

Proliferation of C6 glioma cells is blunted by the increase in gap junction communication caused by tolbutamide

Rosa Sánchez-Alvarez^{a,1}, Arantxa Tabernero^{a,b,1}, Luis I. Sánchez-Abarca^c, Alberto Orfao^c, Christian Giaume^d, José M. Medina^{a,*}

^aDepartamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Salamanca, Edificio Departamental, Pza Doctores de la Reina s/n, 37007 Salamanca, Spain

^bUnidad de Investigación, Hospital Universitario de Salamanca, Salamanca, Spain

^cServicio de Citometría, Universidad de Salamanca, Salamanca, Spain

^dNeuropharmacologie, INSERM U 114, Collège de France, Paris, France

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Abstract We have previously reported that tolbutamide prevents the inhibition of gap junction communication in astrocytes. Here, we show that tolbutamide increases gap junction communication and connexin 43 expression in poorly coupled C6 glioma cells. The increase in communication is concurrent with the inhibition of the rate of proliferation due to a block of the progression of C6 glioma cells through the S phase of the cell cycle. The effects of tolbutamide were quantitatively similar to that found after the elevation of intracellular cAMP. Furthermore, the effects of tolbutamide and cAMP were additive. The possible beneficial effect of tolbutamide on gene therapy for gliomas is discussed. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Brain; Intercellular communication; Glia; Sulfonyleurea; Gene therapy; Tumor

1. Introduction

Gap junction communication has long been proposed as a mechanism required to maintain the control of cell growth [1]. In this context, we have recently shown that the inhibition of gap junction communication triggers astrocyte proliferation and the metabolic pathways required for nucleic acid synthesis [2]. Furthermore, the neoplastic phenotype of several tumors includes the absence of gap junction communication [3]. In fact, several connexins, the proteins which form gap junction channels, have been proposed as tumor suppressor proteins [4]. For instance, the expression of connexin 43, the main connexin in astrocytes [5], correlates inversely with the degree of glioma malignancy [6–8] and the transfection of connexin 43 in glioma cells decreases their rate of growth [9,10]. Therefore, the expression of connexin 43 and hence the establish-

ment of gap junction communication decrease glioma cell proliferation.

Gliomas are the most common brain tumors and are generally highly malignant with a bad prognosis [11]. Because traditional treatments such as chemotherapy or radiotherapy have failed to improve this prognosis [12,13], new strategies such as that based on gene therapy are currently being developed [14]. One of these approaches involves the herpes simplex virus thymidine kinase (HSV *tk*) gene combined with gancyclovir (GCV), currently in clinical trials [15]. GCV is an antineoplastic drug that is only activated after phosphorylation, which takes place in cells transfected with the HSV *tk* gene. Since current methods of transfection do not reach a high percentage of cells, GCV is active only in a reduced number of glioma cells. Cell communication through gap junctions allows the diffusion of the active form of GCV to neighboring cells through the phenomenon known as the ‘bystander effect’ [16,17]. Therefore, the higher the level of gap junction communication between glioma cells, the higher the efficiency of HSV *tk*-GCV gene therapy, since active GCV spreads to more extensive areas [18,19].

As described above, malignant glioma cells express low levels of communication through gap junctions. The induction of connexin expression in these tumoral cells could lead to two beneficial effects on tumor control, bystander effect mediation and tumor suppression. Thus, the development of new strategies to increase gap junction communication in glioma cells may influence the outcome of cancer therapy with HSV *tk*-GCV. We have previously reported that tolbutamide and other sulfonyleureas increase gap junction communication in astrocytes treated with several gap junction inhibitors [20,21]. Consequently, in the present work we were prompted to investigate whether tolbutamide might increase gap junction communication in C6 glioma cells and hence decrease the exacerbated rate of proliferation.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, poly-L-lysine, tolbutamide, 2'-O-dibutyryladenine 3':5'-cAMP (dbcAMP), 5-bromo-2'-deoxyuridine (BrdU), monoclonal antibodies against BrdU, protease inhibitors, polyacrylamide, anti-mouse Ig biotin, anti-mouse Ig FITC, avidin-peroxidase conjugate, the AEC substrate staining kit, propidium iodide and Lucifer yellow

*Corresponding author. Fax: (34)-923-29 45 64.
E-mail address: medina@usal.es (J.M. Medina).

¹ These authors contributed equally to this work.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BrdU, 5-bromo-2'-deoxyuridine; dbcAMP, 2'-O-dibutyryladenine 3':5'-cAMP; HSV *tk*, herpes simple virus thymidine kinase; GCV, gancyclovir

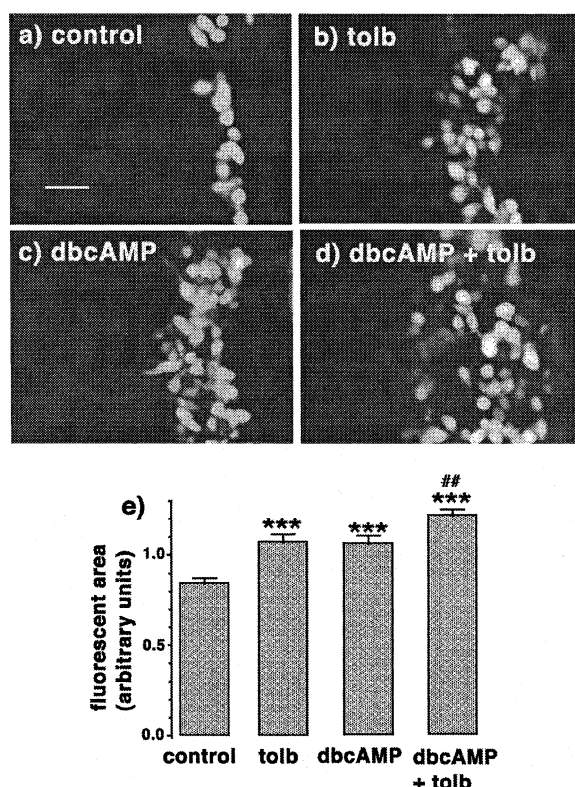


Fig. 1. Effect of tolbutamide and dbcAMP on gap junction permeability among C6 glioma cells. Where stated, C6 glioma cells were preincubated with 400 μ M tolbutamide (tolb) for 30 min, 1 mM dbcAMP for 24 h or 1 mM dbcAMP for 24 h plus 400 μ M tolbutamide for 30 min. Tolbutamide was maintained at the same concentration for the rest of the experiment. a–d: Gap junction permeability was determined by the scrape-loading technique. Loading was performed from the right side of the photographed field. e: The fluorescent area was quantified; the results are means \pm S.E.M. ($n=12$). Statistical differences as compared to controls are given as *** $P < 0.001$ and those compared to the presence of dbcAMP alone as ## $P < 0.01$. Bar = 50 μ m.

were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). Fetal calf serum (FCS), DNase and trypsin were obtained from Boehringer Mannheim (Barcelona, Spain). Monoclonal antibodies against connexin 43 were from Chemicon International Inc. (Temecula, CA, USA). Nitrocellulose sheets were from Bio-Rad (Madrid, Spain). Other chemicals were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain) or Merck (Barcelona, Spain).

2.2. Cell culture

Rat glioma C6 cells were cultured as previously described [22]. Briefly, cells were cultured in DMEM supplemented with 7.5% (v/v) FCS, penicillin (50 U/ml) and streptomycin (37.5 U/ml) on poly-L-lysine-coated Petri dishes. Cells were maintained in culture for 5 days until confluence. 24 h before the experiments, the culture medium was replaced by fresh serum-free DMEM.

2.3. Assessment of junctional communication

Gap junction permeability was determined by the scrape-loading/dye transfer technique described by El-Fouly et al. [23], adapted to our experimental conditions [22]. Briefly, the culture medium was removed and cells were washed with an ionic solution (130 mM NaCl, 2.8 mM KCl, 1 mM CaCl_2 , 2 mM MgCl_2 and 10 mM HEPES, pH 7.2) and preincubated at room temperature in the ionic solution. After 10 min, this solution was removed and cells were further incubated in the same ionic solution without Ca^{2+} [24] for 2 min. Scrape-loading was performed by scraping the cell layer with a broken razor blade in the Ca^{2+} -free ionic solution containing 1 mg/ml of Lucifer yellow CH (Sigma), a highly fluorescent dye that passes through gap

junctions from loaded cells to neighboring ones, but not through the plasma membrane. After 2 min, the dye solution was removed and the cells were carefully washed with excess ionic solution. 8 min after scraping, fluorescence photomicrographs were captured with a digital video camera (Leica DC100) connected to an inverted fluorescent microscope equipped with appropriate filters (Diaphot, Nikon).

To estimate gap junction permeability, six photomicrographs of the center of the dish were taken. Data were obtained by measuring the fluorescent areas of the images using image-analyzer software (NIH Image, kindly supplied by Wayne Rasband, NIH, Bethesda, MD, USA).

2.4. Western blotting

Connexin 43 expression was followed by Western blotting as described previously [2]. Proteins were extracted from the cells using 2% sodium dodecyl sulfate (SDS) in 5 mM Tris-Cl, pH 6.8, containing 2 mM ethylene glycol-bis[β -aminoethyl ether]- N,N,N',N' -tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml antipain, 0.5 μ g/ml pepstatin, 0.5 μ g/ml amastatin, 0.5 μ g/ml leupeptin, 0.5 μ g/ml bestatin and 0.5 μ g/ml of trypsin inhibitor. The protein extract (90 μ g) was applied to a 10% SDS-polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose sheet, as described [25]. The nitrocellulose sheet was blocked with 10% fat-free dried milk in phosphate buffered saline (PBS) and then exposed to monoclonal connexin 43 antibody (1:1000) for 12 h overnight. Anti-mouse Ig biotin (1:1000) was applied for 4 h, followed by conjugated avidin-peroxidase (1:2000) for 1 h. The AEC staining substrate kit, prepared according to the manufacturer's instructions, was then applied for 10 min. Stained nitrocellulose sheets were scanned and the intensity of the bands analyzed using image-analyzer software (NIH Image).

2.5. BrdU labeling for measurement of DNA synthesis

Cell proliferation was determined by counting the number of cells incorporating BrdU, as previously described [2]. C6 glioma cells were incubated with 10 μ M BrdU for 24 h prior to the immunocytochemical detection of this thymidine analogue. Then, C6 cells were incubated with the monoclonal antibody against BrdU (1:100) in PBS containing 0.1% Triton X-100 for 12 h. After washing, C6 glioma cells were incubated with anti-mouse Ig FITC (1:100) for 24 h. The total number of cells and proliferative nuclei were counted in at least 10 fields per Petri dish using image-analyzer software (NIH Image). Each experiment was carried out in six separate Petri dishes.

2.6. Cell cycle studies

The C6 glioma cell cycle was analyzed by flow cytometry following the method described by Vindelov et al. [26] with minor modifications [27]. Briefly, cells were harvested and the cell suspensions were incubated with a sodium citrate buffer solution containing 0.05% (w/v) RNase and 0.1% (w/v) trypsin inhibitor for 10 min. Then, 1.5 ml of the same solution containing 0.021% (w/v) propidium iodide was added and incubation was continued for 15 min at room temperature. At least 20 000 events were analyzed for each experiment on a FACS-can flow cytometer. For the calculation of the distribution of the cells along the different cell cycle phases ModFit mathematical model was used.

2.7. Statistical analyses

Statistical analyses were performed using Student's *t*-test.

3. Results

3.1. Tolbutamide increases gap junction permeability and connexin 43 expression in C6 glioma cells

To test the effect of tolbutamide on gap junction communication in C6 glioma cells, scrape-loading experiments were carried out and the level of expression of connexin 43 was followed by Western blotting. It is well established that the increase in intracellular levels of cAMP upregulates gap junction communication in C6 glioma and other tumoral cells [28–30]. The effect of tolbutamide was therefore studied in parallel and compared with that of dbcAMP. Similarly to other tu-

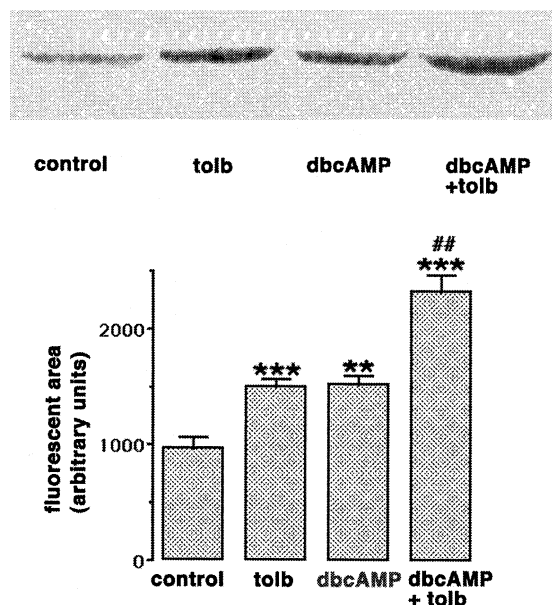


Fig. 2. Effect of tolbutamide and dbcAMP on connexin 43 expression in C6 glioma cells. Where stated, C6 glioma cells were pretreated with 400 μ M tolbutamide (tolb), 1 mM dbcAMP or 400 μ M tolbutamide plus 1 mM dbcAMP. After 24 h, Western blotting of connexin 43 (Cx43) was carried out (upper panel). The intensity of the bands was quantified (lower panel) with a detection limit of 50.8 ± 6.2 arbitrary units. Results are means \pm S.E.M. ($n=4$) and are expressed in arbitrary units. The coefficient of analytical variation was always lower than 14%. Statistical differences as compared to controls are given as *** $P < 0.001$ and ** $P < 0.01$ and those compared to the presence of dbcAMP alone as ### $P < 0.01$.

moral cells, C6 glioma cells are characterized by a low level of communication through gap junctions (Fig. 1a, see also [9]). In this work we show that tolbutamide increases gap junction communication between C6 glioma cells (Fig. 1b). Thus, using C6 glioma cells the scrape-loading experiments revealed that Lucifer yellow diffusion is restricted to the first row of cells (Fig. 1a). In the presence of tolbutamide, however, Lucifer yellow reached approximately three rows of C6 cells (Fig. 1b). Our results indicated that the presence of tolbutamide increased communication through gap junctions by about 20%, as judged by quantification of the fluorescent areas obtained after scrape-loading experiments (Fig. 1e). Our results (Fig. 1c and e) confirm that dbcAMP increases gap junction permeability in C6 glioma cells [19] and also show that the effect of tolbutamide on gap junction communication is sim-

ilar to that obtained with dbcAMP. In addition, when C6 cells were incubated with tolbutamide plus dbcAMP, the communication through gap junction increased by about 30% as compared to the controls and this increase was significantly higher than that obtained with dbcAMP alone (Fig. 1d and e).

Western blot analyses (Fig. 2) revealed that tolbutamide increased the expression of connexin 43, the protein forming the gap junction channel [5], by about 40%. Our results show

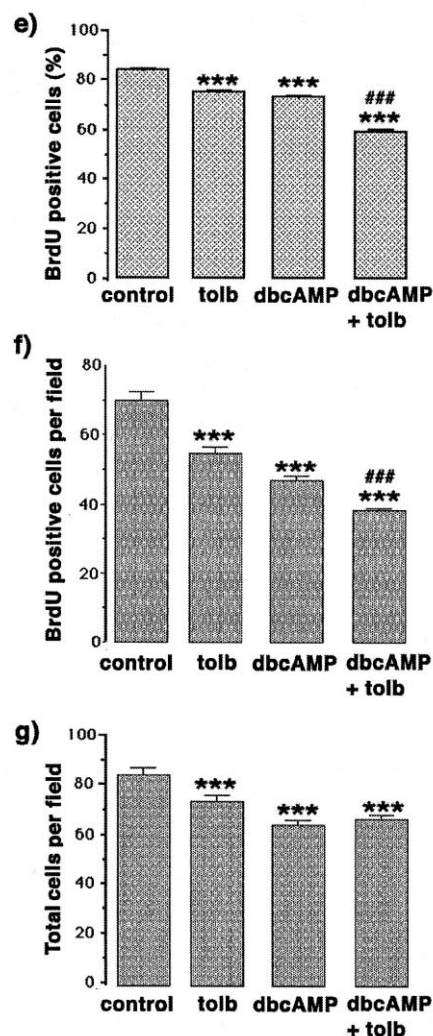
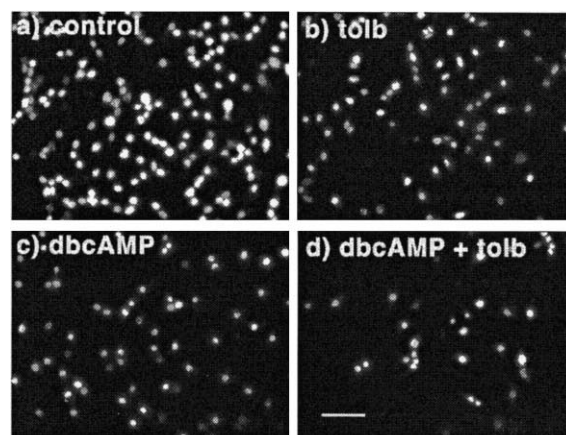


Fig. 3. Effect of tolbutamide and dbcAMP on the rate of C6 glioma cell proliferation. Where stated, C6 glioma cells were pretreated with 400 μ M tolbutamide (tolb), 1 mM dbcAMP or 400 μ M tolbutamide plus 1 mM dbcAMP for 18 h. These treatments were maintained at the same concentration for the rest of the experiment. Cells were further incubated with 10 μ M BrdU for 24 h. Incorporation of BrdU was determined by immunocytochemistry. a–d: Immunocytochemistry showing only fluorescent BrdU-positive nuclei. Note that the presence of tol and dbcAMP reduced the number of BrdU-positive nuclei per field. This effect was stronger when both agents were added together. e: Percentages of cells incorporating BrdU. f: Number of cells incorporating BrdU per field. g: Total number of cells per field. Results are means \pm S.E.M. from at least eight experiments. Statistical differences as compared to controls are given as *** $P < 0.001$. Statistical differences as compared to the presence of dbcAMP alone as ### $P < 0.001$. BrdU, bromodeoxyuridine. Scale bar = 50 μ m.

that the increase in connexin 43 expression promoted by tolbutamide was very similar to that found with dbcAMP (Fig. 2). In addition, the expression of connexin 43 was nearly doubled by the presence of tolbutamide plus dbcAMP in the culture medium (Fig. 2).

3.2. Tolbutamide decreases the rate of C6 glioma cell proliferation

C6 glioma cell proliferation was estimated by quantifying BrdU incorporation by immunocytochemistry (see Fig. 3a–d, as representative experiments). The presence of tolbutamide clearly decreased the number of BrdU-positive nuclei (white nuclei; Fig. 3b). The percentage of C6 cells incorporating BrdU decreased by about 10% in the presence of tolbutamide (Fig. 3e). As has been previously reported [31], the presence of dbcAMP inhibited C6 glioma cell proliferation (Fig. 3c and e). Our results show that the effect of dbcAMP on C6 glioma cell proliferation was very similar to that obtained with tolbutamide (Fig. 3e). Furthermore, the presence of tolbutamide plus dbcAMP further decreased (by about 30%) the percentage of cells incorporating BrdU (Fig. 3d and e). It should be mentioned that the decrease in proliferation promoted by tolbutamide, dbcAMP and dbcAMP plus tolbutamide was higher than the above mentioned. Indeed, the number of cells incorporating BrdU per field was significantly decreased by the presence of tolbutamide, dbcAMP and dbcAMP plus tolbutamide: about 22, 33 and 46%, respectively (Fig. 3f). The differences found between these results are due to the fact that the presence of tolbutamide, dbcAMP and tolbutamide plus dbcAMP significantly decreased the number of cells per field by about 12.5, 24 and 21% respectively (Fig. 3g), suggesting that they induced C6 glioma cell death.

In order to know which cell cycle phase was affected by tolbutamide, cells were analyzed by flow cytometry. Our results indicated that the presence of tolbutamide reduced the percentage of cells in the G2–M phases of the cell cycle by about 25% (Fig. 4a, b and e), with a concomitant increase in those in S phase (from 3.59 ± 0.39 to $6.84 \pm 0.37\%$; $P < 0.001$). The presence of dbcAMP reduced the percentage of S phase cells (from 3.59 ± 0.39 to $2.10 \pm 0.34\%$; $P < 0.05$), while the percentage of cells in the G0–G1 phases increased (by about 12%). Treatment with tolbutamide plus dbcAMP decreased the number of cells in the G2–M phases (by about 26%) while it increased those in G0–G1 phases (by about 14%).

4. Discussion

We have previously reported that tolbutamide and other sulfonylureas prevent the inhibition of gap junction communication caused by gap junction uncouplers in astrocytes [20,21]. Here, we show that tolbutamide also increases gap junction permeability in the poorly coupled C6 glioma cells (Fig. 1a and b). In addition, tolbutamide increased the expression of connexin 43 (Fig. 2), the protein forming the gap junction channel [5]. The effect of tolbutamide on gap junction communication was quantitatively very similar to that found with dbcAMP (Figs. 1 and 2), a well-known activator of gap junction communication in several tumoral cells, including C6 glioma cells [28–30]. However, our findings indicate that the pathways followed by tolbutamide and dbcAMP to increase gap junction communication and connexin 43 expression must be different, since their effects were additive

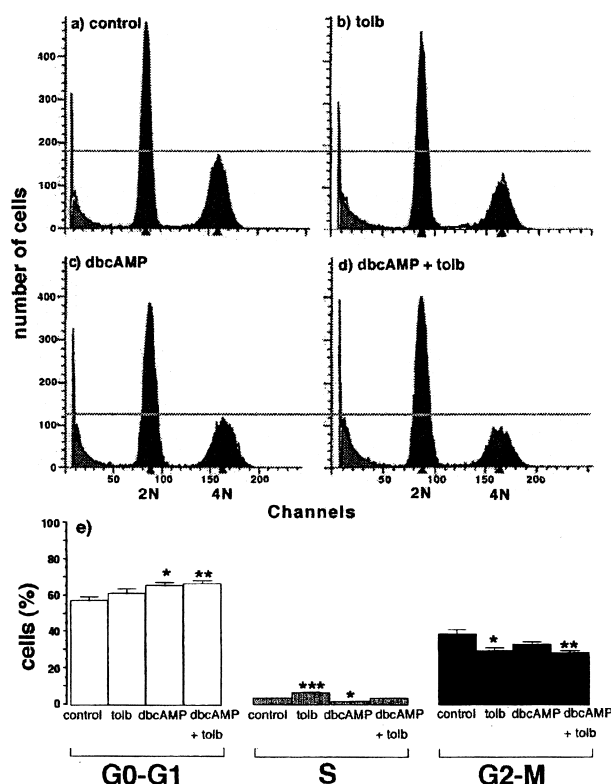


Fig. 4. Effect of tolbutamide and dbcAMP on the C6 glioma cell cycle analyzed by flow cytometry. Where stated, C6 glioma cells were pretreated with 400 μ M tolbutamide (tolb), 1 mM dbcAMP or 400 μ M tolbutamide plus 1 mM dbcAMP. After 48 h cells were harvested and their nuclei stained with propidium iodide. a–d: 20000 events were used to analyze the cell cycle by flow cytometry. Note that the presence of tolbutamide reduced the peak corresponding to cells in the G2–M phases of cell cycle (gray line). e: Integration of the areas corresponding to each phase of the cell cycle. Results are expressed as percentage of cells found in the different cell cycle phases and are means \pm S.E.M. of five to six experiments. Statistical differences as compared to controls are given as *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

(Figs. 1 and 2). Therefore, these results clearly indicate that the combination of these agents could result in an important increase in both the expression of connexin 43 and the communication through gap junction in C6 glioma cells. Since the efficiency of HSV *tk*-GCV gene therapy for gliomas depends on the level of gap junction communication among tumoral cells (for a review see [16,32]), it is reasonable to suggest that tolbutamide, a clinically tolerable drug, either alone or in combination with agents that promote an increase in the intracellular levels of cAMP could act as adjuvants for HSV *tk*-GCV therapy by increasing the spread of the active anti-neoplastic form of GCV.

Gap junction communication not only improves gene therapy but also plays an important role in tumor suppression. Thus, the transfection of connexin 43 in glioma cells decreases their rate of growth [9,10]. Moreover, an elevation in intracellular cAMP levels induces glioma cell differentiation, characterized by the increase in gap junction communication and the decrease in proliferation [31]. Consequently, the establishment of gap junction communication in the poorly coupled cells of gliomas recovered the control of cell growth. In agreement with this, our results show that together with increasing gap junction communication and connexin 43 expression

(Figs. 1 and 2), tolbutamide significantly decreases the rate of C6 glioma cell proliferation and this effect was additive with that of cAMP (Fig. 3b, c and e). In agreement with this, different cell cycle phases were affected by these two agents (Fig. 4). Thus, whereas dbcAMP decreased the percentage of cells in the S phase, correlating with an accumulation of C6 glioma cells in the G0–G1 phases, tolbutamide decreased proliferation by inhibiting progression through the S phase of the cell cycle (Fig. 4). Indeed, tolbutamide significantly decreased the percentage of cells in the G2–M phases coinciding with an increase in the number of cells in the S phase.

Our results are insufficient to recommend the use of tolbutamide or other sulfonylureas for the treatment of gliomas. Nevertheless, two potential beneficial effects of tolbutamide as a coadjuvant for antineoplastic therapy should be mentioned. First, tolbutamide could improve the efficiency of gene therapy since it increases gap junction communication and hence the ‘bystander effect’. Second, tolbutamide inhibits the rate of glioma cell proliferation which is the goal of antineoplastic drugs. Finally, the effect of tolbutamide takes place through transduction signals other than cAMP, allowing its combination with agents which increase intracellular concentrations of cAMP.

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