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Modulation of Myogenic Differentiation in a Human Rhabdomyosarcoma Cell Line by a New Derivative of 5-Fluorouracil (QF-3602)

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The *in vitro* study of mechanisms involved in drug-induced maturation has made it possible to use differentiation-based therapy in clinical practice. The goal of this new therapy is the development of specific agents to induce cancer cells to stop proliferating and express characteristics of normal cells. Recently, by structural modifications of 5-fluorouracil (5-FU), we synthesized a new pyrimidine acyclonucleoside-like compound, 1-{[3-(3-chloro-2-hydroxypropoxy)-1-methoxy]propyl}-5-fluorouracil (QF-3602), which showed in rhabdomyosarcoma cells a low toxicity and time-dependent growth inhibition. In this work, we compared the degree of myogenic differentiation of RD rhabdomyosarcoma (RMS) cells after treatment with QF-3602 and 5-FU. Scanning and transmission electron microscopy (SEM and TEM) and immunocytochemical analyses showed that QF-3602 induced the appearance of myofilaments along the myotube-like giant RD cells, an increase in fibronectin and a decrease in vimentin expression. In contrast, only minor changes were observed with 5-FU. Moreover, polymerase chain reaction (PCR) analyses showed that QF-3602 did not induce overexpression of the *mdr 1* gene, a resistance mechanism that frequently appears in classical cytotoxic therapy in these tumors. Compounds obtained by structural modifications of 5-FU may be useful in differentiation therapy as a new approach to the treatment of RMS.

Key words: Differentiation therapy — Acyclonucleoside prodrugs — Fibronectin — Vimentin — Rhabdomyosarcoma

Cancer differentiation therapy focuses on the development and use of specific agents designed to selectively engage the process of terminal differentiation, leading to the eventual elimination of tumorigenic cells and rebalance of normal cellular homeostasis.[1)] To avoid both cytotoxic effects and the appearance of multidrug resistance (MDR) associated with classical chemotherapy, recent years have seen the design and synthesis of new differentiative drugs from well-known chemical structures.[2–4)] In this connection, novel prodrug derivatives of 5-fluorouracil (5-FU), such as pyrimidine acyclonucleosides, possess a broader spectrum of antitumor activity and fewer toxic effects than 5-FU. Therefore, 5-FU acyclonucleoside-like compounds mimic the action of 5-FU without producing toxicity *in vivo*.[5)]

Rhabdomyosarcomas (RMS), the most common soft tissue sarcoma in children, are characterized by their poor response to cytotoxic treatment and significant morbidity.[6)] The frequent failure of cytotoxic therapy in these tumors may be related to the development of drug resistance.[7)] Significant increases in *mdr 1*/P-glycoprotein expression have been detected in RMS after chemotherapy *in vivo*[8)] and *in vitro*.[9)] However, the cytotoxic treatment of RMS has demonstrated that these tumor cells can be induced to re-enter the differentiation process,[10, 11)] supporting the hypothesis that RMS arises from skeletal muscle cells which are arrested along the normal myogenic pathway to maturation.[11–13)] These findings suggest the potential applicability of differentiation therapy in these tumors, although the development of the MDR phenotype may represent a major limitation in this new approach to the treatment of RMS.[7)] Recently, pyrimidine analogues such as ara-C have been used *in vitro* and *in vivo* to evaluate their potential role for differentiation therapy.[14)] We have previously studied the effect of 5-FU derivatives such as 1-{[3-(3-chloro-2-hydroxypropoxy)-1-methoxy]propyl}-5-fluorouracil (QF-3602), a new 5-FU acyclonucleoside-like prodrug, in the RD RMS cell line. This new pyrimidine derivative at 90 μM showed little toxicity *in vivo* or *in vitro*, and led to time-dependent growth inhibition in comparison with 5-FU.[5, 15)]

In this study we used the RD RMS cell line as an *in vitro* model to evaluate the induction of myogenic differ-

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entiation and drug resistance after treatment with the new 5-FU acyclonucleoside-like compound QF-3602. Although *in vivo* studies are needed, our results suggest that 5-FU acyclonucleoside derivatives such as QF-3602 may be useful for differentiation therapy of these tumors.

MATERIALS AND METHODS

Cell culture and drug treatments The RMS cell line RD,[16] derived from a human embryonal RMS, was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured according to Melguizo *et al.*[9] After synthesis and purification of QF-3602,[15] stock solutions of QF-3602 and 5-FU were prepared. The drugs were dissolved in distilled water, sterilized by filtration, and stored at −20°C. RD cells (1×10^6) were exposed continuously to 90 μM 5-FU or QF-3602, whose lower toxicity *in vivo* and *in vitro* was previously demonstrated.[15] Parallel cultures of RD cells in medium without drugs were used as controls. The medium in both control and drug-treated cultures was replaced every 48 h, and the cultures were maintained and examined every 24 h for 6 days. All experiments were performed in triplicate and repeated twice.

Scanning electron microscopy (SEM) Adherent tumor cells on coverslips were fixed for SEM with 2 % glutaraldehyde, dehydrated in graded concentrations of ethanol, and dried using the critical point method. These preparations were coated with platinum and observed with a Hitachi S-800 SEM (Hitachi, Tokyo). To study the modifications of RD cells in suspension, cells were resuspended at a rate of 2×10^5 cells per 100 ml of a 1 *M* phosphate-buffered solution plus 2.5% glutaraldehyde, for the first fixation. Then the cells were washed 3 times in a 0.1 *M* sodium cacodylate-buffered solution (pH 7.4) and post-fixed with osmium tetroxide in an aqueous solution at room temperature. The cells were washed with water, collected with a coverslip and left to dry at room temperature so that they remained adhered to the glass. After this, the cells were processed as described in the preceding paragraph.

Transmission electron microscopy (TEM) RD cells were observed with a TEM before and after treatment with the drugs. For TEM, RD cells were treated according to Melguizo *et al.*[9] and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The sections were contrasted with uranyl acetate-lead citrate and examined in a Hitachi H-7000 TEM.

Immunofluorescence cytochemistry The parental RD cells, after treatment for 6 days with QF-3602 and 5-FU, were washed several times with PBS at pH 7.2, fixed with methanol at 4°C for 10 min, washed 3 times in phosphate-buffered saline (PBS) and incubated with FN-15 anti-fibronectin monoclonal antibody (mAb) (dilution 1:200) (Sigma, St. Louis, MO) and V9 anti-vimentin mAb (dilution 1:200) (Sigma) at 37°C for 15 min in a humidified chamber. A second incubation at 37°C was done with a 1:50 dilution of FITC-conjugated goat anti-mouse IgG to fibronectin and anti-mouse IgM to vimentin (Sigma) during 30 min. After a final washing in PBS, the slides were mounted at pH 7.2 in FA mounting fluid (Difco Laboratories, Detroit, MI). All cells were examined under a Nikon HFX-IIA light microscope (Nikon, Tokyo) for epifluorescence studies.

Polymerase chain reaction (PCR) evaluation of *mdr 1* mRNA levels Total RNA was obtained according to Maniatis *et al.*[17] and reverse transcription was done with *mdr 1* or β-actin primers as described by Wu *et al.*[18] Levels of *mdr 1* mRNA were estimated relative to β-actin mRNA using a modification of the RNA PCR method.[19] The PCR products obtained were loaded onto 2% agarose gels and visualized by ethidium bromide staining.

RESULTS

Analysis of RD cells with SEM The study of parental RD cells in suspension showed rounded and polygonal cells, which frequently have a flat surface without membrane prolongations. Some cells (<15%) showed shallow membrane folds of different sizes (Fig. 1A). Most of the RD cell population (79%) treated during 6 days with QF-3602 had numerous deep surface folds (desert-rose cells, Fig. 1B). The most evident morphological change in comparison with parental RD cells was the appearance of elongated cells with cytoplasmic prolongations and an increased number of filopodia (Fig. 1C). In contrast, many 5-FU treated cells lacked membrane folds and a few exocytotic bodies were visible (Fig. 1D).

The analysis of adherent parental RD cells showed a variety of more or less elongated and polygonal shapes (Fig. 2A). RD cells treated with QF-3602 showed an evident increase in the cytoplasm, which adhered strongly to the substrate. In these cells the size and number of filopodia and membrane folds were also increased (Fig. 2B). The most important characteristic of myogenic differentiation was the appearance of myotube-like giant cells in which a parallel arrangement of myofibrils along the longitudinal axis was evident (Fig. 2C). In contrast, 5-FU led to the appearance of rounded cells with a very irregular contour and deep surface invaginations (Fig. 2D).

Analysis of RD cells with TEM Untreated RD cells typically contained a single nucleus and few organelles, with no evidence of skeletal muscle differentiation. RD cells treated with QF-3602 showed typical ultrastructural features of myogenic differentiation: multiple nuclei consistent with giant cell formation and cytoplasm that occupied large areas (Fig. 3A) and contained a large number of glycogen deposits in clusters, elongated mitochondria with

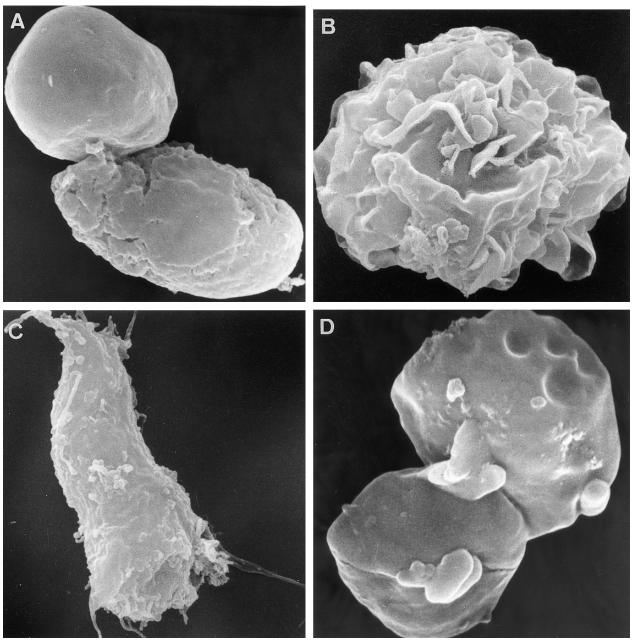

Fig. 1. Analysis by SEM of RD cells in suspension. Morphological characteristics of RD cells (A) growing in standard medium; (B and C) treated with QF-3602: these cells showed deep folds in the cell membrane and are elongated and bear filopodia; and (D) treated with 5-FU. Note the appearance of exocytotic bodies on the membrane. (Magnification: A, ×7000; B, ×6000; C, ×4000; D, ×13 000).

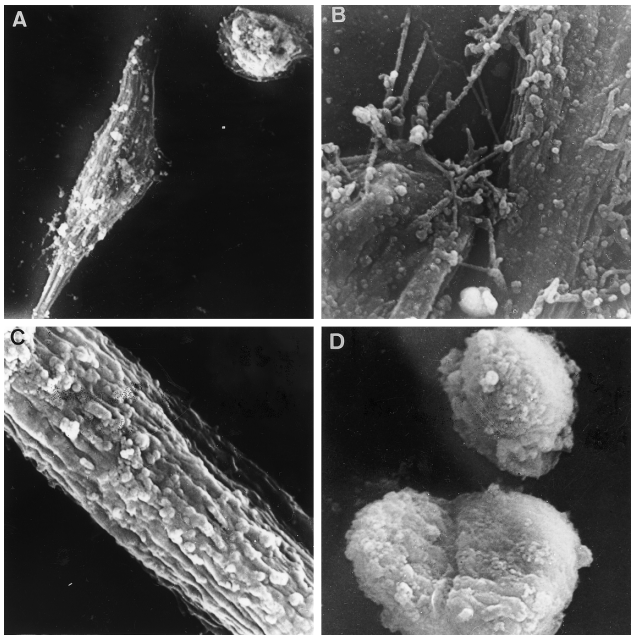

Fig. 2. Analysis by SEM of adherent RD cells. Morphological characteristics of RD cells (A) growing in standard medium; (B and C) treated with QF-3602, showing increased numbers of filopodia and cell-cell contacts and myofibrils along their longitudinal axis; and (D) treated with 5-FU, showing a rounded outline and an irregular surface. (Magnification: A, ×1000; B and D, ×3000; C, ×5000).

clear matrices and dilated cristae (Fig. 3B), and intermediate filaments (Fig. 3C). The most evident characteristic of myogenic differentiation was the appearance of parallel myofilaments in well-defined bundles and the arrangement in structures reminiscent of Z-bands (Fig. 3D). The cultures treated with 5-FU showed few characteristics of myogenic differentiation and a larger proportion of cells with reduced viability (Fig. 3E).

Immunocytochemical analysis We used immunohistochemical staining to evaluate modifications in the expression of the intermediate filament vimentin and the adhesion protein fibronectin. Vimentin expression was widely distributed in parental RD cells, forming a visible nuclear ring from which separate arrays of filaments departed, branching out throughout the cytoplasm (Fig. 4A). In contrast, the expression of fibronectin was practically undetectable, and was distributed in small plaques (Fig. 4B).

Cells induced with QF-3602 displayed low vimentin expression, some cells showing a halo of labelling around the nuclear membrane which did not spread into the cytoplasm (Fig. 4C). However, labelling of the adhesion plaques for fibronectin was greater than in control cells, and was seen throughout the length of the cell. In more differentiated cells we observed incipient extracellular expression on the rim of the cytoplasmic prolongations (Fig. 4D). No modification of vimentin or fibronectin expression was found in RD cells treated with 90 μM 5-FU (not shown).

Expression of *mdr 1* gene A weak PCR product for *mdr 1* was exhibited by the parental RD cell line. After induction of RD cells for 6 days with any of the drugs, there were no modifications in the PCR product for *mdr 1* (Fig. 5A). To demonstrate the integrity of the RNA preparations, PCR was performed using β-actin primers, and all preparations showed similar intensity. PCR amplification of β-actin demonstrated that the difference in the numbers of transcripts in the parental RD cell line and in RD cells treated with QF-3602 and 5-FU was not the result of RNA degradation (Fig. 5B).

DISCUSSION

The use of new drugs that induce differentiation *in vitro* as potential clinical differentiation therapy agents for cancer suggests that an understanding of the cellular and

molecular biology of cell differentiation should advance the development of new cancer therapies. In our laboratory we have synthesized a series of pyrimidine acyclonucleoside-like compounds that act as prodrugs of 5-FU, which can release other substances with antitumor activity such as acrolein,[20] and which have low acute and chronic toxicity *in vivo*.[5]

We studied the action of QF-3602 against a human tumor cell line (RD) to elucidate the phenotypic events associated with myogenic differentiation induced by the

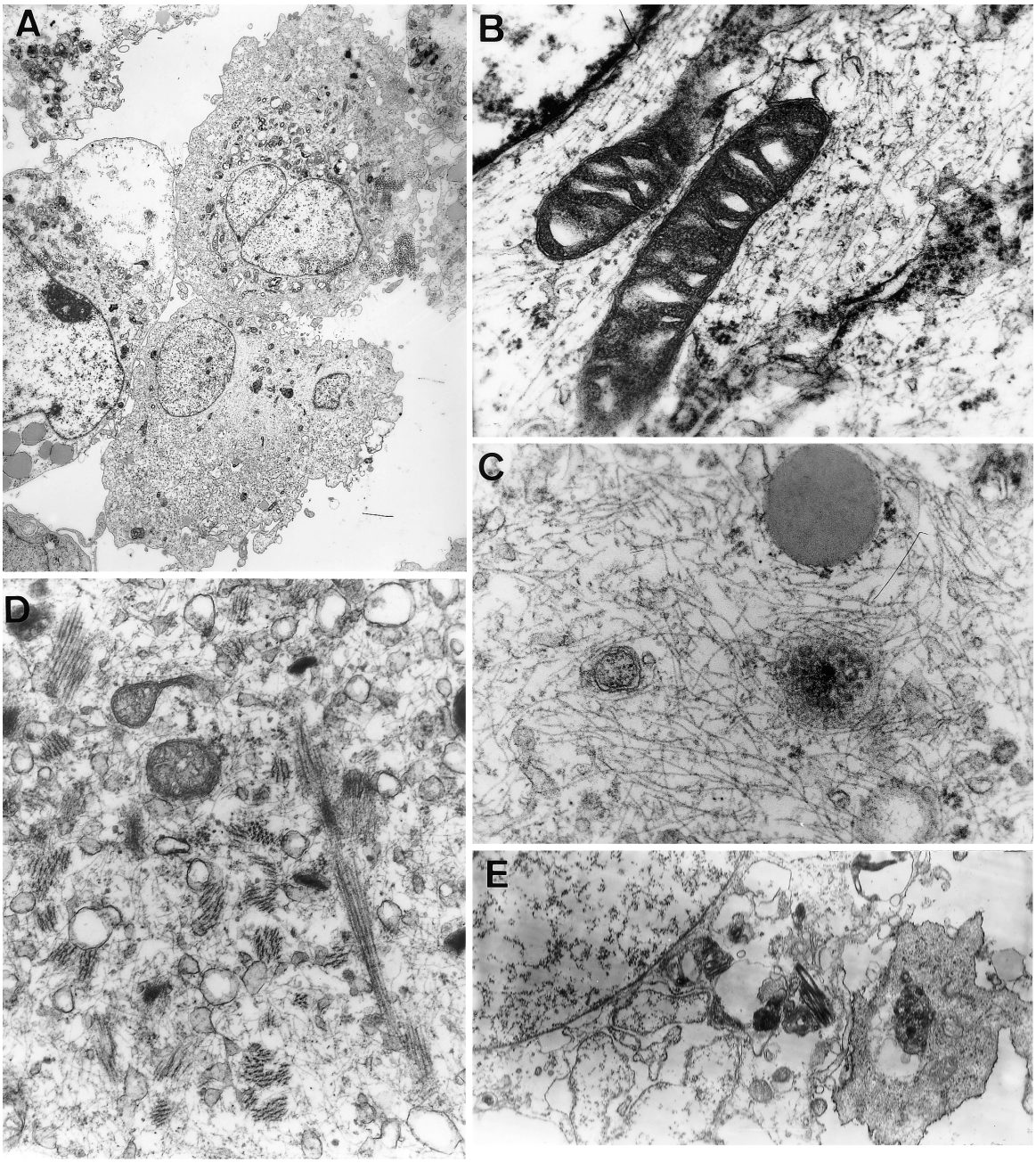

Fig. 3. Analysis by TEM of RD cells treated with QF-3602, which were enlarged and showed multiple nuclei and an increase in the cytoplasm (A) and organelles; (B) elongated mitochondria with dilated cristae; (C) organized intermediate filaments and (D) electron-dense zones similar to Z-bands. (E) RD cells treated with 5-FU, showing evidence of degeneration of the nucleus and cytoplasm. (Magnification: A, ×2500; B and C, ×40 000; D, ×25 000; E, ×8000).

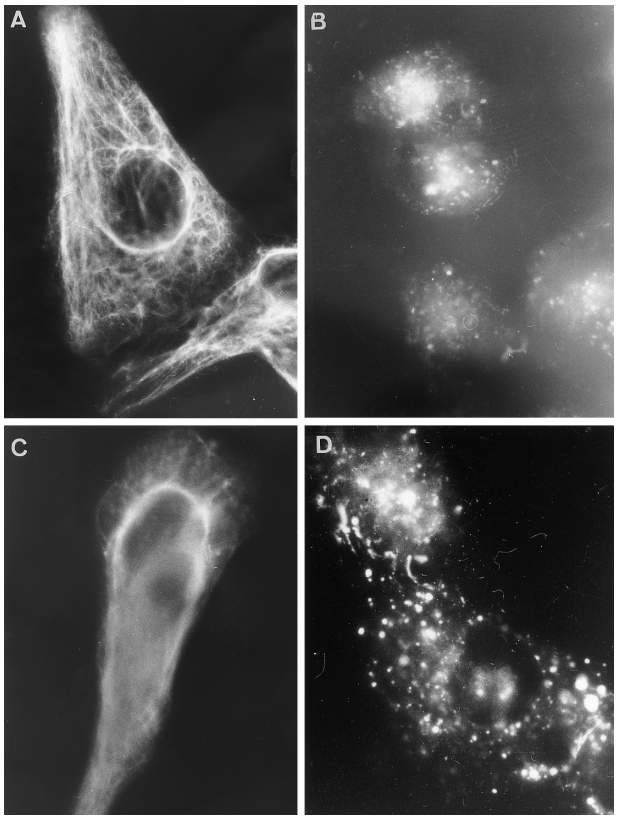

Fig. 4. Immunofluorescence micrographs of untreated and QF-3602-treated RD cells. (A) High expression of vimentin (×100) and (B) low expression of fibronectin (×50) in RD cells growing under standard conditions. (C) Very low expression of vimentin and (D) intense labelling for fibronectin in QF-3602-treated RD cells (×50).

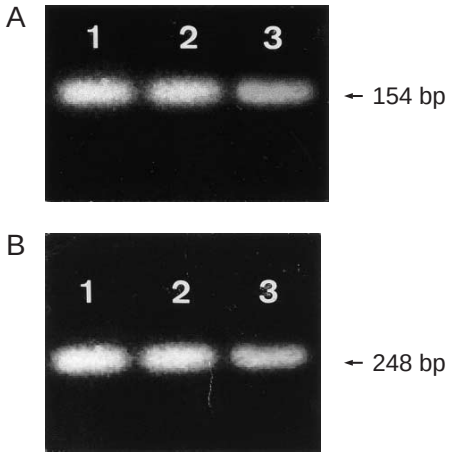

Fig. 5. (A) RT-PCR of *mdr 1* mRNA expression in untreated and treated RD cells. (B) β-Actin mRNA determined as a control for the PCR technique. Lane 1, untreated RD cells; lane 2, RD cells treated with 90 μM 5-FU; lane 3, RD cells treated with 90 μM QF-3602.

new pyrimidine drug. Analyses with SEM indicate that perturbation in the maturation of RD cells may be modulated by QF-3602, which induces characteristics of adult muscle cells. The significant presence of extensive thin sheetlike processes or lamellipodia and filopodia scattered throughout the cytoplasm of RD cells reflected a greater adhesion capacity[21] and a high degree of differentiation, as shown by the appearance of myofibrils in the myotube-like giant RD cells, and that has been described in *in vitro* myogenesis from adult skeletal muscle cells.[22] These phenotypic features were confirmed in TEM images in which RD cells contained abundant myofilament bundles arranged in structures reminiscent of Z-bands. Similar features of myogenic differentiation have been shown with drugs used to treat RMS.[11, 14]

Morphological changes were accompanied by modifications in the expression and distribution of vimentin, which were weak and disrupted after treatment with QF-3602. This protein has been used as a differentiation marker in RMS, and the loss of expression indicates that RD cells had reached a high degree of differentiation.[10] Moreover, QF-3602 induces a significant increase in the markers of myogenic differentiation, desmin, actinin and tropomyosin.[15] The increase in the expression and size of fibronectin plaques in RD cells treated with QF-3602 indicates that this fibronexus protein is essential for morphological and structural changes during muscle cell maturation and differentiation.[23, 24] In fact, during development fibronectin participates in the early phases of alignment and fusion of muscle cells and in the formation of intercellular junctions between myoblasts.[22, 25, 26] Moreover, cell-substrate contacts and cell-cell interaction depend on the expression of fibronectin, which is related to metastatic capacity.[21] The higher degree of muscular maturation and the greater adherence to the substrate found in treated RD cells suggest a lower capacity for invasion and therefore for producing metastases.[27, 28]

We have shown that QF-3602 inhibits the proliferative activity of RD cells.[15] This effect, together with the cytodifferentiation seen after treatment, suggest that the clinical effect of such treatment would be favorable, as was demonstrated by Coffin *et al*.[29] in patients with RMS. *In vivo* experiments are in progress to examine whether a correlation exists between the stage of maturation of RMS cells induced by QF-3602 and their tumorigenic and metastatic capacities.

Drugs classically used to treat RMS induce cell differentiation, but also lead to *mdr 1* overexpression,[13, 30] preventing complete recovery of the normal process of muscular differentiation. Because the overexpression of

mdr 1 is related to the frequent failure of cytotoxic therapy in RMS, we examined the development of resistance in the case of QF-3602 treatment. The use of QF-3602 in RD cells did not increase expression of the *mdr 1* gene, which indicates that the new acyclonucleoside does not induce resistance, at least not via increased *mdr 1* expression.

Although *in vivo* studies will be necessary, the findings of the present study suggest that the new pyrimidine acyclonucleoside-like compound QF-3602 may be useful as an agent for differentiation therapy against RMS. Ultratructural modifications of treated RD cells indicate that QF-3602 induces re-entry into the pathway of myogenic differentiation. Our results, as well as the increase in and reorganization of maturation markers and the absence of evidence that the MDR phenotype was induced, suggest that there may be significant potential advantages in the use of new differentiating agents for the treatment of these tumors.

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