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Indole Family and Neomycin Sulfate: Inductors of Differentiation in C2C12 and RD Cell Lines

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Abstract

Rhabdomyosarcoma (RMS) is a highly aggressive tumor primarily affecting the pediatric population, that generally originates from a failure in the embryonic differentiation of myogenic precursor cells. Standard treatment involves surgery, chemotherapy and radiation therapy, with a poor prognosis, especially when it spreads to other parts of the body, highlighting the need for new treatment approaches. In this study, cellular differentiation was investigated as a potential therapeutic approach for an embryonal subtype of RMS (eRMS) employing a human eRMS cell line, RD; and comparing it with a normal murine myoblast cell line, C2C12. We observed that not only a serum free or a low serum concentration in the cultured medium induce morphological changes towards differentiations, but also staurosporine, bisindolylmaleimide, and neomycin sulfate (direct or indirect protein kinase inhibitors) induces differentiation in RD and C2C12 cell lines, suggesting a crucial role of PKC, PLC and its signaling pathways in a proliferation/differentiation switch.

These findings underscore the importance of exploring cellular differentiation as a therapeutic strategy against RMS and highlight the need for further research into the underlying mechanisms of kinase inhibitor-induced cellular differentiation as a potential cancer treatment.

Keywords: Staurosporine, Bisindolylmaleimide, Neomycin sulfate, RD, C2C12, Skeletal muscle, Differentiation

Introduction

The analysis of differentiation, prevalence, and proliferation of cancer is important for understanding the molecular details of transcription control, as well as providing insights into how these process contribute to cell identity and function. Cancer cells gradually acquire distinct biological capabilities, referred to as "hallmarks," enabling tumor establishment and progression [1,2]. These capabilities include the activation and maintenance of proliferative signals, evasion of growth-suppressive signals, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis mechanisms (such as aberrant Wnt-β-catenin signaling pathway) and the reprogramming energy metabolism and evasion of the immune system [1,2]. Genomic instability and mutations are key facilitators of cancer hallmark acquisition, generating genetic variations responsible for these distinct capabilities. Inflammation, which introduces bioactive molecules into the tumor microenvironment, is another facilitating characteristic of tumor development [2].

Cancer is a leading cause of childhood mortality, being rhabdomyosarcoma (RMS) the most prevalent type diagnosed, particularly the embryonal subtype, eRMS [3]. The incidence of RMS is approximately four new cases per million children under the age of 20, without a specific geographical predilection [4]. RMS is characterized by its primitive mesenchymal origin and a propensity for differentiation into striated muscle tissue [5,6]. Histologically, RMS is classified into two subtypes: alveolar (aRMS) and embryonal (eRMS) [4]. aRMS constitute 20-25% of RMS diagnoses and is characterized by smaller, rounder cells resembling pulmonary alveoli. This subtype displays a more aggressive behavior than others RMS [7]. eRMS is characterized by a heterogeneous population of cells in different stages of differentiation towards a skeletal muscle phenotype [7]. The World Health Organization (WHO) also recognizes another rarer RMS subtype, that is, the Pleomorphic RMS (pRMS), a morphological variant of RMS that typically occurs in adults.

Disease classification of RMS subtypes has been further refined by the identification of ‘fusion positive´ RMS (FPRMS) and ´fusion negative´ RMS (FNRMS) [7]. In terms of immunochemical features, eRMS cells exhibit nuclear localization of β-catenin in addition to its typical cytosolic and proximal membrane localization. They also express high levels of N-cadherin and integrin-α9, both of which are positively regulated by the Notch pathway. These molecular characteristics enhance cell mobility, invasiveness, aggressiveness, and the maintenance of an undifferentiated state within the tumor [8].

During myogenesis, pluripotent mesodermal precursor cells commit to the myoblast lineage, proliferate, differentiate, fuse into multinucleated myotubes and mature to form myofibers. This process is regulated by a family of conserved basic helix-loop-helix (bHLH) transcription factors called MRFs (myogenic regulatory factors) that includes MyoD (myogenic differentiation protein 1), Myf5 (myogenic factor 5), Myogenin, and Mrf4 (also known as Myf6, myogenic factor 6) [9]. The molecular mechanisms that control myogenesis at the transcriptional level are well characterized. MRFs such as MyoD, Myf5, Myogenin, and Mrf4 are expressed in different stages of myogenesis for a greater control in the transcription of the numerous muscle-specific genes, reorganizing metabolism, and mediating muscle contraction processes. In other matters, during embryonic development, three types of muscles are formed: the skeletal, cardiac, and smooth. The mature fiber of skeletal muscle is a complex multinucleated cell specialized in contraction. The precursors of the muscle lineage (myogenic cells), under the action of growth factors and the accumulation of myogenic regulatory factors, fuse with other similar cells, generating a multinucleated myotube that then advances to the final stage of differentiation, the muscle fiber [10].

MyoD and Myogenin are expressed in RMS and thus have been used as diagnostic markers to identify the disease. Among the MFRs, MyoD is considered a master myogenic transcription factor, as its activity can convert non muscle cells into the muscle lineage [9]. Recent studies have shown that the inability of RMS to properly differentiate might be linked to the improper binding of MyoD to its target genes [11].

As an experimental model, we have previously used a murine muscle cell line, C2C12, to investigate the molecular mechanisms of apoptosis. These cells have also provided a good *in vitro* system for studying the major steps of myoblast proliferation, differentiation and apoptosis [12-16]. C2C12 cell line is able to differentiate in both serum-containing (2% horse serum, HS) and serum-free media [12]. Cultures were checked for myotube formation, the activity of creatine phosphokinase and the presence of sarcomeric actin. In C2C12 cells, the extent of differentiation was greater in the serum-free than in the serum-containing system. In both media, C2C12 cells produced sarcomeric actin, showing the presence of sarcomere structure in the myotubes. Also, C2C12 cell line can differentiate into a skeletal muscle fiber in a medium containing 1α,25(OH)$_2$D$_3$ (the active form of vitamin D$_3$) or in a medium with 1α,25(OH)$_2$D$_3$-glycosides enriched natural product (Solbone A) from *Solanum glaucophyllum* leaf extract [16]. Both cultured medium, increased creatine kinase activity and myogenin and myosin heavy chain (MHC) protein expression [16].

Bisindolylmaleimide is an organic compound that forms the core chemical structure of a variety of biologically active compounds. This core structure includes a central maleimide group with two indole groups attached. It is a cell-permeable potent inhibitor of protein kinase C (PKC).

Staurosporine has the bisindolylmaleimide core structure of indole carbazole. It was discovered while screening for microbial alkaloids, and has been shown to possess various biological activities, including hypotensive activity and inhibitory activity of platelet aggregation and antifungal activity [17,18]. It is a broad-spectrum serine/threonine kinase inhibitor, [19] that can affect the activity of PKC [18].

Neomycin is a widely used phospholipase C (PLC) inhibitor, binds to phosphatidylinositol-4,5-bisphosphate (PIP2) and inhibits enzyme activity. The PLC hydrolyzes PIP2 to generate diacylglycerol (DAG) and IP3. DAG remains near the plasma membrane and activates PKC; IP3 diffuses into the cytoplasm and binds to calcium channels, which releases Ca^{2+} that has multiple targets. Therefore, inhibition of PLC with Neomycin indirectly affects PKC. Modulation of these cellular signaling pathways (PKC-PLC) would ultimately lead to the regulation of transcription factors related to myogenesis and differentiation towards a skeletal muscle fiber, as it was reported for NF-AT (nuclear factor of activated T cells), C1, C2, and C3 [20,21].

So, the aim of this study was to investigate the effects of the indole family compounds (staurosporine and bisindolylmaleimide) and neomycin sulfate on myogenic differentiation of human eRMS cells, RD (a tumor cell line) compared with C2C12 cells (a non-tumor cell line), both derived from a mesenchymal lineage and both lines with a tendency to differentiate into skeletal muscle fiber in a non-proliferative medium.

Materials and Methods

Materials

MitoTracker (MitoTracker® Red CMXRos) dye, 4',6-diamidino-2-phenylindole (DAPI) stain and Alexa Fluor 488-conjugated anti-mouse secondary antibody were from Molecular Probes (Eugene, U.S.A.), integrin-α9 (2Q954) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Staurosporine and bisindolylmaleimide components were purchased from Sigma Aldrich Co. (St. Louis, MO). Neomycin sulfate was obtained from Calbiochem. High Pure RNA Isolation kit (11828665001) was from Roche Diagnostics (Mannheim, Germany). High Capacity cDNA Reverse Transcription Kit (4368814) and SYBR® Select Master Mix were purchased from Applied Biosystems. Primer sets were from Invitrogen (Carlsbad, CA, USA). All other reagents used were of analytical grade.

Staurosporine, bisindolylmaleimide and neomycin sulfate characteristics

Staurosporine **(Figure 1A)** is a potent, cell-permeable, reversible, ATP-competitive, and broad-spectrum inhibitor of protein kinases, especially for PKC.

Bisindolylmaleimide **(Figure 1B)** is an organic compound that forms the core chemical structure of a variety of biologically active compounds. This core structure includes a central maleimide group with two indole groups attached. Bisindolylmaleimide is a cell-permeable potent and, as staurosporine, a selective inhibitor of PKC. These two drugs are part of the indole family.

Neomycin sulfate **(Figure 1C)** is the sulfate salt of a kind of neomycin sulfate, an aminoglycoside antibiotic that inhibits translation by binding to the small subunit of prokaryotic ribosomes. Blocks voltage-sensitive Ca^{2+} channels without affecting the Na^{+}- Ca^{2+} antiporter in neurons.

Cell culture and treatments

Human rhabdomyosarcoma cell line, RD, (ATCC number: CCL-136™) and C2C12 murine myoblast cell line (ATCC number: CRL-1772™) were cultured in a growth medium (Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% nistatine, and 2% streptomycin, without phenol red. This medium was called: proliferative medium (PM). Cells were incubated at 37°C under a humidified atmosphere of 5% CO_2 in air.

In RD culture, it can be observed cells at different stages of differentiation with different shapes or morphologies like rounded, star-shaped, spindle; which demonstrates not only their mesenchymal origin but also their tendency to differentiate into muscle fibers.

C2C12 cell line is a subclone of C2 cell line; and resembles in shape and function the satellite cells that surround the mature myofibers. They can proliferate and differentiate to repair muscle tissue when a cellular injury exists [22].

To promote cell differentiation, RD and C2C12 cells were cultured in PM (DMEM with 10% FBS) until 70%-80% of confluence, then PM was replaced by a differentiation medium (DM). The DM was DMEM supplemented with 1% Horse Serum (1%HS) or with 1% Fetal Bovine Serum (1%FBS) or DMEM without serum (serum-free media). The cells were then maintained in DM until typical morphological changes of differentiation were observed (approximately one week). DM was changed every day. Differentiation into a skeletal muscle fiber in a medium with 2% HS or serum-free media was also demonstrated in C2C12 cell line [12] The differentiation of these cells *in vitro* requires a switch from a serum-rich medium to a less rich medium after the cells have reached confluence [12].

To investigate the effects of staurosporine, bisindolylmaleimide, and neomycin sulfate, RD and C2C12 cells were first cultured in a PM (DMEM with 10%SFB) until 70-80% of confluence was reached.

Staurosporine, bisindolylmaleimide, and neomycin were dissolved in isopropanol, then diluted in DMEM to a final concentration of 1 nM for staurosporine, 0.05 mM for bisindolylmaleimide and 6 mM for neomycin sulfate.

When cultures reach 70%-80% confluence, the cultured medium was changed to a DMEM with 10% SFB (PM) containing staurosporine, bisindolylmaleimide, and neomycin sulfate at the concentration described above. As a differentiation control, RD and C2C12 cell lines were cultured in a DM: DMEM with 1% HS, or DMEM with 1% SFB, or DMEM without serum (serum- free media).

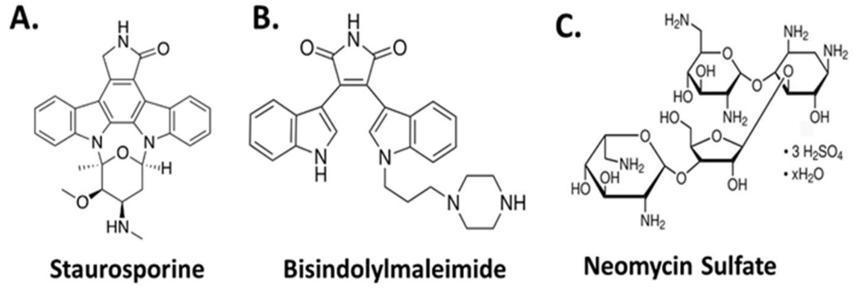

Figure 1. Chemical structure of staurosporine, bisindolylmaleimide, and neomycin sulfate.

Untreated cells (Controls) were cultured in a PM with the respective vehicle (isopropanol). Cells were cultured in plates (Greiner Bio-One, Frickenhausen, Germany). In all cases, the culture medium was renewed every day until the cells showed a morphological change (approx. a week). The drug and serum concentrations used in this study were determined based on prior dose-response studies conducted in other experiments [12,16,19].

MitoTracker Red (MTT) and DAPI staining

MTT was employed for selective stain of active mitochondria. After a week, the cells attached to the plates were stained with MTT, which was prepared on dimethyl sulfoxide (DMSO) and added to the cell culture medium at a final concentration of 1 µmol/L (1:10000, prepared in DMEM without serum). After 30 minutes of incubation at 37°C, the cells were washed with PBS 1X (pH 7.4, 8 g/L NaCl, 0.2 g/l KCl, 0.24 g/L KH_2PO_4, 1.44 g/L Na_2HPO_4) and then they were fixed and permeabilized with methanol at -20°C for 30 minutes. Cells were then washed 3 more times with PBS 1X to remove any methanol residue, and then stained with DAPI (1:500 dilution from a stock solution of 5 mg/ml), dissolved in PBS 1X, during 30 minutes at room temperature in darkness and with gentle stirring. The cells were then mounted on slides for microscopic analysis. A fluorescence microscope (NIKON Eclipse TiS) equipped with standard filter sets was employed to capture fluorescent signals. Images were collected using a digital camera.

Immunocytochemistry

After a week, cells were washed with serum-free phenol red-free DMEM and then fixed and permeabilized for 20 minutes at -20°C with methanol to allow intracellular antigen labeling. After fixation, cells were rinsed 3 times with PBS. Non-specific sites were blocked for 30 minutes in PBS that contained 5% bovine serum albumin. Cells were then incubated overnight at 4°C, in the presence or absence (negative control) of primary antibody (1:50 dilution). The primary antibody was recognized by fluorophore-conjugated secondary antibody. Finally, the coverslips were analyzed by conventional fluorescence microscopy using a fluorescence microscope (NIKON Eclipse TiS) equipped with standard filter sets to capture fluorescent signals. Images were collected using a digital camera.

Quantitative Real Time RT-PCR

After a week, total RNA (10^6 RD and C2C12 cells/condition) was extracted using the High Pure RNA Isolation kit and approximately 2 µg of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer´s instructions. Quantitative measurement of real-time PCR was done using Select Master Mix (2X) under the standard conditions recommended by the manufacturer. Primers pairs (sequences listed 5´to 3´) sets to amplify cDNAs used in the analysis were as follows: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) *Mus musculus*-F: 5'-CGTCCGTAGACAAAATGGT-3', GAPDH *Mus musculus*-R: 5'-TTG ATGGCAACAATCTCCAC-3', GAPDH human-F: 5'-TGCACCACCAA CTGCTTAGC-3', GAPDH human-R: 5'-GGCATGGACTGTGGTCCA TGAG-3', MHC1 *Mus musculus*-F: 5'-ACCGAAGGCGGAACTACTG -3', MHC1 *Mus musculus*-R: 5'-ATGCAGGTGGGTCATCATGG-3'; MHC1 human-F: 5'-GCGGTCTGCAAAGCAAGATT-3'; MHC1 human-R: 5'-CTCCTCAATGCGGGCTTGTA-3'; MYO-D *Mus musculus*-F: 5'-GCACTACAGTGGCGACTCAGAT-3'; MYO-D *Mus musculus*-R: 5'-TAGTAGGCGGTGTCGTAGCCAT-3'; MYO-D human-F: 5'TCCGC GGCATGATGGACT3'; MYO-D human-R: 5’-TGGGCGCCTCGTTGT AGTAG-3’; Integrin-α9 *Mus musculus*-F: 5'-TGTCGTGTCCATACC AACCC-3'; Integrin-α9 *Mus musculus*-R: 5'-ATGGGCACAGGCCA AAACAC-3', Integrin-α9 human-F: 5'-CACGTCCATCACCGGAAT CA-3'; Integrin-α9 human-R: 5'-TGGGCTGAAAGTGAC ACTCC-3'. The specificity of PCR products was confirmed by melting curve analysis. Relative quantification of gene expression was determined by the comparative $2^{-\Delta\Delta CT}$ method [23,24].

Statistical analysis

Results are shown as means ± S.D. Statistical differences among groups were determined by ANOVA followed by a multiple comparison post hoc test. Data were considered significant at $p < 0.05$.

Results

Low serum concentration or non-serum (DM) induce differentiation in C2C12 and RD cell lines

We first evaluated the morphological changes that occurred in C2C12 and RD cell lines switching cultured conditions from PM to DM (1% FBS or 1% HS, or medium without serum). After 7 days, the typical morphological changes towards a muscle fiber phenotype, such as alignment, elongation, and fusion of mononucleated cells to multinucleated myotubes, were observed in both cell lines **(Figure 2)**. The comparison revealed clearly distinct morphologies between cells cultured in DM compared to those in PM **(Figure 2)**.

Staurosporine, Bisindolylmaleimide and neomycin sulfate treatments induces morphological changes associated with differentiation in C2C12 and RD cell lines

Then we investigate if the indole family drugs (staurosporine and bisindolylmaleimide, PKC inhibitors) and neomycin sulfate (a PLC inhibitor) induce cell differentiation in both C2C12 and RD cell lines. For this, cells were grown in a PM until 70%-80% confluence and then staurosporine, bisindolylmaleimide or neomycin sulfate was added into the PM as it was described in Methods.

In **Figure 3A**, C2C12 cells treated with staurosporine showed elongation with enhanced alignment and directional uniformity. The cells were elongated and aligned in a coordinately way, a biological phenomenon indicative of

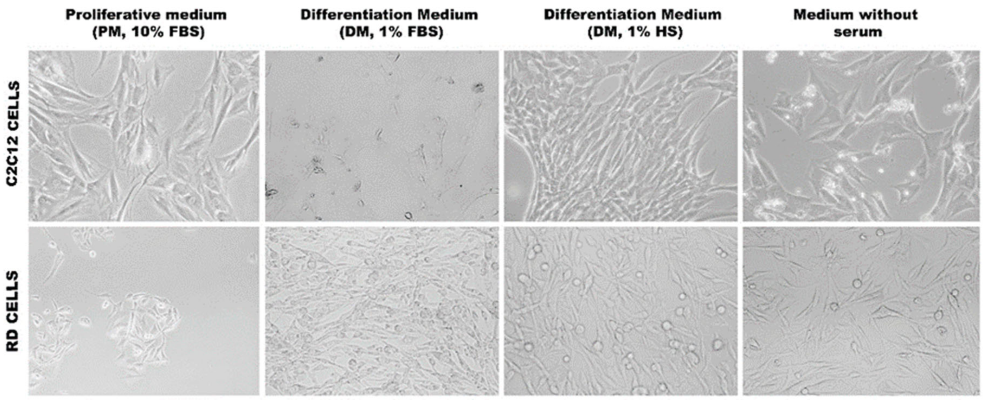

Figure 2. Low serum concentration or non-serum (DM) induces differentiation in C2C12 and RD cell lines. C2C12 and RD cells were cultured in proliferative medium (PM: DMEM with 10% FBS). Once they reached 70% to 80% of confluence, the medium was switched into a DM (DMEM with 1% FBS, DMEM with 1% HS or medium without serum) or maintained in a PM (controls). Photographs were captured to visualize morphological changes. Magnification: 20X.

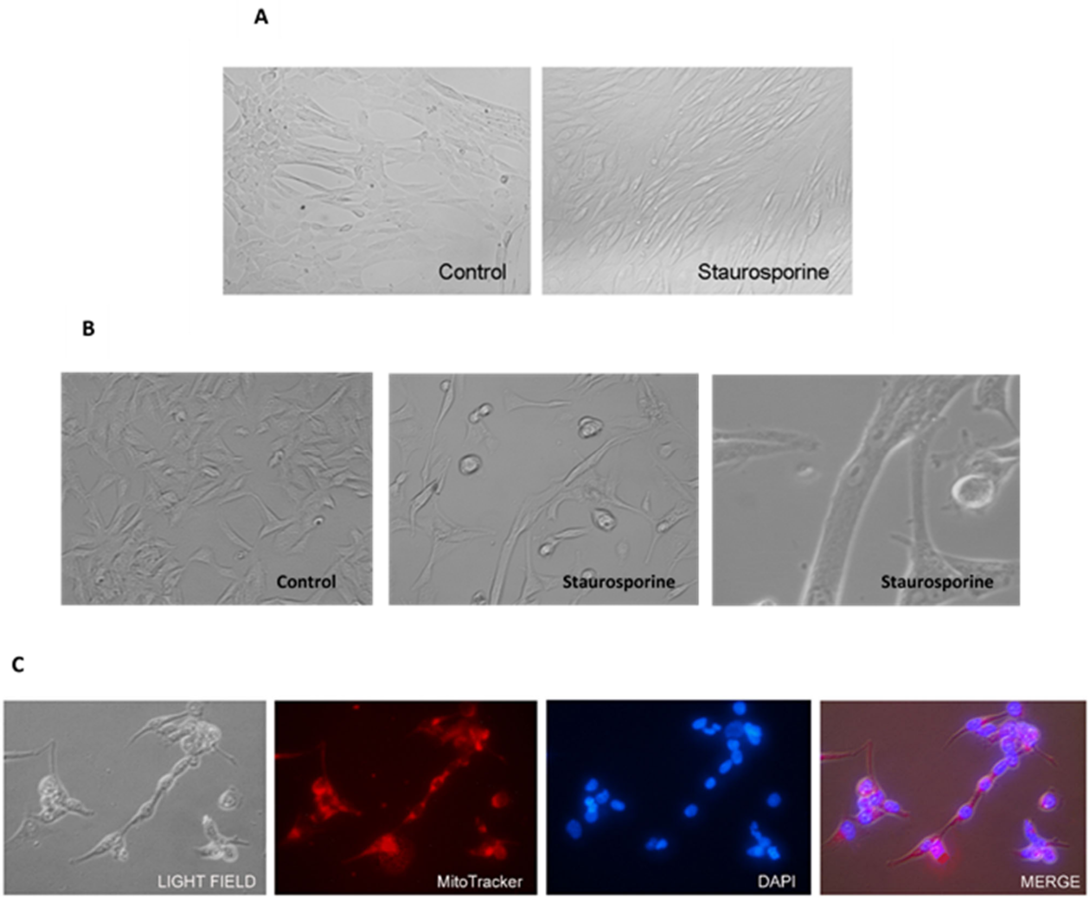

Figure 3. Staurosporine treatment induces morphological changes associated with differentiation in C2C12 and RD cell lines. The cells were cultured in a proliferative medium (PM) until 70%-80% confluence. Then the cultures were treated with 1 nM of staurosporine in PM or the vehicle isopropanol dissolved in PM (Control), for one week. After a week the cells were analyzed under a fluorescence microscope. **A.** C2C12 cell line. **B:** RD cell line non-stained. **C:** RD cell line stained with MITT and DAPI. Magnification: 20X. Figure B on the right, magnification: 40X.

impending differentiation. Similarly, after one week of treatment, RD cells were observed and then stained with MTT and DAPI. Visible and fluorescence images were captured to demonstrate the effects of 1 nM staurosporine on RD cells. A composite image combines various views, vividly illustrating the characteristic biological phenomenon of differentiation, the formation of multinucleated cells resembling myotubes. RD cells treated with staurosporine for one week displayed elongation and fusion, transitioning from mononucleated cells to multinucleated, marking the onset of cellular differentiation **(Figure 3B** and **3C)**. In **Figure 3B**, RD cells undyed are observed. The image on the right shows in detail the fused cells in the transformation process towards a multinucleate muscle fiber. This effect was further visible after MTT and DAPI staining, confirming the morphological changes observed **(Figure 3C)**.

Following this, we observed in C2C12 and RD cells treated with bisindolylmaleimide in a **PM**, a similar response to those cells treated with staurosporine. Bisindolylmaleimide induces noticeable changes including enhanced alignment, elongation, and the presence of multinucleated cells, as it is illustrated in **Figures 4A** and **4B**.

Neomycin sulfate, an antibiotic that inhibit the PLC activity, also induced, in C2C12 and RD cell lines, significant morphological changes towards a skeletal muscle fiber phenotype **(Figures 5A** and **5B)**.

Staurosporine, bisindolylmaleimide and neomycin sulfate regulates MHC1, MyoD and integrin-α9 gene expression in C2C12 and RD cells

Then we quantified the relative levels of mRNA of Myosin Heavy Chain 1 (MHC1), MyoD and Integrin- α9, in C2C12 and RD cell cultures. Experiments were carried out in the presence or absence of staurosporine, bisindolylmaleimide, and neomycin sulfate in a PM, or a medium without serum (DM); as it was described in Methods. Total RNA was isolated, transcribed into cDNA and used to determine the expression levels of the different genes mentioned using real-time PCR, as it was described before. GAPDH was used as a reference gene.

We first evaluate the gene expression level of MHC1. MHC1 is considered a specific marker of differentiation towards a skeletal muscle phenotype. **Figure 6** shows the relative expression level of MHC1 in C2C12 and RD. We observed a significant increase in MHC1 mRNA in C2C12 and RD cultured cells in PM treated with staurosporine, bisindolylmaleimide or neomycin sulfate compared to control samples. As it was expected, C2C12 and RD cells cultured in serum-free

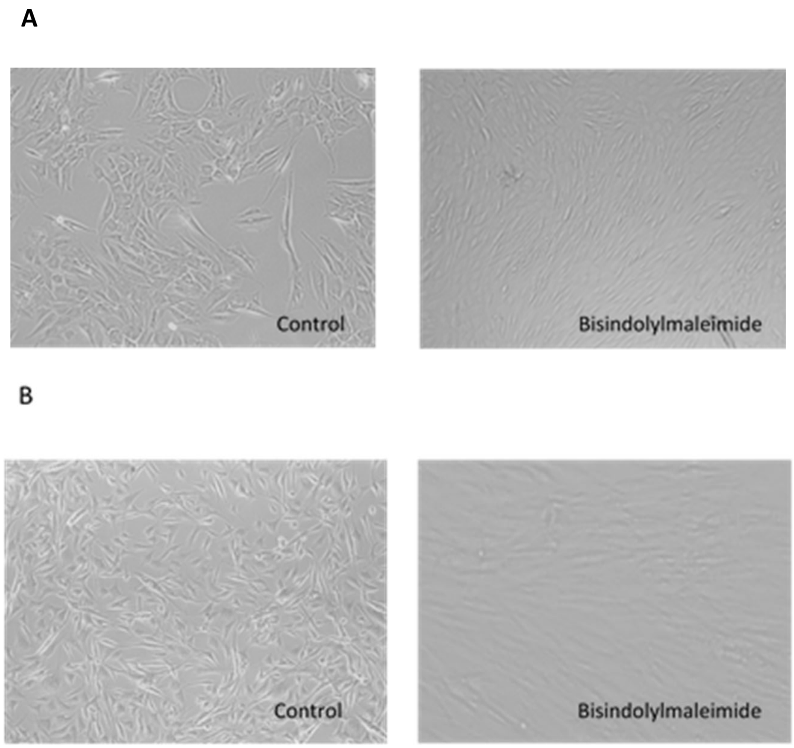

Figure 4. Bisindolylmaleimide treatment induces morphological changes associated with differentiation in C2C12 and RD cell lines. The cells were cultured in PM until 70%-80% confluence. Then the cultures were treated with 0.05 mM of bisindolylmaleimide for a week in PM medium or the vehicle isopropanol dissolved in PM (Control). **A.** C2C12 cell line. **B**: RD cell line. Magnification: 20X.

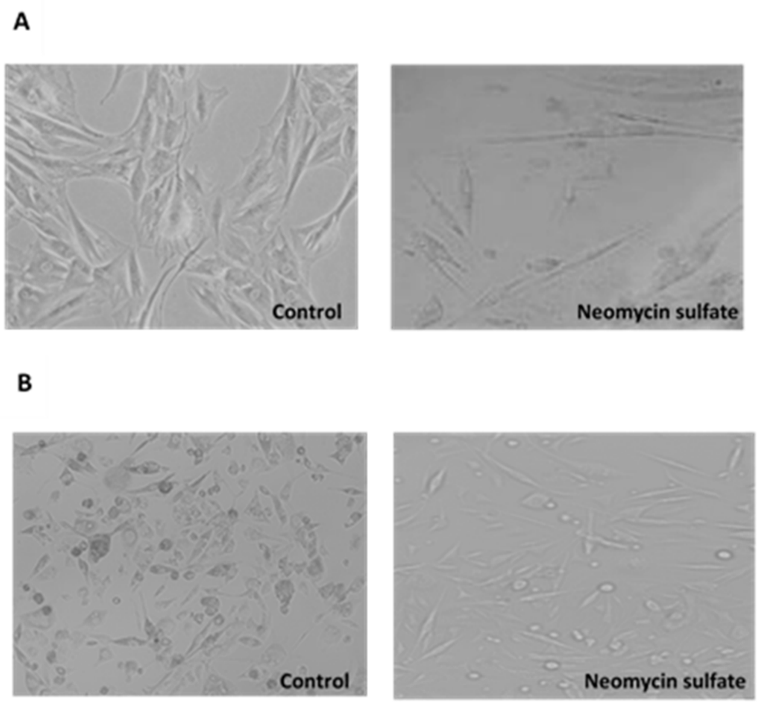

Figure 5. Neomycin sulfate treatment induces morphological changes associated with differentiation in C2C12 and RD cell line. The cells were cultured in PM until 70%-80% confluence. Then the cultures were treated with 6 mM of Neomycin sulfate for a week in PM medium, or the vehicle isopropanol dissolved in PM (Control). **A.** C2C12 cell line. **B**: RD cell line. Magnification: 20X.

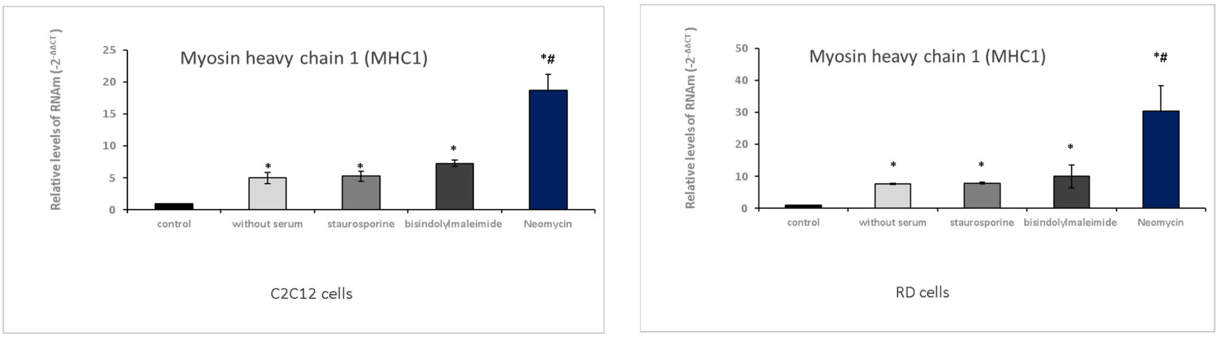

Figure 6. Staurosporine, bisindolylmaleimide, and neomycin sulfate upregulate MHC1 gene expression in C2C12 and RD cells. Transcript levels of MHC1 were determined by Real Time PCR as it was described in Methods. The cells were cultured in **PM** until 70%-80% confluence, then the cultures were treated with staurosporine, bisindolylmaleimide, and neomycin sulfate for a week in PM medium, or in a medium without serum (DM). Control: untreated cells (vehicle, isopropanol, dissolved in PM). Transcription levels were normalized to the expression level of GAPDH gene. The relative expression $2^{-\Delta\Delta CT}$ was used for quantification. Averages ± S.D. are given; * $p<0.05$ respect to control condition; # $p<0.05$ respect to: without serum, staurosporine and bisindolylmaleimide treatments.

medium, showed an increase in MHC1 gene expression. These observations are derived from the analysis of three independent experiments, where the $2^{-\Delta\Delta CT}$ value was obtained as a measure. In C2C12 and RD It is evident that the expression of MHC1 significantly increase with the different treatments; compared to the control condition; being higher with neomycin sulfate.

We also evaluated the expression of the MyoD gene. MyoD is expressed in proliferating myoblasts and together with myogenin begins to be expressed in stages of primary differentiation from myoblasts to myotubes. **Figure 7** shows the mRNA levels of MyoD gene in C2C12 and RD cells treated with 1 nM of staurosporine, 0.05 mM of bisindolylmaleimide or 6 mM of neomycin sulfate or in a medium without serum (DM) during one week, respect to control cells, as it was previously described in Methods. As it was expected, different treatments (PM with PKC/PLC inhibitors or medium without serum) induced an increased expression of MyoD, being higher for bisindolylmaleimide and neomycin sulfate treatments in both cell lines.

Integrins are heterodimeric transmembrane glycoproteins formed by α and βsubunits. They interact with glycoproteins of the extracellular matrix such as fibronectin. Upon ligand binding, integrins can activate signal transduction pathways that regulates cell adhesion, migration, invasion, and epithelial-mesenchymal transition (EMS), a critical process in embryonic development. We aimed to assess the expression of the Integrin-α9 gene which encodes the integrin-α9 protein, a highly expressed integrin in RD cell line [8]. Upon examining the gene expression level in both C2C12 and RD cells treated with staurosporine, we observed downregulation of its expression in C2C12 but the results were highly variable in RD, compared to the control counterpart **(Figure 8)**.

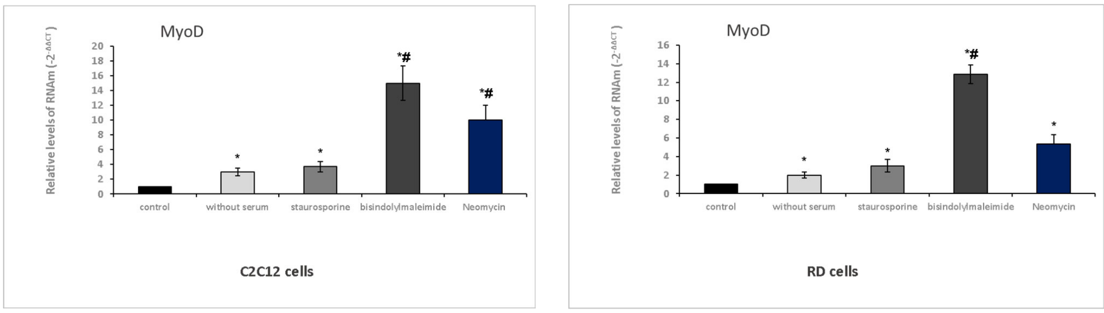

Figure 7. Staurosporine, bisindolylmaleimide, and neomycin sulfate upregulate MyoD gene expression in C2C12 and RD cells. Transcript levels of MyoD were determined by Real Time PCR as it was described in Methods. The cells were cultured in **PM** until 70%-80% confluence, then the cultures were treated with staurosporine, bisindolylmaleimide, and neomycin sulfate for a week in PM medium, or in medium without serum (DM). Control: untreated cells (vehicle, isopropanol, dissolved in PM). Transcription levels were normalized to the expression level of GAPDH gene. The relative expression $2^{-\Delta\Delta CT}$ was used for quantification. Averages ± S.D. are given* $p<0.05$ respect to control condition; # $p<0.05$ respect to: without serum and staurosporine treatments (C2C12), respect to: without serum, staurosporine, and neomycin treatments (RD).

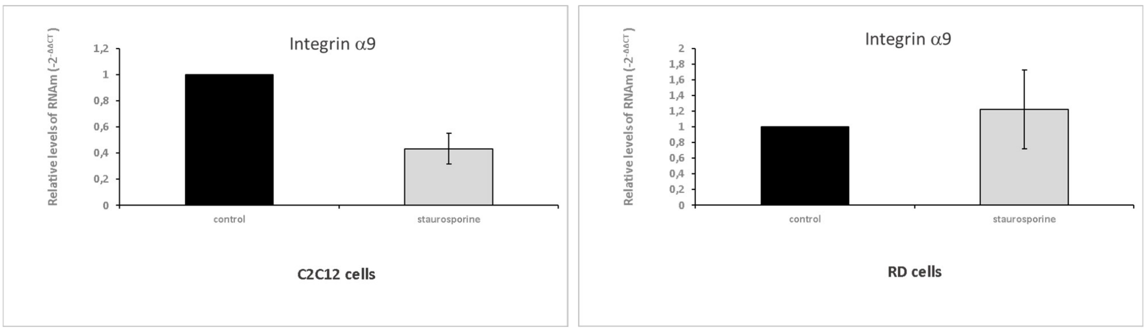

Figure 8. Staurosporine regulates integrin-α9 gene expression in C2C12 and RD cells. Transcript levels of integrin-α9 were determined by Real Time PCR as described in Methods. The cells were cultured in PM until 70%-80% confluence, then the cultures were treated with staurosporine, for a week in PM medium. Control: untreated cells (vehicle, isopropanol, dissolved in PM). Transcription levels were normalized to the expression level of GAPDH gene. The relative expression $2^{-\Delta\Delta CT}$ was used for quantification. Averages ± S.D. are given.

Staurosporine, bisindolylmaleimide and neomycin sulfate regulates integrin-α9 protein level in RD cells

As the real-time PCR results for integrin-α9 were highly variable in RD **(Figure 8)**, we evaluated protein expression level by immunocytochemistry using a specific antibody against integrin-α9. **Figure 9** shows the result obtained by immunocytochemistry assay employing a specific antibody against integrin-α9 and a secondary antibody conjugated with Alexa 488 as it was described in Methods. A slight decrease in labeling and therefore in protein level of integrin-α9 were observed in different treatments (staurosporine, bisindolylmaleimide and neomycin sulfate) compared to the protein level in the control condition. A clear labeling on the plasma membrane can be observed in control cells (A and B). In B the labeling on the plasma membrane is shown in detail (magnification 60X), where cytoplasmic projections on the membrane are clearly observed.

RD cells treated with the PKC and PLC inhibitors, show a decreased fluorescent signal, compared to the control condition **(Figure 9A)**, especially RD cells treated with bisindolylmaleimide or neomycin sulfate, suggesting a decreased integrin-α9 protein level. Although cells treated with staurosporine show less fluorescent signal compared to control cells, but more fluorescent signal compared to bisindolylmaleimide and neomycin sulfate conditions.

Discussion

Rhabdomyosarcoma (RMS) is a highly aggressive tumor that primarily occurs in childhood and originates from immature skeletal muscle cells. Its prognosis varies depending on the tumor's location, size, and the presence of metastasis, among other factors. Standard treatment typically involves a combination of surgery, chemotherapy, and radiation therapy. In this study, we focused on the embryonal subtype of RMS (eRMS), which tends to localize in the head and neck, genitourinary tract, and perirectal area in children and young adults. Morphologically, eRMS exhibits a wide range of cellular characteristics, including cross-striations, large nuclei, and pleomorphism. In some cases, embryonal structures such as rosette formation, tubular structures, and alveolar growth patterns can be observed. Additionally, a high rate of cellular proliferation has been noted, complicating both the diagnosis and treatment of this disease [4,5].

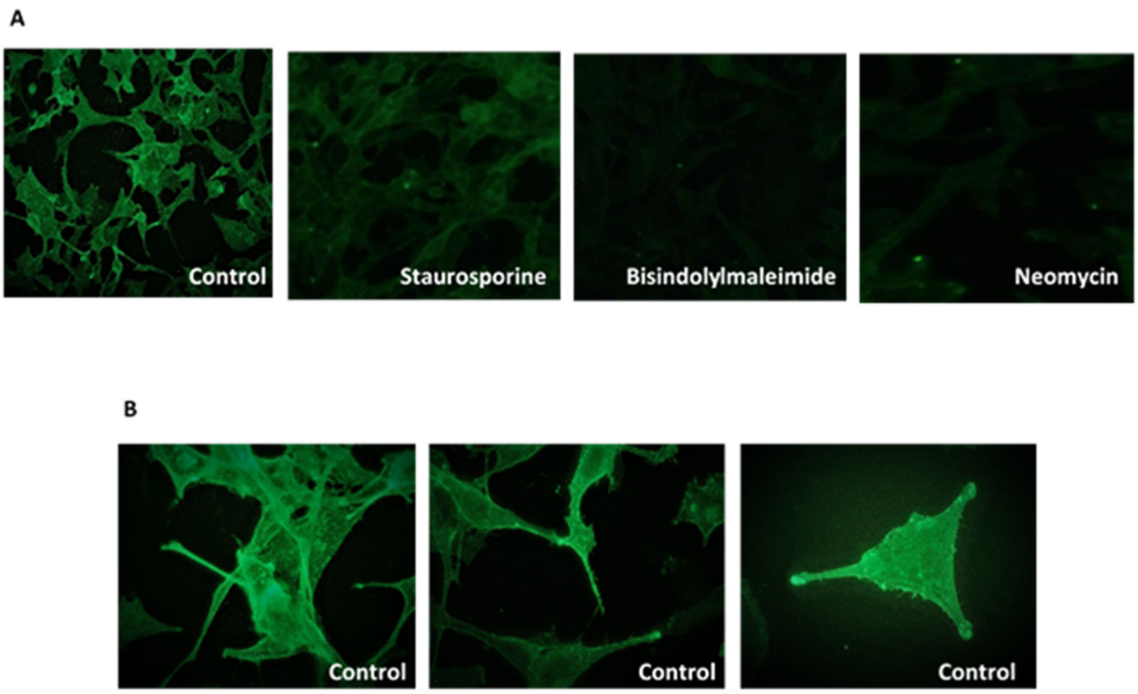

Figure 9. Staurosporine, bisindolylmaleimide, and neomycin sulfate regulate integrin-α9 protein level in RD cells. Protein levels of integrin-α9 were determined by immunocytochemistry as it was described in Methods. The cells were cultured on coverslips in PM until 70%-80% confluence, then the cultures were treated with staurosporine, bisindolylmaleimide, and neomycin sulfate for a week in PM medium. Control: untreated cells (vehicle, isopropanol, dissolved in PM). The cells were then fixed, permeabilized, and incubated with a primary antibody specific for integrin-α9 and a secondary antibody conjugated with Alexa 488 fluorophore. Plasmatic membrane localization was clearly observed in control cells (**A** and **B**). Lower fluorescence was detected in treated cells (**A**). **A**: magnification 20X; **B**: magnification: 60X.

Differentiation of RMS cells refers to the acquisition of mature and specific characteristics of muscle cells. The induction of cellular differentiation can have beneficial effects like inhibition of tumor growth and sensitivity to certain treatments. Several studies have investigated certain approaches to induce differentiation in RMS cells [25,26]. When a cell differentiates, it means that acquires a specialized form, structure, and function to carry out specific tasks within a multicellular organism. Differentiation is essential during the embryonic and postnatal development of an organism, as well as in the renewal and repair of tissues. This process is regulated by a combination of intrinsic genetic signals and extrinsic environmental factors. Once a cell differentiates, it acquires specific morphological, biochemical, and functional characteristics that distinguish it from other cells.

Previous works reported that C2C12 cells effectively differentiate in a cultured medium containing lower levels of horse serum or in serum free medium [12]. They form myotubes that successfully produce components of sarcomeric structures, and the myotubes generated are readily visually apparent.

In this work we not only observed a differentiation of C2C12 cell line into a muscle cell phenotype when cells were cultured during a week in 1% of Horse serum (HS) or 1% Fetal Bovine Serum (FBS) or in a serum free medium; but also RD cell line shows a differentiation phenotype into a muscle cell fiber in these cultured conditions.

Since many of the proteins present in the fetal bovine serum are not identified, it is very difficult to pinpoint the protein absence from serum-free media that is essential for maintaining cells in an undifferentiated stage. We also hypothesize that a lower serum concentration (1%) or a serum free medium may offer more conducive conditions for differentiation by restraining cell proliferation and promoting the expression of differentiation-associated genes. Additionally, a reduced serum concentration could induce a deceleration in the cell cycle, facilitating quicker cellular differentiation. Moreover, a lower serum concentration may enhance cell adhesion to the substrate, thereby fostering cellular differentiation.

The absence of serum or a low serum concentration in the culture medium affects cell proliferation. This can be interpreted as follows: cells that are in a proliferation medium (PM, 10% FBS) and undergoes into a change in the culture conditions, like a low serum content or a serum free medium, this switch could cause a change in cellular behavior, then cells have to take a different development path since they enters into a stressful situation due to lack of some growth factors. This stress has at least two possible pathways: cell death or cell survival following the differentiation pathway, with lower energy expenditure since the cell leaves the cell cycle and enters in a stage similar to the G0.

In addition, C2C12 and RD cells, growing in PM treated with staurosporine, bisindolylmaleimide or neomycin sulfate developed similar morphological changes towards a skeletal muscle phenotype, that those observed in cells cultured in a low serum concentration medium or medium without serum (DM). The cells change their shape, they stretched, became directed, and it was observed, during the time that the culture was maintained (one week), cell fusion and development of multinucleated fibers. This discovery holds significance specially for RD cell line, because cell differentiation entails not only the loss of the cell potential to transform into another cell type, but also, as would be the case for RD cell line (malignant and invasive phenotype), the loss of the biological capabilities of the tumor cells, referred to as "hallmarks," that enabling a tumor to establish and progress. Importantly, this effect was observed under proliferative conditions (10% FBS), indicating that the differentiation induced by the PKC or the PLC inhibitors (staurosporine, bisindolylmaleimide or neomycin sulfate; respectively), it is specific to the presence of the inhibitor in the cultured medium.

The process of myogenesis involves the commitment and differentiation of pluripotent primitive mesodermal cells into skeletal muscle and is regulated by members of the myogenic regulatory factors family, MRFs. Members of the MRF family include MyoD, Myf5, myogenin, and Mrf4 (Myf6), transcription factors with basic helix-loop-helix domains that act sequentially in the myogenic differentiation process.

MyoD, a transcription factor that plays a pivotal role in muscle differentiation and is one of the earliest markers when myogenesis occurs; It is expressed in activated satellite cells, but not in quiescent ones. The function of MyoD in development is to recruit mesoderm cells to differentiate into a muscle cell line, and then regulate the entire process. MyoD could also play a role in muscle repair. One of the main actions of MyoD is to enhance p21 transcription levels and thus take cells out of the cell cycle and get them into a differentiation pathway.

C2C12 have been shown to contain a heterogeneous population of cells, most of which are deficient in the production of proteins involved in myoblast differentiation [22]. Instead, in rhabdomyosarcoma cells, especially in RD cell line, cells can be observed in different stages of differentiation with a tendency to differentiate into skeletal muscle fiber. The cell population in RD is much more heterogeneous than in C2C12 cell line, since in RD there are cells in different morphological stages clearly marked, that coexist all together in the same cell culture (rounded and totally undifferentiated cells, mesenchymal cells and elongated or spindle-shaped cells).

We have detected, in basal conditions for both cell lines, that MyoD gene is expressed, suggesting the ability of both cell lines to differentiate towards a mscular lineage. The presence or absence of MyoD in some cells population in RD cultures, may result from genetic mutations, epigenetic alterations, or other aberrant signaling pathways that modulate its expression.

MHC1 is one of the major contractile protein that converts chemical energy into mechanical energy through the hydrolysis of ATP. It is found in striated muscle and also detected in mature myotubes; therefore, MHC1 gene expression is a clear marker of the commitment of cells towards a skeletal muscle phenotype. It could be said that it is a specific marker of differentiation towards a skeletal muscle fiber.

When transcription levels of genes involved in muscle differentiation towards a skeletal muscle fiber were analyzed by real-time PCR, we observed that not only a free serum medium but also different treatments with different inhibitors in a PM increased their expression. Specifically, we evaluated mRNA levels for MyoD and MHC1 genes; in addition to the expression of mRNA for integrin-α9, a transmembrane protein overexpressed in RD cell line, essential for cell matrix adhesion, migration, and blood vessels extravasation.

Real-time PCR assays demonstrated that the observed morphological changes towards a skeletal muscle phenotype in C2C12 and RD cell lines, were coincident with the expression of genes that lead to myogenesis (such as MyoD) and the expression of genes that are specific for differentiation towards a skeletal muscle fiber (MHC1).

For integrin-α9, regarding the transcription levels, the result was conclusive for C2C12 cells, where staurosporine treatment decreased the mRNA level of this transmembrane protein. This was expected taking into account that the integrin's proteins play a fundamental role in cell migration and are expressed mainly in mesenchymal cells. Also, plays a crucial role in regulating the differentiation of muscle cells and the formation of muscle tissue [27,28].

In RD cell line, that in basal conditions overexpressed integrin-α9, a conclusive result was not obtained by real-time PCR. However, immunocytochemistry assays showed a decrease in integrin-α9 protein expression in different treatments, compared to control condition. In this tumor cell line, this receptor has a fundamental role in providing some of the malignant phenotypic characteristics, like giving the tumor cells the ability to migrate and transfer blood vessels. The overexpression of the receptor is directly related to the aggressive (metastatic) phenotype.

It has been shown that activation or inhibition of PKC can have significant effects on cell differentiation in various cell types, including muscle cells, neurons, and epithelial cells [29]. The PKC pathway plays an important role in cellular differentiation by modulation of gene expression and signaling pathways affecting cellular organization. Here, bisindolylmaleimide, staurosporine, and neomycin sulfate were identified as effective agents for inducing cell differentiation in C2C12 and RD cell lines. Bisindolylmaleimide, staurosporine can directly inhibit PKC; and neomycin indirectly through the PLC pathway, by binding to negatively charged phospholipids in the cell membrane, leading to changes in its composition and affecting PLC activation. Also the PLC pathway could affect various other enzymes, proteins, and ion channels activities.

If a PKC inhibitor induces cell differentiation, it could be indicative that the PKC signaling pathway could be exerting an inhibitory effect on differentiation rather than promoting it. Something relevant to consider in the context of PKC and cellular differentiation is that some PKC inhibitors can modulate the expression of MyoD [30].

Therefore, it is inferred that the signaling pathway used by all the compounds studied (staurosporine, bisindolylmaleimide, and neomycin sulfate) to induce differentiation in C2C12 and RD cell lines possibly involves the PKC signaling pathway.

Modulation of these pathways would ultimately lead to the regulation of transcription factors related to myogenesis and differentiation towards a skeletal muscle fiber, as it is the case of NFAT (nuclear factor of activated T cells), C1, C2 and C3; already reported [20,21].

Actually we are analyzing the transcription factors that can be regulated by the PLC/PKC pathways and that could be involved directly or indirectly in myogenesis.

The role of Sirtuins are important in myogenesis, muscle development and survival of tumor and non-tumor myogenic cells as it was demonstrated for C2C12 and RD cell lines.

Sirtuins (SIRT) are a family of deacetylases (class III histone deacetylases) and ADP-ribosyl transferases with evolutionarily conserved functions in cellular metabolism and chromatin regulation. There are 7 types of SIRTs (SIRT1-SIRT7) in mammals, that can regulate cellular processes such as metabolism, cell survival, differentiation, DNA repair and are involved in the pathogenesis of solid tumors and leukemias. They are also active on substrates other than histones, being able to deacetylate transcription factors associated with cancer, like SIRT 1 deacetylates p53, favoring its degradation [31]. SIRT1 has a primary localization in the nucleus and its deacetylase action targets PCAF/MyoD and MEF2 [32]. SIRT2 is involved in maintaining DNA integrity, forcing damaged cells to undergo apoptosis [31].

C2C12 studies show that SIRT1 and SIRT2 promote myoblast proliferation. SIRT1 increases muscle cell proliferation by regulating cell cycle regulators, such as inhibiting p21WAF/CIP1 or increasing p27Kip1 expression, and activating the Wnt signaling pathway. SIRT2 increases muscle cell proliferation by activating the ERK1/2 pathway [32].

SIRT1 shows decreased expression during C2C12 differentiation. Overexpression of SIRT1 negatively affects myogenesis by downregulating myogenin, MEF2C, MHC, and other differentiation-related genes.

On the other hand, in C2C12 cell line, SIRT3 expression increases significantly during early differentiation, and its depletion inhibits myoblast differentiation, leading to decreased myogenin and MyoD expression and a lack of polynucleated myotubes.

SIRT1 is overexpressed in synovial sarcoma tumors and soft tissue sarcoma cell lines (including the RD cell line), compared to normal mesenchymal cells. It was observed that inhibition of SIRT1 and SIRT2 has an antiproliferative effect on rhabdomyosarcoma cells [31]. In RD cells, silencing of SIRT1 and SIRT2 impairs RD proliferation. The Inhibition of SIRT1 and SIRT2 affects the autophagy process leading to rhabdomyosarcoma cells death. SIRT1 would have a protective role in rapidly growing tumors where the supply of blood, oxygen, and nutrients becomes limited. The expression of SIRT1 and SIRT2 is crucial for the survival of these sarcoma cells [31].

Our next studies will be focused on the role of SIRTs, especially SIRT 1, 2, and 3. We will analyze the expression levels of the genes during different treatments, basically, the effects of staurosporine, bisindolylmaleimide, and neomycin sulfate on SIRT 1, 2, and 3 gene expression and the regulation of myogenesis.

This study provides evidence that cellular differentiation in C2C12 and RD cell lines can be induced by staurosporine, bisindolylmaleimide, and neomycin sulfate, suggesting a key role of PKC and its signaling pathways in cell differentiation. These findings carry important implications for the development of novel therapeutic strategies focusing on cellular differentiation in undifferentiated malignant cells.

Conclusions

In conclusion, this study sheds light on the mechanisms underlying cellular differentiation in C2C12 and RD cell lines; RD being a highly aggressive tumor predominantly found in childhood. The embryonal subtype of RMS (eRMS) exhibits a diverse array of cellular characteristics, including cross-striations and pleomorphism, along with embryonal structures like rosette formation and tubular structures. Cellular differentiation, marked by the acquisition of mature muscle cell characteristics, is pivotal in impeding tumor growth and enhancing sensitivity to treatments. Our findings show that staurosporine, bisindolylmaleimide, and neomycin sulfate induce differentiation, implicating the PKC/PLC signaling pathways. Notably, neomycin sulfate, although not a direct kinase inhibitor, that impacts various signaling cascades and ion channels, shows its potential role in modulating cell differentiation. Understanding these mechanisms holds promise for the development of targeted therapeutic strategies to reverse the malignant phenotype.

Conflicts of Interest

All the authors declare that they have no conflict of interest.

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