Protective effect of the dopamine D_3 receptor agonist (7-OH-PIPAT) against apoptosis in malignant peripheral nerve sheath tumor (MPNST) cells

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Abstract. Emerging evidence indicates that the dopamine D_3 receptor (D_3R) mediates protective roles both in neuronal and non-neuronal cell lines. In a previous study we proposed that neurofibromin, a large tumor suppressor protein encoded by the neurofibromatosis type 1 gene (NF1), may increase susceptibility to apoptosis after serum deprivation in malignant peripheral nerve sheath tumor (MPNST) cells, thus acting as a proapoptotic gene. In addition, it has been observed that D_3Rs are functionally correlated to neurofibromin. In this study, we examined whether 7-OH-PIPAT, a potent dopamine D_3R agonist, exerts an antiapoptotic role under the same culture conditions and then correlated this effect to changes in NF1 expression. Results showed that serum deprivation caused a significant reduction of cell viability (MTT assay) both after 24 and 48 h ($p<0.001$). Treatment with increasing concentrations of 7-OH-PIPAT (10^{-9}-10^{-5} M) induced a progressive increase in cell viability both after 24 and 48 h as compared to vehicle-treated cells, with significant changes at the highest concentrations tested (10^{-6} and 10^{-5} M). Consistently, at the latter two concentrations, a significant reduction in oligonucleosomes formation was observed, thus suggesting an antiapoptotic role of 7-OH-PIPAT. These results were confirmed by Hoechst 33254 nuclear staining. To investigate whether these effects were correlated to changes in NF1 transcript and protein expression, quantitative real-time PCR, Western blot and immunofluorescence analyses were performed. Results demonstrated that the upregulation of NF1 transcripts and protein levels induced by serum withdrawal were remarkably attenuated by 10^{-6} and 10^{-5} M agonist treatment within 24 h ($p<0.01$ and $p<0.001$, respectively), whereas similar effects were observed already at a lower concentration (10^{-7} M) after 48 h treatment ($p<0.001$). In conclusion, these results suggest that D_3R might mediate the protective response to serum deprivation in MPNST cells through the inhibition of NF1 gene expression, further underlying a subtle role of these receptors in MPNST development.

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Introduction

Neurofibromatosis type 1 (NF1) is the most common autosomal-dominant neurogenetic disorder, with an incidence of ~1 in 3500. The disease is characterized by pigmentary skin alterations and the growth of a variety of benign and malignant tumors that develop in association with both the peripheral and central nervous system (1,2). NF1 patients develop multiple benign dermal neurofibromas that do not become malignant, while 30-50% of NF1 patients also develop plexiform neurofibromas that usually grow along a major nerve. NF1 patients have an 8-13% lifetime risk of developing a malignant peripheral nerve sheath tumor (MPNST), a highly aggressive malignant neoplasm in which Schwann cells represent the primary neoplastic cell type (3,4). Aberrant mutations within the NF1 gene lead to the functional inactivation of the encoded tumor suppressor protein neurofibromin. Neurofibromin contains a central domain (the NF1-GAP-related domain or NF1-GRD) that shares extensive sequence homology with other GAPs that regulate Ras (5). Ras is a small GTPase which plays a central role in cell survival, proliferation and differentiation by transducing responses to growth stimuli initiated at the cellular surface to several intracellular signalling molecules (6). Neurofibromin acts as a GAP, which terminates Ras signalling. In addition to its role in controlling cell proliferation, a proapoptotic role of neurofibromin has been proposed in mouse embryonic fibroblasts (7) and in MPNST cells (8), suggesting that besides its Ras-dependent growth inhibition, neurofibromin may exert tumor suppression by conferring sensitivity to apoptosis.

The cloned D_3R is an autoreceptor that belongs to the D_2-like subfamily of dopamine receptors, also comprising the D_2R and D_4R (9). This receptor contains seven transmembrane regions (9-11), a feature commonly associated with the

members of the superfamily of G-protein-coupled receptors (12). Besides their well-established role as neurotransmitters in synaptic transmission (13), D_3Rs are actively expressed in oligodendrocyte progenitor cells (14), raising the possibility that these receptors might be involved in alternative biological functions. To support this, several findings have demonstrated that dopamine receptors mediate protective actions in astrocytes (15), oligodendrocytes (16) and pheochromocytomas (17). However, the cellular mechanisms for protection triggered by D_3Rs remain unexplored. The previous identification of a functional correlation between the D_3R and neurofibromin (18) and the subsequent proposals of NF1 as a candidate proapoptotic gene (7,8) led us to hypothesize that the D_3R might also mediate a protective action in response to serum deprivation in MPNST cells, possibly through the interaction with neurofibromin. To test this hypothesis, the biological role of the selective D_3R agonist 7-OH-PIPAT was assessed in serum-starved MPNST cells and the effect was correlated to changes in NF1 expression. The present data may provide new insights into the comprehension of the role of D_3R in MPNST development and further highlight the potential interaction between dopamine receptors and tumor suppressor genes.

Materials and methods

Cell culture. The study was carried out in rat malignant peripheral nerve sheath tumor (MPNST) cells (CRL-2768) obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 200 μg/ml streptomycin (Sigma-Aldrich, USA). Cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2. Once cells reached confluence, they were switched to serum-free medium with or without 0.1% DMSO (vehicle) for subsequent testing.

MTT test. To assess cell viability, we used the cell proliferation kit I (MTT) following manufacturer's instructions (Roche). Cells were cultured either in presence of 10% FBS, in total absence of serum or additioned with raising concentrations (10^{-9}-10^{-5} M) of (RS)-trans-7-hydroxy-2-[N-propyl-N-(3'-iodo-2'-propenyl)amino]tetralin maleate (7-OH-PIPAT) (Tocris Bioscience, UK) for 24 and 48 h. To exclude any protective effects attributable to the presence of the vehicle used to deliver the D_3R agonist, cell viability was also measured in serum-starved cells added with 0.1% DMSO.

Cells were seeded into 96-well plates at a concentration of $1x10^4$ cells/well. DMEM containing 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was added in each well. Following incubation for 4 h at 37˚C, medium was removed, and 100 μl of DMSO was added. Formazan formed by the cleavage of the yellow tetrazolium salt MTT was measured spectrophotometrically by absorbance change at 550-600 nm using a microplate reader.

Apoptotic assay by immunodetection of oligonucleosomes. Mononucleosomes and oligonucleosomes released from the nucleus into the cytoplasm of apoptotic cells were detected with the use of a sandwich enzyme-linked immunosorbent assay (Cell Death Detection ELISA^{PLUS} 10X) (Roche Applied Sciences). The assay is based on quantitative sandwich enzyme-linked immunosorbent assay principle, with a rat monoclonal antibody directed against DNA histones, respectively. For sample preparation, 10% serum-cultured MPNST cells, serum-starved cells with or without vehicle and added with different concentrations of 7-OH-PIPAT (10^{-9}-10^{-5} M) were harvested by trypsinization. Cells were then lysed in incubation buffer for 30 min. The lysate was centrifuged at 20,000 rpm x 10 min. The supernatant was diluted to yield $1x10^4$ cell equivalents/ml and used for immuno-detection. The assay was performed as follows: i) an antibody that reacts with the histone H1, H2A, H2B, H3 and H4 was fixed on the wall of the microplate module provided with the kit; ii) samples prepared as described above were added to the plate containing the immobilized anti-histone antibody; iii) anti-DNA monoclonal antibodies conjugated to peroxidase were added, to allow their binding to the DNA part of nucleosomes; and iv) after removal of unbound peroxidase conjugate, the amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2'-azino-di(3-ethylbenzthiazoline sulfonate) as a substrate.

Fluorescence microscopic analysis of cell death. Cells cultured in the presence of 10% FBS, in serum-free medium and added with either vehicle or growing concentrations of 7-OH-PIPAT (10^{-7}-10^{-5} M) for 48 h were analyzed for typical morphological features of apoptotic degeneration by the use of fluorescence microscopy with the nuclear dye Hoechst 33258 (19). Briefly, cells were fixed with a solution of methanol:acetic acid (3:1 v/v) for 30 min, washed three times in PBS and incubated for 15 min at 37˚C with 0.4 μg/ml Hoechst 33258 dye. After being rinsed in water, cells were visualized for determination of nuclear chromatin morphology with an Axiovert 40 fluorescence microscope (Zeiss). Apoptotic cells were recognized on the basis of nuclear condensation and/or fragmented chromatin. Each condition was reproduced in three dishes per experiment. Representative photomicrographs of both apoptotic and normal cells were taken from three fields per dish in a fixed pattern.

Analysis of mRNA expression by RT-PCR. Total RNA extracts were isolated by 1 ml TRIzol reagent (Invitrogen) and 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. Pellet was washed with 75% ethanol and air dried. Single-stranded cDNAs were synthesized incubating total RNA (5 μg) with SuperScript III RNase H-reverse transcriptase (200 U/μl) (Invitrogen); Oligo-$(dT)_{20}$ primer (100 nM) (Invitrogen); 1 mM dNTP mix (Invitrogen), dithiothreitol (DTT, 0.1 M), recombinant RNase-inhibitor (40 U/μl) at 42˚C for 1 h in a final volume of 20 μl. Reaction was terminated by incubation of samples at 70˚C for 10 min.

Aliquots of cDNA were amplified using specific primers for NF1, D_1R, D_2R, D_3R, D_4R, D_5R and S18 ribosomal subunit. Oligonucleotide sequences are listed in Table I. Each PCR reaction contained 0.4 μM specific primers, 200 μM dNTPs, 1.25 units AmpliTaq Gold DNA polymerase and GeneAmp buffer containing 2.5 mM $MgCl^{2+}$ (Applied

Table I . Primer sequences.

Gene	Forward	Reverse	bp length
NF1 Accession no. NM_010897.2	TTCGATACACTTGCGGAAAC	CACATTGGCAAGAGCCATAG	114
Dopamine D_1 receptor Accession no. NM_010076.1	GAGCAGGACATACGCCATTT	GCTTCTGGGCAATCCTGTAG	101
Dopamine D_2 receptor Accession no. NM_010077.1	TGCCATTGTTCTTGGTGTGT	GTGAAGGCGCTGTAGAGGAC	111
Dopamine D_3 receptor Accession no. NM_007877.1	GGGGTGACTGTCCTGGTCTA	AAGCCAGGTCTGATGCTGAT	110
Dopamine D_4 receptor Accession no. NM_007878.2	CTGCAGACACCCACCAACTA	CCTGGACCTCGGAGTAGACA	100
Dopamine D_5 receptor Accession no. NM_013503.1	GGCTATTTCCAGACCCTTCC	TGAGTTGGACCGGGATAAAG	116
Ribosomal protein S18 Accession no. NM_011296.2	GAGGATGAGGTGGAACGTGT	GGACCTGGCTGTATTTTCCA	115

Forward and reverse primers were selected from the 5' and 3' region of each gene mRNA. The expected length of each PCR amplification product is indicated in the right column.

Biosystem). PCR was performed using the following three cycle programs: i) denaturation of cDNA (1 cycle: 95˚C for 12 min); ii) amplification (40 cycles: 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 45 sec); iii) final extension (1 cycle: 72˚C for 7 min). Amplification products were separated by electrophoresis in a 1.8% agarose gel in 0.045 M Tris-borate/1 mM EDTA (TBE) buffer.

Real-time quantitative PCR analysis. Aliquots of cDNA (400 ng) from cells grown in medium containing both normal or in total absence of serum and cells subsequently treated with either 0.1% DMSO or 10^{-7}, 10^{-6} and 10^{-5} M of 7-OH-PIPAT for 24 and 48 h were amplified in parallel reactions with external standards at known amounts (purified PCR products, ranging from 10^2-10^8 copies) using specific primers recognizing NF1 mRNA (Table I). To normalize data, mRNA levels of S18 ribosomial subunit (reference gene) were measured in each amplification. Each PCR reaction (final volume of 20 μl) contained 0.5 μM primers, 1.6 mM Mg^{2+}, 1X Light Cycler-FastStart DNA Master SYBR-Green I (Roche Diagnostic). Amplifications were performed using a Light Cycler 1.5 instrument (Roche Diagnostic) using the following program setting: i) cDNA denaturation (1 cycle: 95˚C for 10 min); ii) quantification (45 cycles: 95˚C for 10 sec, 57˚C for 7 sec, 72˚C for 5 sec); iii) melting curve analysis (1 cycle: 95˚C for 0 sec, 65˚C for 15 sec, 95˚C for 0 sec); iv) cooling (1 cycle: 40˚C for 30 sec). Each amplification was carried out in triplicates in three different experiments. The temperature transition rate was 20˚C/sec, except for the third segment of the melting curve analysis where it was set to 0.1˚C/sec. Quantification was obtained by comparing the fluorescence emitted by PCR products at unknown concentration with the fluorescence emitted by external standards at known concentration. For this analysis, fluorescence values, measured in the log-linear phase of amplification, were estimated with the second derivative maximum method using Light Cycler Data Analysis software. PCR products specificity was evaluated by melting curve analysis followed by gel electrophoresis.

To assess the different expression levels we employed the ΔCt comparative method (20). We analyzed the mean of the crossing points (or crossing threshold = Ct) of each sample. The Ct represents the number of cycles needed to detect a fluorescence above a specific threshold level and it is inversely correlated to the amount of nucleic acids template present in the reaction. The ΔCt was calculated by normalizing the mean Ct of each sample to the mean Ct of the reference gene measured in the same experimental conditions. For the quantification of each gene we considered 10% FBS-cultured MPNST cells as positive samples (calibrator sample). The ΔΔCt of each sample was calculated by subtracting calibrator ΔCt to sample ΔCt. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the fold-change.

Western blot analysis. Crude extracts were prepared by homogenizing cells in a buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics) using a Teflon-glass homogenizer and then sonicated. Protein concentrations were determined by the Bradford method (21) using bovine serum albumin (BSA) as a standard. Sample proteins (50 μg) were diluted in 4X

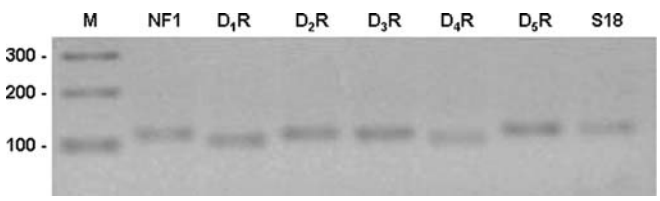

Figure 1. Expression of NF1 and dopamine receptors in MPNST cells by RT-PCR analysis. Amplification products obtained using specific primers which recognized NF1, APP, D_1R, D_2R, D_3R, D_4R and D_5R generated bands of the expected length (Table I). Ribosomal protein S18 was used as a control in each PCR amplification. A 100-bp DNA ladder is shown on the left side of each gel (lane M) with bands labelled in bp units.

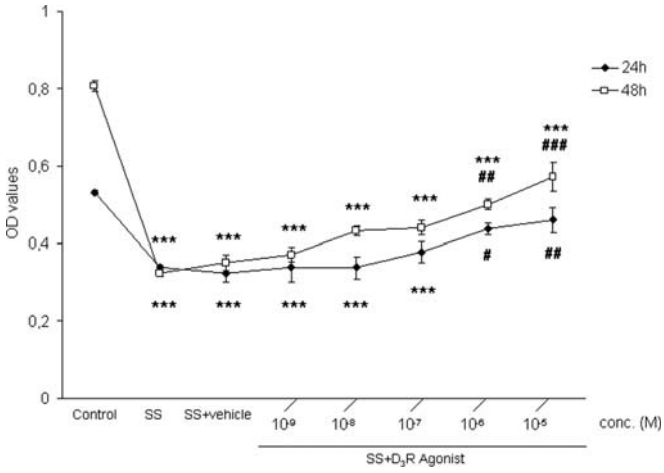

Figure 2. Dose-response effects of 7-OH-PIPAT treatment on cell viability. Induction of cell viability in serum-starved MPNST cells treated with increasing concentrations of the D_3R agonist after 24 (◆) and 48 h (□). Cells cultured in presence of 10% FBS (control), in total absence of serum (SS), additioned with 0.1% DMSO (SS + vehicle) or 7-OH-PIPAT (SS + D_3R agonist) were used for MTT measurements as described in Materials and methods. Values are expressed as mean ODs (n=6) ± SEM. Results are representative of at least three independent experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$ or $^{\#\#\#}p<0.001$ as compared to serum-starved cells treated with vehicle. $^{***}p<0.001$ as compared to control groups.

sodium dodecyl sulphate (SDS) protein gel loading solution (Invitrogen), boiled for 5 min, separated on 4-12% Bis-tris gel (Invitrogen) by electrophoresis and processed as previously described (22). Immunoblot analysis was performed by using a rabbit polyclonal antibody raised against a peptide mapping within the C-terminus of neurofibromin of human origin (sc-67, Santa Cruz Biotechnology Inc.) and a rabbit polyclonal antibody raised against amino acids 210-444 of ß-tubulin of human origin (sc-9104, Santa Cruz Biotechnology Inc.), which was used as loading control. All primary antibodies were diluted 1:200, while the secondary antibody (goat antirabbit IgG2b HRP, Amersham Biosciences) was used at 1:10000. Blots were developed using the enhanced chemiluminescence technique (Amersham Biosciences). No signal was detected when the primary antibody was omitted (data not shown).

Immunofluorescence analysis. Cells were permeabilized in 0.1% sodium citrate with 0.1% Triton X-100 detergent (Sigma, St. Louis, MO). After pretreatment in a blocking solution consisting of 4% normal goat serum (Sigma), 0.25% BSA (Sigma-Aldrich,) in 0.1 M/l PBS-TX for 2 h at room temperature, permeabilized cells were incubated for 20 h at 4˚C in a primary antibody raised against a peptide mapping within the C-terminus of neurofibromin of human origin (sc-67). Cells were then incubated in a secondary antibody conjugated to a fluorescent tag (Alexa Fluor 488, Molecular Probes, Inc., Eugene, OR) for 1.5 h at room temperature and shielded from light. After a series of PBS and double-distilled water washes, the fixed cells were cover-slipped with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Ten fields from randomly selected slides were visualized using an Axiovert 40 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) at x20 magnification and images of each field were captured using a digital camera (Canon, Japan).

Statistical analysis. Data are reported as mean ± SEM. Oneway analysis of variance (ANOVA) was used to compare differences among groups followed by Tukey test to evaluate which groups were statistically different. The level of significance for all statistical tests was $p \leq 0.05$.

Results

Identification of NF1 and dopamine receptors transcripts by RT-PCR analysis. RT-PCR analysis was performed in MPNST cells with the aim to evaluate whether this cell line expresses both NF1 and dopamine receptors mRNAs. Amplification products obtained using specific primer pairs (Table I) demonstrated that both NF1 and dopamine receptor genes are expressed in this cell line (Fig. 1). Primers for S18 ribosomal subunit were used as control in each PCR amplification and generated bands of the expected length.

Effect of treatment with different concentrations of 7-OH-PIPAT on cell viability after serum deprivation. Removal of serum from culture medium produced a significant decline in cell viability as compared to 10% FBS-cultured cells both after 24 and 48 h [$F(15, 95) = 31.92$; $p<0.001$] (Fig. 2). Treatment with increasing concentrations of 7-OH-PIPAT (10^{-9}-10^{-7} M) induced a slight increase in cell viability when compared to serum-deprived cells both after 24 and 48 h, although not reaching significant values. However, at the highest concentrations tested (10^{-6} and 10^{-5} M) significant increases in cell viability were observed both after 24 h ($p<0.05$ and $p<0.01$, respectively) and 48 h ($p<0.01$ and $p<0.001$, respectively) (Fig. 2).

Effect of treatment with different concentrations of 7-OH-PIPAT on oligonucleosome formation following serum withdrawal. The presence of an apoptotic process in serum-starved MPNST cells was confirmed by means of a non-radioactive assay based on quantitative sandwich enzyme-linked immunosorbent principle. Mononucleosomes and oligonucleosomes released from the nucleus into the cytoplasm of apoptotic cells significantly increased both after 24 and

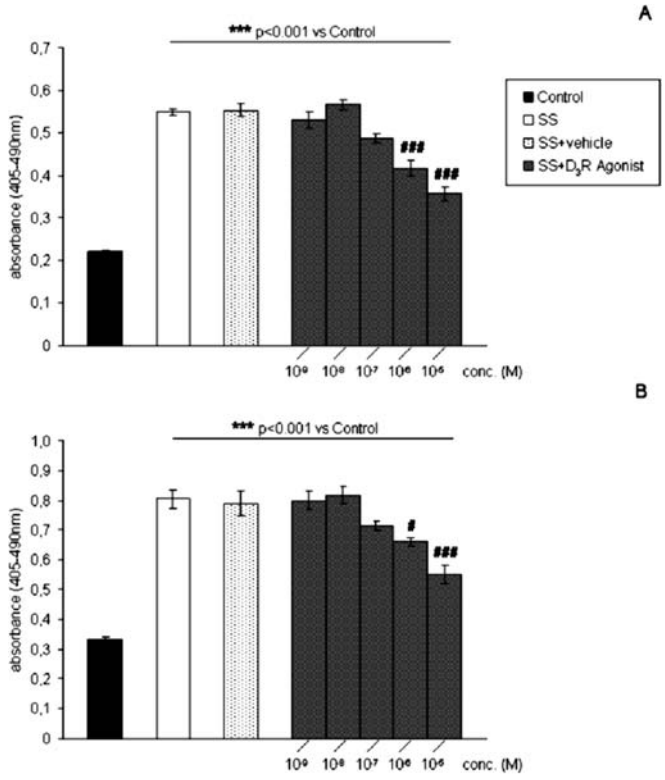

Figure 3. Dose-response effects of 7-OH-PIPAT treatment on oligonucleosome formation. Inhibition of apoptosis in serum-starved MPNST cells treated with increasing concentrations of the D_3R agonist after 24 (A) and 48 h (B). Cells cultured in presence of 10% FBS (control), in total absence of serum (SS), added with 0.1% DMSO (SS + vehicle) or 7-OH-PIPAT (SS + D_3R agonist) were used for oligonucleosome formation measurements as described in Materials and methods. Values are expressed as mean ODs (n=6) ± SEM. The bar graph shows the results of three independent experiments. $^{\#}p<0.05$ or $^{\#\#\#}p<0.001$ as compared to serum-starved cells treated with vehicle. $^{***}p<0.001$ as compared to control groups.

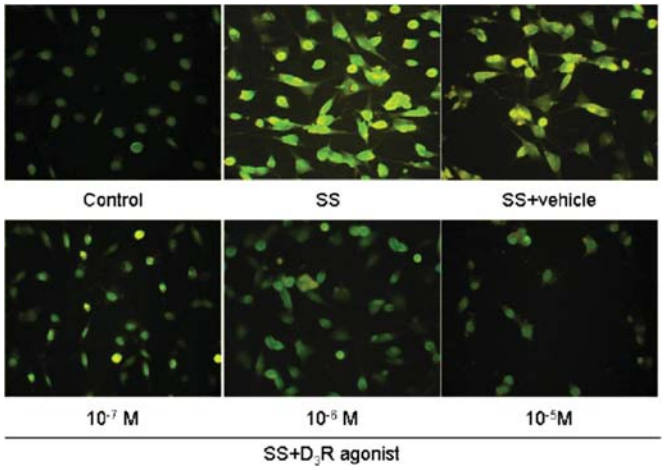

Figure 4. Hoechst 33258 nuclear staining of serum-starved MPNST cells treated with 7-OH-PIPAT. Representative photomicrographs of MPNST cells cultured with 10% serum (control), serum-starved (SS), 0.1% DMSO (SS + vehicle) or additioned with the indicated concentrations of 7-OH-PIPAT (SS + D_3R agonist) after 48 h exposure. Cells were stained with the fluorescent nuclear dye Hoechst 33258 and viewed at x40 magnification. Hoechst 33258 binds to nuclear DNA and emits an intense fluorescence in apoptotic cells due to nuclear condensation and/or chromatin fragmentation. Each condition was reproduced in three dishes per experiment. Representative photomicrographs of both apoptotic and normal cells were taken from three fields per dish in a fixed pattern.

48 h of serum deprivation (Fig. 3A and B). Consistent with the results obtained from viability tests, both 10^{-6} and 10^{-5} M 7-OH-PIPAT concentrations were able to significantly reduce apoptosis in serum-starved cells after 24 [$F(7, 41) = 50.04$; $p<0.001$] (Fig. 3A) and 48 h [$F(7, 41) = 26.65$; $p<0.05$ and $p<0.001$, respectively] (Fig. 3B).

Morphological observation of nuclear condensation/fragmentation in serum-starved MPNST cells treated with different concentrations of 7-OH-PIPAT. To evaluate the effect of 7-OH-PIPAT on morphological nuclear condensation and/or fragmented chromatin induced by serum withdrawal, we performed Hoechst 33342 staining on MPNST cells cultured either in 10% FBS (control), in total absence of serum or additioned with increasing concentrations of the D_3R agonist (10^{-7}-10^{-5} M, respectively) for 48 h (Fig. 4).

Results demonstrated that while 10% serum-cultured cells show apparent normal morphology and no evidence of DNA fragmentation, serum deprivation induced a remarkable enhancement of the fluorescent signal, which was still maintained in presence of 0.1% DMSO (vehicle). Interestingly, nuclear condensation progressively decreased in serum-free cultured cells additioned with raising concentrations of 7-OH-PIPAT (10^{-7}-10^{-5} M) (Fig. 4), further supporting a D_3R-mediated antiapoptotic role.

NF1 mRNA and protein expression in MPNST cells treated with 7-OH-PIPAT. To correlate the previously observed antiapoptotic effects exerted by 7-OH-PIPAT treatment to changes in both NF1 mRNA and protein levels, quantitative real-time PCR and Western blot analyses were carried out on MPNST cells treated as described above both after 24 and 48 h exposures.

Our findings revealed that the low NF1 expression observed in presence of 10% FBS was significantly increased by serum withdrawal both after 24 and 48 h (Fig. 5A-D). The addition of 7-OH-PIPAT to serum-free cultured cells caused a significant reduction of NF1 mRNA and protein expression both at 10^{-6} and 10^{-5} M concentration after 24 h [$F(5,17) = 58.64$; $p<0.01$ and $p<0.001$, respectively], whereas much more evident decreases were observed already at a lower concentration (10^{-7} M) after 48 h [$F(5,17) = 36.99$; $p<0.001$] (Fig. 5A and B). Although a strict correlation between transcript and protein levels may not always be found (23), the representative bands obtained from neurofibromin protein analysis carried out after 24 and 48 h (Fig. 5C and D, respectively) showed high correspondence with the results obtained by mRNA measurements.

Neurofibromin immunofluorescence analysis in MPNST cells treated with 7-OH-PIPAT. To evaluate whether D_3R agonist treatment induced changes in protein cellular localization after serum starvation, immunofluoresce analysis of neurofibromin protein was performed in MPNST cells cultured in 10% FBS, in serum-free medium with or without 0.1%

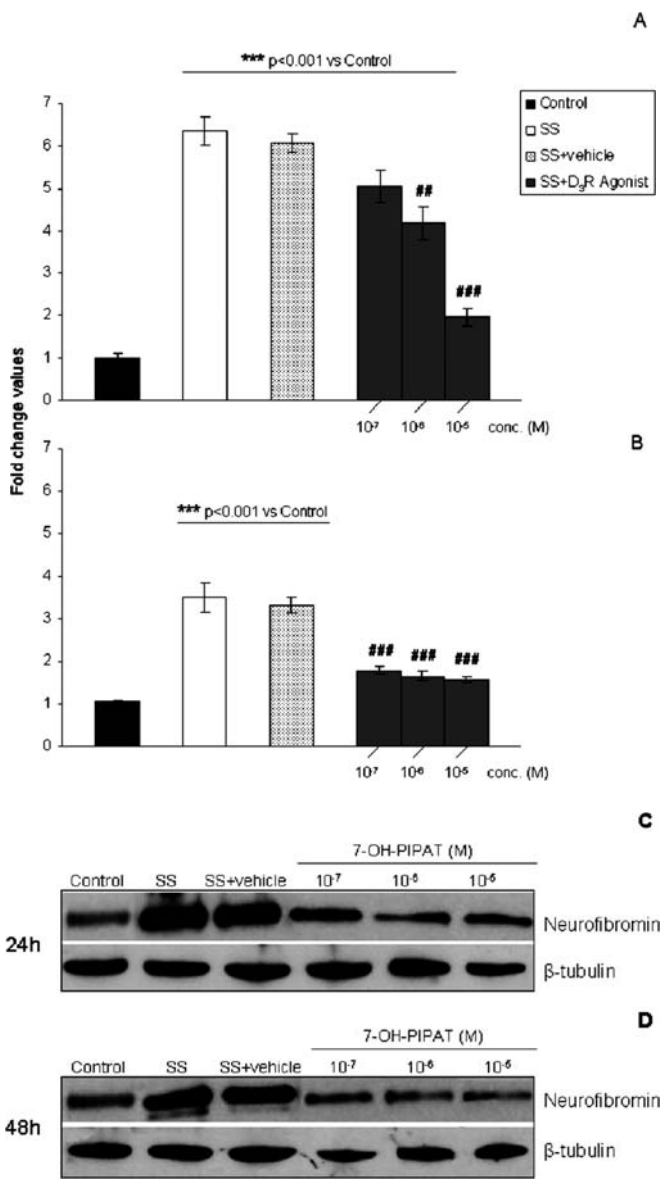

Figure 5. Quantitative real-time PCR and Western blot analyses of NF1 and neurofibromin expression in serum-starved MPNST cells treated with 7-OH-PIPAT. Results are presented as mean fold-changes of 10% FBS-cultured MPNST cells (control, n=4) ± SEM. Relative fold-changes of the NF1 gene after treatment with the indicated concentrations of the D_3R agonist after 24 (A) and 48 h (B) were normalized to the endogenous ribosomal protein S18 (house-keeping gene) and then calculated using the comparative ΔCt method (20). Baseline expression levels of the control groups were set to 1. ($^{##}p<0.01$ or $^{###}p<0.001$ as compared to serum-starved cells treated with vehicle; $^{***}p<0.001$ as compared to control groups). Representative neurofibromin immunoblots (C and D) obtained using 50 μg of homogenates from MPNST cells cultured under the same experimental conditions for mRNA measurements showed comparable changes in neurofibromin expression both after 24 (C) and 48 h (D). ß-tubulin was used as loading control in each experiment. The diagrams show the results of three independent experiments.

DMSO (vehicle) and added with the maximum concentration of 7-OH-PIPAT (10^{-5} M) for 48 h (Fig. 6).

In agreement with our previous work (8), MPNST cells cultured in presence of normal levels of serum displayed a low intense immunosignal, which raised significantly in serum-deprived cells after 48 h. The addition of 0.1% DMSO

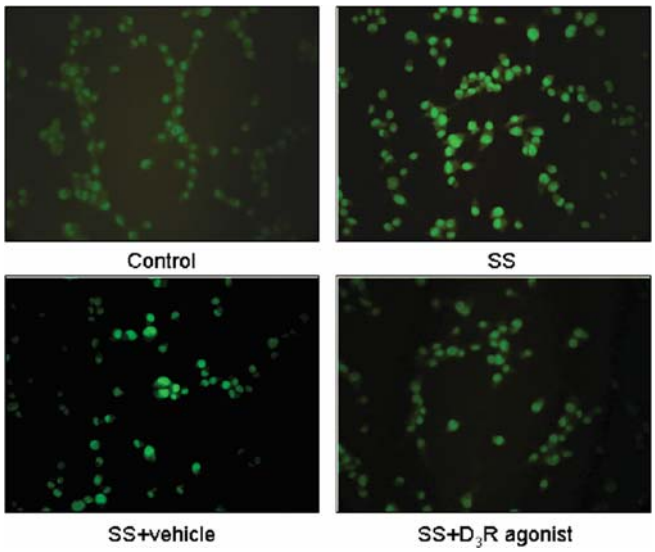

Figure 6. Immunofluorescence analysis of neurofibromin in serum-starved MPNST cells treated with 7-OH-PIPAT. Representative photomicrographs showing neurofibromin immunolocalization in MPNST cells cultured with 10% serum (control), serum-starved (SS), 0.1% DMSO (SS + vehicle) or additioned with the maximum concentration used (10^{-5} M) of the D_3R agonist (SS + D_3R agonist) after 48 h exposure. Photomicrographs shown are representative results taken from ten different fields from randomly selected slides. Magnification x20.

(vehicle) in the absence of serum did not produce appreciable changes on neurofibromin reactivity. Treatment with 10^{-5} M D_3R agonist induced a notable reduction of the immunosignal as compared to serum-starved cells added with vehicle only after 48 h exposure (Fig. 6).

Discussion

It has been reported that tumor cells undergoing serum starvation *in vitro* partially mimic metabolically stressed cells trying to adjust to a changed environment *in vivo* by inducing signal transduction and gene expression so that the tumor continues to grow (24). These adaptatory responses seem to be activated in the attempt to preserve tumor cells from death.

Our research group has previously shown that serum-starved MPNST cells undergo apoptosis (25) Thereafter, we have correlated this effect to changes in the expression of the NF1 tumor suppressor gene, suggesting that besides its Ras-dependent control on cell proliferation (26,27), NF1 might exert tumor suppression by conferring sensitivity to apoptosis (7,8). Interestingly, a functional link between the D_3R and neurofibromin have been proposed, corroborating the idea that D_3Rs might mediate a protective action after serum deprivation in MPNST cells through the interaction with the NF1 gene (18). To support this, several observations have already demonstrated that different non-selective dopamine-receptor agonists exert protective roles in response to various insults in different non-neuronal cell types (15-17), although the underlying mechanisms still need to be elucidated.

In the present study we showed the protective effects exerted by 7-OH-PIPAT, a selective D_3R agonist, on apoptosis

induced by serum deprivation in MPNST cells and correlated this effect to changes in NF1 mRNA and protein expression. We found that treatment of serum-deprived cells with raising concentrations (10^{-9}-10^{-5} M) of 7-OH-PIPAT induced a progressive increase in viability both after 24 and 48 h, although significant changes were observed only at the highest concentrations tested (Fig. 2). Consistent with these results, apoptotic tests performed under the same experimental conditions revealed a significant decrease in oligonucleosome formation at the latter two concentrations, thus suggesting that the increased viability observed was probably due to a D_3R-mediated antiapoptotic activity (Fig. 3). However, it was not clear why in our experiments with 7-OH-PIPAT produced significant effects only at the highest concentrations employed, although other studies have shown similar effects even at higher doses using non-selective compounds (17,28). To our knowledge, it is likely that D_3R-independent mechanisms should also be activated in order to completely rescue starved cells from apoptosis (29). For instance, it has been reported that some dopaminergic agents exert protective effects via trophic factors such as FGF-2 (30), NGF and GDNF (31). However, based on these findings, subsequent experiments were carried out using the concentrations of the D_3R agonist (ranging from 10^{-7}-10^{-5} M) that showed efficacy against apoptosis induced by serum deprivation. Morphological analyses of DNA fragmentation indicated that pretreatment with 7-OH-PIPAT partially reversed the evident apoptotic process activated by serum withdrawal after 48 h (Fig. 4).

The protective mechanism exerted by 7-OH-PIPAT against serum deprivation was then correlated to changes in both NF1 mRNA and protein expression by means of quantitative real-time PCR and Western blot analyses. Consistent with our hypothesis, the induction of NF1 expression as a consequence of serum deprivation was significantly reduced by D_3R agonist treatment both after 24 and 48 h (Fig. 5A-D). These results were confirmed by immunofluorescence analysis (Fig. 6). Although further investigations should be warranted in order to clarify the mechanisms by which D_3Rs are involved in the regulation of NF1 expression and its role in apoptosis, the common NF1 gene and protein induction observed during serum starvation and the subsequent inhibition induced by 7-OH-PIPAT treatment support the interaction proposed by Donarum *et al* (18) and suggest that D_3R agonists might play an important role in MPNST development.

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