

Pharmacological targeting of β -adrenergic receptor functions abrogates NF- κ B signaling and MMP-9 secretion in medulloblastoma cells

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Abstract: Targeting of the vascular endothelium compartment explains, in part, the therapeutic efficacy of the nonselective β -adrenergic antagonist propranolol against common endothelial tumors such as hemangiomas. In vitro, the antiangiogenic biological activity of propranolol was shown to inhibit human brain microvascular endothelial cell tubulogenesis. However, possible interference of propranolol with cell signaling associated with the tumoral compartment remains unexplored. We therefore assessed the potency of propranolol against a pediatric brain tumor-derived DAOY medulloblastoma cell model. Gene expression of β_1 -, β_2 -, and β_3 -adrenergic receptors was confirmed in DAOY cells by semiquantitative RT-PCR. We next found that propranolol dose-dependently inhibited induction of the key extracellular matrix-degrading and blood–brain barrier disrupting enzyme matrix metalloproteinase-9 (MMP-9) by phorbol 12-myristate 13-acetate (PMA). Propranolol not only inhibited PMA-induced phosphorylation of the extracellular signal-regulated kinase (Erk), but also that of I κ B, preventing the I κ B phosphorylation which is a prerequisite for I κ B degradation. Propranolol inhibition of I κ B phosphorylation was shown to occur with optimal efficacy at 30 μ M. Although propranolol, at up to 100 μ M, did not affect cell viability, it potentiated PMA-mediated signaling that ultimately led to diminished phosphorylation of Akt. The anti-Erk and anti-Akt phosphorylation effects are both suggestive of antiproliferative and antisurvival signaling, respectively. Our data are therefore indicative of a pharmacological role for propranolol against β -adrenergic receptor signaling functions involving the nuclear factor-kappaB-mediated regulation of MMP-9.

Keywords: medulloblastoma, β -adrenergic receptors, MMP-9, NF- κ B

Introduction

The expression of matrix metalloproteinase-9 (MMP-9) is significantly increased during tumor progression and is considered as a major contributor to the opening of the blood–brain barrier (BBB).¹ Although human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components of the BBB, it is unclear whether MMP-9 that causes its disruption originates from the vascular or the tumoral compartment. Recent evidence from adenoviral-mediated MMP-9 downregulation demonstrated a key role for MMP-9 in endothelial cell network organization as human dermal microvascular endothelial cell migration and capillary-like tube formation were reduced in cell wounding and spheroid migration assays.² Aside from involvement in angiogenesis, MMP-9 is also known to be required for tumor vasculogenesis,³ an alternative pathway for neovascularization that is increasingly being found in a variety of states characterized by vascular growth such as hemangioma.⁴ In the latter, MMP-9 was among the increased hypoxia-induced

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mediators characterizing the stem/progenitor cells in children with hemangioma.⁵

Any therapeutic strategies leading to specific targeting of MMP-9 is therefore likely to be of utility in treating common endothelial tumors such as hemangiomas of infancy. Accordingly, therapeutic targeting of β -adrenergic receptor functions with propranolol was found to efficiently inhibit neovascularization during the proliferative phase of infantile hemangioma.^{6,7} The exact mechanism and signaling pathways involved in this inhibition of MMP-9 expression still remain undefined, and it is believed that marrow-derived endothelial progenitor cells may be partly involved.⁵ While recent studies delineated a unique brain endothelial phenotype in which MMP-9 secretion by HBMEC was increased upon treatment with the tumor-promoting agent phorbol 12-myristate 13-acetate,^{8–10} the effects of propranolol and the contribution of β -adrenergic receptor function to the regulation of MMP-9 secretion by the tumor compartment itself has received little attention. In fact, we have shown that MMP-9 is secreted by numerous cell types and that its presence is often indicative of an invasive phenotype during tumor development.^{8,11–14} Leakiness of the vascular endothelium is among the best known of the deleterious brain tumor-associated effects.^{15,16} Whether any β -adrenergic receptor-mediated functions are involved in such events is unknown.

In this study, we used the pediatric brain tumor-derived DAOY cell line model to assess the potential contributions of β -adrenergic receptor functions regulating MMP-9 secretion. Propranolol's pharmacological effects were tested and we provide molecular evidence showing that inhibition of nuclear factor-kappaB (NF- κ B)-mediated brain tumor signaling specifically reduces the secretion of MMP-9.

Material and methods

Materials

Propranolol, sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON, Canada). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON, Canada). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA, USA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL, USA). The polyclonal antibodies against phospho-ERK, Akt and phospho-Akt were purchased from Cell Signalling (Danvers, MA, USA), the polyclonal anti-ERK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibody against GAPDH was from Advanced Immunochemical Inc. (Long Beach, CA). Horseradish peroxidase-conjugated donkey

antirabbit and antimouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Sigma-Aldrich Canada.

Cell culture

The human DAOY medulloblastoma cell line was purchased from American Type Culture Collection and was maintained in Eagle's Minimum Essential Medium containing 10% (v/v) calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37°C, with 95% air and 5% CO₂.

cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from cultured DAOY cells using TRIzol reagent. For cDNA synthesis, ~1 μ g total RNA was reverse-transcribed into cDNA using an oligo dT primer and the iScript reverse transcriptase cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). cDNA was stored at –20°C for PCR (Applied Biosystems Inc, Foster City, CA). Human primers for β_1 - (QT00204309), β_2 - (QT00200011), and β_3 - (QT00200004) adrenergic receptors and for Peptidylprolyl isomerase A (PPIA, QT01866137) were from QIAGEN. Semi-quantitative RT-PCR analysis was performed starting with 1 μ g cDNA, followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON, Canada). PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification and the products were resolved on 1.8% agarose gels containing 1 μ g/mL ethidium bromide.

Gelatin zymography

Gelatin zymography was used to assess the extent of proMMP-2 and proMMP-9 activity as previously described.¹⁰ Briefly, an aliquot (20 μ L) of the culture medium was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in a gel containing 0.1 mg/mL gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H₂O. Gels were further incubated at 37°C for 20 hours in 20 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H₂O. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblotting procedures

Proteins from control and treated cells were separated by SDS–PAGE. After electrophoresis, proteins were

electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1-hour incubation with horseradish peroxidase-conjugated antirabbit or antimouse IgG (1/2,500 dilution) in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfé, QC, Canada).

Cytotoxicity and cell proliferation assays

To assess the effect of propranolol on DAOY cell viability, the release of lactate dehydrogenase (LDH) upon damage of the plasma membrane was analyzed in the same condition media that was used for gelatin zymography. LDH activity was measured at 30°C by a continuous optical test based on the extinction change of pyridine nucleotide at 340 nm as described by the manufacturer's instructions (Promega). The cleavage of the tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate} by mitochondrial dehydrogenases (Roche Diagnostics, Laval, QC, Canada) was also used to assess cell proliferation.

Results

Expression of β -adrenergic receptor transcripts in medulloblastoma-derived DAOY cells

β_1 -, β_2 -, and β_3 -adrenergic receptor gene expression was first assessed for medulloblastoma-derived DAOY cells from which total RNA had been extracted. The design of primers enabled measurement of the expression levels for each of the individual human β -adrenergic genes and of the house keeping gene PPIA. This was validated by visualization of a single cDNA amplicon product obtained from total RNA by semi-quantitative RT-PCR on an agarose gel (Figure 1). This confirms that β -adrenergic receptors are expressed in DAOY cells.

Propranolol inhibits phorbol 12-myristate 13-acetate (PMA)-induced secretion of MMP-9 in DAOY medulloblastoma cells

DAOY cells were serum-starved and treated for 18 hours with various doses of propranolol in the presence or absence of a fixed (1 μ M) PMA concentration (Figure 2A); other cells were treated with various doses of PMA in the presence or absence of 30 μ M propranolol (Figure 2B). The conditioned media

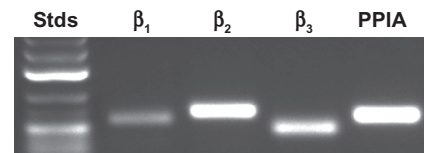


Figure 1 Gene expression analysis of β -adrenergic receptor expression in DAOY medulloblastoma cells. Total RNA was extracted from medulloblastoma-derived DAOY cells and semi-quantitative RT-PCR performed as described in the Methods section. cDNA amplicons were resolved on an agarose gel in order to confirm a single amplification product.

were harvested to measure the levels of MMP-9 by gelatin zymography. While MMP-9 activity was undetectable under basal conditions (Figure 2A, upper panel), it was significantly increased in PMA-treated cells (Figure 2A, lower panel). When DAOY were treated with combined PMA and propranolol, MMP-9 was dose-dependently inhibited with an IC_{50} of ~ 3.1 μ M propranolol (Figure 2C). Maximal MMP-9 secretion was achieved with PMA at 1 μ M (Figure 2B, upper panel). This induction was significantly inhibited in the presence of 30 μ M propranolol (Figure 2D). Collectively, these results suggest that propranolol selectively inhibits MMP-9 in response to carcinogenic-promoting conditions.

Propranolol reverses PMA-mediated I κ B decrease

Among MMP-9 expression regulators, the nuclear factor- κ B (NF- κ B) signaling pathway has been demonstrated to link cancer to inflammatory diseases.¹⁷ We therefore first assessed whether this signaling was activated upon PMA treatment and whether it was reflected in I κ B degradation. Cells were treated with 1 μ M PMA for 18 hours, lysates were isolated and I κ B expression was assessed through Western blotting. PMA signaling led to decreased I κ B expression (Figure 3, black bar). Increasing doses of propranolol were found to dose-dependently reverse the PMA-mediated decrease of I κ B, suggesting possible signaling interference by propranolol of I κ B, whose phosphorylation is essential for its degradation.¹⁸

Propranolol inhibits PMA-induced I κ B phosphorylation that leads to I κ B degradation

PMA-mediated phosphorylation of I κ B was next assessed in order to show whether this explains the subsequent decrease in I κ B expression. DAOY cells were treated for 45 minutes with 1 μ M PMA following preincubation with either vehicle or 30 μ M propranolol. Preincubation with vehicle followed by PMA treatment rapidly led to I κ B phosphorylation and to a concomitant decrease in I κ B (Figure 4A, left panel). When

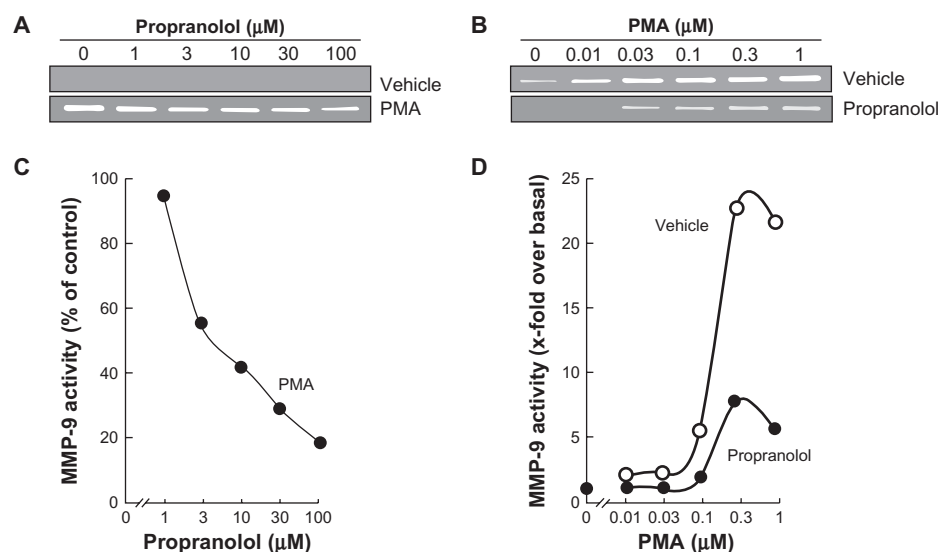


Figure 2 Propranolol inhibits PMA-induced matrix metalloproteinase-9 (MMP-9) secretion in DAOY medulloblastoma cells. Medulloblastoma-derived DAOY cells were serum-starved in the presence of various concentrations of propranolol in combination with vehicle or 1 μ M PMA for 18 hours (A), or in the presence of various concentrations of PMA in combination with vehicle or 30 μ M propranolol for 18 hours (B). Scanning densitometry was used to quantify the extent of proMMP-9 gelatinolytic activity for each set of data (C, D). Data shown is representative of two independent experiments.

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

DAOY cells were preincubated with propranolol, PMA was unable to induce I κ B phosphorylation and, consequently, I κ B protein levels remained unchanged throughout the 45-minute treatment (Figure 4A, right panel). The corresponding levels of phosphorylated I κ B (Figure 4B) and of total

I κ B (Figure 4C) expression were quantified by scanning densitometry.

Propranolol inhibits PMA-induced phosphorylation of Erk, and potentiates PMA-mediated Akt dephosphorylation

Alternative signaling pathways known to be triggered by PMA include the Erk pathway as well as the Akt pathway.^{19,20} Although Erk/Akt signaling cross talks are well documented, the former is involved in cell proliferation²¹ while the latter regulates cell survival.²² Pharmacological β -adrenergic blockade strategies specifically aimed at targeting these two signaling pathways may provide additional tools to reduce DAOY cell proliferation and/or survival. DAOY cells were therefore treated under conditions similar to those shown in Figure 4 (ie, stimulated for 45 minutes with 1 μ M PMA following preincubation with either vehicle or 30 μ M propranolol). We found that preincubation with vehicle followed by PMA treatment led to increased Erk phosphorylation with no effect on Akt phosphorylation status (Figure 5A, left panel). When DAOY cells were preincubated with propranolol, Erk phosphorylation by PMA was significantly reduced, while phosphorylation levels of Akt decreased as quickly as 10 minutes following PMA stimulation (Figure 5A, right panel). The corresponding ratios of phosphorylated Erk/total Erk (Figure 5B) and of phosphorylated Akt/total Akt (Figure 5C) were quantified by scanning densitometry. Cell proliferation

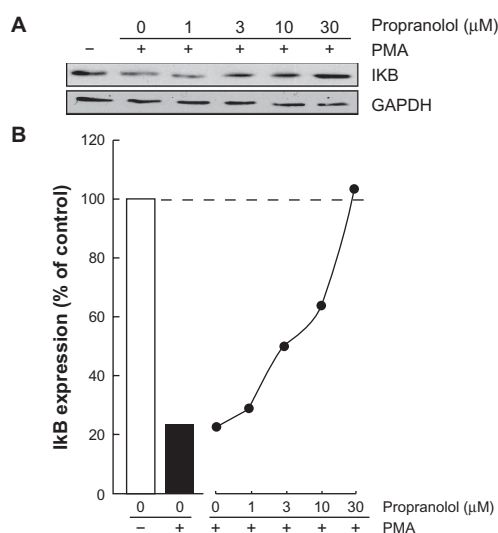


Figure 3 Propranolol reverses PMA-mediated I κ B degradation. Medulloblastoma-derived DAOY cells were serum-starved in the presence of various concentrations of propranolol in combination with vehicle or 1 μ M PMA for 18 hours. A) Lysates were isolated, electrophoresed via sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunodetection of I κ B and GAPDH proteins was performed as described in the Methods section. B) Quantification was performed by scanning densitometry of the autoradiograms. Data were expressed as the percent (%) expression of untreated basal conditions.

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

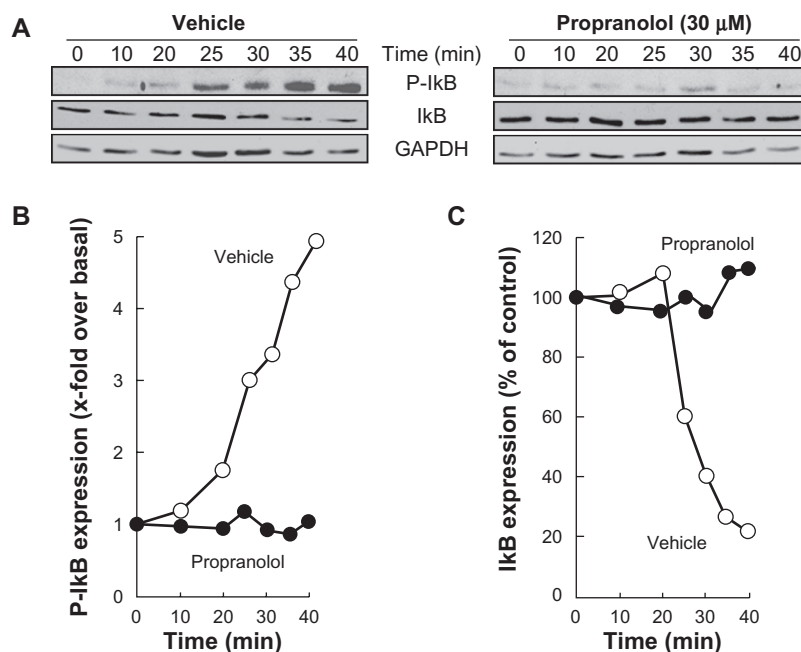


Figure 4 Propranolol inhibits PMA-induced I κ B phosphorylation that leads to I κ B degradation. **A)** Medulloblastoma-derived DAOY cells were serum-starved for 30 minutes in the presence of vehicle or 30 μ M propranolol. Cells were then incubated for the indicated time with vehicle or 1 μ M PMA. Lysates were isolated, electrophoresed via sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunodetection of phosphorylated I κ B (P-I κ B), I κ B, and of GAPDH proteins was performed as described in the Methods section. **B, C)** Quantification was performed by scanning densitometry of the autoradiograms. Data were expressed as x-fold induction over basal untreated cells for P-I κ B, and as the percent (%) expression of untreated basal conditions for I κ B.

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

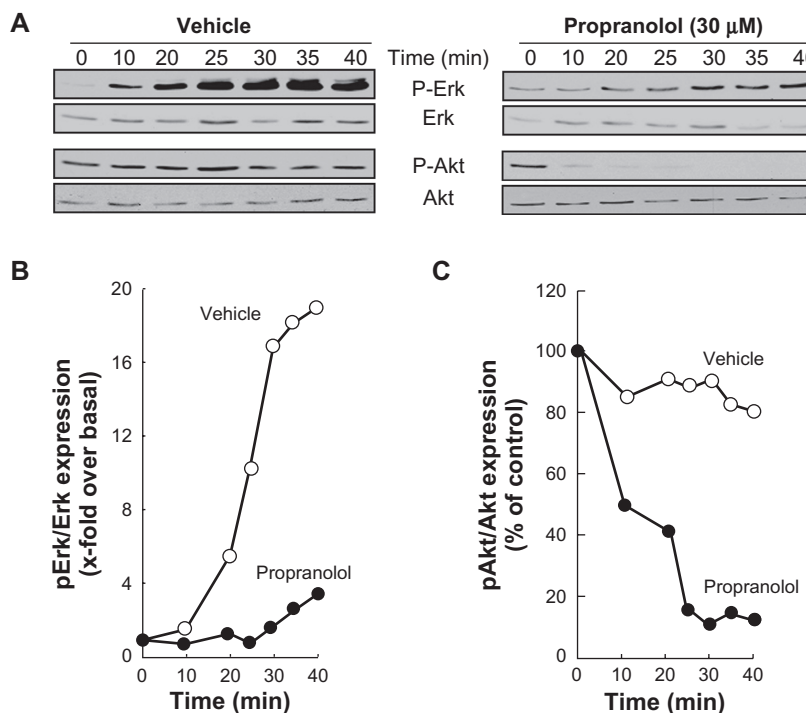


Figure 5 Propranolol inhibits PMA-induced phosphorylation of Erk, and potentiates PMA-mediated Akt phosphorylation. **A)** Medulloblastoma-derived DAOY cells were serum-starved for 30 minutes in the presence of vehicle or 30 μ M propranolol. Cells were then incubated for the indicated time with vehicle or 1 μ M PMA. Lysates were isolated, electrophoresed via sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunodetection of phosphorylated Erk (P-Erk), Erk, phosphorylated Akt (P-Akt), and of Akt proteins was performed as described in the Methods section. **B, C)** Quantification was performed by scanning densitometry of the autoradiograms. Data were expressed as x-fold induction over basal untreated cells for P-Erk/Erk, and as the percent (%) expression of untreated basal conditions for P-Akt/Akt.

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

assays confirmed the antiproliferative effect of propranolol (Figure 6A), while no cell death was induced at up to 100 μ M propranolol (Figure 6B). Collectively, this experimental evidence suggests that β -adrenergic blockade rather exerts strong antiproliferative effects combining the converging signaling originating from Erk/Akt pathways.

Discussion

Propranolol is a nonselective β -adrenergic antagonist that crosses the BBB and which is widely used clinically for various conditions including hypertension, anxiety and excessive sympathetic responses that often characterize patients during the perioperative period.²³ Clinical benefits have been observed in combination with COX-2 inhibitors in postoperation cancer patients, in whom perioperative treatment resulted in improved immune competence and in reduced risk of tumor metastasis.²⁴ It was therefore inferred that blockade of β -adrenergic receptor functions would affect tumor development, an effect that was confirmed by the inhibition of experimentally induced pulmonary adenocarcinoma development.²⁵ The contribution of β -adrenergic receptor functions to tumorigenesis was also reflected by the suggested antiangiogenic effects of β -blockers on a tumor-associated endothelial cell model. As such, evidence for increased expression of β_2 -adrenergic receptors in the brain tumor-derived vascular compartment was demonstrated, and adrenergic blockade with propranolol resulted in the inhibition of HBMEC tubulogenesis.²⁶ Targeting brain tumor-associated endothelial cell functions with β -blockers, as part of cancer treatments, may therefore become an appealing prospect to be further investigated.

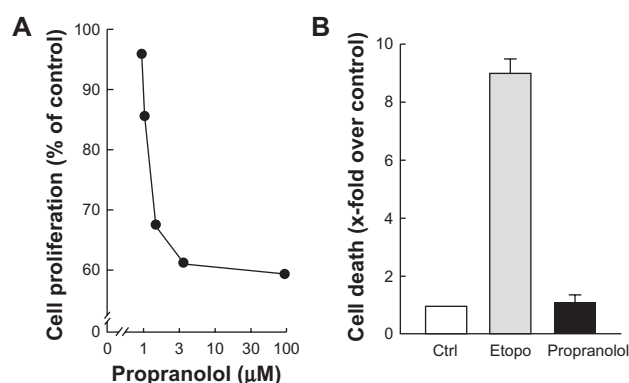


Figure 6 Propranolol inhibits PMA-induced cell proliferation but not cell survival. **A)** Medulloblastoma-derived DAOY cells were treated as described in Figure 2 and left to grow for 48 hours. Cell proliferation assay was performed as described in the Methods section. **B)** Cell death was assessed through the release of LDH into the conditioned media and assessed as described in the Methods section of serum-starved DAOY cells treated with vehicle (white bar), 50 μ M etoposide (grey bar), or 100 μ M propranolol (black bar).

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

To date, no human clinical study has documented the specific chemotherapeutic effect of propranolol in anticancer therapy. In vivo clinical data will ultimately provide definitive proof as to the therapeutic efficacy of propranolol in anticancer treatments, and may benefit from our in vitro demonstration and elucidation of propranolol's molecular mechanism of action. Among the strongest evidence, and perhaps the best in vivo study that supports our current data, is the demonstration that MMP-9 and the pro-angiogenic factor VEGF are both inhibited by propranolol in nasopharyngeal carcinoma tumor cells.²⁷ Several other in vivo approaches have also shed light on the chemopreventive actions of propranolol in reducing pancreatic ductal adenocarcinoma growth in animal models²⁸ and in reducing metastatic development of PC-3 prostate cancer in nude mice.²⁹ Such published data strongly suggest a potent anticancer action in line with those we infer in this study using a DAOY pediatric brain tumor-derived cellular model.

One major implication of our study relates to the documented relationship between inflammation and cancer. Increasing evidence suggests that the inflammatory microenvironment in and around tumors is an indispensable participant in the neoplastic process.³⁰ NF- κ B plays an important role in the regulation of inflammatory responses and where NF- κ B signaling can be activated by diverse stimuli including proinflammatory cytokines, infectious agents and cellular stresses.³¹ It may therefore be appealing in both the prevention and treatment of brain cancers to target NF- κ B signaling that regulates, in part, MMP-9 expression. Accordingly, targeting capacity of several pharmacological agents has led to inhibition of MMP-9; these agents include numerous nutraceutical molecules. Among these, sulforaphane,^{8,32} epigallocatechin-gallate,^{14,33,34} curcumin,^{35,36} resveratrol,^{37,38} proanthocyanidins³⁹ and lycopene⁴⁰ have all been proved to inhibit MMP-9 expression/secretion. More interestingly, all of the above-mentioned diet-derived molecules also abrogated the NF- κ B signaling pathway which regulates MMP-9 expression.⁴¹

PMA-induced I κ B phosphorylation and subsequent degradation, which together results in the release of NF- κ B p65 and p50 subunits followed by their nuclear translocation, subsequently regulates MMP-9 transcription.⁴² We show that inhibition of I κ B phosphorylation by propranolol accordingly results in diminished downstream expression of MMP-9 expression. Finally, our data also provide support to the Erk/Akt signaling crosstalk regulating cell proliferation. It is well documented that Erk activation is required for cell proliferation to proceed.⁴³ Furthermore, natural biological regulation of Erk nuclear localization has been demonstrated

to regulate cell proliferation, while overactivation of Akt has been shown to prevent the nuclear translocation of Erk by stabilizing endogenous PEA15, resulting in cell proliferation restriction.⁴⁴ Inhibition of such signaling crosstalk, as we observe for the effect of propranolol, may therefore be viewed as a double check-point control since propranolol not only inhibits Erk phosphorylation status, but would also possibly prevent Akt-mediated Erk nuclear translocation, the overall effect of which will result in blocking cell proliferation, in agreement with Figure 6A.

We have previously reported that medulloblastoma-derived cancer stem cells possessed increased MMP-9 expression in neurosphere cultures,¹³ and that members of the low-density lipoprotein receptor-related proteins, which also exhibit important functions in MMP-9 recycling,^{45,46} provided a differential molecular signature between parental and CD133+ DAOY medulloblastoma cells.⁴⁷ Increased MMP-9 expression was also associated with colospheres derived from colon cancer cultures.⁴⁸ Collectively, these data suggest that cancer stem cell targeted strategies involving MMP-9 expression may possibly be envisioned. Whether β -adrenergic blockade would be involved remains to be determined. In support of our current data with brain tumor cells, β_2 -adrenergic antagonists suppressed pancreatic cancer cell invasion by inhibiting NF- κ B and MMP-9 expression,⁴⁹ while MMP-9 levels were decreased upon β_1 - and β_2 -adrenoceptor blockade.⁵⁰ In summary, our data are indicative of a role for propranolol against carcinogen-mediated signaling that leads to the secretion of the BBB disruptor enzyme MMP-9. Our results also illuminate the alternative roles that excessive MMP-9 expression may play in inflammatory diseases and in inflammation associated with tumor development.

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Disclosure

The authors declare they have no competing interests.

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