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Brief Communication

**BDNF/TrkB Signaling as a Potential Novel Target in Pediatric Brain
Tumors: Anticancer Activity of Selective TrkB Inhibition in
Medulloblastoma Cells**

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Abstract

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5 Medulloblastoma (MB) is the most common malignant pediatric brain tumor.
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7 Deregulation of BDNF/TrkB signaling has been associated with increased proliferative
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9 capabilities, invasiveness and chemo-resistance in several types of cancer. However, the
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11 relevance of this pathway in MB remains unknown. Here, we show that the selective
12
13 TrkB inhibitor ANA-12 markedly reduced the viability and survival of human cell lines
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15 representative of different MB molecular subgroups. These findings provide the first
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17 evidence supporting further investigation of TrkB inhibition as a potential novel
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19 strategy for MB treatment.
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26 **Keywords** TrkB • Brain-derived neurotrophic factor • Medulloblastoma • Brain tumor •
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Introduction

Brain tumors represent the main leading cause of cancer-related death in childhood. Medulloblastoma (MB), the most common type of brain cancer afflicting children, is an embryonal solid tumor that usually arises in the cerebellum and spreads through the cerebrospinal fluid (CSF), leading to metastasis (Brandes et al. 2015; Roussel et al. 2011). The use of next-generation sequencing and other advanced molecular biology approaches has recently revolutionized our understanding of MB biology, leading to the current consensus that MB represents a heterogeneous group of tumors that can be divided into four distinct molecular subgroups – WNT, SHH, Group 3 and Group 4 (Taylor et al. 2012). MB subgroups display distinct cellular origins, mutations, gene expression signatures, methylation profiles as well as clinical course (Northcott et al. 2012; Rusert et al. 2014.). Despite the remarkable recent advances in the understanding of MB biology, one-third of patients still have low chance of being cured. Contemporary therapeutic approaches are highly toxic, and survivors often suffer from treatment-related neurological disabilities (Samkari et al. 2015). Therefore, the development of novel specific therapies is urgently needed.

Emerging therapeutic targets for cancer treatment include receptors for neurotrophins (NT), which are also important for the normal development and function of the CNS. The activities of NTs are mediated by NGF, BDNF, NT-3 and NT-4/5 binding to their related tropomyosin kinase receptors, TrkA, TrkB and TrkC respectively (Huang and Reichardt 2003; Nakagawara, 2001). Trk activation or mutations have been detected in several types of cancer, including tumors of neural origin, such as neuroblastoma and MB (Thiele et al. 2009; Tan et al. 2014). BDNF and

1 TrkB overexpression or TrkB activation have been characterized in neuroblastoma
2 (Brodeur et al. 2009), lung (Sinkevicius et al. 2014), colorectal (de Farias et al. 2010),
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4 prostate (Bronzetti et al. 2008) and breast cancers (Vanhecke et al. 2011). TrkB-positive
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6 cells are linked to increased proliferative capabilities, anoikis resistance, metastasis,
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8 invasiveness and chemo- resistance (de Farias et al. 2012; Desmet and Pepper, 2006; Li
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10 et al 2007; Thiele et al. 2009).

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15 Expression of BDNF and TrkB has been detected in both MB tumor samples and
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17 MB cell lines (Chou et al. 1997; Schmidt et al. 2010). Previous reports have shown that,
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19 under certain experimental conditions, human recombinant BDNF alone or in
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21 combination of HDAC inhibitors, is able to decrease cell viability in MB cell lines (Nör
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23 et al. 2011; Schmidt et al. 2010). However, the biological role and clinical significance
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25 of BDNF/TrkB signaling in MB remain poorly understood, and previous studies have
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27 not verified whether TrkB inhibition affects MB growth.

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33 A small-molecule selective TrkB inhibitor, ANA-12, has been recently
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35 developed and has become a useful tool for examining the involvement of BDNF/TrkB
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37 signaling in physiological and pathological processes (Cazorla et al. 2011). Here, we
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39 investigated the effects of ANA-12 in human pediatric MB cell lines representative of
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41 different molecular subgroups. ANA-12 reduced cell viability and clonogenic survival
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43 in a dose-dependent manner. These findings provide the first evidence suggesting TrkB
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45 inhibition as a potential targeted therapy for MB.

Materials and Methods

Reagents

ANA-12 (N-[2-[[[Hexahydro-2-oxo-1H-azepin-3-yl] amino] carbonyl] phenyl]-benzo [b] thiophene-2-carboxamide), human recombinant BDNF, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Annexin V-FITC was obtained from Santa Cruz Biotechnology (Dallas, TX, USA.). Propidium iodide (PI), trizol, SuperScript® III Reverse Transcriptase and low DNA mass ladder were provided by Invitrogen-Life technologies (Carlsbad, CA, USA). GoTaq® Hot Start Polymerase and RQ1 RNase-Free DNase were supplied by Promega (Madison, WI, USA). Ethidium bromide was purchased from Biotium (Hayward, USA). All culture materials were obtained from Gibco-Life technologies (Grand Island, NY, USA). Cisplatin was donated by the Kaplan Oncology Institute (Porto Alegre, Brazil).

Cell Culture and Treatments

Human MB cell lines Daoy, D283, ONS-76 and UW-228 were kindly donated by Dr. Michael D. Taylor (The Hospital for Sick Children, Toronto, Canada). Daoy, D283 and ONS-76 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose, while UW-228 cell was cultured in DMEM: Nutrient Mixture F-12 (DMEM/F-12), both media supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1% (v/v)

1 penicillin/streptomycin. Cells were incubated in a humidified atmosphere of 5% CO₂ at
2 37°C.
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5 Cells were treated with increasing concentrations of ANA-12 (5, 20 or 30 μM)
6 or BDNF (50 ng/ml) in complete medium for 48 hours. ANA-12 was dissolved in
7 DMSO. The concentration of the vehicle DMSO was used as control and did not exceed
8 0.5% (v/v). ANA-12 concentrations were based in a previous *in vitro* study using this
9 inhibitor (Sinkevicius et al. 2014).
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21 **Cell Viability**

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27 Cell viability was assessed by trypan blue cell counting as described previously (Jaeger
28 et al. 2013; Nör et al. 2011). Daoy, ONS-76, UW-228 and D283 cells were seeded at a
29 density of 3x10³ cells per well in complete medium into 96-well plates (TPP®
30 Switzerland). After overnight culture in complete medium, cells were treated with
31 ANA-12. After 48 hours of treatment, the medium was removed, cells were washed
32 with PBS and 50 μl of 0.25% trypsin/EDTA solution was added to detach cells. Cell
33 suspension was homogenized with 0.4 % Trypan blue 1:1 and counted immediately in a
34 hemocytometer. Experiments were performed at least four times in quadruplicates for
35 each treatment. Cell viability was normalized to the control DMSO.
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54 **Cell Survival**

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1 For evaluation of cell survival, UW-228 and D283 cells were plated at 400 cells per
2 well in six-well plates (NEST®, China). Cells were allowed to adhere and then were
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4 incubated overnight in complete medium at 37°C, and were then exposed to ANA-12 or
5
6 BDNF. After 48 h the cells were washed with standard medium to remove ANA-12 or
7
8 BDNF and cultured for another week, with the medium being changed every 2 days.
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10 Cells were then fixed with 70 % ethanol and counterstained with 0.5 % crystal violet.
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12 Colony numbers and colony size were assessed by ImageJ plugin, “ColonyArea” as
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14 previously described by Guzmán et al (2014).
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23 **Cell Cycle**

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30 UW-228 cell was plated at 15×10^3 cells per well in 24-well plate (NEST®, China),
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32 followed by drug treatments as describe above. After 48h of treatment, both floating and
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34 attached cells were harvested, washed twice with PBS and marked with a solution
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36 containing 50 µg/ml PI, 0.1% Triton X-100 and 0,1% sodium citrate for 15 min, in the
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38 dark, at room temperature. Cells were analyzed by flow cytometer (Attune® applied
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40 biosystems). Single cells were gated using width and area parameters. An area
41
42 parameter histogram was used to determine the percentage of cells in Sub-G1/G0, G1, S
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44 and G2 phases.
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54 **Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

1 Total RNA from UW-228 cell was extracted using trizol reagent, in accordance with the
2 manufacturer's instructions, quantified in NanoDrop (Thermo Scientific), treated with
3 DNase and reverse-transcribed with superscript® III First-Strand Synthesis supermix.
4 BDNF, TrkB and β -actin primers used for RT-PCR amplification were designed
5 according to the corresponding GenBank sequence and are shown in Table 1. The
6 expression of β -actin was measured as an internal control.
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15 PCR conditions for experiments were 1.5 mM MgCl₂, 0.4 μ M for each primer,
16 0.2 dNTPs, 1.25u GoTaq® Hot Start Polymerase, and 1 μ l cDNA template. All assays
17 were carried out in a total volume of 15 μ l using 35 cycles for amplification that
18 consisted of 1 min at 95 °C, denaturation at 94 °C for 30 s, annealing at 58–60 °C,
19 accordingly to the specific primer, for 30 s, and extension of primers at 72 °C for 45 s,
20 followed by a final extension at 72 °C for 10 min. The products of BDNF, TrkB and β -
21 actin were electrophoresed through 1.5 % agarose gels containing ethidium bromide and
22 visualized with ultraviolet light. The fragments' length was confirmed using a low DNA
23 mass ladder. For each set of PCR reactions, a negative control was included.
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41 **Statistical Analysis**

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48 Data are shown as mean \pm standard error of mean (SEM). Statistical analysis was
49 performed by one-way analysis of variance (ANOVA) followed by tukey post-hoc test
50 for multiple comparisons of at least three independent experiments for each
51 experiments; *P* values under 0.05 was considered significant. Analyses were conducted
52 using the GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).
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Table 1 should be included here

Results

TrkB Inhibition Reduces MB Cell Viability

The cytotoxic activity of the selective TrkB inhibitor ANA-12 was evaluated by trypan blue cell counting assay at 48 hours under standard growth conditions (Fig. 1). Analysis with ANOVA indicated that ANA-12 produced a reduction of MB cell viability at all doses tested ($F = 2.182$, $df = 9$, $P = 0.0392$). Further analysis with Tukey tests comparing control and drug-treated cells showed that ANA-12 at 5 μM was ineffective in ONS-76, UW-228 and Daoy cells, but significantly reduce cell viability in D283 cells ($29.8 \pm 9.03\%$; $P < 0.01$). Treatment with ANA-12 at 20 μM reduced viability in all cell lines (ONS-76: $62.95 \pm 4.8\%$, $p < 0.001$; UW-228: $57.69 \pm 5.3\%$, $P < 0.001$; Daoy: $59.41 \pm 9.27\%$, $P < 0.01$; D283 $62.51 \pm 4.08\%$, $P < 0.0001$). The maximal decrease in cell viability was produced by ANA-12 at 30 μM (ONS-76: $75.43 \pm 6.9\%$, $P < 0.0001$; UW-228: $94.80 \pm 4.16\%$, $P < 0.001$; Daoy: $90.10 \pm 3.52\%$, $P < 0.001$; D283: $93.58 \pm 0.82\%$, $P < 0.0001$).

Figure 1 should be included here

TrkB Inhibition Reduces MB Cell Survival

The effects of ANA-12 and BDNF on colony formation were analyzed in UW-228 and D283 cells (Fig. 2). ANA-12 reduced colony formation in both cell lines over one week, after 48h of treatment. BDNF alone did not affect colony formation. In UW-228 cells, ANA-12 decreased colony number at 20 μ M ($10.53 \pm 5.69\%$, $P < 0.01$) and 30 μ M ($3.03 \pm 0.87\%$, $P < 0.001$), when compared to both control ($33.4 \pm 1.75\%$) and BDNF-treated cells ($21.26 \pm 3.91\%$), whereas colony size was not significantly affected (Fig. 2a, 2c). In D283 cells, colony number was reduced only by the dose of 30 μ M of ANA-12 ($3.76 \pm 2.39\%$, $P < 0.05$) when compared with either control ($22.71 \pm 5.09\%$) or BDNF-treated cells ($22.16 \pm 2.42\%$). This dose of ANA-12 also decreased colony size ($1.53 \pm 0.99\%$, $P < 0.05$) in comparison with either control cells ($9.94 \pm 2.51\%$) and cells exposed to BDNF ($10.03 \pm 1.66\%$) (Fig. 2b, 2d).

Figure 2 should be included here

TrkB Inhibition alters MB cell cycle

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5 UW-228 cells were used to assess cell cycle distribution after 48h of ANA-12
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7 treatment. ANA-12 induced sub-G1 cell cycle arrest in a dose-dependent manner (Fig.
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9 3). Intracellular PI fluorescence intensities are presented (Fig. 3a) and the percentage of
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11 cells in the sub-G1 phase was significantly increased after treatment with ANA-12 at 30
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13 μM (sub G1: $17.3 \pm 3.99\%$, $P < 0.001$) compared to either controls (sub-G1: $1.73 \pm$
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15 0.07%) and BDNF-treated cells (sub-G1: $1.09 \pm 0.55\%$). In addition, the percentage of
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17 cells in the G1 phase was inhibited by ANA-12 at 30 μM (G1: $36.86 \pm 4.57\%$, $P <$
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19 0.001) compared to controls (G1: $65.3 \pm 4.90\%$) and BDNF-treated cells (G1: $64.50 \pm$
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21 2.83%) (Fig. 3b). BDNF alone did not affect the cell cycle. These data suggest that
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23 ANA-12 induced cell cycle arrest in UW-228 cell.
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UW-228 MB cells express mRNA for TrkB and BDNF

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37 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis confirmed that
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39 UW-228 cell express mRNA for BDNF and TrkB. Two transcripts with 130 bp and 123
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41 bp of size, representing a fragment of TrkB and BDNF respectively, were identified
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43 (Fig. 3c). BDNF and TrkB expression for Daoy, D283 and ONS-76 MB cells were
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45 demonstrated in previous reports (Nör et al. 2011; Schmidt et al. 2010).
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Figure 3 should be included here

Discussion

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7 Aggressive multimodal therapy in MB patients is generally associated with long term
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9 side effects, therefore novel antitumor strategies targeting deregulated pathways need to
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11 be explored (Gottardo et al. 2014). Given that BDNF/TrkB signaling has been shown to
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13 promote tumor cell proliferation, survival and increase chemo-resistance in several type
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15 of cancers (Tan et al. 2014), we hypothesized that this pathway could regulate cell
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17 viability in pediatric MB. To study BDNF/TrkB signaling in MB cells, we employed a
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19 small molecule, ANA-12, which blocks TrkB selectively (Cazorla et al. 2011). We used
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21 MB cell lines that were recently characterized as representative of different MB
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23 molecular subgroups lines representative of different molecular groups of MB. Daoy,
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25 ONS-76 and UW-228 cells show features of SHH tumors, whereas D283 displays *MYC*
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27 amplification and is classified as Group 3 MB subgroup 3 (Xu et al. 2015). Our results
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29 indicate that TrkB inhibition can reduce cell viability and survival of MB cells.
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37 The suggestion that BDNF/TrkB might be relevant in MB first came from early
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39 studies examining this pathway in tumor samples and cell lines. Segal et al. (1994)
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41 found that the level of TrkB mRNA expression was not associated with extent of
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43 disease progression or patient survival. Washiyama et al. (1996) evaluated 27 samples
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45 from MB patients and found 67% of tumors expressing TrkB and 22% expressing
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47 BDNF. However, the molecular classification of MB was only recently defined (Xu et
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49 al. 2015), and the relationship between expression levels of NTs and Trk receptors in
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51 different subgroups remains to be characterized. Expression of BDNF and TrkB was
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53 previously identified in Daoy, ONS-76 and D283 cell lines (Schmidt et al. 2010), and in
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the present study we demonstrate that UW-228 cell likewise express mRNA for both TrkB and BDNF.

The present report is the first demonstration that selective TrkB inhibition displays antitumor effect in MB cells. Moreover, it was previously demonstrated that a pan-Trk inhibitor can reduce MB xenografts growth (Evans et al. 1999). Because we did not aim to address candidate downstream signaling components possibly involved in mediating the effects, any discussion of mechanisms remain speculative at this point. TrkB activation initiate multiple signaling cascades, including mitogen-activated protein kinase (MAPK) pathway, phosphatidyl-inositide 3-kinase (PI3K) pathway, and phospholipase C-gamma (PLC- γ). All these pathways play important roles in cell proliferation, differentiation and survival, consistent with a role for TrkB in these cellular processes (Boulle et al. 2012).

The sub-G1 accumulation observed in cell cycle analyses may be related to apoptotic cells, which can be identified on DNA frequency histograms as cells with fractional DNA content (Kajstura et al. 2007). A previous report using leukemia cells found apoptosis and reduction of GSK-3 β phosphorylation after ANA-12 treatment (Polakowski et al. 2014). In neurons, GSK-3 β activation counteracts the effects of BDNF, and specific downstream signaling of TrkB phosphorylation pathway converge to the inactivation of GSK-3 β (Phukan et al. 2010). The involvement of GSK-3 β in a variety of cellular responses, including cytoskeleton regulation, cell cycle progression, apoptosis and cell adhesion, is well established (McCubrey et al. 2014). It has been also postulated that constitutive phosphorylation of GSK-3 β , found in many tumor cell types, including MB, improves cell survival and contributes to malignant transformation

1 (Urbanska et al. 2007). However, the functional role of ANA-12 in apoptosis of MB
2 cells remains to be characterized.
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5 In summary, the present study found pronounced dose-dependent inhibitory
6 effects of a selective TrkB inhibitor, ANA-12, on cell viability and survival in pediatric
7 MB cell lines *in vitro*. These results provide the first evidence that selective TrkB
8 inhibition may be a promising strategy worth further investigation in experimental MB.
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Table 1. Forward and reverse primers used for RT-PCR amplification

Gene	Primer sequence	PCR product size (bp)
TrKB	Forward: 5'-TGGTGCATTCCATTCACTGT-3'	130
	Reverse: 5'-CGTGGTACTCCGTGTGATTG-3'	
BDNF	Forward: 5'-GGCTATGTGGAGTTGGCATT-3'	123
	Reverse: 5'-CTTCAGAGGCCTTCGTTTTG-3'	
β-actin	Forward: 5'-GAGACCTTCAACACCCCAG 3'	190
	Reverse: 5'-GCTACAGCTTCACCAGCAG 3'	

Legends for figures

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7 **Fig. 1.** A TrkB inhibitor dose-dependently reduces MB cell viability. ANA-12
8 decreased the viability of (a) ONS-76, (b) UW-228, (c) Daoy, and (d) D283 cells. Cells
9 were treated with increasing concentrations of ANA-12. After 48 h of drug exposure the
10 cell viability was assessed by trypan blue counting assay. Data are expressed by mean \pm
11 SEM percentage of control (the average value among replicates was assumed as 100%)
12 and represent four independent experiments performed in quadruplicates. Statistically
13 significant differences are marked by asterisks as follows: ** $P < 0.01$, *** $P < 0.001$,
14 and **** $P < 0.0001$.
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30 **Fig. 2.** TrkB inhibition reduces colony formation in MB cells. UW-228 and D283 cells
31 were exposed to BDNF (50 ng/ml) or ANA-12 (5, 20 or 30 μ M) for 48 hours and
32 subsequently maintained in standard growth medium for 7 days. Colony formation and
33 colony size were assessed using ImageJ version 1.47n software. (a and c) Data are mean
34 \pm SEM % colony number and colony size. Data represent at least three independent
35 experiments. Statistically significant differences are marked by asterisks, * $P < 0.05$ and
36 ** $P < 0.01$. (b and d) Representative images of colonies formed under the different
37 treatment conditions.
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54 **Fig. 3.** TrkB induced sub-G1 cell cycle arrest in UW-228 MB cell. Cells were exposed
55 to BDNF (50 ng/ml) or ANA-12 (5, 20 and 30 μ M) for 48 hours and subsequently the
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1 cell cycle distribution was determined by propidium iodide staining. (a) Representative
2 DNA fluorescence histograms of UW-228 cell following the treatments and control
3 (DMSO). (b) Data are mean \pm SEM % of cells in each cycle phase. Statistically
4 significant differences are marked by asterisks, *** $P < 0.001$. The data represents three
5 independent experiments. (c) UW-228 MB cell express TrkB and BDNF. mRNA was
6 extracted from UW-228 cells and RT-PCR was performed. A transcript with 130 bp,
7 representing a fragment of TrkB gene, was identified. A transcript with 123 bp,
8 representing a fragment of BDNF, was identified. B-actin was used as control.
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