Progesterone Inhibits the Growth of Human Neuroblastoma: *In Vitro* and *In Vivo* Evidence

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We investigated the antitumorogenic effects of progesterone (P4) in a human neuroblastoma (SK-N-AS) cell line *in vitro* and in a mouse xenograft model of neuroblastoma. The safety of P4 was tested in rat primary cortical neurons and human foreskin fibroblasts (HFF-1). At high doses, P4 significantly ($P < 0.05$) decreased SK-N-AS cell viability *in vitro*, and this effect was not blocked either by 5α-reductase inhibitor, finasteride or the P4 receptor antagonist RU486. Even at very high doses, P4 did not induce any cell death in healthy primary cortical neurons or HFF-1. The bioavailability of P4 24 h after the last injection in the serum of treated animals was significantly ($P < 0.05$) higher (10–33 μg/mL) than in untreated animals. In nude mice, P4 (50 and 100 mg/kg) inhibited neuroblastoma growth by ~50% over 8 d of treatment. No drug toxicity was observed in the mice, as measured by body weight and activity. P4 suppressed the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP-9, MMP-2), which are involved in tumor vascular development. High-dose P4 inhibited tumor growth by suppressing cell proliferation and inducing apoptosis, as evidenced by the expression of proliferating cell nuclear antigen and cleaved caspase-3. P4 significantly increased the expression of P4 receptor isoform-A and suppressed phospho-Akt (Ser437) expression. In conclusion, at high doses, P4 effectively inhibits the growth of solid neuroblastoma tumor and has high bioavailability, selective toxicity and a high margin of safety, making it a possible candidate for further study as a potential clinical treatment of neuroblastoma.

INTRODUCTION

Neuroblastoma is the most common tumor among children under 1 year of age, accounting for about 28% of all cancers diagnosed in European and U.S. infants, and frequently recurs in high-risk cases (1). Despite a number of phase III and IV clinical trials for new strategies against neuroblastoma, a safe and effective drug without the adverse side effects associated with high drug doses, radiation or chemotherapy has not yet been found.

One such agent may be the neurosteroid progesterone (P4), a neuroprotective and pleiotropic drug with several mechanisms of action. We recently reported that a combination of P4 with vitamin D hormone affords better neuroprotection than P4 alone in an *in vitro* excitotoxicity model of brain damage (2). In the course of establishing this experimental model, we observed that high concentrations of P4 (20, 40 and 80 μmol/L) were neuroprotective against a cytotoxic challenge in primary cortical neurons but caused significant cell death in a tumor cell line, PC-12 (pheochromocytoma). These observations led us to consider the possibility that P4 may have selectively toxic effects capable of inhibiting neuroblastoma growth.

P4 supports normal development by protecting the fetus from immune inflammatory attack during gestation. Fetal growth must be well controlled for normal development. The outer ring of placenta cells are extremely aggressive, behaving much like tumor cells as they invade the uterine wall and tap into the mother's blood vessels. Among the numerous factors that control cell cycle machinery to check abnormal growth, P4 plays a pivotal role in meiosis and mitosis (3) by regulating the activity of positive regulators (cyclins and CDKs) and inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$) of the cell cycle through the P4 receptor (PR) (4). High natural P4 levels during pregnancy are associated with a lower incidence of maternal breast cancer and also appear to exert a long-term protective effect against breast cancer (5). The antiproliferative and apoptotic effects of P4 have been reported for breast, endometrial, ovarian, colon and salivary gland tumors

in vitro and *in vivo* (6–9). Taken together, these facts suggest ways in which natural P4 might function as a chemotherapeutic agent against neurogenic tumors such as neuroblastoma.

In this chemotherapeutic study, we used natural P4 because increasing evidence suggests that it has several advantages over synthetic progestins, which can exert non–P4-like effects (10,11). Liang *et al.* (12) reported convincing evidence that synthetic progestins do not act the same way as P4 and in fact may promote breast cancer development in human patients and other species, which can be suppressed by blocking vascular endothelial growth factor (VEGF) expression (13). The combination of some progestins with estrogen in hormone replacement therapy has resulted in an increased risk of breast cancer because these agents tend to increase cell proliferation in breast tissue (14). Recently, several clinical trials found that, in hormone replacement therapy, the combination of estrogen with a progestin such as medroxyprogesterone acetate (MPA) increased the risk of breast cancer (15). Two cohort studies followed over mean durations of 7 and 9 years showed no increase in breast cancer risk when estrogen was combined with natural P4, but there was an additional risk when synthetic progestins were used (16,17). Further, MPA has differential effects on cell proliferation and growth inhibition in benign and malignant human breast epithelial cells (18). There may be substantive metabolic differences between native and synthetic forms of P4 that could account for some of the conflicting clinical trial outcomes. The metabolism of natural P4 in normal breast tissue generates products that have anticarcinogenic activities (19), but there is also a risk that chronic dosing with low-dose P4-implanted pellets can stimulate growth of breast cancer tumor cells implanted into nude mice (12). Moreover, natural P4 and MPA have different target genes in breast cancer cell lines, as evidenced by a cDNA microarray study of 3,000 genes (20).

In this report, we present proof-of-concept for P4's antitumor effects against neuroblastoma and propose that high-dose P4 may be worth considering as a safe and effective treatment for this disease. We asked the following: (a) As observed in PC-12 cells, does natural P4 at high concentrations induce cell death in neuroblastoma? (b) Does P4 mediate its cytotoxic effects through its metabolite allopregnanolone (ALLO)? (c) Does P4 cause cytotoxicity via the classic P4 receptor (PR)? (d) Can a vehicle (control) agent at given high concentrations also induce nonspecific cell death in tumor cells *in vitro*? (e) Is P4 effective in killing tumor cells *in vivo*? We hypothesized that P4 would selectively induce cell death in tumor cells *in vitro* but would have no damaging effects in healthy primary cortical neurons at high concentrations. The next step was to determine whether P4 treatment could suppress tumor development in a live animal model of neuroblastoma. Accordingly, we implanted a neuroblastoma cell line in healthy nude mice and then examined the effect of 8 days of P4 treatment on the following: tumor growth, markers of tumor proliferation (proliferating cell nuclear antigen [PCNA]), vascularization (VEGF), matrix metalloproteinases (MMPs, CD31), apoptosis (cleaved caspase-3, transferase-mediated dUTP nick-end labeling [TUNEL]), and finally, the expression of PR isoforms A and B (PR-A, PR-B) and phospho-Akt (Ser473) signaling. These were some of the possible mechanisms of P4's action in reducing tumor growth.

MATERIALS AND METHODS

Cell Culture

Rat pheochromocytoma (PC-12) and human neuroblastoma (SK-N-AS) cell lines were purchased from ATCC (Manassas, VA, USA). E18 rat primary cortical neurons were procured from Genlantis (N200200, San Diego, CA, USA). Human primary fibroblasts (HFF-1) were a gift from Prof. Erwin Van Meir (Neurosurgery, Emory University School of Medicine, Atlanta, GA, USA). Cells were grown in multiwell plates in their respective culture medium, as directed by the manufacturer at 37°C under 5% CO_2 environment. Primary neurons were cultured in Neurobasal medium (GIBCO/Invitrogen, Carlsbad, CA, USA) containing a B27 supplement (GIBCO) for 8–10 d before experiments.

Cell Viability and P4, Mifepristone and Finasteride Treatments

Tumor cells were kept under starvation for 24 h before use. For all *in vitro* experiments, P4 (P3972; Sigma, St. Louis, MO, USA), RU486 (mifepristone; M8046; Sigma) and finasteride (F1293; Sigma) stocks were prepared in absolute dimethylsulfoxide (DMSO) and further diluted in culture medium. The final concentration of DMSO was kept at <5 μL/mL. Cells were exposed to different concentrations of P4 (0.1, 1, 5, 10, 20, 40 and 80 μmol/L) either as a single exposure for 48 h or as repeated exposures for 3 and 6 d. For single exposure experiments, P4 was added once and cell viability was evaluated 48 h after exposure. For repeated exposure, the culture medium was replaced daily and P4 was added to the cells every day. RU486 and finasteride were tested in the same manner as P4, either alone or in combination with the different doses of P4. The doses of RU486 were the same as P4. For the finasteride study, we combined 10 μmol/L of P4 with either 10 or 20 μmol/L of finasteride and 20 μmol/L P4 with either 20 or 40 μmol/L finasteride. On the third and sixth day, cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 20 μL MTT solution (5 mg/mL phosphate-buffered saline [PBS]) was added per well and incubated at 37°C for 4 h until purple precipitate was visible. DMSO (50 μL) was added to solubilize the crystals, and the absorbance was read at 570 nm with a microplate reader (EL808; BIO-TEK Instruments, Winooski, VT, USA).

Inoculation of Mice and P4 Treatment

To replicate our findings, we conducted two separate experiments in

mouse xenograft models of human neuroblastoma. SK-N-AS cells (3×10^6) were mixed 1:1 with Matrigel (BD Biosciences, San Jose, CA, USA) and injected subcutaneously into the right flanks of female athymic nude mice (Hsd: Athymic Nude-*Foxn1^{nu}*; Harlan, Indianapolis, IN, USA). Protocols (204–2009) were approved by the Institutional Animal Care and Use (IACU) Committee, Emory University. Tumors were allowed to grow to 200–230 mm^3, and tumor-bearing animals were then separated into three groups (n = 12): vehicle, P4 50 mg/kg (P50) and P4 100 mg/kg (P100). Two additional groups of non–tumor-bearing mice served as drug controls for the two different doses of P4 (50 and 100 mg/kg) and to check drug toxicity on the basis of survival, body weight and activity. P4 was prepared as a homogenous suspension in saline solution, and a single peritumoral injection of either P4 suspension or vehicle alone was given daily for 8 d.

Tumor Measurement

Tumors were monitored by caliper measurement daily, and tumor volume (*V*) was calculated using the formula $[V = 0.4 \times a \times b^2]$, where *a* is the largest tumor diameter and *b* is the smallest tumor diameter (21).

Activity Scoring

Activity was measured using the following scale: 0 = no activity, unresponsive to stimulation; 1 = light movements, lethargic; 2 = moderately alert, moving around, no standing up; 3 = very active, highly alert, standing up.

P4 Bioavailability

Serum P4 assay was done at the Biomarkers Core Laboratory, Yerkes National Primate Research Center, Emory University, by radioimmunoassay using a commercially prepared kit (Siemens Health Care, Los Angeles, CA, USA).

Immunohistochemistry

Tumors were removed by excision, weighed and then cut in half. Half of each tumor was then fixed in 10% buffered for-

malin and the other half was snap-frozen in liquid nitrogen for Western blot analysis. Samples were fixed for 24 h, embedded in paraffin, sectioned at 5 μm and immunostained for CD31 (immunofluorescence), cleaved caspase-3 (Asp175) and phospho-Akt (Ser473), which were purchased from Cell Signaling Technology (Danvers, MA, USA). Briefly, sections were deparaffinized, rehydrated and treated with 3% hydrogen peroxide in distilled water for 20 min. The sections were labeled by the avidin-biotin-peroxidase procedure (22). Primary antibodies were applied at 4°C overnight. The reaction was revealed by incubating the sections with 3,3 diaminobenzidine (Vector Laboratories, Burlington, CA, USA) for 1–2 min; the sections were counterstained for 1 min with hematoxylin. The images were captured with an Olympus BX41 microscope using Image-Pro software.

Western Blot Analysis

Protein was extracted from tumor tissue using T-per extraction buffer (Pierce, Rockford, IL, USA) with protease inhibitors and assayed for protein concentration by BCA microplate protein assay (23225; Pierce). Protein samples (50 μg) were separated under reducing and denaturing conditions by 4–20% acrylamide Criterion gel (Bio-Rad, Hercules, CA, USA) at 200 V for 1 h and transferred to a polyvinylidene difluoride membrane at 100 V for 30 min. The nonspecific binding sites of the membrane were blocked with 5% nonfat dry milk in PBS-T (PBS containing 0.05% Tween-20). The membranes were probed with VEGF (A-20), MMP-2 (H-76), MMP-9 (C-20), PR (AB-52), PCNA (PC-10; Santa Cruz Biotech, Santa Cruz, CA, USA) and β-actin (AC74; Sigma) primary antibodies overnight at 4°C and then incubated in their respective horseradish peroxidase–conjugated secondary antibodies. Blots were developed using a chemiluminescent substrate (Pierce) for 5 min. Chemiluminescent bands were detected on a Kodak autoradiography film in a darkroom, and their densities were measured using Bio-Rad Gel-Doc Quantity-One 4.6.1 software.
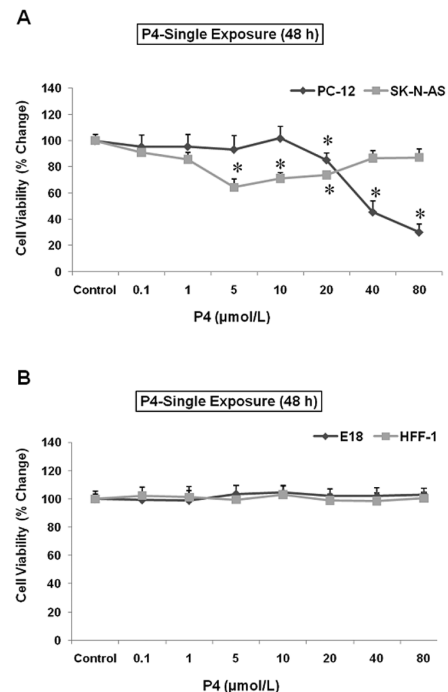

Figure 1. Effect of a single P4 exposure on the viability of PC-12 and SK-N-AS (A) and primary cortical neurons and HFF-1 cells (B). Cells were exposed to different concentrations of P4 (0.1, 1, 5, 10, 20 and 80 μmol/L) for 48 h. The stock solution of P4 was prepared in DMSO (final concentration <5 μL/mL medium). Cell death was measured by MTT assay. Data are expressed as means ± standard deviation (SD) of three independent experiments. Significant difference: *$P < 0.05$ compared with vehicle group.

TUNEL Assay

Detection of apoptosis in neuroblastoma tumor tissue taken from P4-treated or vehicle-treated mice was done using an *in situ* cell death detection kit, fluorescein (11684795910; Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions.

Statistical Analysis

The statistical analyses of data were performed with analysis of variance (ANOVA) followed by Dunnett's test to compare several treatment groups to a single control group. The significance of results was set at $P < 0.05$. For densitometric and cell counting analysis, data
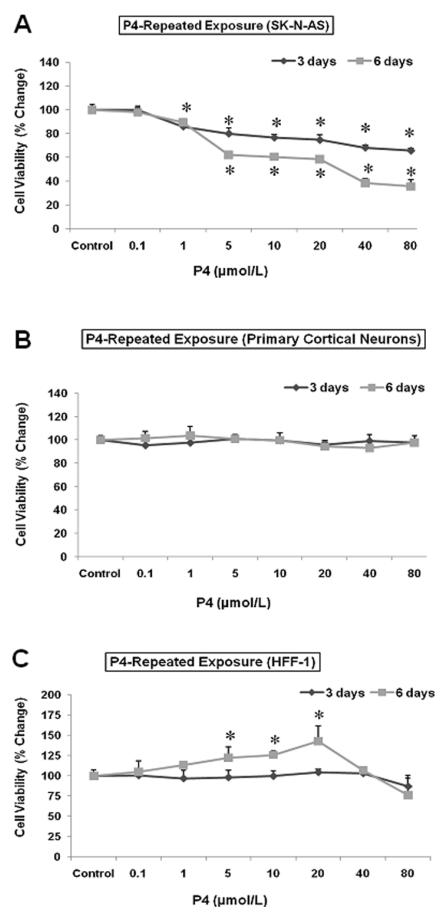
Figure 2. Effect of repeated exposures of P4 on the viability of SK-N-AS (A), primary cortical neuron (B), and HFF-1 cells (C). Cells were exposed to different concentrations of P4 daily for 3 and 6 d by replacing the culture medium containing P4. The stock solution of P4 was prepared in DMSO (final concentration <5 μL/mL medium). Cell death was measured by MTT assay. Data are expressed as means ± SD of three independent experiments. Significant difference: *$P < 0.05$ compared with vehicle group.

were analyzed using a two-tailed unpaired *t* test.

RESULTS

Single Exposure to P4 in PC-12 and SK-N-AS Cells

A single 48-h exposure to P4 at a range of concentrations (0.1, 1, 5, 10, 20, 40 and 80 μmol/L) caused significant ($P < 0.05$) cell death at higher concentrations (20, 40

and 80 μmol/L) in PC-12 cells, but no toxicity was observed at lower concentrations (0.1, 1, 5 and 10 μmol/L) (Figure 1A). SK-N-AS cells exposed to P4 produced a U-shaped response curve for viability. There was significant ($P < 0.05$) loss at 5, 10 and 20 μmol/L, but other concentrations did not induce cell death (see Figure 1A).

Single P4 Exposure in Primary Cortical Neurons (E18) and Fibroblasts (HFF-1)

Healthy primary rat cortical neurons were exposed to different concentrations of P4 as described above. No cell death was observed at any concentration (Figure 1B). We took this to indicate that P4 is safe in healthy tissue. Because primary cortical neurons are nondividing cells, we wanted to test the effect of P4 on healthy dividing cells. No toxicity was observed *in vitro* in human fibroblasts (HFF-1) exposed to the different concentrations of P4 for 48 h (see Figure 1B).

Repeated P4 Exposure in SK-N-AS, Primary Cortical Neurons and HFF-1 Cells

SK-N-AS cells were exposed to different concentrations of P4 for 3 and 6 d. A significant ($P < 0.05$) increase in cell death was observed after both 3 and 6 d of exposure (Figure 2A). Maximum cell death was observed at the highest concentration (80 μmol/L). It is important to emphasize that repeated exposures of P4 for 3 and 6 d did not produce any death in either primary cortical neurons or HFF-1 cells (Figures 2B, C). Instead P4 showed significant proliferative effects in HFF-1 fibroblasts at 5, 10 and 20 μmol/L concentrations over the 6 d of exposure.

2-Hydroxypropyl-β-Cyclodextrin (HBC) in PC-12 and SK-N-AS Cells

To test the hypothesis that a control vehicle at high concentrations could induce death in tumor cells, we used 2-hydroxypropyl-β-cyclodextrin (HBC), an inert cyclic oligosaccharide commonly used in animals as a vehicle for lipophilic drugs such as P4. Different concentrations of HBC (0.1, 1, 5, 10, 20,
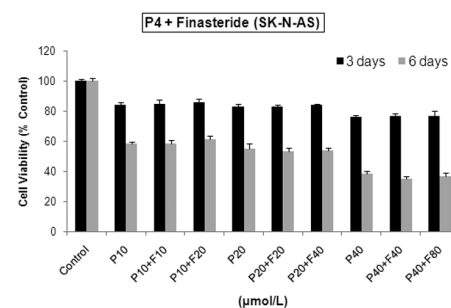

Figure 3. The cytotoxic effect of P4 does not require metabolization. SK-N-AS cells were repeatedly exposed to either three higher cytotoxic concentrations (10, 20 and 40 μmol/L) of P4 alone or combined with different concentrations of finasteride for 3 and 6 d by replacing the culture medium daily containing drugs. Cell death was measured by MTT assay. Data are expressed as means ± SD of three independent experiments. Finasteride (F) could not block the cytotoxic effect of P4 (P) at any concentration.

40 and 80 μmol/L) were exposed to PC-12 and SK-N-AS cells for 3 and 6 d. As measured by MTT assay, no increase in death was found in any of the cell lines at any concentration of HBC (data not shown).

Cytotoxic Effect of ALLO in Repeated P4 Exposure in SK-N-AS Cells

To test whether P4 exerts cytotoxic effects in SK-N-AS cells through its active metabolite ALLO, SK-N-AS cells were exposed to high concentrations of P4 (10, 20 and 40 μmol/L) alone or combined with the 5α-reductase inhibitor, finasteride (10, 20, 40 and 80 μmol/L) to block the conversion of P4 into ALLO. At days 3 and 6, we observed that finasteride could not block or even modulate the cytotoxic effects of P4's high concentrations in SK-N-AS cells (Figure 3). These findings suggest that the cytotoxic effect of P4 is not mediated by P4 conversion to ALLO.

Repeated Exposure of P4 and RU486 in SK-N-AS Cells

Figures 4A and B show the effects of repeated exposure of P4, RU486 and
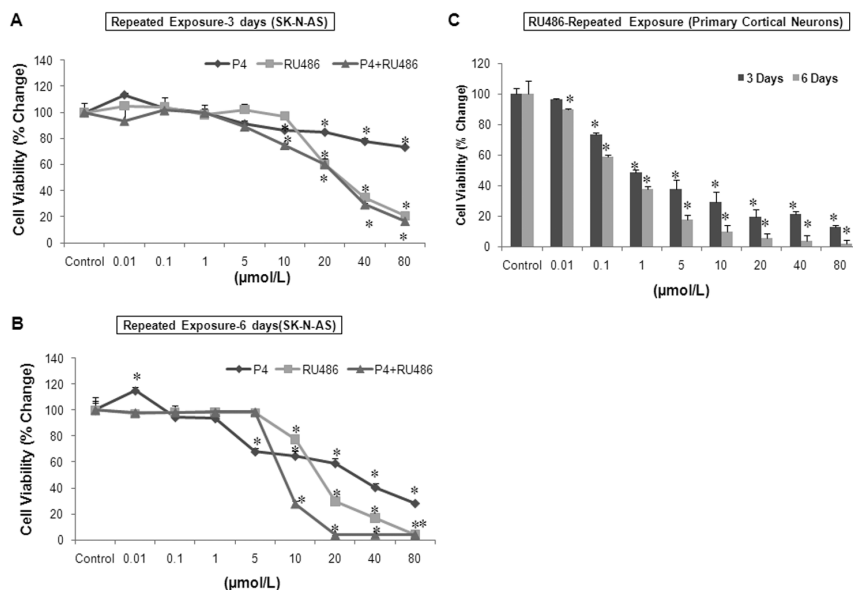
Figure 4. Effect of repeated exposures of P4, RU486 and their different combinations on the viability of SK-N-AS cells (A) and the effect of RU486 on the viability of primary cortical neurons (B). Data are expressed as means ± SD of two independent experiments. Significant difference: *$P < 0.05$ compared with control (vehicle) group.

their different combinations at 3 and 6 d. In the presence of a low concentration (0.01 μmol/L) of P4, SK-N-AS cells showed a slight nonsignificant increase in cell viability/proliferation above control values at day 3, which became significant ($P < 0.05$) at day 6. But the proliferative effect was significantly decreased ($P < 0.05$) and cell death was observed at high concentrations of P4 (5, 10, 20, 40 and 80 μmol/L) at both 3 and 6 d. The addition of RU486 blocked the proliferative effect of low P4 concentrations but failed to block the cytotoxic effect of P4 at high concentrations. RU486 alone showed a P4 mimetic effect at high concentrations but, when P4 and RU486 were combined, a marked enhancement of the toxic effect was observed.

Repeated RU486 Exposure in Primary Cortical Neurons

We tested whether RU486 is safe at high concentrations in rat primary cortical neurons. Unlike P4, repeated exposure of primary cortical neurons to RU486 resulted in significant ($P < 0.05$) cell death in a concentration-dependent manner over 3 and 6 d (Figure 4C).

P4 in Tumor Growth *In Vivo*

In light of the findings from our cell culture experiments, we sought to determine whether P4 would be effective in inhibiting neuroblastoma growth in a nude mouse xenograft model of human neuroblastoma. Mice with induced tumors were treated daily with P4 doses of 50 or 100 mg/kg or saline solution (n = 12/group). When data from two iterations of this procedure were combined, after 1 wk of peritumoral injections of P4, we observed an ~50% inhibition of tumor growth, as measured by caliper ($P < 0.05$) at both doses, compared with mice given only vehicle (Figures 5A, D). No difference in tumor growth was noted between the two P4 doses.

Toxicity of P4 was assessed by survival, activity and daily measures of body weight. All animals survived the P4 treatment without evidence of toxic effects and the mice gained 10% to 15% of their pretreatment body weight. Further, non–tumor-bearing mice receiving only P4 (50 or 100 mg/kg) did not show any signs of toxicity or ill effects. There was a significant decrease ($P < 0.05$) in tumor weight in the 50 and 100 mg/kg groups compared

with the vehicle group (Figure 5B). We observed no difference in tumor weight between the two doses of P4.

Bioavailability of P4

Analysis of serum collected 24 h after the last treatment revealed very high levels of P4 in both the 50 and 100 mg/kg groups (Figure 5C). Average serum P4 levels were 14.91 ng/mL in mice given 50 mg/kg, whereas mice given 100 mg/kg showed 31.14 ng/mL P4. The vehicle group level was 5.16 ng/mL.

P4 Affects the Expression of MMP-2, MMP-9, VEGF and CD31

Western blot analysis of tumor tissue revealed a significant ($P < 0.05$) decrease in the expression of MMP-2 (pro- and active), MMP-9 and VEGF in both P4-treated groups compared with vehicle (Figures 6A–C). There was no significant difference between the two doses of P4 in modulating the expression of MMPs and VEGF. Qualitative analysis of CD31 immunostaining, a marker of endothelial cells in tumor tissue, revealed that P4 at both doses reduced the expression of CD31 compared with vehicle (Figure 7). Because MMP-2, MMP-9 and CD31 play positive roles in activating angiogenesis, these findings raise the possibility that P4 has antiangiogenic effects in neuroblastoma.

P4 Inhibits Neuroblastoma Cell Proliferation

Densitometric analysis of Western data revealed a significant ($P < 0.05$) decrease in the expression of PCNA in P4-treated groups compared with vehicle (Figure 6D). P4 at both doses reduced cell proliferation by ~41%.

P4 Modulates the Expression of PR-A and PR-B in Neuroblastoma

We examined the protein expression of PR-A and PR-B in mouse tumor tissue. P4 treatment produced a significant ($P < 0.05$) increase in PR-A expression of ~82% and 93% at 50 and 100 mg/kg doses, respectively (Figure 6E). Although we observed ~26% and 21% decreases in PR-B expres-
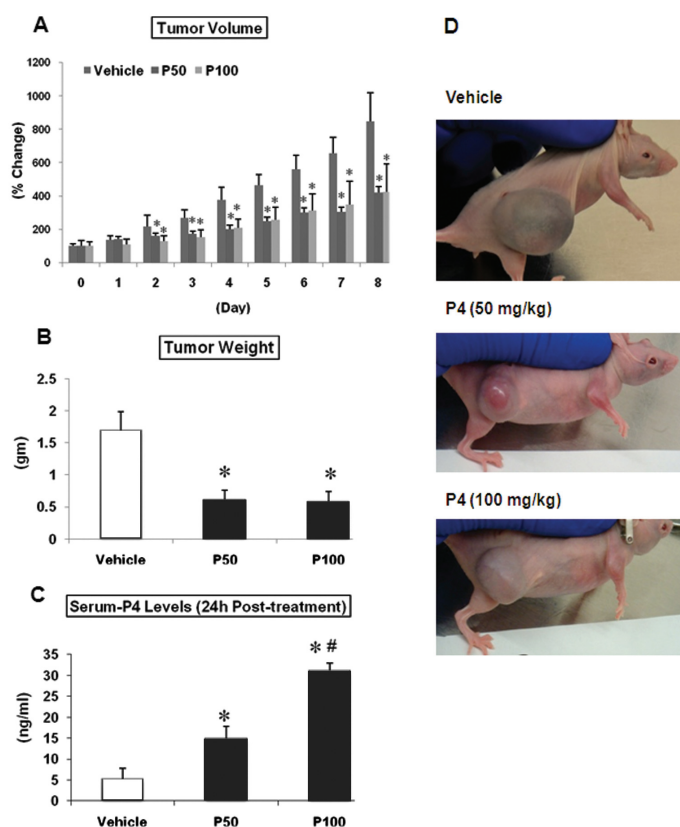
Figure 5. Tumor inhibitory effects of P4 against neuroblastoma (n = 12/group). Tumor volume (A), tumor weight (B), serum P4 levels (C) and representative photographs of tumors (D) in different groups after P4 treatment are shown. Data are expressed as percent change in means ± standard error of the mean (SEM) of tumor volume in different groups (n = 12) from two independent experiments. Significant difference: *$P < 0.05$ compared with vehicle group.

sion with 50 and 100 mg/kg doses of P4, respectively, they were not significant.

P4 Induces Apoptosis in Neuroblastoma

Immunohistochemistry for cleaved caspase-3 in the tumor tissue as a marker of apoptosis revealed a highly significant ($P < 0.05$) increase in apoptotic cells in both P4-treated groups (Figure 8A) compared with vehicle. We further confirmed this effect by TUNEL assay to detect apoptotic cells in tumor tissue in the different groups. We observed very few TUNEL-positive cells in the vehicle group (Figure 8B). After treatment with P4 at both doses, a marked increase in TUNEL-positive cells was observed, suggesting that P4 exerts an apoptosis-inducing effect in neuroblastoma.

P4 Suppresses Akt Signaling in Neuroblastoma Cells

Immunostaining of tumor tissue showed that P4 treatment at both doses (Figures 9B, C) suppressed Akt signaling, as evidenced by a significant decrease in phospho-Akt cells compared with vehicle-treated mice (Figure 9A). This observation indicates that Akt signaling is involved in the antitumor effects of P4.

DISCUSSION

Taken together, our findings strongly indicate that, at high doses, P4 suppresses the growth of neuroblastoma, *in vitro* and *in vivo*, without having toxic effects on healthy neural cells and fibroblasts. Over the past several decades, researchers and clinicians have developed pediatric cancer therapies that show ro-

bust survival with low- and intermediate-risk neuroblastoma, but the overall survival for high-risk neuroblastoma remains below 50% (23). One of the major problems associated with chemotherapeutic agents is their cytotoxic actions not only on cancer cells but also on healthy tissue. An agent that selectively kills cancer cells but remains safe for healthy cells could be an important breakthrough for future chemotherapeutic strategies.

In this study, when healthy primary cortical neurons and human fibroblasts were exposed to different doses of P4 in either single or repeated exposures, no cell death was observed at any concentration. Our findings can be taken to suggest some P4 specificity in inducing the death of tumor cells. Thus, the hormone apparently can differentiate between normal healthy cells and tumor cells and specifically induces cell death in neuroblastoma while remaining nontoxic or protective for normal cells. The exact signaling mechanisms for this action remain to be identified. We previously reported that P4 at high concentrations (10, 20, 40 and 80 μmol/L) prevented neuronal death in primary cortical neurons exposed to glutamate toxicity. Maximum protection was seen at 20 μmol/L (2). In contrast, here we report cytotoxic effects of P4 at the same high concentrations (10, 20, 40 and 80 μmol/L) in neuroblastoma cells, suggesting that P4 has pleiotropic actions. Our findings with finasteride suggest that P4 itself regulates cytotoxicity in SK-N-AS cells; its conversion to ALLO is not necessary to obtain these effects.

Having observed promising results *in vitro*, we tested two doses of P4 (50 and 100 mg/kg) in a mouse xenograft model of neuroblastoma using human SK-N-AS cells. We selected the high P4 doses on the basis of our *in vitro* data and our previous reports that P4 is neuroprotective at lower doses (8 and 16 mg/kg) against traumatic brain injury and stroke (24–26). In the current experiment, we found a potent inhibitory effect of high-dose P4 on neuroblastoma tumor growth in nude
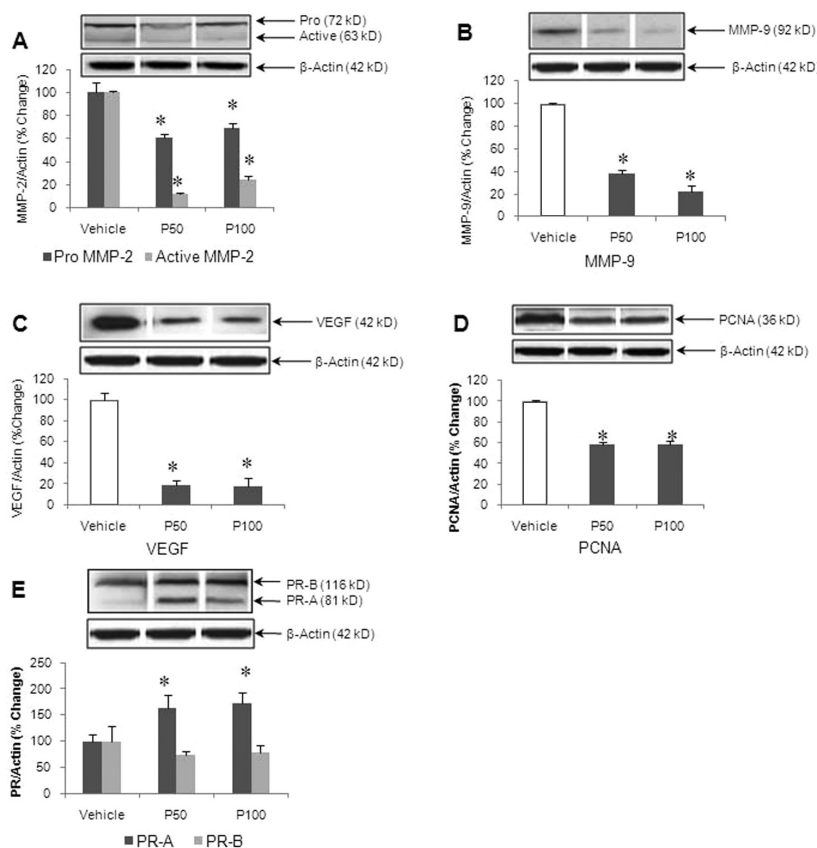
Figure 6. P4 modulates proliferation and vascularization of neuroblastoma tumor. Western blot and densitometric analysis of the expression of vascularization (A–C) and proliferation (D) markers and PR (E) in neuroblastoma are shown. Significant difference: *$P < 0.05$ compared with vehicle group. P50, P4 (50 mg/kg); P100, P4 (100 mg/kg).

mice. After only 8 d of treatment, P4 at both high doses reduced the average tumor weight and tumor volume by ~50% compared with vehicle. No significant difference in tumor volume reduction was observed between the two high doses of P4. Because treatment began when the tumor was already large (~200–230 mm^3), we surmise that P4 can reduce the growth of large neuroblastoma tumors of SK-N-AS origin.

In a comparative study, Wiehle *et al.* (27) tested RU486, P4 and CDB-4124 (nor-progestin) against mammary tumors *in vivo* and observed that P4 treatment at 10 mg/kg for 28 days increased the size and number of tumors in female rats. We speculate that P4's tumor-promoting effect in the Wiehle *et al.* study could be because of the lower dose used compared with the present study,

demonstrating a hormetic effect of the hormone. In our study, tumor suppression required 5–10× the dose in the experiments performed by Wiehle *et al.*, further suggesting that P4 has a bimodal effect.

In another study, Benakanakere *et al.* (11) reported that both MPA and natural P4 accelerate 7,12-dimethylbenz(*a*) anthracene (DMBA)-initiated mammary tumors and increase angiogenesis in rats. They showed that P4 treatment 4 weeks after DMBA treatment increases the incidence of mammary tumors but does not affect the latency period, whereas MPA treatment significantly reduced the latency period and increased tumor incidence. We speculate that the high incidence of tumors Benakanakere *et al.* observed with P4 is attributable to the low dose (10 mg/60-day release,

0.166 mg/day) they used compared with our study (~2.2–2.5 mg/day). Although our experimental design is different, the results of Benakanakere *et al.* support our *in vitro* findings showing a proliferative effect of a very low concentration (0.01 µmol/L) of P4 in SK-N-AS cells.

Serum P4 analysis 24 h after the last injection revealed very high levels of P4 in treated animals, indicating high bioavailability of P4. Our experiments demonstrate that high serum P4 levels are sufficient to reduce tumor growth of SK-N-AS origin. Toxicity of treatment was assessed by survival, activity and daily body weight in the tumor-bearing mice. We also injected P4 at both 50 and 100 mg/kg doses in non–tumor-bearing healthy nude mice to check any possible toxicity of P4. We observed no mortality, and all nontumor animals survived the high P4 doses with no evidence of toxic side effects. In fact, the mice gained >10% of baseline body weight values. These findings suggest a positive safety profile for high doses of P4.

Because P4 is a pleiotropic agent with a variety of molecular mechanisms, it is likely that multiple gene or receptor-specific mechanisms are involved in its antitumor action against SK-N-AS neuroblastoma. In support of the notion that P4 is pleiotropic, a recent microarray study reported that high doses of P4 applied to endometrial tumor cells affected the expression of 247 genes, including many involved in the control of the cell cycle, proliferation, differentiation and the immune inflammatory response (28). To elucidate the mechanisms underlying its antitumoral activity in our model, we measured the effects of P4 on markers of tumor proliferation (PCNA), vascularization and angiogenesis (VEGF, MMP-2 and MMP-9) and apoptosis (cleaved caspase-3). It is well established that MMPs facilitate tumor cell proliferation by creating a vascular stroma. Increased expression of MMP-2 and MMP-9 was reported in advanced stages of neuroblastoma (29). Our data support this finding, since we observed overexpression of pro-/active MMP-2, MMP-9 and
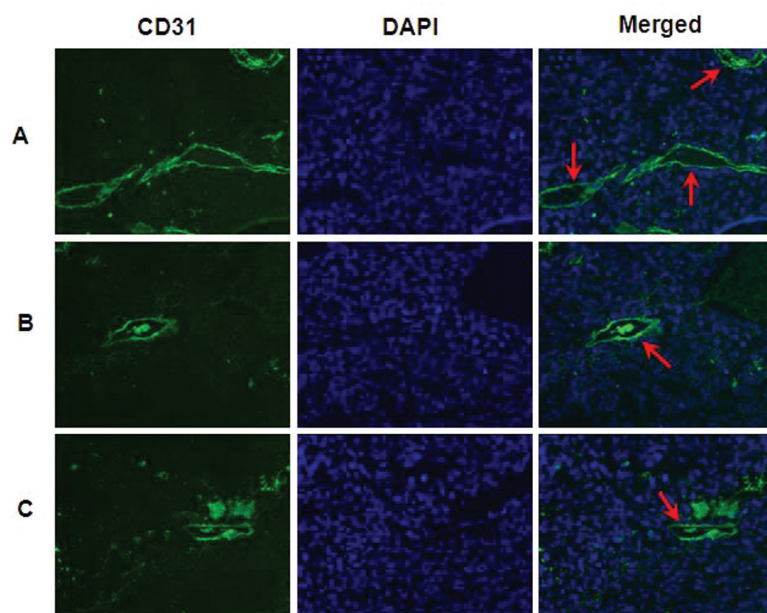
Figure 7. Effect of P4 treatment on tumor vascularization. Tumor tissue sections were fluorescence stained with anti-CD31 antibody (endothelial marker) and counter-stained with di aminido phenyl indol (DAPI). Representative photomicrographs of different groups are shown: vehicle (A), P4 (50 mg) (B) and P4 (100 mg) (C). The arrow shows CD31-positive endothelial cells.

VEGF in vehicle-treated animals. P4 at high doses significantly inhibited the expression of all these factors in the tumor tissue, thus depriving the tumors of vascular support. We confirmed this effect by staining with CD31, an endothelial marker, and observed a marked decrease in CD31-positive endothelial cells in P4-treated groups.

We noted a marked decrease in tumor cell proliferation and an induction of apoptosis in tumor tissue after 8 days of P4 treatment in nude mice, as evidenced by PCNA expression, cleaved caspase-3 and TUNEL assay, respectively. P4 has previously been reported to inhibit proliferation and induce apoptosis in a variety of tumor cell lines other than neuroblastoma (9,30). We tested whether these antiproliferative and apoptotic effects of P4 were associated with Akt signaling. Akt is a crucial regulator of cell proliferation, differentiation, metabolism and apoptosis (31). Akt/PI3K signaling is reported to control apoptosis in neuroblastoma, and its caspase-dependent destruction irreversibly inactivates survival signaling (32). We noted high phospho-Akt (Ser473) levels in the vehicle group, which was significantly inhibited by P4 treatment at both doses. It appears that P4 has potent antiproliferative and proapoptotic effects in neuroblastoma, which may be, at least in part, mediated by Akt signaling.

We analyzed the expression of PR-A and PR-B in neuroblastoma tumors of SK-N-AS origin *in vivo*. The two forms of the PR (PR-A and PR-B) are translated from the same gene but have individual effects (33) and unique sets of target genes (34). The development, invasiveness and metastatic potential of carcinoma cells may thus be influenced by the functional and transcriptional differences of the PR. PR expression has been inversely correlated with PCNA expression in patients with malignant melanoma (35). It was suggested by Inoue *et al.* (36) that P4 may promote the growth of tumor cells via PR-B while inhibiting growth via PR-A in meningiomas and astrocytic tumors. These authors suggest that the introduction of selective PR

modulators could contribute to the treatment of neurogenic tumors. Similarly, Kim and Chapman-Davis (30) reported that PR-A plays a role in reducing tumor proliferation in the uterus of laboratory mice, while PR-B induces cell growth, providing indirect support for what we observed in our tumor model. In the present study, we found that PR-B expression was more pronounced than that of PR-A in tumor tissue *in vivo*. We emphasize that PR-A expression was significantly higher in P4-treated groups compared with vehicle, but no significant difference among groups was observed in PR-B expression. These findings can be interpreted to suggest that PR-A and PR-B are differentially involved in the growth of SK-N-AS tumors and that a relationship between high doses of P4 and PR-A expression could be one of the mechanisms of P4-mediated reduction in tumor growth.

We further evaluated the effect of RU486 alone and in combination with P4 *in vitro* to test whether the cytotoxic effects of P4 in SK-N-AS cells are PR mediated. We observed that P4 alone at a very low concentration (0.01 μmol/L) showed an increase in cell proliferation, and this proliferative effect was inhibited by RU486. However, higher doses of P4 (5, 10, 20, 40 and 80 μmol/L) were cytotoxic, and RU486 did not show any inhibitory response to the cytotoxic effect of P4. Administration of RU486 alone showed a P4 mimetic effect at high concentrations. Interestingly, when P4 and RU486 were combined, a marked enhancement of the antiproliferative effect was observed. Future research might demonstrate that, with proper dosing, tumor suppression can be further enhanced by this combinatorial therapy. These findings strongly suggest both genomic and nongenomic steroid actions at low and high concentrations, respectively. The genomic proliferative effect of P4 at low concentration was inhibited by RU486, whereas the antiproliferative/cytotoxic effect of high concentrations of P4 is likely due to nongenomic mechanisms that are still to be determined. Our findings are in agreement
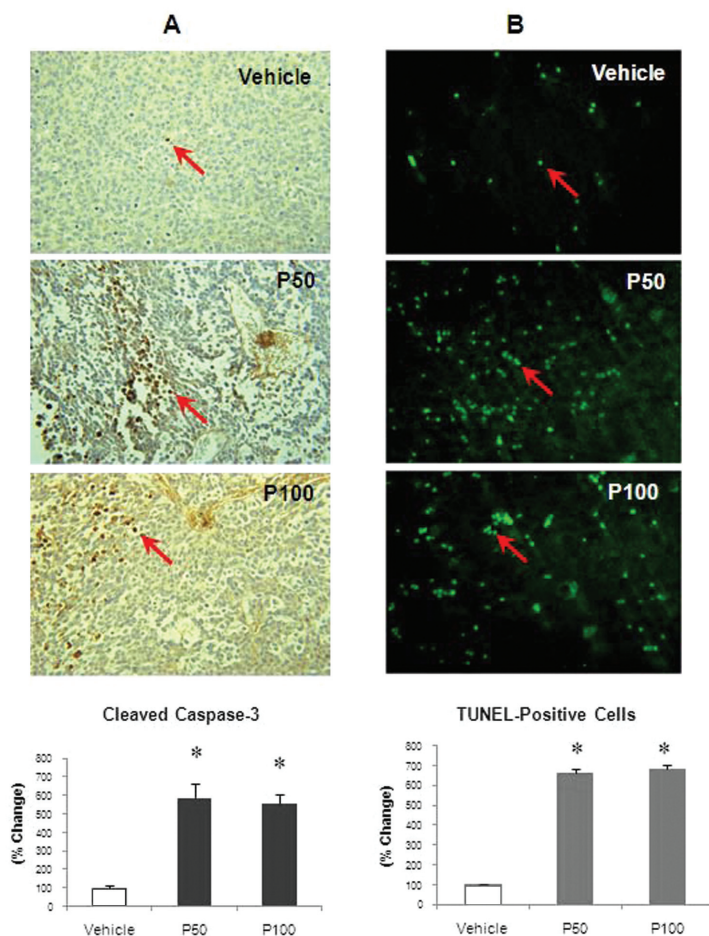
Figure 8. P4 inhibits neuroblastoma growth by inducing apoptosis. (A) Representative photomicrographs (40×) of immunohistochemistry and quantification of cleaved caspase-3–positive cells in tumor sections. The arrow shows the brown-stained cleaved caspase-3–positive cells. TUNEL assay was performed to confirm apoptotic effect of P4 in the neuroblastoma tumor. (B) Representative photomicrographs (20×) and quantification of TUNEL-positive cells in different groups. Data are expressed as means ± SD. Significant difference: *$P < 0.05$ compared with vehicle group. P50, P4 (50 mg/kg); P100, P4 (100 mg/kg).

Figure 9. P4 modulates AKT signaling in human neuroblastoma. (A) Representative photomicrographs (40×) of immunohistochemistry for phospho-Akt (Ser473) in tumor sections. The arrow shows the brown-stained phospho-Akt (Ser473) active cells. (B) Quantification of phospho-Akt (Ser473)-positive cells in different groups. Significant difference: *$P < 0.05$ compared with vehicle group. P50, P4 (50 mg/kg); P100, P4 (100 mg/kg).

with previous reports (37,38) suggesting that P4 at high concentrations has nongenomic actions. We speculate that the proliferative effect of a low concentration of P4 may be PR dependent, and this can be inhibited by RU486. Conversely, the antiproliferative/cytotoxic effects of P4 at a high concentration are nongenomic and not glucocorticoid receptor mediated, since RU486 also blocks the glucocorticoid receptor. In a recent study, Wang *et al.* (39) found that RU486 could partially reduce apoptosis in human lens epithelial cells, and "because of its toxicity," it did not prevent glucocorticoid-induced

cataracts. This partial effect could be dose dependent or receptor specific because, in cells from mammary tumors, low doses of progestins and RU486 were proliferative, whereas higher doses were inhibitory. Because RU486 showed a better cytotoxic effect in SK-N-AS cells than P4, we tested whether RU486 is a selective cytotoxic agent such as P4 and whether it is also safe in healthy primary cortical neurons. Unlike P4, repeated exposure to RU486 for 3 and 6 days significantly killed primary cortical neurons in a concentration-dependent manner. Given the marked differences in cell death out-
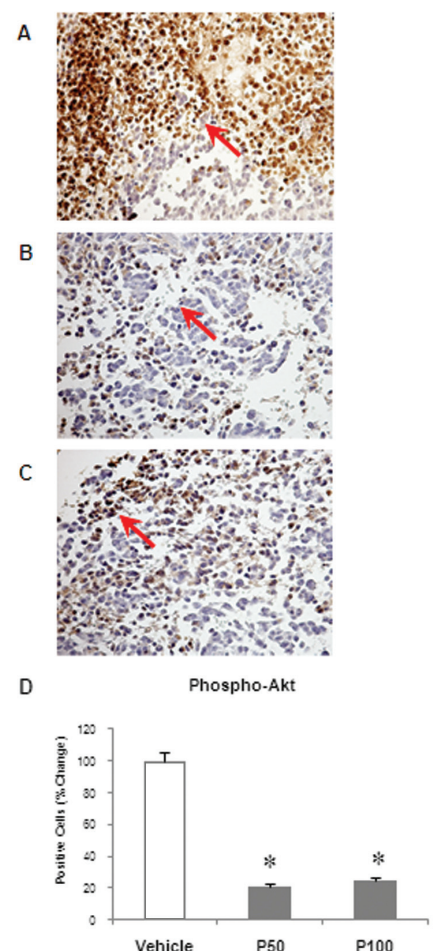
comes between RU486 and P4, it is highly likely that the two agents act through different receptor mechanisms.

Our *in vitro* findings suggest that P4 exerts a nongenomic cytotoxic action that is not PR dependent. Conversely, our Western blot data showed an increase in PR-A in tumor tissue *in vivo*. RU486 is not a specific PR antagonist because it also blocks glucocorticoid receptors. The mechanism by which RU486 inactivates

the PR is complex and not completely understood, although its use in reproductive medicine is based on its actions on the PR. With respect to tumor proliferation, RU486 can inhibit the growth of a number of different cancer cell lines. Recently, Tieszen *et al.* (40) reported that RU486 can inhibit cancer cells independently of the expression of the nuclear PR. In another study, Fjelldal *et al.* (37) exposed breast cancer and uterine cervix cancer cells to P4 or RU486 and found that, depending on the dose of both agents, the treatments could reduce cell density *in vitro* and were not due to their actions on the intranuclear P4 receptor. However, there are some data showing that RU486 may not be as highly specific as a P4 antagonist because of its partial PR-agonist activity (41,42). Here, we can also speculate that one of the reasons for its complex and sometimes contradictory effects is that RU486 is a selective blocker of PR-B only and does not block PR-A. Also, a much purer and stronger anti-progestagen, Org 31710, with low anti-glucocorticoid activity and weak androgenic/antiandrogenic and anabolic properties (43,44) would be worth studying, but this is a question for further targeted research. It would be interesting to use PR-A and PR-B knockout mice to investigate the exact role of PR isoforms in the antitumoral activity of P4.

High doses of P4 can induce death in neurogenic tumor cells, significantly inhibit tumor growth in a xenograft model of neuroblastoma of SK-N-AS origin and exhibit very high bioavailability. Our *in vitro* and *in vivo* studies demonstrate inhibitory effects of P4 on markers of tumor proliferation, vascularization, angiogenesis and apoptosis induction in tumor tissue, suggesting some possible mechanisms of P4 against neuroblastoma tumors. We also showed an association between the antitumor effects of P4 on Akt signaling and PR-A/PR-B expression in tumor tissue.

It would be interesting to investigate the genomic and pharmacological relationships between P4 and antitumor activity via PR and Akt signaling. Tumors of neurogenic origin often have a poor prognosis. Most chemotherapy drugs are highly toxic and nonselectively kill tumor cells as well as normal healthy cells. Our data show not only the selective cytotoxic effects of high concentrations of P4, but also the safety profile of these high concentrations in primary cortical neurons and human fibroblasts. Although much more research is needed before going to clinical trial, we should consider whether P4 could be used alone or in conjunction with reduced levels of radiation, surgery or chemotherapy to improve patient survival and functional outcome.

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DISCLOSURE

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