement with previous studies using other types of human cancer cells [21]. It appears that a threshold level of Cx43 is required for the modulation of chemosensitivity and GSH levels since tRA alone had a small effect on Cx43 (while it induced GJIC to the same degree as db-cAMP) and no significant effect on IC_{50} values and GSH levels. On the other hand, db-cAMP treatment was associated with an increase in Cx43 levels and GJIC, as well as a decrease in GSH levels and IC_{50} values. Interestingly, a combination of db-cAMP and tRA resulted in a potent enhancement of Cx43 expression and phosphorylation as well as of GJIC, as compared to treatment with db-cAMP or tRA alone. The effect of these two mediators on anticancer agent sensitivity followed a similar pattern, in which they further reduced IC_{50} values and the level of GSH. Doxorubicin was the agent by which cells were most sensitized in the presence of the two compounds. Melphalan and BCNU were influenced by db-cAMP and tRA to a lesser extent.

GJIC is the gap junctional communication sum of at least 14 connexin genes. However, Cx43 is the only gene known to be induced by cAMP, although we cannot exclude the possibility that other connexins may also be regulated by cAMP. Treatment with tRA, as compared to db-cAMP, led to a lower level of induction of Cx43 and a comparable induction of GJIC. Therefore, it is possible that tRA could induce GJIC by principally upregulating other connexin genes, while the db-cAMP-induced GJIC occurs mostly through the upregulation of Cx43. When used in combination, tRA may potentiate the db-cAMP induction of Cx43 and GJIC, as well as the reduction in GSH and IC_{50} values. It has also been shown that retinoids can potentiate the effect of cAMP on gene expression [42]. It is therefore possible that a Cx43-dependent induction of GJIC is more important to GSH depletion and chemosensitization than a Cx43-independent induction of GJIC. Previous work has shown that GJs formed by different connexin genes have varying permeabilities and modes of regulation [8, 35], which also supports the idea that activation of Cx43 may be more relevant for cell chemosensitization to cytotoxic agents.

Our results demonstrate that exposure to db-cAMP and tRA results in a dramatic GSH reduction and increased cell sensitization of the human neuroblastoma line IMR-32. A possible mechanism by which Cx43-dependent upregulation of GJIC could be important in chemosensitivity is by allowing GSH intercellular transport. GSH is a major detoxification agent involved in resistance to chemotherapy drugs, including doxorubicin, melphalan, and BCNU [1, 39, 40]. GSH depletion has been shown to sensitize tumor cells to cytotoxic drugs, while a GSH-depleting agent, L-buthionine sulfoximine, is currently in clinical trials [2]. The sharing of GSH among metabolically coupled cells may be quite important in generating a ''mass buffering'' effect for a number of toxins and their reactive byproducts, e.g. free radicals. Therefore, chemical stimulation of GJIC and establishment of enhanced cell communication might play an important role in reducing the level of GSH and metabolic cooperation. This could be explained by active diffusion of GSH through GJs to surrounding cells resulting in a dilution of the GSH concentration among cells with higher GJIC and greater associated cell sensitivity to chemotherapy.

Another possible mechanism of this enhanced cytotoxicity could involve the transport of anticancer agents or their active metabolites to adjacent cells through GJs, thereby targeting a greater proportion of the cell population. This phenomenon is known as the ''bystander effect'' and it has already been demonstrated for activated gancyclovir in the context of suicide gene therapy [18]. The bystander effect depends on GJ permeability to the cytotoxic agents or their metabolites. In our case, the chemotherapeutic drugs were too large to pass through GJs, which generally allow the passage of molecules up to 1–2 kDa in size. However, drug intermediates such as free radicals produced by doxorubicin could readily pass through GJs to neighboring cells, thereby affecting a greater fraction of the cell population. Indeed Cx43 has been previously implicated in the bystander effect by allowing activated gancyclovir to reach neighboring cells [12].

Alternatively, one could argue that connexins and GJIC are not the main mechanism by which tRA and db-cAMP affect chemosensitivity and GSH reduction. Although there is no work on the combined role of tRA and cAMP in chemotherapy, we cannot rule out the possibility that they modulate other signaling pathways that also lead to chemosensitivity. Given the complexity of both transcriptional and post-transcriptional regulation of connexins, these experiments are going to be very challenging.

The cAMP signaling pathway may offer an important target for cancer therapy. Most of the designed cAMP analogues are too toxic to be used in the clinical setting. However, recent early clinical studies of 8-chloro-cAMP have shown positive results [36]. Similarly, retinoids are already used for cancer therapy [15, 45]. However, there are no studies of the combination of cAMP, retinoids, and chemotherapy. Our study highlights the potential benefit of combining GJIC modulators and chemotherapeutic agents.

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