5-Fluorouracil Derivatives Induce Differentiation Mediated by Tubulin and HLA Class I Modulation

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Abstract: Neoplastic cells exhibit defects in their ability to differentiate; therefore, differentiation therapy represents a viable option to control cancer growth and progression. Rhabdomyosarcomas (RMS), a malignant tumor of skeletal muscle, is the most common soft tissue sarcoma in children and is characterized by its poor response to cytotoxic treatment and significant morbidity. Since modulation of α-tubulin and human leukocyte antigen (HLA) class I expression has been detected during malignant transformation, we analyzed in this study the expression pattern of both kinds of proteins after the treatment with 5-FU derivatives in the human RMS RD cell line. Cytotoxic assays, scanning and transmission electron microscopy, flow cytometry and immunocytochemical analyses were used. The compounds analyzed belonged to the following three categories: (a) symmetrical bis(5-fluorouracil-1-yl) derivatives with a linker that connects the N^1 atoms of both pyrimidine moieties by means of two amide bonds; and (b) an ester with the 5-FU base. The whole structure corresponds to the terminal fragment of the molecules included in (a) and (c) 5-fluorouracil acyclonucleoside-like structures. 1-{[3-(3-Chloro-2-hydroxypropoxy)-1-methoxy]propoxy]propyl}-5-fluorouracil (**2**), that belongs to the class (a) produced the highest increment of tubulin and its intense capillary distribution throughout the cytoplasm. On the other hand, *N,N*-bis[3-(5-fluorouracil-1-yl)-3-methoxypropanoyl]-α,α–diamino-*m*-xylene (**5**) and **2** that are included in the class (c) caused the major percentage of marked cells by the HLA class I proteins. In short, our results showed that the 5-FU derivatives increase HLA class I expression and showed greater microtubule stability with an important network of tubulin beams related with the degree of differentiation of RD cells. These results could mean a more favorable prognosis of the patients affected with these tumors.

Key Words: Acyclonucleoside-like compounds, amide bond, 5-Fluorouracil, symmetrical "double-headed" *O,N*-acetals.

INTRODUCTION

There is a complex relationship that exists between growth, differentiation, neoplastic transformation and the expression of genes and tumoral suppressor genes. The proliferation control occurs partly by means of the regulated progression of cells through the consecutive stages of cellular differentiation [1]. In tissues with a fast cellular turnover, the cells that have reached the higher level of differentiation undergo apoptosis. Thus, terminal differentiation represents a form of negative control of growth and regulating molecules interact to produce a correct balance between cellular multiplication and differentiation [2].

Neoplastic transformation does not necessarily destroy the potential for the expression of the differentiated characteristics. So, the differentiation therapy represents a viable option to control cancer growth and progression.

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma not only in children [3], but also in adolescents and young adults, accounting for 20% of all sarcomas [4,5]. The main feature of this tumor is the presence of cells that histologically resemble normal fetal skeletal muscles [6]. It occurs as a consequence of regulatory disruption of the growth and differentiation of skeletal muscle progenitor cells and is characterized by its poor response to cytotoxic treatment and significant morbidity [7]. Therefore, the diagnosis of RMS is based on the detection of specific features of skeletal muscle lineage, such as cross-striations, by light and electron microscopy. In addition, the analysis of muscle-specific markers is also useful, especially in the absence of cross-striations. Traditionally, the markers for muscle-specific such as actin, desmin and myoglobin have been widely used to study differentiation of RMS. We have previously shown that novel 5-fluorouracil (5-FU) acyclonucleoside prodrugs induce typical structural differentiating modifications in several tumoral cell lines, including RD and RMS [8,9].

Skeletal muscle differentiation involves a complete reorganization of the microtubule network, which shows a redistribution of the microtubule organizing center from the centrosome to the nuclear membrane. The parallel disposition of the microtubules is found within the morphogenetic events that take place during myogenesis. The microtubules lie adjacent to intermediate filaments and stress fibers throughout

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the longitudinal axis of the elongated myoblasts and myotubes [10,11]. Before their fusion and differentiated myotubes, myoblasts alter their morphology and acquire a disorganized myofibrilar pattern when the cytoplasmic microtubules are altered by the agents that cause depolymerization [12]. Due to detyrosinated and acetylated tubulin, an increase in the stability of the microtubules is necessary at the beginning of the muscular differentiation [13].

Modulation of the HLA expression has been detected during malignant transformation. Total or selective losses of HLA class I antigens have been reported in different human tumor samples. The rates of HLA class I losses in some tumors are close to 100%, being 96% in cervical carcinomas [14] or 96% in breast carcinomas [15]. In fact, the MHC antigens downregulation has recently been associated with improved survival and antitumor immune response in lung and breast cancer, respectively [17,18]. This finding indicates that the modulation of HLA antigen expression and its determination in the diagnostic, as also during the course of the disease, may be a prognostic factor in some tumors.

Modulation of α-tubulin and human leukocyte antigen (HLA) class I expression has been detected during malignant transformation. For this reason, in this study we analyzed the expression pattern of both kinds of proteins after the treatment with 5-FU derivatives.

CHEMISTRY

5-Fluorouracil (5-FU, **1**) is a fluorinated pyrimidine described by Duschinky and Pleven in the Hoffmann-La Roche laboratories in 1957 [19]. Its great activity as an antineoplastic and immunosuppressor was demonstrated soon after [20]. Nowadays, it is employed primarily in the treatment of some adenocarcinomas of the gastrointestinal tract (colon, pancreas, stomach), breast and less efficaciously, in certain hepatomas and ovarian, prostate, cervix, urinary bladder and oropharynx carcinomas. It is also effective for the treatment of superficial basal cell carcinomas when applied topically.

Nevertheless, in spite of the important clinical usefulness of 5-FU, it presents a great toxicity, which has led to constant research with the object of finding more potent but less toxic derivatives. The compounds designed, synthesized and biologically evaluated belong to the following three categories: (a) 5-FU acyclonucleoside-like structures (**2** and **3**); (b) symmetrical bis(5-fluorouracil-1-yl) derivatives with a linker that connects the *N*-1 atoms of both pyrimidine moieties by means of two amide bonds (**4** and **5**); (c) a β-hemiaminal carboxylic ester with the 5-FU base (**6**): in this case, the structure corresponds to the terminal fragment of the molecules included in (a), but changing the amide by an ester group (Fig. (**1**)).

The area of acyclonucleosides or seco-nucleosides (nucleoside analogs in which the "sugar" is linear instead of cyclic as is normal) has been widely explored due to the successful development of the antiherpes drug acyclovir [21]. As part of a program for the development of 5-FU derivatives, we described the formation of ayclonucleoside analogs *via* a tin (IV) chloride-mediated regiospecific opening of alkoxy-1,4-diheteroepanes [22]. Compound **2** proved to be 2-fold more active than 5-FU (IC_{50} = 45 μM) against HEp-2 cells in culture (human larynx tumor). In view of

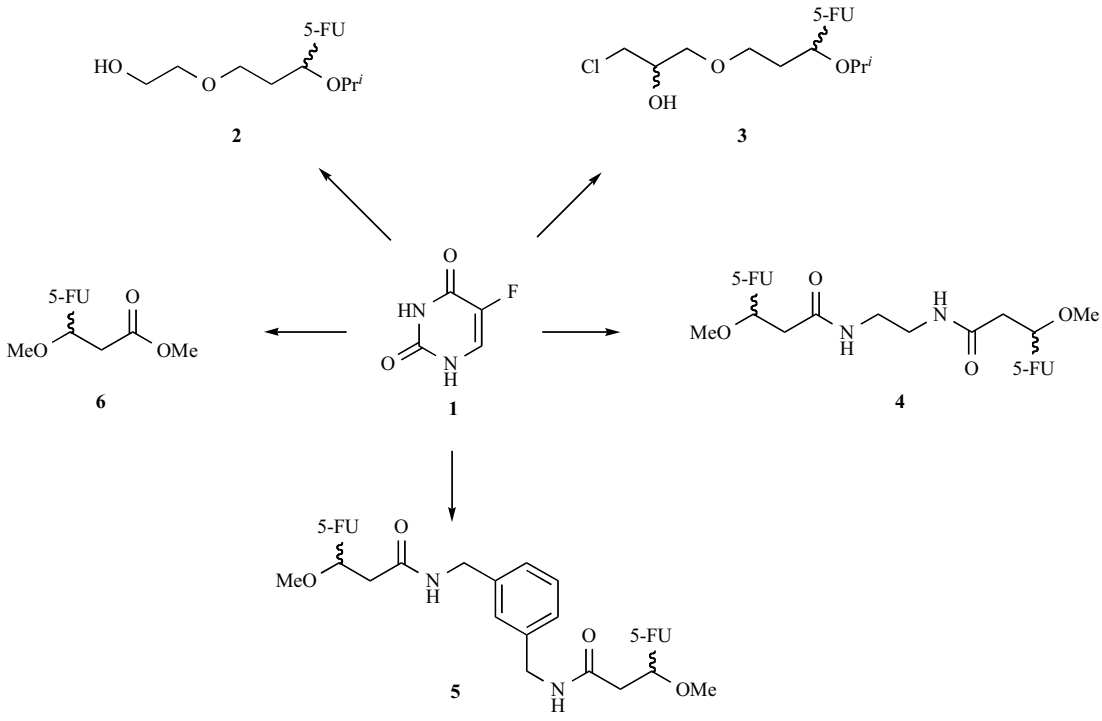

Fig. (1). Structures of the 5-FU derivatives evaluated biologically herein. In all the cases the attachment of the 5-FU moiety occurs at the *N*-1 atom of the uracil ring.

the chemotherapeutic activities exhibited by the acyclonucleosides 1-{[3-(2-hydroxyethylhetero-1-alkoxy]propyl}-5-fluorouracils [21], we explored new acyclic nucleosides in which their 3-hydroxyethoxypropyl moiety was chemically modified and we obtained structure **3** with the chlorhydrin fragment in the two-carbon atom acyclic chain [23]. Interestingly, a human embryonal cell line RD derived from RMS treated with **3** at a concentration of 90 μM concentration released the neoplastic cells from their blockade, allowing them to recover their normal myogenic development.

Prodrugs in which the 5-FU has been attached to amino acids [24] or even peptides [25] have also been reported. Moreover, compounds bearing two or more molecules of 5-FU in their structures as well as 5-FU supported on a polymeric matrix have also been described [26]. The amide bond is an important constituent in many biologically active compounds and the preparation of substituted amides has received much attention. Some amides with 5-FU attached to them have been reported [27]. The rationale for compounds **4** and **5** was based on the following aspects:

1. The possibility to release two moles of 5-FU per mole of the symmetrical "double-headed" *O,N*-acetal with the subsequent increase in activity and
2. The chemical fragment bearing the 5-FU molecules and might improve the pharmacokinetic characteristics of the cytostatic agent 5-FU.

Methyl 3-methoxy-3-(5-fluorouracil-1-yl)propanoate (**6**) was prepared from 3,3-methyl dimethoxypropanoate under our standard conditions [28]. Compounds **4** and **5** induced morphological and phenotypical differentiation in the RMS cell line at 4.5 and 3.5 μM, respectively. Compounds **4**, **5** and **6** significantly proved to increase two-fold the desmin expression in comparison with that of parental cells. However, the percentage of vimentin-positive cells decreased significantly after 6 days of treatment [28].

MATERIALS AND METHODS

Cell Culture and Drug Treatment

The RMS cell line RD, derived from a human embryonal rhabdomyosarcoma, was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured according to Melguizo *et al*. [29]. After the synthesis and purification of **2-5**, stock solutions were prepared. The drugs were dissolved in distilled water, sterilized by filtration and stored at -20 ºC. RD cells (1×10^6) were exposed continuously to drugs at fifty percent of the inhibition concentration (IC_{50}). The μM IC_{50} values against the RD human RMS cancer cell line were the following: 90 ± 0.02 for **1**, 45 ± 0.23 for **2**, 90 ± 0.11 for **3**, 9 ± 0.54 for **4**, 7 ± 0.12 for **5**, and 25 ± 0.92 for **6** [8,9,28]. 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 hr and the cultures were maintained and examined every 24 hr for 6 days. All experiments were performed in triplicate and repeated twice.

Scanning Electron Microscopy Analysis

The cells were grown on sterile 10 × 10 cm coverslips for scanning electron microscopy. They were fixed for 2 hr at 40 °C with 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, containing KCl 0.7 M, $CaCl_2$ 1.24 mM and $MgCl_2$ 1.24 mM. They were then washed and treated for 1 hr with 1% tannic acid in 0.1 M cacodylate buffer at 4°C and briefly washed again in the same buffer. Fixed cells were then reconcentrated by centrifugation in 1% agarose and postfixed in 2% OsO_4. Cell cultures were dehydrated in amyl acetate/ethanol, followed by critical point drying and coating with gold. A Zeiss DSM 950 scanning electron microscope, equipped with microanalysis system by X Ray dispersive energy (Oxford Isis 300) was used for observations. The analyses were obtained during 50 s of live time, with a dead-time rate not superior to 10%. The beam currents used were approximately 2-5nA with 20kV of acceleration.

Transmission Electron Microscopy Analysis

RD cells were observed with a TEM before and after treatment with drugs. For TEM, RD cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr at room temperature. The pellet and monolayer were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1 hr at room temperature and dehydrated in ethanol. Cells were detached from the culture vessel by rapid treatment with propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed 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 H7000 transmission electron microscope.

Immunofluorescence Cytochemistry

The parental RD cells, after treatment for 6 days with **2-5**, were washed several times with PBS at pH 7.2, fixed with methanol at 4 ºC for 10 min, washed 3 times in PBS and incubated with anti-tubulin monoclonal antibody (mAb) (dilution 1:400) (Sigma, St. Louis, MO) at 37 ºC for 15 min in a humidified chamber. A second incubation at 37 ºC was done with a 1:50 dilution of FITC-conjugated goat anti-mouse IgG to tubulin (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.

FACScan Analysis

Briefly, RD cells before and after treatment with drugs were transferred to universal screw cap tubes containing PBS, then washed and centrifuged at 225*g* for 5 min. To determine HLA class I expression, the cells were fixed with 2% formaldehyde for 10 min at –20 °C and immediately washed three times in PBS at 4 °C. The cells were then incubated for 30 min at 4 °C with the two monoclonal antibodies (mAbs) W6/32 and GRB1. The rest of the procedure was conducted as described above. The results obtained were evaluated as mean fluorescence. The percentage increase in mean fluorescence was calculated by the formula: (MFI-MFB/MFB) × 100, where MFI is the mean induced fluorescence and MFB is the mean basal fluorescence.

Statistical Analyses

All statistical analyses were done with the SPSS, release 7.5 (SPSS, Chicago, IL, USA). The results were compared

with Student´s *t* test. All data are expressed as the mean ± SD. The differences were considered statistically significant at a *P* value of < 0.05.

RESULTS AND DISCUSSION

Neoplastic transformation does not necessarily destroy the potential for the expression of differentiated characteristics, including the cessation of proliferation under appropriate environmental conditions [30]. The understanding of mechanisms that control growth and differentiation has allowed the design, development and use of specific agents that involve the process of terminal differentiation leading to the elimination of tumorigenic cells both *in vitro* and in cancer patients [31,32]. We developed a series of 5-FU prodrugs such as pyrimidine acyclonucleoside-like compounds that possess antitumor activity and present low toxicity *in vivo* [21,33]. We used specific markers of normal muscular differentiation to decide if these compounds led to the re-entry of the tumoral cells into the normal pathway of development. These 5-FU derivatives induced myogenic differentiation in the human RMS cells [8,9].

In this study, we evaluate the connection between myogenic differentiation of RMS RD cells treated with 5-FU derivatives and HLA class I and tubulin expression. Greater signs of differentiation were displayed by scanning (SEM) and transmission electron microscopy (TEM) analysis of RD cells treated with the IC_{50} value of **2** and with twice the IC_{50} values of **3** and **6**. The untreated human RMS cells displayed a more or less extended variety and polygonal shapes. The surface appeared without membrane prolongations and a greater thickening in the central part of the cells was observed due to the presence of the nucleus (Fig. (**2A**)). RD cells treated with the derivatives showed an elongated shape with numerous cytoplasmic prolongations and an increased number of filopodia. The most important characteristic of myogenic differentiation was the appearance of a parallel arrangement of myofibrils along the myotube-like giant cells (Fig. (**2B**)), and that has been reported in *in vitro* myogenesis from adult skeletal muscle cells [34].

The transmission electron microscopy (TEM) analysis showed that unlike control RD cells, the RMS cells treated for 6 days with certain drug concentrations displayed ultrastructural changes. These have been described by De Luna *et al.* [35] as clear signs of myogenic differentiation. After **4**, **5** or **6** treatments, RD cells were elongated and multinucleated with the displaced nuclei towards an extremity (Fig (**3A**)). Unlike the non-treated cells, the cytoplasm occupied a great extension of the cellular surface. It contained an increased number of organelles among which we emphasized: polyribosomes, a moderate amount of expanded rough endoplasmic reticulum and occasional cisterns of the Golgi apparatus, abundant elongated mitochondria with dilated cristae and clear matrices (Fig. (**3B**)) or many lipid vesicles throughout the cytoplasm and near the nucleus (Fig. (**3C**)). As shown in (Fig. (**3D**)) the most evident characteristic of myogenic differentiation was the appearance of organized intermediate filaments and well-defined bundles of parallel myofilaments. Similar features of myogenic differentiation have been shown by us with **2** and **3** and suggest that treated RMS cells

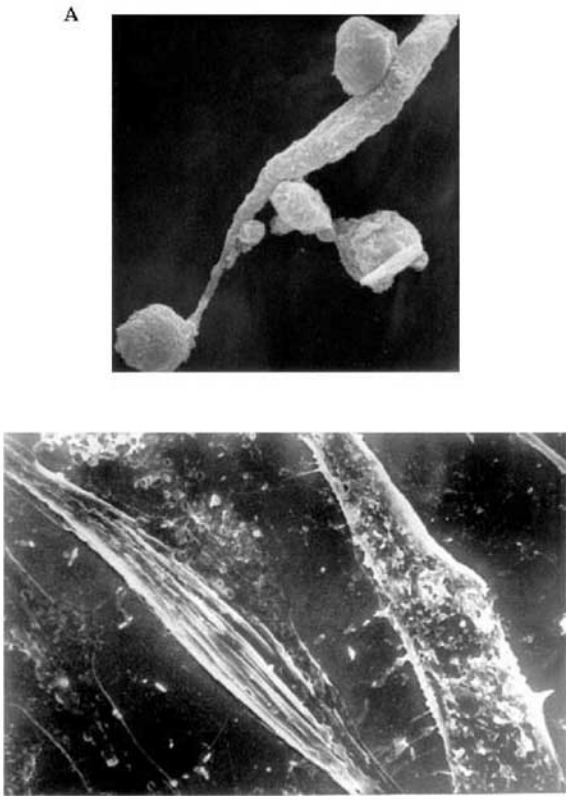

Fig. (2). Scanning electron micrograph images of untreated and treated RD cells for 6 days with the 5-FU derivatives. (**A**) Morphological characteristics of RD cells growing in standard medium (× 1000). (**B**) Appearance of myotube-like giant cells showing myofibrils along the longitudinal axis in RD cells treated with 9 μM of **4** (× 3000).

re-entered the programme of muscle maturation and subsequently underwent a process similar to myogenesis [8,9,36].

Microtubules have been implied in the myogenesis by virtue of their juxtaposition in the formation of myofibrils [37] and by the effect that the antagonists of the microtubules have on the differentiation of muscular cells. For this reason, we analyzed the expression pattern of tubulin after treatment with the drugs. As shown in (Fig. (**4A**)) the immunocytochemical staining showed that untreated RD cells displayed an unequal cellular distribution of microtubules: in a small proportion of cells, where the tubulin is more evident, its distribution is near the centrioles and is extended in a short space, diminishing as the periphery of the cells is approached; and a significant number of cells with a non-clear distribution and with an increase of the cytoplasmic spaces can be seen. This pattern seems to indicate that the microtubule stability is not very great and that the microtubules survive for a very short period of time due to a fast and continuous polymerization and depolymerization process. Thus, according to the findings of Gundersen *et al.* [13], there exists an increase in the microtubule dynamism that supposes a high level of myogenic undifferentiation, with an increased mitotic capacity.

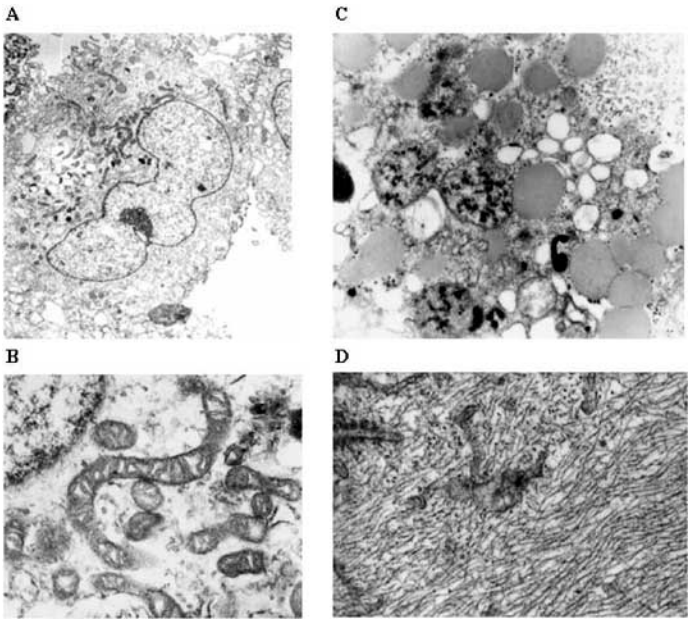

Fig. (3). Transmission electron micrographs of RD cells treated for 6 days with **4, 5** and **6**. (**A**) Multinucleated giant cell with nucleoli and increased numbers of cytoplasmic organelles (× 2500). (**B**) Elongated mitochondria with dilated cristae (× 32500). (**C**) Numerous lipid vesicles (× 5000). (**D**) Highly organized intermediate filaments in the cytoplasm (× 20000).

5-FU derivatives seem to equilibrate the microtubules production and its stability in the doses that produced more differentiation, as is observed in (Fig. (**4B**)) which shows an optimal centriolar organization and a distribution of large, medium and small microtubule bundles parallel to the longitudinal axis, along all the cellular soma. Moreover, the presence of regular hollows intimately related to the microtubule network is characteristic of the cells treated with both pyrimidine derivatives, which suggests an interaction between these intracellular components and other structures. This situation is necessary to allow the mobilization of these structures that in the case of the Golgi apparatus has an important entailment in the myogenic differentiation process [38]. Microtubules constitute a scaffold that guides myosin filaments in the process of assembly and organization during sarcomere constitution [39] and therefore, confirm the importance of these elements of the cytoskeleton in the acquisition of differentiated characteristics of muscular cells.

In addition, within the group of these drugs, attention was drawn to the dose corresponding to twice that of the IC_{50} of **3** that markedly increased the tubulin expression and its distribution by capillary forms in the cytoplasm (Fig. (**4C**)), which indicated the greater stability of the microtubules. These findings are compatible with a greater degree of differentiation, because during the normal myogenesis when the myoblasts merge to form myotubules, the microtubules are rearranged forming parallel longitudinal fibers under the surface of syncytium [40].

All the data previously shown indicate that the modifications found in the tubulin after the induction of the RD cells with the different pharmacological agents, make their use as an excellent marker of myogenic differentiation. This can serve as a pattern for thin filaments in the formation of the myofibrils, characteristic of the mature myotubes [13]. The

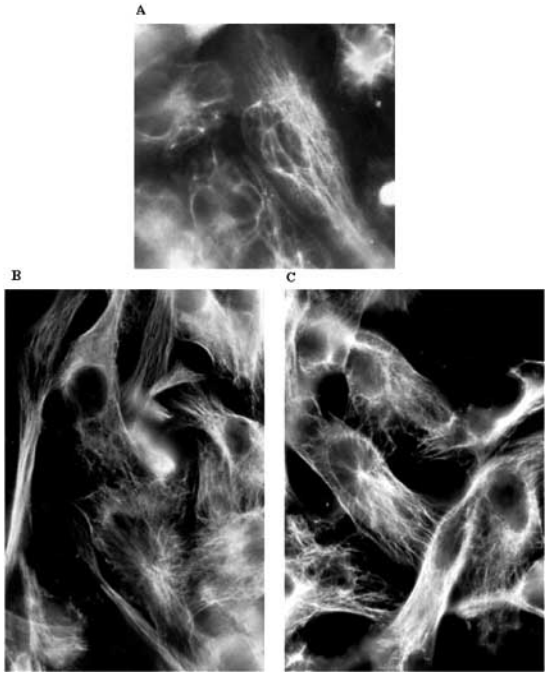

Fig. (4). Immunofluorescence micrographs of untreated and treated human RD cells. (**A**) Cellular distribution of tubulin in RD cells growing under standard conditions (× 50). (**B**) Treated RD cells with 7 μM of **6** showing intense labeling and a distribution of large, medium and small microtubule along the longitudinal axis of the cells (× 50). (**C**) Increased tubulin expression and distribution by capillary of 90 μM of **3** treated RD cells (× 50).

Table 1. FACScan Analysis of HLA Class I Antigen Expression in the Human RD Rhabdomyosarcoma Cell Line After Treatment with Pyrimidine Derivatives

Compound	½ × IC_{50}	IC_{50}	2 × IC_{50}
2	94.14±1.8[a]	93.95±1.4[a]	74.95±2.0
3	90.03±1.0[b]	96.71±2.2[a]	95.23±2.3[a]
4	90.62±1.5[b]	79.12±2.1	67.45±1.2[b]
5	86.56±1.7	97.12±2.5[a]	54.15±2.8[a]
6	71.27±2.9	90.22±1.2[b]	81.10±2.7

HLA class I value for control RD cells: 78.43±1.0

All data are %±S.E.M. of four different determinations. Significance was determined by comparison of the means with Student´s *t*-test.[a] Significantly different ($p<0.001$) compared with control RD cells. [b] Significantly different ($p<0.005$)compared with control RD cells.

compounds presented in this article could act as other drugs that in general comprise the group of microtubule-stabilizing antimitotic agents such as taxanes and epothilones, which promote their assembly and stabilize them and therefore lead to a mitotic interruption and apoptosis induction [41-43].

Finally, in order to determine whether HLA is modulated by the 5-FU derivatives and its relationship to the degree of myogenic differentiation, FACS analysis provided quantitative data on the changes in HLA class I and class II expression in the RD cell line after 6 days of treatment with different concentrations of all drugs. The class I molecules encoded by the major histocompatibility complex (MHC) are cell surface glycoproteins that play a fundamental role in the regulation of immune responses. HLA class I molecules are necessary for the presentation of peptide antigens to cytotoxic T-lymphocytes (CTLs) [44] and for the immune regulatory activity exerted by NK cells [45]. We used the monoclonal antibody W6/32 that recognizes a common determinant of HLA class I and the monoclonal antibody GRB1 that recognizes HLA class II. The analysis of HLA class II expression showed low levels of this antigen in the parental line, not displaying significant modifications after treatment with the drugs (data not shown).

There was an increase of HLA class I expression that was not equal in all the derivatives used, but with different modifications depending on the concentration. Due to the great variability of results obtained for each drug, we have reflected the results in Table **1**.

After treatment with 4.5 μM of **4** and 7 μM of **5**, the percentage of HLA class I RD cells increased significantly to 90.62% and 97.12%, respectively. However, RMS cells treated with 18 μM of **4** and 14 μM of **5** showed a reduction in the labeling for HLA class I in relation to control RD cells (Table **1**). The lower concentration of **2** induced a significant increase of HLA class I expression (94,14% against the 78.43% of the control cells). However, **3** caused the major percentage of HLA class I marked cells (96.71% of positive cells for 90 μM). Only 25 μM of **6** increased the expression of HLA class I. These increases agree with the doses of drugs that induced greater phenotypic differentiation, which suggest an important relationship between HLA class I and myogenic differentiation. Evidence indicates that both HLA class I and class II are constituently expressed in cultures derived from normal human skeletal muscle biopsies. Their expression can be modulated by agents *in vitro* and the presence of these antigens can be related to the process of myoblastic fusion *in vitro* [46,47]. Moreover, restoration of the normal tumor HLA class I phenotype may be a new way to restore an efficient immune response in cancer patients and to improve the prognosis and metastatic potential [48]. It has recently been shown that the increase of HLA expression in human RMS makes it a good candidate for tumor-specific immunotherapy [49].

CONCLUSION

In short, our results show that the 5-FU derivatives induce phenotypic myogenic differentiation in RMS cells that is accompanied by greater microtubule stability and an important increase in the HLA class I expression. The increased expression and network of tubulin beams in RD-treated cells suggest its use as an excellent marker for myogenic differentiation. Moreover, the increased HLA class I expression seems to be related to both the antitumor immune response and the re-entry into the normal myogenesis program. These results reveal the potential use of 5-FU derivatives to improve the prognosis and to diminish metastatic capacity of RMS in patients.

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