RESEARCH ARTICLE

Anti-proliferative effect of curcumin on melanoma cells is mediated by PDE1A inhibition that regulates the epigenetic integrator UHRF1

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Scope: Curcumin inhibits proliferation of many cancer cells. Cyclic nucleotide phosphodiesterases (PDEs), by hydrolyzing intracellular cyclic adenosine-3′,5′-monophosphate (cAMP) and/or cyclic guanosine-3′,5′-monophosphate (cGMP), play a pivotal role in signalling pathways involved in cell proliferation. Therefore, this study investigated PDE1–5 participations in the anti-proliferative properties of curcumin in B16F10 murine melanoma cells.

Methods and results: We report that curcumin inhibits PDE1–5 activities ($IC_{50} \cong 10^{-5}$ M), indicating that curcumin acts as a non-selective PDE inhibitor. In melanoma cells, PDE4 and PDE1 represent the major cAMP-PDEs and cGMP-PDEs activities, respectively. Curcumin treatment decreased PDE1 and PDE4 activities and dose dependently increased intracellular cGMP levels, whereas cAMP levels were unchanged. Curcumin inhibited cell proliferation and cell cycle progression by accumulating cells in the S- and G2/M-phases with enhanced expressions of cyclin-dependent kinase inhibitors. In contrast, expressions of PDE1A, cyclin A and the epigenetic integrator ubiquitin-like containing PHD and Ring Finger domains 1 (UHRF1) and DNA methyltransferase 1 (DNMT1) were decreased by curcumin. Interestingly, PDE1A overexpression increased UHRF1 and DNMT1 expressions and rescued the B16F10 cells from curcumin anti-proliferative effects. Nimodipine, a PDE1 inhibitor, mimicked the curcumin effects.

Conclusion: Curcumin exerts its anti-cancer property by targeting PDE1 that inhibits melanoma cell proliferation via UHRF1, DNMT1, cyclin A, p21 and p27 regulations. This suggests that natural PDE1 inhibitors present in food might be effective in preventing cancer.

Keywords:
cAMP/cGMP / Cancer / DNMT1 / Nimodipine / Rolipram

1 Introduction

Curcumin is a polyphenolic compound extracted from turmeric (*Curcuma longa*), commonly used in curry powder and has been used for centuries in indigenous medicine. This natural compound has been reported to possess anti-inflammatory, anti-oxidant and anti-cancer activities. Their anti-cancer properties have been shown in vitro and in vivo models; yet, the precise mechanism of action is still unknown but probably involves the induction of differential expression of cell cycle regulators [1–4]. Cell cycle progression is governed by cyclin-dependent kinases (CDKs) that are activated by cyclin binding and inhibited by CDK inhibitors [5]. CDKs regulate checkpoints that integrate mitogenic and growth inhibitory signals, coordinating cell cycle transitions. In various human tumor cell lines, anti-cancer properties of curcumin are mediated by delaying the cell cycle progression via expressions of CDK inhibitors, $p16^{INK4a}$, $p21^{WAF1/CIP1}$ and $p27^{/KIP1}$ via inhibition of cyclin E and cyclin D1 expressions [6–9].

Interestingly, the expression of UHRF1 (Ubiquitin-like containing PHD and ring finger domains 1) is regulated by

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Abbreviations: cAMP, cyclic adenosine-3′,5′-monophosphate; **CaM,** calmodulin; **CDK,** cyclin-dependent kinase; **cGMP,** cyclic guanosine-3′,5′-monophosphate; **DMPPO,** 1,3-dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl)-pyrazolo[3,4-*d*]pyrimidin-4-(5*H*)-one; **DNMT1,** DNA methyltransferase 1; **PDE,** cyclic nucleotide phosphodiesterase; **RT-PCR,** real-time PCR; **UHRF1,** ubiquitin-like containing PHD and ring finger domains 1

$p21^{CIP/WAF}$ and p53, and controls the expression of $p16^{INK4A}$ [10, 11]. UHRF1, initially named and discovered as ICBP90 [12], is a protein with a central function in epigenetic modulation by transmitting, with the help of DNA methyltransferase 1 (DNMT1), the methylation status from the mother cells to daughter cells [13–15]. Previous reports from our laboratory and others showed that UHRF1 is essential for cellular proliferation [12, 16]. These findings could explain why UHRF1 is up-regulated in cancer [17, 18].

Cyclic adenosine-3′,5′-monophosphate (cAMP) and cyclic guanosine-3′,5′-monophosphate (cGMP) are important second messengers that mediate various cellular responses to external stimuli. cAMP and cGMP are synthesized by adenylyl and guanylyl cyclases and specifically hydrolyzed by cyclic nucleotide phosphodiesterases (PDEs). The family of PDEs consists of 11 isozyme families (PDE1–PDE11) that are expressed in different mammalian cell types [19]. Their specific contributions to tissue function in physiological or physiopathological conditions designate them as new therapeutic targets [20, 21]. Notably, our team showed that some PDE isozymes are up-regulated in angiogenesis and that their specific inhibition overcomes this pathophysiological process by acting on p21 and p27 [22, 23]. Various reports have indicated overexpression of individual PDE isozymes in various tumour tissues suggesting that inhibitors specific for individual PDE isozymes may selectively restore normal intracellular signalling, providing antitumour therapy with reduced adverse effects [24]. Interestingly, we recently showed in Jurkat cells, a model of acute lymphoblastic leukemia, that thymoquinone, from *Nigella sativa*, induces apoptosis by decreasing PDE1A and UHRF1 expressions [25, 26].

In the present study, we investigated the potential role of curcumin toward PDE subtypes in the anti-proliferative properties of curcumin in melanoma B16F10 cell line. Our study shows, for the first time, that anti-proliferative effect of curcumin on B16F10 melanoma cells is specifically mediated by PDE1A inhibition that down-regulates UHRF1 and DNMT1. This suggests that natural PDE1 inhibitors present in food might be effective in preventing cancer and might participate to epigenetic regulation of various genes.

2 Methods and materials

2.1 PDE activity assay

PDE activity and PDE isozyme contribution were determined by a radioenzymatic assay as described previously [27]. Total cAMP-PDE and total cGMP-PDE activities were assessed at 1 μM substrate concentration. The contribution of PDE isozymes was determined by using selective inhibitors as described before [27]: 10 μM nimodipine for PDE1, 20 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) for PDE2, 1 μM cilostamide for PDE3, 10 μM rolipram for PDE4, 0.1 μM 1,3-dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl)-pyrazolo[3,4-*d*]pyrimidin-4-(5*H*)-one (DMPPO) for PDE5. cAMP-PDE and cGMP-PDE activities insensitive to 3-isobutyl-1-methylxanthine (IBMX, 100 μM) were considered as PDE8 and PDE9 activities, respectively. The residual activity was, therefore, considered as other PDE activities. Specific activities were expressed as pmol min^{-1} mg^{-1} protein.

2.2 Assessment of curcumin effect on purified PDEs

PDE1, PDE3, PDE4 and PDE5 were isolated by anion-exchange chromatography from bovine aortic smooth muscle cytosolic fraction as previously described [28]. PDE2 was isolated from human platelets [29]. Purified PDEs were stored as aliquots at −80°C until use. The concentration of curcumin that produced 50% inhibition of substrate hydrolysis (IC_{50}) was calculated through non-linear regression analysis (GraphPad Prism, San Diego, CA, USA) of concentration–response curves performed with 1–300 μM of curcumin and included six different concentrations of curcumin.

2.3 Cell culture and treatment

The mouse B16F10 melanoma cell line, strain C57BL/6J was obtained from ATCC (American type culture collection) Manassas, VA, USA. Curcumin, rolipram and nimodipine were prepared as a 100 mM solution in 100% DMSO. Appropriate working concentrations were prepared with the cell culture medium. The final concentration of DMSO was 0.1% in both control and in treated cells.

2.4 Cell viability and cell cycle analysis

Cell viability rate was determined by cell counting using the Trypan blue exclusion method (Invitrogen). The viability rate was obtained by dividing the number of Trypan blue-negative cells by the total number of cells. For cell cycle distribution, cells were seeded in 6-well plates at a density of 3×10^5 cells and grown for 24 h, then exposed to tested substances for a further 24-h period. Cells were then harvested by centrifugation ($200 \times g$ for 5 min at room temperature), washed with phosphate-buffered saline (PBS) solution and resuspended in 70% ethanol. After 1 h incubation at 4°C, cells were centrifuged at 200*g* for 10 min at 4°C and washed once with PBS buffer. Cells were then resuspended in 500 μL PBS buffer containing 50 μg/mL propidium iodide and 50 μg/mL RNase solutions and were kept in dark for 30 min at 37°C. Cellular DNA content was assessed by capillary cytometry (Guava EasyCyte Plus HP system).

2.5 Intracellular cAMP and cGMP assays

Cells were plated at 5×10^5 cells/60-mm dish and allowed to adhere for 24 h. Then, the cells were incubated with medium containing indicated concentration of curcumin for 24 h. Cells were then harvested, washed with PBS and intracellular cAMP and cGMP levels were determined by a competitive enzyme immunoassay system with a kit from Assay Designs, following manufacturer's recommendations. Cyclic nucleotide levels were expressed in pmol mg protein^{-1}.

2.6 Cell extracts

Cells were plated at 5×10^5 cells/60-mm dish and allowed to adhere for 24 h. Then treated for 24 h. Cells were centrifuged at 200*g* for 5 min at RT. Cell pellets were then washed thrice with PBS and resuspended in the homogenizing buffer (20 mM Tris, 5 mM EGTA, 150 mM NaCl, 20 mM Na glycerophosphate, 1 μM H-89, 10 mM NaF, 1 mM $NaVO_3$, 1% Triton X-100, 0.1% Tween 20, 166 μM Pefabloc, 133 μM aprotinin, 8.3 μM bestatin, 2.5 μM E64, 3.3 μM leupeptin, 1.6 μM pepstatin-A) and were sonicated thrice for 15 s. The cell homogenates were centrifuged at 14 000*g* for 10 min at 4°C and the supernatants were stored as aliquots at −80°C until used. Protein concentration was determined by the Lowry method [30]. These cell extracts were used for PDE activity assay and Western blot.

2.7 Western blot analysis

Proteins samples (30 μg) were denatured and solubilized for 5 min at 95°C in Laemmli buffer, electrophoresed on SDS-polyacrylamide gels (8 or 12% acrylamide) and electro-transferred onto polyvinylidene fluoride membranes, as previously described [31]. Membranes were immunoblotted with antibodies against: PDE1A (1:2500), PDE1B (1:2500), PDE1C (1:2500); p21 (1:2000); p27 (1:2000); Cyclin E (1:2000); Cyclin A (1:2000); UHRF1 (0.04 μg/mL); DNMT1 (2 μg/mL); β-actin (1:60 000). Immobilized antigens were detected by chemiluminescence using horse radish peroxidase-conjugates as secondary antibodies (Promega; 1:60 000), an ECL kit (GE Healthcare) and autoradiography films. Autoradiography signals were captured on a Gene-Genius Bio Imaging System (Syngene) using the GeneSnap software and analyzed using the GeneTool software.

2.8 Assessment of PDE mRNA expression by quantitative RT-PCR

Harvested cells were homogenised with a glass–glass Potter 3 × 30 s at 1500 rpm in Qiazol lysis buffer (RNeasy® kit). Total RNA was extracted with RNeasy® kit following manufacturer's instructions, including a RNase-free DNase treatment to prevent co-amplification of genomic DNA. The integrity of purified RNA was verified by 4% agarose gel electrophoresis, and its quantity assessed by spectrophotometry at 260 nm (Genequant Pharmacia). About 1 μg of total RNA was retro-transcribed in cDNA using iScript cDNA Synthesis Kit (Bio-Rad). β-actin, as a housekeeping gene, was used for normalization. Specific primers were designed using consensus sequences of all isoforms of each type of PDE family. Consensus sequences were retrieved from NCBI nucleotide database and were obtained in multiple alignments by using Mafft version 6 http://align.bmr.kyushu-u.ac.jp/mafft/online/server/. Specific primers for PDEs and β-actin were designed with Primer3 Input (version 0.4.0) and their sequences are presented in Table 1. The transcript levels of PDE1 isozymes and β-actin were quantified by real-time PCR performed in the MyiQ® single-color detector system using iQ SYBR® Green Supermix containing the hot-start i*Taq* DNA polymerase. The amplification conditions were: 95°C for 5 min, 40 cycle at 94°C for 10 s, 60°C for 10 s and 72°C for 15 s. Specificity of amplification products was assessed by melting curve analysis and their sizes, comprised between 150 and 200 bp, were checked by 4% agarose gel electrophoresis. Samples were processed in triplicate according to manufacturer's guidelines. Relative gene expression was calculated using the comparative threshold (Ct) method [32]. The results were calculated as $2^{-\Delta\Delta Ct}$, ΔCt being normalized with β-actin.

2.9 Transient transfection

Cells were seeded in 24-well plates at a density of 5×10^3 per well. Transfections were performed using lipofectamine (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Cells were transfected using the Qiagen Expression Kit/mammalia (Qiagen 39003) with either 1 μg of HS_PDE1A_IM_1 plasmid or 1 μg of HS_CDC2_IM_1 plasmid (control vector) as control (Qiagen, Courtaboeuf, France). Untransfected cells were also used as control. Cells were allowed to grow for 24 h and were then treated for 24 h with curcumin (10 μM) or vehicle (buffer with 0.1% DMSO).

2.10 Statistical analysis

Data are expressed as mean ± SEM from three independent experiments and analyzed with the one-way ANOVA test

Table 1. Primer sequences used for real-time PCR experiments

PDE1A forward	5′ CAC TGG CTC ACT GAA CTG GA 3′
PDE1A reverse	5′ GAC GTG GTG ATT CTC AAG CA 3′
PDE1B forward	5′ TCA AGA ACC TGG ACC TCT GG 3′
PDE1B reverse	5′ GGC CTC CAG AAA ACT CAT CA 3′
PDE1C forward	5′ GGT TCA AGA GCA TCG TCC AT 3′
PDE1C reverse	5′ TGA TCT CCA CTG GCC TCA TT 3′

with the Tukeys post-test. Significance was defined as $P \leq 0.05$.

2.11 Drugs and chemicals

Curcumin was generously given by Pr. Annelise Lobstein, (CNRS UMR 7200, Université de Strasbourg, France). Rolipram and cilostamide were, respectively, synthesized by Dr. J. J. Bourguignon [33] and Dr. C. Lugnier [34]. DMPPO (a PDE5 inhibitor, [35]) was generously given by Dr. P. Grondin (Laboratoires GlaxoSmithKline, Les Ulis, France). Nimodipine was a generous gift from Bayer (Berlin, Germany). EHNA and IBMX were from Sigma-Aldrich. Propidium iodide and RNase solution were from Sigma-Aldrich (St. Louis, MO, USA). Cocktail protease inhibitor cocktail (set III 539134) was from Calbiochem.

Antibodies sources were as follows: PDE1A, PDE1B and PDE1C antibodies were from FabGennix, Frisco, TX, USA; cyclin E and cyclin A antibodies manufactured by Neomarkers were from Thermo Fisher scientific (Illkirch, France); p21 and p27 antibodies were from Santa Cruz Biotechnology; UHRF1 antibody was obtained as described elsewhere [12]; DNMT1 antibody (Stressgen Biotechnologies, Victoria, BC, Canada); β-actin and β-tubulin antibodies were purchased from Sigma-Aldrich and GAPDH antibody from Chemicon.

3 Results

3.1 Effect of curcumin on purified PDE1–5

To investigate the potential contribution of PDEs in the mechanism of action of curcumin effect, this natural compound was tested on purified PDE1–PDE5 activities. These isozymes are characterized by their substrate specificity (cAMP and/or cGMP) and sensitivity to intracellular mediators (cGMP, calmodulin (CaM)). PDE1 hydrolyses mainly cGMP and is activated by Ca^{2+}/CaM complex. PDE2 hydrolyses both cAMP and cGMP, and its cAMP hydrolyzing activity is stimulated by cGMP, whereas PDE3 mainly hydrolyses cAMP and this hydrolysis is competitively inhibited by cGMP. PDE4 specifically hydrolyses cAMP, whereas PDE5 specifically hydrolyses cGMP [21]. Table 2 shows that PDEs were inhibited by curcumin with an IC_{50} of 18 ± 1, 12 ± 2, 16 ± 2, 22 ± 2 and 35 ± 2 μM for CaM-activated PDE1, cGMP-activated PDE2, PDE3, PDE4 and PDE5, respectively, indicating that curcumin acts as a non-selective PDE inhibitor in the micromolar range. Furthermore, it should be noticed that basal PDE1 activity was threefold less sensitive to curcumin indicating that curcumin might interact in a CaM-dependent way.

3.2 Pharmacological characterization of PDE isozyme contributions in B16F10 cells

In B16F10 cells, total cAMP-PDE activity (40.7 ± 0.6 pmol $min^{-1} mg^{-1}$; Fig. 1A) was about four times higher than total cGMP-PDE activity (8.7 ± 0.12 pmol $min^{-1} mg^{-1}$; Fig. 1B). The cAMP-PDE activity was mostly due to PDE4 (64%) and PDE8 (19%) and to a lesser extend to PDE3 (9%) and ''others'' (8%) that represented essentially PDE1 and PDE2 (Fig. 1A). The cGMP-PDE activity was mainly due to PDE1 (35%), PDE9 (23%) and PDE5 (13%), while PDE2 contributed for only 2%. Others (28%) might represent PDE10 and PDE11 (Fig. 1B). This result shows that PDE4 and PDE1 are the major cAMP- and cGMP-PDEs activities in B16F10 cells, respectively.

3.3 Effect of curcumin and rolipram treatment on PDE activities of B16F10 cells

Curcumin (10 μM) treatment of B16F10 cells for 24 h significantly decreased by 45% ($P < 0.01$) total cAMP-PDE activity, whereas rolipram (10 μM, a specific inhibitor of PDE4) increased cAMP-PDE activity by 56% ($P < 0.001$) (Fig. 2C). These changes are due to their respective effects on PDE4 activity (Fig. 2E): a 35% decrease ($P < 0.05$) induced by curcumin treatment and an 85% increase induced by rolipram treatment ($P < 0.01$). In the presence of rolipram, curcumin did not overcome increases either in total cAMP-PDE or PDE4 activities (Fig. 2C and E).

Curcumin treatment decreased significantly the total cGMP-PDE activity by 60% ($P < 0.001$) (Fig. 2D) and this effect was due to a drastic decrease in PDE1 activity (82% ($P < 0.01$, Fig. 2F). Predictably, rolipram treatments did not prevent the inhibitory effect of curcumin on the total cGMP-PDE and cGMP-PDE1 activities (Fig. 2D and F).

Table 2. Effects (IC_{50} values, μM) of curcumin on purifed PDEs (PDE1–PDE5)

Isoform	PDE1 basal	PDE1 activated	PDE2 basal	PDE2 activated	PDE3	PDE4	PDE5
Modulator	EGTA	Ca/CaM	–	cGMP			
Substrate	cGMP	cGMP	cAMP	cAMP	cAMP	cAMP	cGMP
Curcumin	63 ± 3	18 ± 1	15 ± 4	12 ± 2	16 ± 2	22 ± 2	35 ± 2

Results are the means ± SEM of three independent experiments.

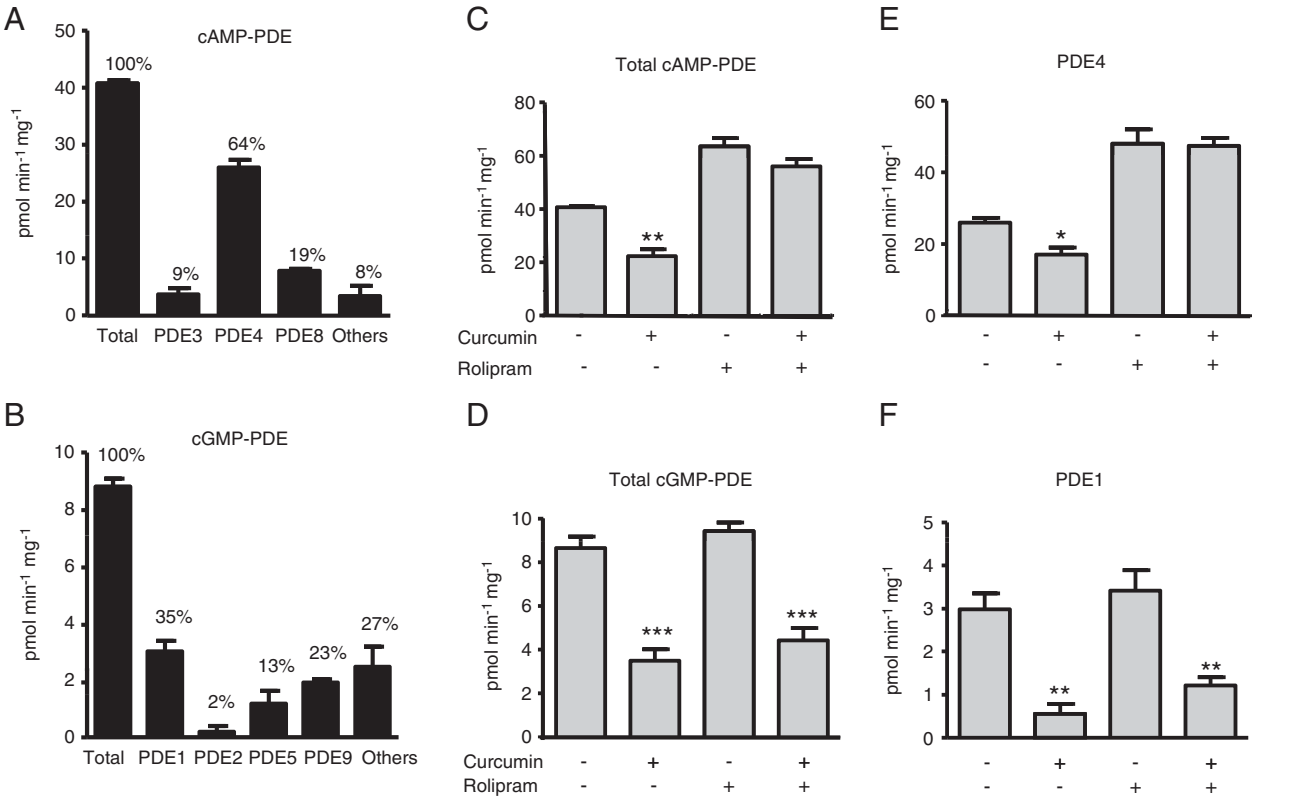

Figure 1. Characterization of PDE isozymes and effect of curcumin and rolipram treatments on PDE isozyme activities in B16F10 melanoma cells. Cell extracts were prepared and their PDE activity profiles were determined as described in Section 2 at 1 μM cAMP (A) and 1 μM cGMP (B). B16F10 cells in culture were incubated for 24 h with vehicle, curcumin (10 μM), rolipram (10 μM) or curcumin (10 μM) and rolipram (10 μM) together. Cell extracts were prepared and their PDE activities were determined at 1 μM cAMP (C and E) and 1 μM cGMP (D and F). Data are expressed in pmol min^{-1} mg^{-1} protein as mean ± SEM of three independent experiments. In comparison with control: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Interestingly, rolipram was not able to decrease PDE1 activity, but overcame partially the inhibitory effect of curcumin on PDE1 activity (23%, $P<0.05$).

3.4 Curcumin effects on B16F10 cell proliferation and cell cycle progression

Since PDE4, specifically hydrolyses cAMP and since total cAMP-PDE in B16F10 cells is mainly due to PDE4, the effect of rolipram was assessed in comparison with curcumin on cell proliferation and cell cycle for 24 h. Rolipram (10 μM) did not affect B16F10 cell proliferation (Fig. 2A) and cell cycle progression (Fig. 2B). However, curcumin (10 μM) significantly inhibited B16F10 cell proliferation ($P<0.001$) (Fig. 2A) and induced a decrease in cell in G1-phase ($P<0.001$) associated with cell accumulation at S- ($P<0.001$) G2/M-phases ($P<0.001$) (Fig. 2B). Curcumin caused cell accumulation in the S- and G2/M-phases of the cell cycle by inducing an increase in the expression of CDK inhibitors $p21^{WAF1/CIP1}$ (904%, $P<0.001$) and $p27^{KIP1}$ (286%, $P<0.001$) (Fig. 3A and B). Conversely, cyclin A (82%, $P<0.001$) and UHRF1 (97%, $P<0.001$) expressions were inhibited, whereas the cyclin E expression was unaffected by curcumin treatment (Fig. 3A and B).

3.5 Effects of curcumin treatment on intracellular cyclic nucleotide levels in B16F10

Surprisingly, intracellular cAMP level in B16F10 cells was unchanged with curcumin treatment (10 and 20 μM) for 24 h (Fig. 4A), although it significantly decreased total cAMP-PDE and PDE4 activity (see Fig. 1C and D). However, curcumin increased significantly and dose dependently cGMP level (10 μM, 183%, $P<0.01$; 20 μM, 298%, $P=<0.001$) (Fig. 4B) in agreement with the decreased total cGMP-PDE activity mainly related to cGMP-PDE1 (see Fig. 1E and F).

3.6 Effects of nimodipine on B16F10 cell proliferation and cell cycle progression

Since, curcumin inhibited PDE1 activity and increased the intracellular cGMP level, we have questioned whether

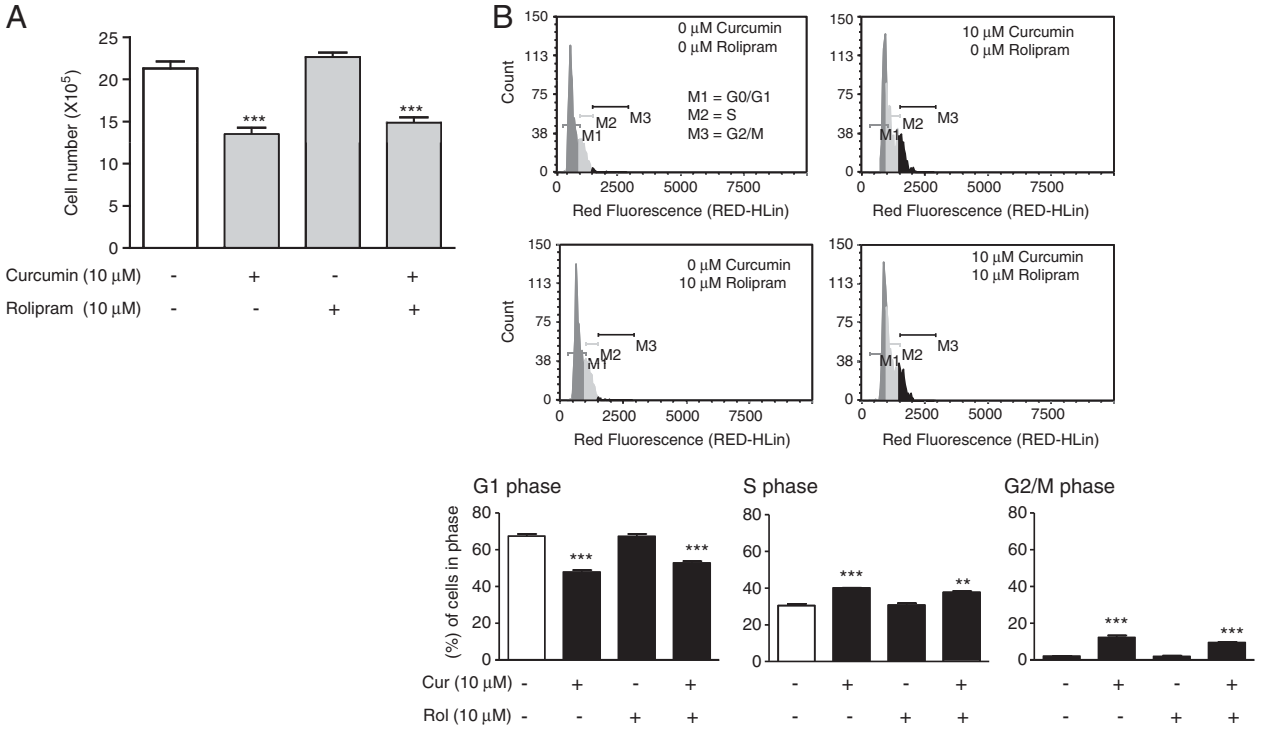

Figure 2. Effect of curcumin and rolipram treatments on B16F10 melanoma cell proliferation and cell cycle progression. Cells were treated for 24 h with vehicle, curcumin (10 μM), rolipram (10 μM) or curcumin and rolipram together. Cell proliferation (A) and cell cycle distribution (B) were assessed as described in Section 2. Cell number is expressed as mean ± SEM ($n = 3$). For each treatment, the results are expressed in % of cells in each phase and are the mean ± SEM of three independent experiments. In comparison with control: $^{**}p < 0.01$; $^{***}p < 0.001$.

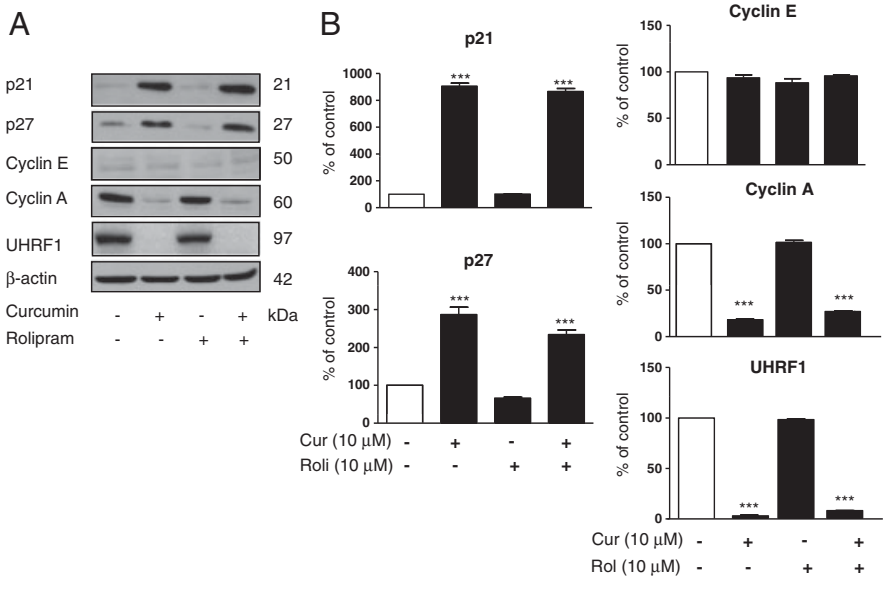

Figure 3. Effect of curcumin and rolipram on the expression of critical proteins involved in B16F10 melanoma cell cycle progression. Cells were treated for 24 h with vehicle, curcumin (10 μM), rolipram (10 μM) or curcumin and rolipram together. Protein expression was assessed by Western blot as described in Section 2. (A) The traces shown are representative of three independent experiments. (B) The results are expressed in % of control and are the mean ± SEM of three independent experiments. In comparison with control: $^{***}p < 0.001$.

curcumin could manifest its anti-proliferative effect through the regulation of PDE1. Treatments of B16F10 cells for 24 h with nimodipine (a selective PDE1 inhibitor, [36]) dose dependently inhibited the cell proliferation (by 36% at 20 μM; by 47% at 30 μM, $P < 0.001$) (Fig. 5A) and accumulated the cell at S–G2M-phase (Fig. 5B) associated with up-regulations of p21 and p27, whereas cyclin A and UHRF1 were down-regulated (Fig. 6A and B).

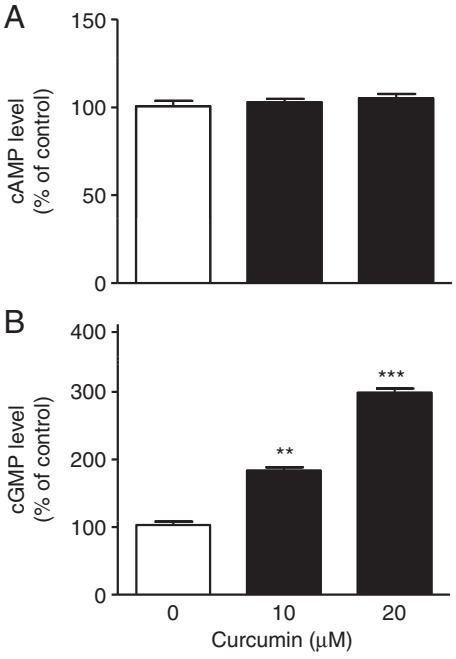

Figure 4. Effect of curcumin on intracellular cyclic nucleotide levels. B16F10 melanoma cells were treated for 24 h with or without curcumin (10 or 20 μM). Intracellular cAMP (A) and cGMP (B) levels were determined as described in Section 2. The results are expressed in % of control and are the mean ± SEM of three independent experiments. In comparison with control: $^{**}p<0.01$; $^{***}p<0.001$.

3.7 Effect of curcumin on PDE1A expression

At this point, it was clear that curcumin and nimodipine have similar anti-proliferative effects on B16F10 cells and a common mechanism of action. To go deeper, we therefore studied PDE1 family expression in B16F10 cells and detected different variants of PDE1A, PDE1B and PDE1C (Fig. 7A). While the expression of the different variants of PDE1B and PDE1C were unchanged with curcumin treatment (Fig. 7A), curcumin did modify the pattern expression of PDE1A. Indeed, curcumin decreased the 75 kDa- and the 55 kDa-PDE1A variants, while the 65 kDa-PDE1A variant was stable (Fig. 7A). In addition, curcumin also inhibited the expression of PDE1A at the transcription level, since the results of RT-PCR showed a decreased level of PDE1A mRNA in the curcumin-treated cells (Fig. 7B). However, in contrast PDE1C mRNA was increased (Fig. 7B).

3.8 PDE1A transfection effects on B16F10 cell proliferation in response to curcumin

To determine whether the anti-proliferative effects of curcumin are mediated by PDE1A inhibition in B16F10 cells, these cells were transfected with the cDNA of PDE1A. For this purpose, cells were transfected with either control vector or PDE1A plasmid and in the presence or absence of curcumin. As shown in Fig. 8A, overexpression of PDE1A rescued the cells from anti-proliferative effects of curcumin by increasing cell number in the PDE1A-transfected-curcumin-treated cells. These data clearly show that decreased level of PDE1A expression by curcumin treatment is critical for B16F10 cell proliferation. Figure 8B attested that curcumin decreases PDE1A expressions and knockdown UHRF1. As expected, the PDE1A transfection resulted in an overexpression of PDE1A proteins (75, 65 and 55 kDa). Interestingly, this overexpression is accompanied by an increase in the UHRF1 expression (Fig. 8B) showing that PDE1A expression is critical for UHRF1 regulation. Interestingly, curcumin treatment decreased DNMT1 expression (Fig. 8B). Accordingly, PDE1 overexpression partially overcame this decrease in DNMT1 expression.

4 Discussion

In the present study, our objective was to verify the hypothesis that PDEs could be one of the curcumin targets and to investigate the implication of PDEs in the anti-proliferative effects of curcumin on B16F10 melanoma cells. For this purpose, curcumin IC_{50} was determined for the first time on five different purified PDEs (PDE1–PDE5). The results showed that curcumin is a non-selective PDE isozymes inhibitor acting in the micromolar concentration range. In B16F10 melanoma cells, PDE4 and PDE1 represent major cAMP (64%) and cGMP (35%) hydrolyzing activities, respectively. Surprisingly, inhibition of PDE4 by rolipram treatment did not affect cell proliferation and cell cycle progression, suggesting that the cAMP pathway is not implicated in the anti-proliferative effects of curcumin on B16F10 melanoma cells. In agreement with our result showing that rolipram treatment increases PDE4 activity, Narita et al. showed that rolipram and forskolin treatments did not inhibit proliferation of human malignant melanoma cells, but conversely enhanced cell proliferation by increasing PDE4 activity [37].

The CDK inhibitors (p21 and p27), which directly inhibit the activities of cyclin-CDK complexes, have been implicated in cell cycle progression of many melanoma cells [38]. Cyclins are often overexpressed in cancer cells, and thereby accelerate cancer cell growth. Cyclin A, which interacts with Cdk2, has been found to play an important role in the regulation of phases S and G2/M [39]. In addition, overexpression of UHRF1 has been shown in many cancer cell lines [17, 40]. Herein, our results show that curcumin treatment of melanoma B16F10 cells inhibited cell proliferation, delayed cell cycle progression and up-regulated p21 and p27, whereas cyclin A, UHRF1 and DNMT1 were found to be down-regulated. It is likely that the cell cycle arrest by curcumin is due to the down-regulation of UHRF1 itself resulting from a PDE1A down-regulation. Indeed, cell cycle

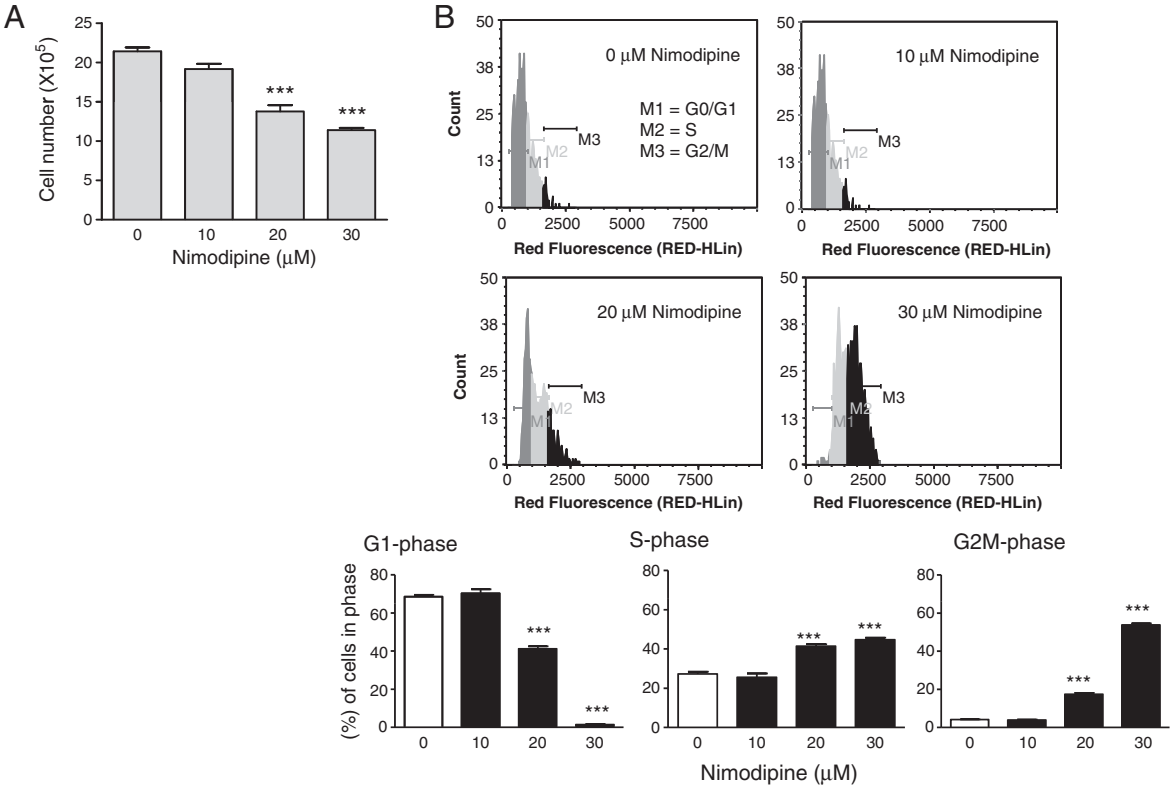

Figure 5. Effect of nimodipine on B16F10 cell proliferation (A) and cell cycle progression (B). Cells were treated with nimodipine (0, 10, 20 and 30 μM) for 24 h, cell proliferation (A) and cell cycle distribution (B) were determined as described in Section 2. For each treatment, the results are expressed in % of cells in each phase and are the mean ± SEM of three independent experiments. In comparison with control: $^{***}p < 0.001$.

progression from G1 to the S-phase is dependent upon E2F1, which is known to regulate UHRF1 expression [17, 41]. However, it is not known whether PDE1A is acting before or after E2F1. Before, is the most likely hypothesis considering that the promoter of UHRF1 has E2F1 binding sites [42]. Thus, our results emphasize the mechanism of action of curcumin on the cell cycle progression by the control of UHRF1 expression. Furthermore, considering that the UHRF1/DNMT1 couple is a key factor of the DNA methylation pattern maintenance, our results support the notion that PDE1A may participate in the epigenetic control of gene expression and that curcumin is a potent regulator of DNA methylation profiles.

Some studies demonstrated that sustained increases in intracellular cGMP concentrations, related to PDE5 inhibition, regulate cell growth and induce apoptosis [43, 44]. The mechanism of this action is incompletely understood, although increased cGMP concentrations appear to arrest cell cycle progression, via G2/M blockade [45–47] and modulate cell cycle regulatory proteins such as p21, p27 and cyclin D1 [48, 49]. In the present study, although curcumin on one hand is a PDE4 inhibitor and on the other hand decreases PDE4 activity-treated melanoma cells, surprisingly cAMP levels were found to be unchanged. However, curcumin treatment increased dose dependently intracellular cGMP levels, suggesting that the anti-proliferative effect of curcumin was mediated by a cGMP-PDE inhibition, more specifically by inhibition of PDE1, which is shown to contribute for 35% to cGMP-PDE activity. Curcumin treatment of B16F10 melanoma cells decreased PDE1 activity by 82%. Interestingly, nimodipine (a PDE1 selective inhibitor, [36]) inhibited dose dependently B16F10 melanoma cell proliferation, cell cycle progression, up-regulated p21 and p27, while cyclin A and UHRF1 were down-regulated. These findings indicate that nimodipine effects are similar to curcumin effects on melanoma B16F10 cells.

The PDE1 family is encoded by three genes (PDE1A, PDE1B and PDE1C). Each of these three genes has alternative promoters and is alternatively spliced to give rise to a multitude of disparate mRNAs and proteins products that differ according to the species origin. The molecular weight of PDE1 enzymes, which exist as dimers, ranges from 58 to 86 kDa per monomer [50]. In B16F10 melanoma cells, our data show that different variants have been detected for all PDE1 families (three PDE1A variants, two PDE1B variants and three PDE1C variants). Amongst PDE1A family, only a 52 kDa-PDE1A variant [51] was previously reported for mouse, but unfortunately the corresponding mRNA for the

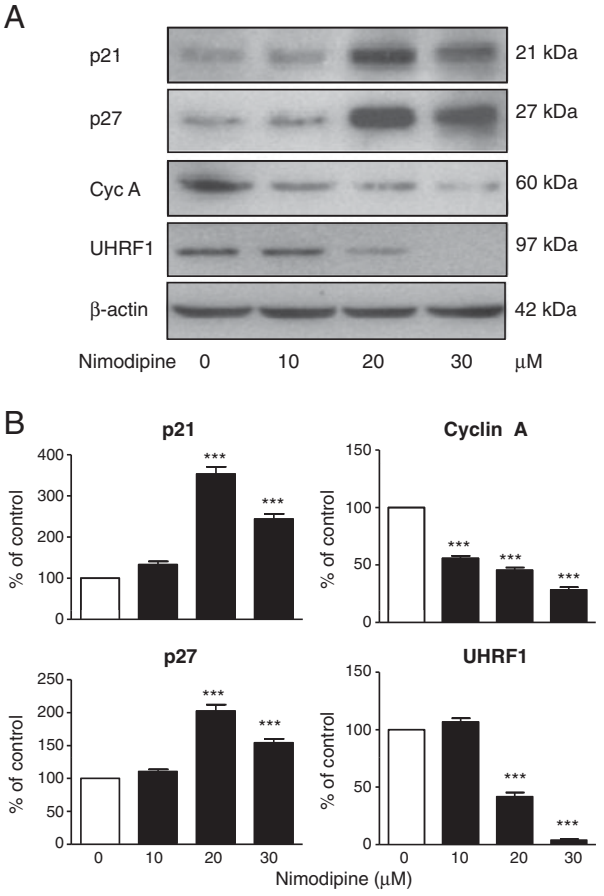

Figure 6. Effect of nimodipine on the expression of critical proteins involved in B16F10 melanoma cell cycle progression. Cells were treated for 24 h with different doses of nimodipine. Protein expression was assessed by Western blot as described in Section 2. (A) The traces shown are representative of three independent experiments. (B) The results are expressed in % of control and are the mean ± SEM of three independent experiments. In comparison with control: $^{***}p<0.001$.

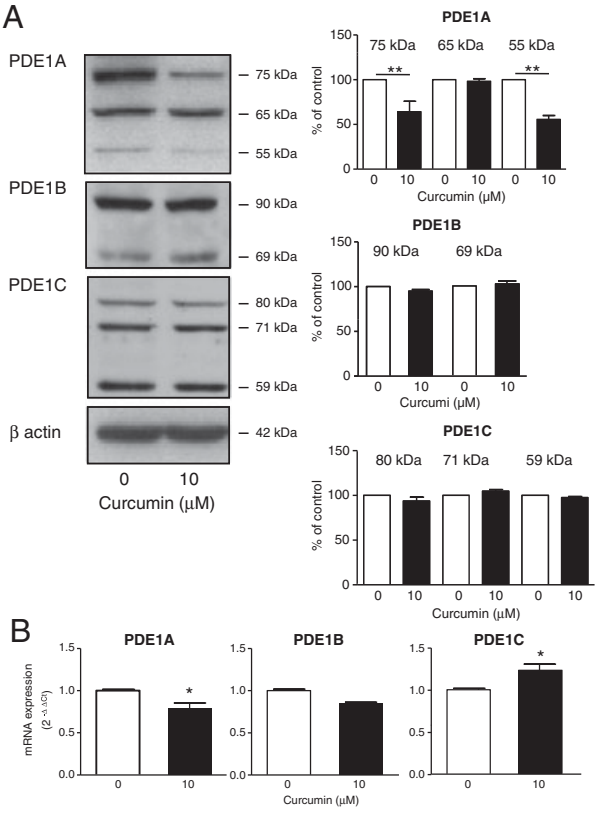

Figure 7. Effect of curcumin treatment on PDE1 protein expressions and on PDE1A, PDE1B and PDE1C mRNA expressions in B16F10 melanoma. Cells were treated for 24 h with or without curcumin (10 μM). Protein expressions were assessed by Western blot as described in Section 2. (A) The traces shown are representative of three independent experiments. (B) The results are expressed in % of control and are the mean ± SEM of three independent experiments. Total RNA was extracted and 1 μg of total RNA was reverse-transcribed in cDNA and mRNA expressions were assessed as described in Section 2. In comparison with control: $^{*}p<0.05$, $^{**}p<0.01$.

other mouse PDE1A variants are unknown. Therefore, their specific expression could not be investigated. Curcumin treatment decreased selectively and significantly the expression of two PDE1A variants (75 and 55 kDa), whereas the PDE1B and PDE1C variants were not affected. Moreover, curcumin decreased selectively PDE1A-mRNA levels, whereas the PDE1B and PDE1C mRNAs levels were not inhibited. Interestingly, transfection of B16F10 cells with the cDNA of PDE1A partially rescued the cells from the anti-proliferative effects of curcumin, increased UHRF1 and DNMT1 expressions indicating that PDE1A is an essential player in the curcumin anti-proliferative effects on B16F10 melanoma cells by regulating UHRF1 and DNMT1 (Fig. 9).

Our data showing anti-proliferative effect of curcumin in B16F10 melanoma cells are in agreement with the previous data obtained on other various melanoma cell lines such as B16 and WM-115 [52], A375 and the relatively resistant G361 malignant human melanoma cell line [53]. Furthermore, curcumin has been shown to prevent in vivo tumour development highlighting the therapeutic potential of our study [54, 55]. In another studies focused on PDE participations in angiogenesis, we have performed in vivo tumorization experiments in C57BL/6N mice with B16F10 melanoma cells, showing that curcumin or a PDE1 inhibitor treatment decreases tumour growth (unpublished observations). This indicates that both compounds are able to prevent tumour growth.

Recently, Shimizu et al. showed that human malignant melanoma cell growth was inhibited by the PDE1 inhibitor vinpocetine and suggested that PDE1C, which was expressed in these cells, may play an important role in human malignant melanoma cells. However, no involvement of PDE1A or any other PDE1 variants in the anti-proliferative

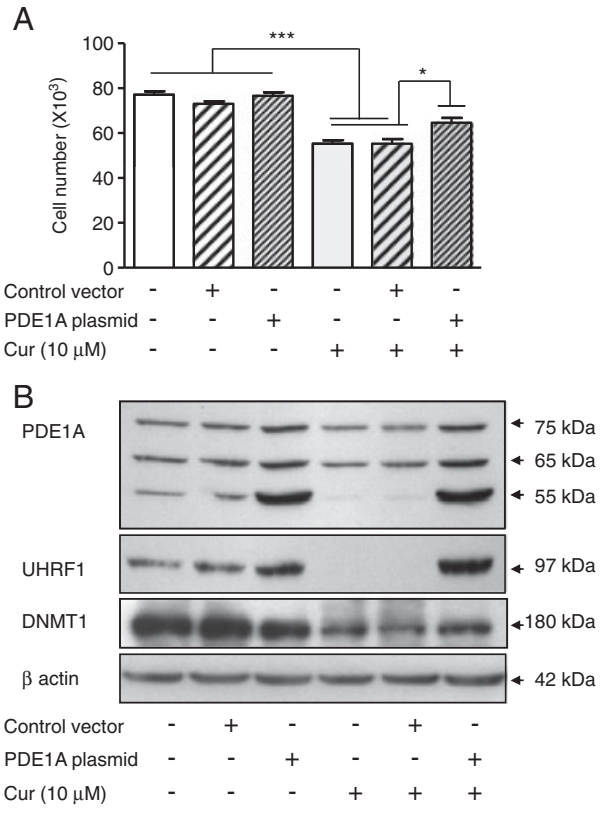

Figure 8. Effect of PDE1A overexpression on cell proliferation (A) and PDE1A, UHRF1 and DNMT1 expressions (B). Cells were transfected with either 1 μg of PDE1A plasmid or 1 μg of control vector. Untransfected cells were also used as control. Cells were allowed to grow for 24 h and then were treated for 24 h with curcumin (10 μM) or vehicle (buffer with 0.1% DMSO). (A) The results of cell proliferation are expressed as mean ± SEM of three independent experiments: $^{*}p<0.05$; $^{***}p<0.001$. PDE1 s, UHRF1 and DMNT1 expressions were assessed by Western blot as described in Section 2. (B) Traces shown are representative of three independent experiments.

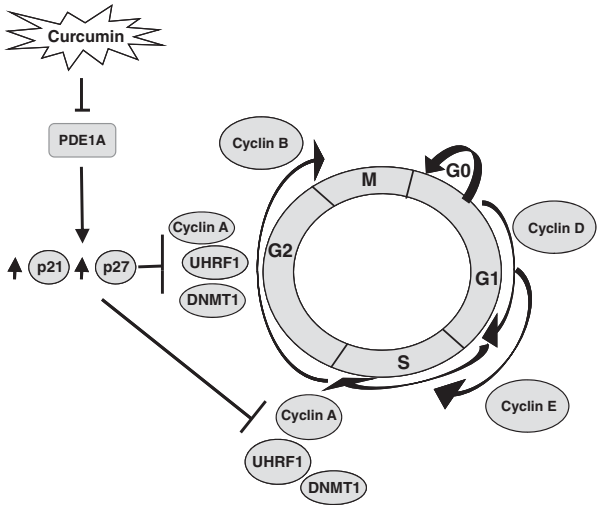

Figure 9. Mechanism of curcumin action via a PDE1A-dependent mechanism involving G2/M cell cycle arrest, down-regulation of UHRF1, DMNT1 and cyclin A as well as p21 and p27 up-regulation.

effect of vinpocetine were investigated in their study [56]. But, it should be noticed that vinpocetine is a poor PDE1 inhibitor [57] and not specific for PDEs since it also activates Ca^{2+}-activated K^{+} channel (BK_{Ca}) in opposite to 8-bromo cGMP that is ineffective [58].

Exisulind (sulindac sulfone), an inactive metabolite of the non-steroidal, anti-inflammatory agent, showed anti-proliferative properties as PDE5 inhibitor in many cancer cell lines [59, 60]. This compound associated with the standard therapy (carboplatin and etoposide) is in phase II of clinical trials in patients with extensive small cell lung cancer [61]. Exisulind showed an IC_{50} of 70 μM for cGMP-PDE1 and 110 μM for cGMP-PDE5 [62], indicating that it is a less potent inhibitor of PDE in comparison with curcumin which is four and three times more potent on PDE1 and PDE5, respectively. These data suggest that exisulind anti-cancer effects may be merely mediated by PDE1 inhibition rather than PDE5. More recently, a study showed that treatment of colorectal cancer cells with curcumin and exisulind resulted in a synergistic inhibitory effect of 50-90% on cell growth associated with G2/M arrest and induction of apoptosis [63], suggesting that curcumin and exisulind inhibited the same target, i.e. PDE1. In opposite, Arozarena et al. reported that, in response to B-Raf kinase (BRAF), down-regulation of PDE5A induces melanoma cell invasion, pointing out the therapeutic potential of PDE1 inhibitors [64].

In this study, we show in B16F10 cells that PDE1A inhibition by curcumin decreases UHRF1 and DNMT1 expressions and decreases cell proliferation, similar to thymoquinone effects reported in our recent study on Jurkat cells [26]. Although, Jurkat cells and B16F10 melanoma cells are characterised by differences in PDE contributions, PDE1A present in both cell lines may represent a common regulator key of cell proliferation in these cells.

In conclusion, the present study shows, for the first time, the involvement of the PDE1A in the anti-proliferative effects of curcumin and suggests a potential role for PDE1A inhibition as a new strategy to prevent and treat hyper-proliferative disorders including cancer. It also proposes a new molecular mechanism by which curcumin present in food might act to prevent cancer occurrence and participate in epigenetic control of gene expression.

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