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# $N^6$-Methyldeoxyadenosine, a nucleoside commonly found in prokaryotes, induces C2C12 myogenic differentiation

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**Abstract**

$N^6$-Methyl-2′-deoxyadenosine (MedAdo) is a nucleoside naturally found in prokaryotic DNA. Interestingly, the $N^6$-methylation of adenine in DNA seems to have been counter-selected during the course of evolution since MedAdo has not been detected in mammalian DNA until now. We show here that treatment with MedAdo induces myogenesis in C2C12 myoblasts. The presence of MedAdo in C2C12 DNA was investigated using a method based on HPLC coupled to electrospray ionization tandem mass spectrometry which is several thousand fold more sensitive than assays used previously. By this procedure, MedAdo is detected in the DNA from MedAdo-treated cells but remains undetectable in the DNA from control cells. Furthermore, MedAdo regulates the expression of p21, myogenin, mTOR, and MHC. Interestingly, in the pluripotent C2C12 cell line, MedAdo drives the differentiation towards myogenesis only. Thus, the biological effect of MedAdo is suppressed in the presence of BMP-2 which transdifferentiates C2C12 from myogenic into osteogenic lineage cells. Taken together these results point to MedAdo as a novel inducer of myogenesis and further extends the differentiation potentialities of this methylated nucleoside. Furthermore, these data raise the intriguing possibility that the biological effects of MedAdo on cell differentiation may have led to its counter-selection in eukaryotes.


*Keywords:* Cell differentiation; $N^6$-Methyl-2′-deoxyadenosine; Myogenesis; C2C12

$N^6$-Methyl-2′-deoxyadenosine (MedAdo) is a nucleoside naturally found in prokaryotic DNA. This modified nucleoside has historically been associated with the bacterial restriction/modification system that ensures the protection of the bacterial genome from invasion by foreign DNA [1]. After that, the prokaryotic adenine methylation was also implicated in DNA mismatch repair, DNA–protein interactions, control of gene expression, initiation of chromosome replication, and bacterial virulence [2,3]. Interestingly, this DNA methylase activity responsible for the $N^6$-methylation of adenine seems to have been counter-selected during the course of evolution since MedAdo is not detected in mammalian DNA [4,5]. However, the transfection in mammalian cells of eukaryotic enhancer or promoter regions which have been methylated by bacterial methylases on some of their adenine residues demonstrates that in eukaryotes this "unnatural" adenine methylation may either create novel steroid hormone response element or impair the binding of eukaryotic transcription factors [6–8]. This point may be of special concern since all the plasmids currently used in gene therapy are subjected to adenine methylation as a consequence of bacterial DNA adenine methylation (dam) activity of *Escherichia coli* [9,10]. Another interesting role of MedAdo in mammalian cells is the property of this methylated deoxynucleoside to induce cell differentiation. Thus, the neuronal differentiation of PC12 pheochromocytoma and P19 teratocarcinoma cells, and the differentiation of the C6.9 glioma cell line towards an oligodendroglial phenotype have been reported to occur

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following MedAdo treatment [11,12]. We present here novel pieces of evidence demonstrating the potentialities of MedAdo to induce cell differentiation in the myogenic C2C12 cell line. The reported findings suggest that MedAdo drug could be the prototype of a new class of differentiation agent with a broad range of therapeutic potential.

## Materials and methods

*Cell culture and differentiation culture conditions.* The C2C12 mouse skeletal cell line was obtained from the American Type Culture Collection. Cells were passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Myogenesis was induced by treating 50–70% subconfluent cells with 750 μM MedAdo. For induction of osteogenic differentiation, the cell culture medium was supplemented with 300 ng/ml bone morphogenetic protein (BMP-2). Alkaline phosphatase (ALP) staining was performed as described by Reyes et al. [13].

*RT-PCR.* Total RNA extraction from C2C12 cells and RT-PCR were, respectively, performed using the Rneasy and Qiagen OneStep RT-PCR kits (Qiagen, Courtaboeuf, France). Analysis of gene expression was performed in C2C12 cells upon differentiation induction for 48 h in the presence of MedAdo. The oligonucleotide sequences were as follows (product length is indicated in brackets): myogenin (424 bp) (sense) 5′-GCTCAGCTCCCTCAACCAG-3′ and (antisense) 5′-ATGTGAATGGGGAGTGGGA-3′ [14]; HPRT (hypoxanthine-guanine phosphorybosyl transferase) (250) (sense) 5′-GCTTGGTTTT GAAAAGGACCTCT-3′ and (antisense) CACAGGACTAGAAC ACCTGC. Genomic DNA contamination was controlled by PCR of non-reverse transcribed RNA (data not shown).

*DNA analysis.* DNA was extracted as detailed recently [15]. Subsequently, DNA was digested with a mixture of endo- and exonucleases [15] that quantitatively liberate MedAdo. This was demonstrated using plasmid DNA that contained a known amount of MedAdo (data not shown). A HPLC–electrospray ionization tandem mass spectrometry (HPLC–MS/MS) method has been developed for the detection of MedAdo as previously described for the measurement of oxidized nucleosides [16,17]. The HPLC–MS/MS system used for that purpose has been described in detail elsewhere [16]. The octodecylsilyl silica gel Uptisphere ODB column (5 μm, 150 × 2 mm i.d.) used for this purpose was obtained from Interchim (Montluçon, France). Typically the separations were achieved using a linear gradient of acetonitrile in 2 mM ammonium formate, starting from 0% and reaching a 30% level within 30 min. The flow rate was set at 200 μl/min. Under these conditions, MedAdo eluted at 21.6 min, whereas the normal nucleosides including dCyd, dGuo, Thd, and dAdo eluted at 12.1, 15.1, 16.2, and 18.2 min, respectively. The methylated nucleoside exhibits, in the positive ionization mode, a pseudo-molecular ion at $m/z = 266$ uma corresponding to the protonated molecule (MW = 265). Collision-induced dissociation of the above-mentioned ion gives rise to a predominant ion at $m/z = 150$ uma corresponding to the loss of the 2-deoxyribose moiety, as usually observed for 2′-deoxyribonucleosides [18]. Therefore, the transition 266–150 was used to selectively detect MedAdo in the so-called multiple reaction monitoring (MRM) mode. The limit of sensitivity for the detection of MedAdo ($S/N = 3$) was found to be close to 20 fmol injected (corresponding to a concentration of 0.5 nM). Quantification was performed by external calibration and the amount of DNA was determined by UV measurement using the peak of dGuo [16].

*Western blot analysis.* Cells were scraped from dishes, lysed in ice-cold lysis buffer (Tris 50 mM, pH 8.0, NaCl 250 mM, EDTA 5 mM, and NP40 0.5%) that contained a protease inhibitor cocktail (Roche, Meylan, France), and subsequently incubated for 15 min on ice. After vortexing, insoluble cellular components were cleared by centrifugation. Protein concentration was determined using Bio-Rad Protein Assay kit. Equal amounts of lysates (80 μg) were heated for 5 min in Laemmli sample buffer, separated on SDS–PAGE gels, and blotted on Hybond ECL membranes (Amersham Biosciences, Orsay, France). After electrophoretic transfer of proteins from SDS–PAGE gels to nitrocellulose membranes, membranes were saturated with 5% skimmed milk in TBS–Tween 0.1% (Tris 20 mM, pH 7.4, NaCl 137 mM, and Tween 20 0.1%) and incubated at room temperature for 3 h. The membranes were then incubated overnight at 4 °C with primary antibodies against p21 clone F-5 (Santa Cruz; dilution 1/200), MHC (Sigma, dilution 1/1000), mTOR (Cell Signaling, dilution 1/1000). After three washes in TBS–Tween–milk solution, the membranes were incubated with the corresponding secondary antibody coupled to horseradish peroxidase (1/5000) (Amersham Biosciences, Orsay, France) for 30 min. Membranes were then processed using the Amersham ECL kit following the manufacturer's instructions.

## Results

### *MedAdo induces the differentiation of C2C12 cells*

In the presence of 10% foetal calf serum (proliferation medium), C2C12 cells proliferate as myoblasts. To investigate the potentiality for MedAdo to induce differentiation of C2C12 cells cultured in this proliferation medium, cells were grown to 50–70% confluency and then MedAdo was added at different concentrations (within the 1 μM–1.5 mM range). Results presented in Fig. 1 show that MedAdo induces morphologic differentiation of C2C12 cells into myotubes with an optimal effect observed upon addition of 750 μM MedAdo. Under these conditions, abundant myotube formation occurred after 4–5 days (Fig. 1B). The effect observed is specific since cell fusion is not observed following treatment with adenine, adenosine, $N^6$-methyladenosine, and 2′-deoxyadenosine (1 nM–1.5 mM) (data not shown). Myogenic differentiation of C2C12 can also be induced by substituting proliferation medium (DMEM plus 10% foetal calf serum) by a differentiation medium (DMEM plus 2% horse serum). Addition of MedAdo to C2C12 cells cultured in 2% horse serum further enhances the cell fusion observed (Figs. 1C and D).

In PC12 cells, several lines of evidence have been presented, suggesting that the neuronal MedAdo-induced differentiation of these cells is mediated through adenosine A2a receptors and via the cAMP and MAPK signalling pathways [12]. Interestingly, these signalling pathways do not seem to be recruited by MedAdo in C2C12 cells since none of the antagonists or inhibitors that have been shown to be efficient in suppressing the MedAdo-mediated effect on PC12 cells worked on C2C12 cells. Thus, pre-treating C2C12 cells with adenosine A2a antagonist ZM241385 or with SQ22536 or PD98059 which, respectively, inhibit adenylate cyclase and MEK1/2 has no effect on the MedAdo-induced myogenic differentiation of C2C12 cells (data not shown). Conversely, no myogenic differentiation was

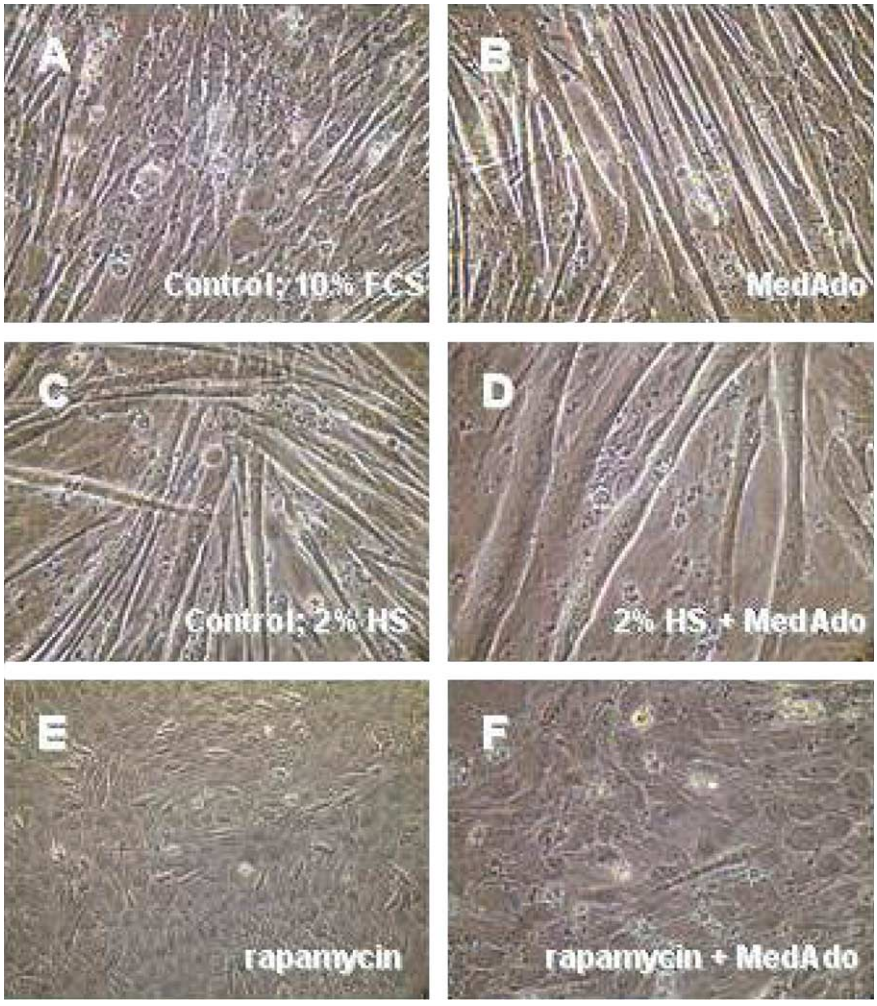


Fig. 1. Effect of MedAdo on the induction of C2C12 myogenesis. C2C12 cells were cultured for 5 days with: (A) 10% foetal calf serum; (B) 10% foetal calf serum + 750 μM MedAdo; (C) 2% horse serum; (D) 2% horse serum + 750 μM MedAdo; (E) 10% foetal calf serum + 20 nM rapamycin; and (F) 10% foetal calf serum + 750 μM MedAdo + 50 nM rapamycin. Phase contrast photographs show the morphologic phenotypes.

observed following either addition of the A2a adenosine receptor agonist DPMA that partially mimics the effect of MedAdo on PC12 cells, or after treatment of C2C12 cells with forskolin, an activator of adenylate cyclase (data not shown). However, the effect of MedAdo on C2C12 cells was suppressed when cells are cultured in the presence of rapamycin, a specific inhibitor of the serine/threonine kinase mammalian target of rapamycin (mTOR) [19] (Fig. 1F).

Bone morphogenetic protein (BMP-2) has been reported to inhibit myogenic differentiation of C2C12 cells and to induce in these cells alkaline phosphatase activity (ALP), a typical marker of osteoblast differentiation [20]. To examine the interaction between muscle and osteoblast differentiation in the presence of MedAdo, C2C12 cells were co-treated with both BMP-2 and MedAdo. Addition of 300 ng/ml BMP-2 totally suppressed the MedAdo effect on cell fusion (compare Fig. 2B vs D), whereas ALP activity was induced in cells treated with BMP independently of the presence of MedAdo (Figs. 2C and D). Interestingly, no ALP positive cells are detected in MedAdo-treated cells in the absence of BMP-2 (Fig. 2B). Hence, in the pluripotent C2C12 cells, MedAdo exerts a specific action and drives the cells towards a myogenic rather than an osteogenic differentiation programme. In addition, BMP-2 antagonizes the myogenic differentiation programme triggered by MedAdo (Fig. 2).

### *MedAdo is incorporated in the DNA of MedAdo-treated cells, but is not detected in control DNA*

MedAdo is a nucleoside naturally found in prokaryotic DNA [2]. To investigate the presence of MedAdo in the DNA of C2C12 cells, we have developed an assay based on the sensitive and specific detection provided by tandem mass spectrometry coupled to HPLC. Using the latter analytical approach, the detection limit for MedAdo is less than 1 MedAdo per million normal nucleosides. Interestingly this is one thousand times more

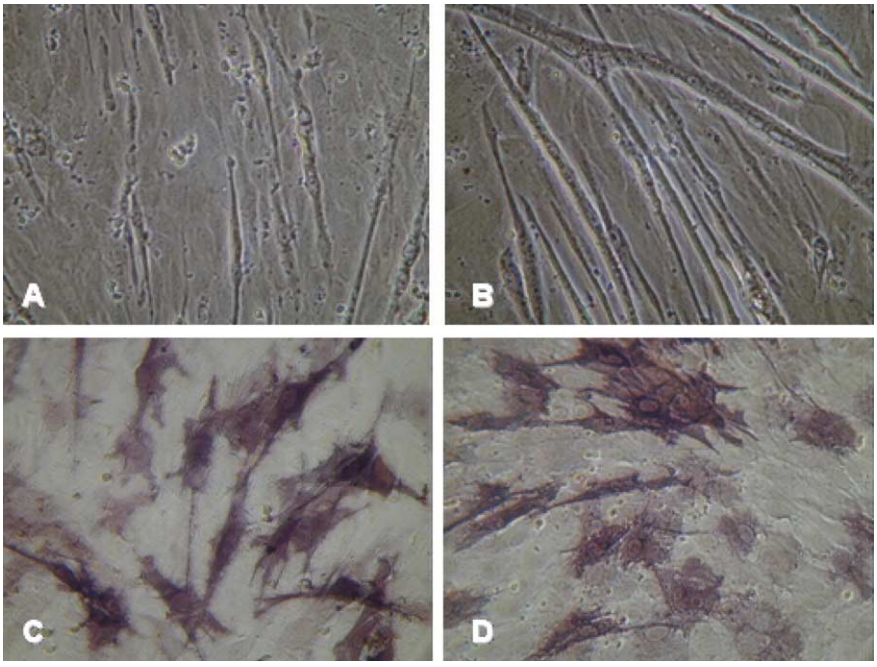


Fig. 2. Effect of BMP-2 on the MedAdo-induced myogenesis of C2C12 cells. C2C12 cells cultured for 5 days with (C,D) or without (A,B) 300 ng/ml BMP-2 in DMEM containing 10% foetal calf serum in the presence (B,D) or absence (A,C) of 750 μM MedAdo. After fixation with methanol, cells were stained for ALP activity as described in Materials and methods. Phase contrast photographs are shown.

sensitive than previous assays that failed to measure detectable amounts of the methylated adenine base in eukaryotic DNA [5,21]. Since mycoplasmal DNA is known to contain MedAdo [22] care was taken, using a sensitive PCR-based detection assay [23], to check for the absence of mycoplasmal contamination. In the absence of MedAdo in the culture medium, the presence of the latter modified nucleoside is not detected in the DNA extracted from either exponentially growing cells or confluent non-fusioning (10% FCS, proliferation medium) or differentiated cells (2% HS differentiation medium). Taking into account the limit of sensitivity of the HPLC–MS/MS assay, it may be suggested that the level of MedAdo in untreated cells is lower (if any) than 0.1 per million nucleosides. The sensitivity of our assay makes possible the detection of MedAdo in the DNA of MedAdo-treated cells (Fig. 3). Following incubation of C2C12 cells with 750 μM MedAdo during 4 days, the level of MedAdo incorporated in genomic DNA is about 13 MedAdo per million nucleosides (Fig. 3). Since a relatively high concentration of MedAdo was used for cell treatment a control experiment was conducted to demonstrate that the detected methylated nucleoside was not due to a contamination of DNA with unincorporated MedAdo. For this purpose, the extracted DNA, dissolved in water, was analysed by HPLC–MC/MS without enzymatic digestion (DNA was removed by filtration). In these experiments no MedAdo was detected indicating that measured MedAdo was indeed due to incorporation into cellular DNA.

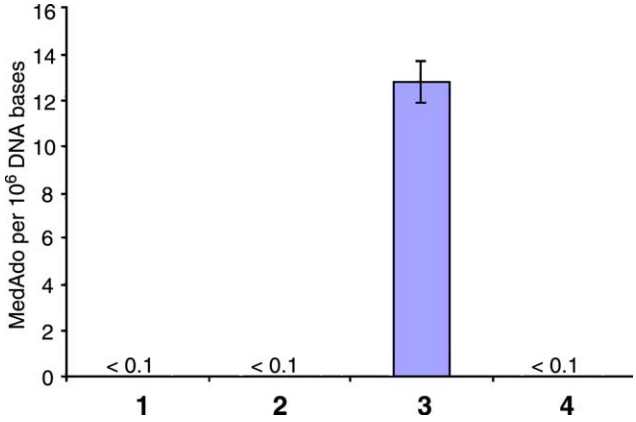


Fig. 3. Relative amounts of MedAdo present in the DNA of C2C12 cells. DNA was extracted from: (1) C2C12 exponentially growing cells in proliferation medium (10% foetal calf serum); (2) confluent cells in proliferation medium (10% foetal calf serum) (see Fig. 1A); (3) MedAdo-treated cells in proliferation medium (see Fig. 1B); and (4) confluent fusioning cells in differentiation medium (2% horse serum) (see Fig. 1C). Quantification was performed as described in Materials and methods. Results represent the average and standard deviation of three independent determinations.

### *MedAdo increases myogenin, p21$^{waf}$, myosin heavy chain protein, and mTOR expressions in C2C12 cells*

The myogenic differentiation follows a series of well-described events including, for example, the expression of myogenin, p21$^{waf1}$, and myosin heavy chain (MHC) [24]. Therefore, the expression of myogenin mRNA

during MedAdo-induced myogenesis was analysed by semi-quantitative RT-PCR. Results presented in Fig. 4A show that myogenin mRNA levels increase following MedAdo addition. Concomitantly, Western blot analyses of protein lysates from control and MedAdo-treated cells revealed an enhanced expression of p21 protein already detected after 48 h of treatment with MedAdo that precedes a dramatic increase in MHC expression (Fig. 4B). In addition, an increase in the synthesis of mTOR, a protein essential for skeletal muscle differentiation [25,26], which has been identified as a target of the inhibitory action of rapamycin on C2C12 myogenesis, is detected at day 2 and 3 (Fig. 4B). Localization of MHC expression by immunofluorescence labelling confirms the presence of MHC in myotubes (Fig. 4C).

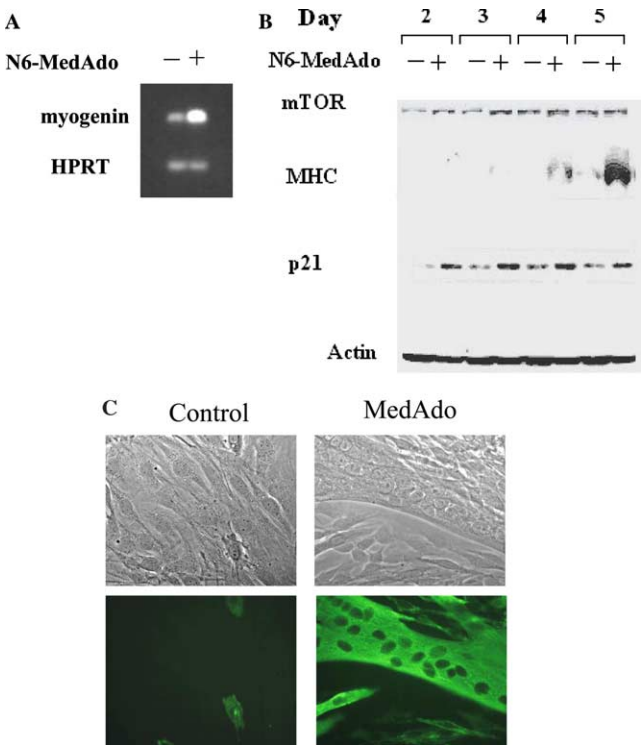


Fig. 4. Effect of MedAdo on myogenin expression and on the synthesis of mTOR, MHC, and p21 proteins. (A) For RT-PCR analysis total RNA was extracted from cells cultured in DMEM supplemented with 10% foetal calf serum for 2 days in the presence (+) or absence (−) of 750 μM MedAdo. Products were amplified (22 cycles) from cDNA as described under Materials and methods and revealed by ethidium bromide staining. (B) Time course analyses of mTOR, MHC, and p21 synthesis during MedAdo treatment. C2C12 cells cultured in DMEM supplemented with 10% foetal calf serum were stimulated for differentiation by addition of 750 μM MedAdo as indicated. (C) Induction and localization of MHC synthesis in C2C12 myotubes. Immunostaining of C2C12 cells with the muscle differentiation marker MHC, demonstrating its localization in myotubes in cells treated with 750 μM MedAdo for 4 days. Immunofluorescence staining and phase contrast photomicroscopy of the same fields are shown.

## Discussion

It is currently assumed that MedAdo is a nucleoside exclusively found in prokaryotic DNA where it is involved in several important biological functions. These include DNA mismatch repair, DNA–protein interactions, control of gene expression, initiation of chromosome replication, and bacterial virulence [1–3]. However, it must be pointed out that the assays previously used with mammalian DNA, which have failed to detect the presence of this methylated base, have focused on the search of the relatively abundant 5-methylcytosine. The HPLC–MS/MS assay developed here for the detection of MedAdo in DNA is about one thousand times more sensitive than previously available methods [21]. Using this essay MedAdo incorporation was demonstrated in the DNA of MedAdo-treated cells. On the other hand, the lack of detectable amounts of MedAdo in non-treated C2C12 cells strongly suggests that MedAdo is at the best extremely rare if present. However, an overall extent of adenine methylation lower than 0.00001% can be biologically relevant if it occurs in a regulated fashion on specific gene regulatory elements such as GA boxes (GGGAGGG) that are known to be involved in myoblast differentiation [27], or TATA or CAAT boxes. This emphasizes the importance to continue the analysis of DNA with more sensitive methods to establish the presence or absence of methylated adenine in mammalian DNA. In this regard it is noteworthy that the "unnatural" adenine methylation of eukaryotic promoters by bacterial $N^6$-adenine methyltransferases can either create functional artificial hormone responsive elements or inactivate some promoters [6–8]. A prerequisite to the "natural" methylation of adenine in mammalian DNA is the existence of an adenine-$N^6$-DNA methyltransferase gene in mammalian genomes. In contrast, to cytosine DNA methyltransferases, which belong to a family of conserved enzymes, bacterial adenine-$N^6$ methyltransferases are much more heterogeneous. Besides a weakly conserved F_G_G amino acid motif shared by all MTases, the latter enzymes only contain one moderately conserved (D/N)PP(Y/F) motif [28]. Through search in the Swiss-Prot database on the basis of the NPPY motif, we identified a human sequence already described as a putative $N^6$-DNA-methyltransferase (Protein PRED28) whose gene is located on chromosome 21 [29]. The demonstration of the functionality of the corresponding enzyme as a true $N^6$-DNA-methyltransferase could provide clues for a biological role of MedAdo in controlling processes such as development, cell differentiation, senescence, and/or transformation. However, although it is tempting to speculate that incorporation of MedAdo in DNA may be involved in the differentiation observed, other modes of action of MedAdo can be envisaged such as, for instance, interferences in the metabolic cycle of AdoMet and AdoHcy [30].

Our results demonstrate that MedAdo induces the muscle differentiation of C2C12 cells maintained in high serum concentration (proliferation medium). In addition, this drug enhances myogenesis in C2C12 cells shifted in low serum concentration (differentiation medium). MedAdo has been previously reported to induce neuronal differentiation of P19 teratocarcinoma and PC12 pheochromocytoma cells as well as oligodendroglial differentiation of C6.9 glioma cell line [11]. In PC12 cells, evidence has been provided, suggesting that MedAdo-induced differentiation is mediated through adenosine A2a receptors and via the cAMP and MAPK signalling pathways [12]. Interestingly, these signalling pathways do not seem to be recruited by MedAdo in C2C12 cells since none of either antagonists or inhibitors efficient to suppress the MedAdo effect on PC12 cells are operative on C2C12 cells. This finding suggests that MedAdo may mediate its effect via several cell-specific signalling pathways and as a result, the precise mode of action of MedAdo should be defined for each cell line. In this regard it is noteworthy that rapamycin which specifically targets the mTOR signalling pathway [19] abolishes the MedAdo effect on C2C12 cells. In addition, our finding that MedAdo increases the expression of myogenin and the synthesis of mTOR, p21, and MHC is in agreement with the proposed model for myogenic differentiation of C2C12 cells [24]. According to this, it is noteworthy that the forced expression of p21 augments the expression of muscle-specific gene in confluent cultures of mitogen-stimulated myoblasts [31]. This is correlated with the establishment of the post-mitotic state in differentiating myoblasts [24].

Another important point is the selectivity of MedAdo action. C2C12 are pluripotent cells that can either differentiate in myotubes or either acquire an osteoblast phenotype in the presence of BMP-2. Thus, it is noteworthy that MedAdo drives the C2C12 cells exclusively towards myogenic differentiation. On the other hand, BMP-2 converts the differentiation pathway of C2C12 myoblasts into osteoblast lineage independently of the presence of MedAdo. The latter observation underlines the specificity of action of MedAdo which is limited, in the pluripotent C2C12 cell line, to the induction of the myogenic programme. The existence of this cross-talk between MedAdo and BMP-2 could be related to a cell cycle-mediated process, since in C2C12 cells, cell cycle withdrawal precedes cell fusion upon myogenesis.

In conclusion this study provides additional evidence on the potency of MedAdo to induce cell differentiation. Thus, in addition to the induction of a neuronal phenotype in PC12 and P19 and to the mediation of oligodendroglial differentiation in the C6 glioma cell line, this methylated nucleoside is able to promote myogenesis in C2C12 cells. In addition, these results point to the specificity of action of the drug which in the pluripotential C2C12 cell line drives the cells towards myogenesis. Another important point is our failure to detect MedAdo as a natural constituent of C2C12 DNA. Hence, the presence or absence of this base in mammalian DNA warrants further studies using more sensitive assays and should be extended to different normal or pathological tissues. In this regard, it is noteworthy that the methylation status of the DNA from *Drosophila melanogaster* has been controversial for several decades until the recent demonstration that this species also contains 5-methylcytosine in its DNA [32,33].

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