Biochemical and Biophysical Research Communications 425 (2012) 328–332

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

BDNF/TrkB signaling protects HT-29 human colon cancer cells from EGFR inhibition

Caroline Brunetto de Farias [a,b,c,d], Tiago Elias Heinen [a,c,d], Rafael Pereira dos Santos [a,c,d], Ana Lucia Abujamra [a,b,d], Gilberto Schwartsmann [a,d,e], Rafael Roesler [a,b,c,d,*]

[a] Cancer Research Laboratory, University Hospital Research Center (CPE-HCPA), Federal University of Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil
[b] Children's Cancer Institute, 90420-140 Porto Alegre, RS, Brazil
[c] Laboratory of Neuropharmacology and Neural Tumor Biology, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, 90050-170 Porto Alegre, RS, Brazil
[d] National Institute for Translational Medicine (INCT-TM), 90035-003 Porto Alegre, RS, Brazil
[e] Department of Internal Medicine, School of Medicine, Federal University of Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 14 July 2012
Available online 25 July 2012

Keywords:
Brain-derived neurotrophic factor
TrkB
Cetuximab
Epidermal growth factor receptor
Colorectal cancer

ABSTRACT

The clinical success of targeted treatment of colorectal cancer (CRC) is often limited by resistance to anti-epidermal growth factor receptor (EGFR) therapy. The neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor TrkB have recently emerged as anticancer targets, and we have previously shown increased BDNF levels in CRC tumor samples. Here we report the findings from *in vitro* experiments suggesting that BDNF/TrkB signaling can protect CRC cells from the antitumor effects of EGFR blockade. The anti-EGFR monoclonal antibody cetuximab reduced both cell proliferation and the mRNA expression of BDNF and TrkB in human HT-29 CRC cells. The inhibitory effect of cetuximab on cell proliferation and survival was counteracted by the addition of human recombinant BDNF. Finally, the Trk inhibitor K252a synergistically enhanced the effect of cetuximab on cell proliferation, and this effect was blocked by BDNF. These results provide the first evidence that increased BDNF/TrkB signaling might play a role in resistance to EGFR blockade. Moreover, it is possible that targeting TrkB could potentiate the anticancer effects of anti-EGFR therapy.

1. Introduction

Resistance to epidermal growth factor receptor (EGFR)-targeted monoclonal antibodies poses a limitation for the therapy of colorectal cancer (CRC). Much recent research has focused on characterizing the molecular mechanisms of acquired resistance to the anti-EGFR antibody cetuximab. In CRC, these mechanisms include mutations in the *KRAS*, *BRAF*, or *NRAS* genes, which might compensate for EGFR blockade by stimulating the mitogen-activated protein kinase (MAPK) pathway [1–4]. It is also likely that resistance can be mediated by amplification of other receptor tyrosine kinases (RTKs), as has been demonstrated for other members of the HER (ErbB) family such as HER2 [5–7]. The identification of additional resistance mechanisms may reveal new avenues to increase the effectivity of EGFR inhibitors.

Signaling mediated by the prototypical neurotrophin, brain-derived neurotrophic factor (BDNF), which acts by activating the RTK TrkB, has recently emerged as an anticancer target [8–10]. In CRC, mutations have been found in two sites of the TrkB kinase domain [11], and we [12] and others [13] have recently described increased levels of BDNF in CRC tumor samples and cultured cells. Interestingly, our previous findings indicated that BDNF/TrkB signaling might play a role in resistance against an antitumor peptide that blocks gastrin-releasing peptide receptor (GRPR), through a mechanism dependent on EGFR [12]. Here, we show for the first time that cetuximab treatment in cultured human colon cancer cells *in vitro* is associated with a reduction in the expression of both BDNF and TrkB. Importantly, BDNF could protect from the effects of cetuximab on cell growth, and inhibiting TrkB increased the sensitivity to cetuximab. These findings provide the first evidence that BDNF/TrkB signaling might play a role in resistance to EGFR blockade, and that TrkB inhibition can potentiate the antitumor effects of anti-EGFR therapy.

2. Materials and methods

2.1. Cell culture and treatments

The HT-29 human colorectal cancer cell line was obtained from the American Type Culture Collection (Rockville, Md., USA) in 2000. Cells were seeded (7×10^3 cells per well) in 96-well polystyrene tissue culture-treated flat-bottom microtiter plates (TPP, Switzerland) in RPMI 1640 (LGC Biotechnology, Cotia, Brazil) medium

* Corresponding author at: Laboratory of Neuropharmacology and Neural Tumor Biology, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, 90050-170 Porto Alegre, RS, Brazil. Fax: +55 51 33083121
E-mail address: rafael.roesler@pq.cnpq.br (R. Roesler).

0006-291X/
http://dx.doi.org/10.1016/j.bbrc.2012.07.091

supplemented with 10% fetal bovine serum (Sorali, Campinas, Brazil), gentamicin (4 mg/ml; Nova Pharma, Jardim Anápolis, Brazil), ampicilin (50 mg/ml; Nova Pharma, Jardim Anápolis, Brazil), and fungizone (250 mg/kg; GIBCO, Grand Island, USA). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO2 in air. The cells were seeded at a density of 1×10^4 cells/well in RPMI 10% FBS into 24-well (cell counting experiments) or 7×10^3 cells per well in 96-well (MTT assay experiments) plates and allowed to grow for 24 h [12].

The medium was replaced and cells were treated with cetuximab (0.1, 1.0, or 10 nm; C225; Merck, Darmstadt, Germany) or medium for 24 h, after which, in the cells in the BDNF co-treatment groups, human recombinant BDNF (10 ng/ml; Sigma–Aldrich, St. Louis, Mo., USA) was added for 48 h. For the evaluation of the co-treatment between cetuximab (0.1 nM) and K252 (10 nM; Sigma–Aldrich, St Louis, USA) cells were seeded in RPMI 1640 10% FBS for 24 h, followed by the treatment for 72 h. The doses of agents were chosen on the basis of pilot dose curves previous experiments [12].

2.2. Cell proliferation and survival assays

For trypan blue cell counting, the medium was removed, cells were washed with Hanks' Balanced Salt Solution (Invitrogen), and 100 μl of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer [12,14]. Proliferation was also measured by MTT assay. MTT was added to each well (11 μl; 5 mg/ml; Sigma- Aldrich, St Louis, USA), and they were then incubated for 4 h at 37 °C. The plate was left at room temperature until it was completely dry. Dimethyl sulfoxide (DMSO) was added and the absorbance was read at 492 nm in a multiplate reader [12,15].

Cell survival was measured with a colony formation assay as previously described [12]. Cells were seeded into 6-well plates (400 cells/well) after treatment with cetuximab (0.1 or 10 nm) and BDNF (10 ng/ml), alone or combined, for 48 h. After incubation, the cells were replaced in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum for 7–10 days. Cells were then fixed with 70% ethanol and counterstained with 0.5% crystal violet. Only colonies containing 50 or more cells were scored under a microscope. The radiation-surviving fraction was then calculated as:

$$SF = \frac{\text{Number of colonies in treatment cells}}{\text{Number of colonies in controls}} \times 100$$

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

HT-29 cells were cultured in the presence of cetuximab (0.1 or 10 nm) for 10 h for RT-PCR reaction, followed by total RNA extraction using Trizol reagent (Invitrogen, São Paulo, Brazil), in accordance with the manufacturer's instructions, and reverse transcribed with superscripttm III First-Strand Synthesis supermix (Invitrogen). The human β-actin, BDNF, and TrkB primers were designed according to the corresponding Gene Bank sequence. The forward and reverse primers used for RT-PCR amplification were as follows: BDNF, 5'-GGCTATGTGGAGTTGGCATT-3' (forward) and 5'-CTTCAGAGGCCTTCGTTTTG-3' (reverse); TrkB, 5'- TGGTGCATTCCAT TCACTGT-3' (forward) and 5'-CGTGGTACTCCGTGTGATTG-3' (reverse); β-actin, 5́-GAGACCTTCAACACCCCAG-3́ (forward) and 5́ -GC TACAGCTTCACCAGCAG-3́ (reverse). All product sizes were 190 bp.

Semiquantitative RT-PCR conditions were optimized to determine the number of cycles that would allow product detection within the linear phase of mRNA transcript amplification. Experiments were performed with 1.5 mm MgCl2, 0.1 μM for each primer, 0.2 mm DNTPs, 1U Taq Platinum (Invitrogen), and 2 μl cDNA template. The expression of β-actin was measured as an internal control. The PCR conditions for β-actin, BDNF, and TrkB experiments were: 2.5 mm MgCl2, 0.1 μM for each primer, 0.2 mM DNTPs, 1U Taq Platinum (Invitrogen), and 1 μl cDNA template. All assays were carried out in a total volume of 15 μl using 35 cycles for amplification that consisted of 1 min at 95 °C, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension of primers at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The products of β-actin (190 bp), BDNF (190 bp), and TrkB (190 bp) were electrophoresed through 1.0% agarose gels containing ethidium bromide (Biotium, Hayward, USA) and visualized with ultraviolet light [12]. The fragments' length was confirmed using a Low DNA Mass Ladder (Invitrogen) and the relative expression of the BDNF and TrkB gene was determined by densitometry using freeware ImageJ 1.37 for Windows. Each experiment was performed in replicate using RNA isolated from independent cell cultures, and representative findings are shown. For each set of PCR reactions, a negative control was included. Semiquantitative data are shown as percentage changes relative to β-actin (the lowest value among replicates in the control group was taken as 100%).

2.4. Statistics

Data are shown as means ± SEM; $n = 3$–6 independent experiments. The lowest value among replicates in the control group was taken as 100%. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) followed by Tukey post hoc tests when appropriate. In all comparisons, $P < 0.05$ was considered statistically significant.

3. Results

3.1. The anti-EGFR monoclonal antibody cetuximab dose-dependently reduces the proliferation of HT-29 human CRC cells

The first experiment examined the effects of cetuximab on the proliferation of HT-29 CRC cells. The expression of EGFR in HT-29 cells was confirmed in a previous report [12]. Treatment with cetuximab at 10, but not 0.1 or 1.0 nM, significantly reduced the proliferation of HT-29 cells assessed by MTT assays ($P < 0.001$ compared to controls; Fig. 1), indicating that EGFR blockade by cetuximab dose-dependently inhibits the proliferation of CRC cells.

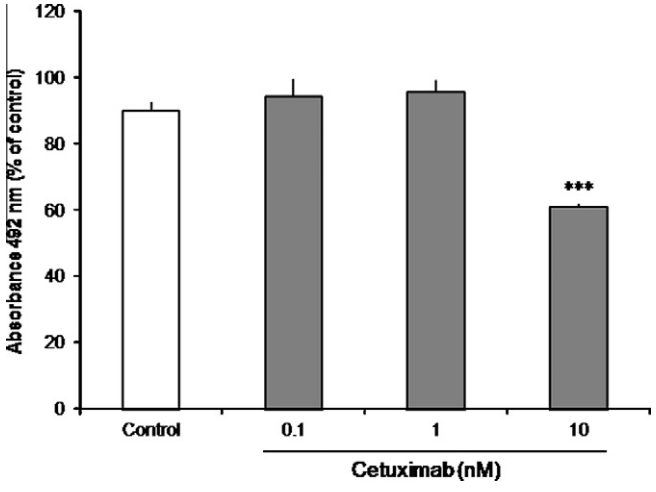

Fig. 1. EGFR blockade by cetuximab dose-dependently reduces CRC cell proliferation. Cells from the HT-29 human CRC cell line were cultured and treated with cetuximab (0.1, 1.0, or 10 nM) as described in **Materials and methods**. Cell proliferation was measured with MTT assays. Data are mean ± SEM absorbance (the lowest value among replicates in the control group was taken as 100%); $n = 4$ independent experiments; $^{***}P < 0.001$ compared to controls.

3.2. The inhibitory effect of cetuximab on CRC cells is associated with a decreased mRNA expression of BDNF and TrkB

The dose of cetuximab shown to inhibit the proliferation of HT-29 cells in the previous experiment also produced reductions of 55.3% and 48.7%, respectively, in the mRNA expression of BDNF ($P < 0.001$ compared to controls; Fig. 2A) and TrkB ($P < 0.001$ compared to controls; Fig. 2B) assessed by RT-PCR. Cetuximab at 0.1 nM, on the other hand, produced reductions of only about 3–4% in the expression of BDNF and TrkB mRNA, although these reductions also were significantly different from controls (BDNF, $P < 0.01$; TrkB, $P < 0.001$). The results suggest that the antiproliferative effect of EGFR blockade in CRC cells involves, and possibly requires, an inhibition of BDNF/TrkB signaling.

3.3. BDNF protects CRC cells from the inhibitory effects of cetuximab

The addition of human recombinant BDNF (10 ng/ml) blocked the effects of cetuximab at 10 nM on HT-29 cell proliferation assessed by trypan blue cell counting (Fig. 3A). Again, treatment with 10 nM cetuximab resulted in a significant reduction of cell proliferation ($P < 0.05$). However, proliferation was not inhibited, and was

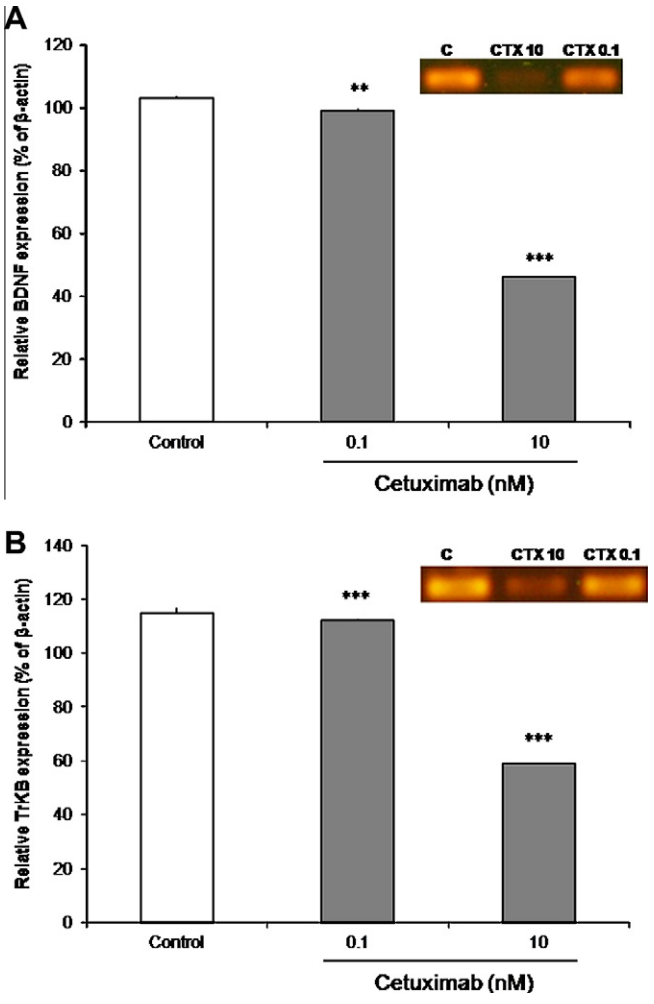

Fig. 2. Cetuximab reduces BDNF and TrkB mRNA expression in CRC cells. HT-29 cells were cultured and treated with cetuximab (CTX, 0.1 or 10 nM) as described in **Materials and methods**. The mRNA expression of BDNF (A) and TrkB (B) was measured by RT-PCR. Data are mean ± SEM relative expression (% of β-actin). Representative results are shown on the top of the graphs; $n = 3$ independent experiments; $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to controls.

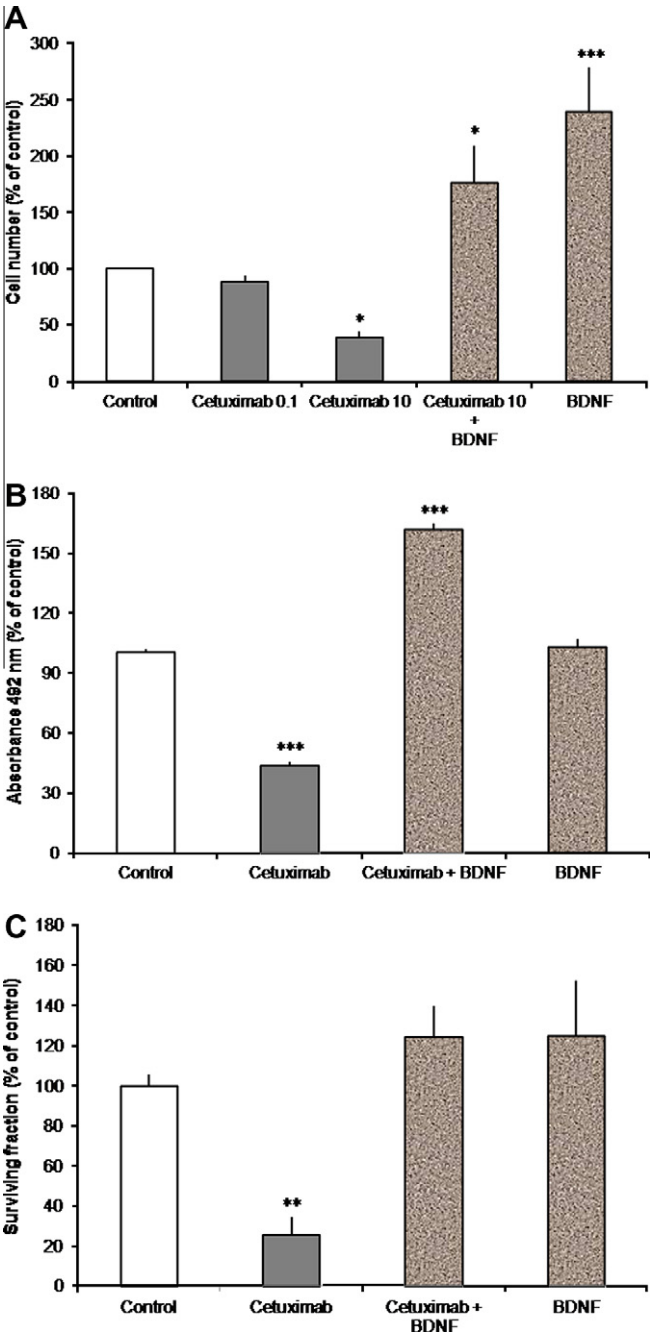

Fig. 3. BDNF protects CRC cells from the antitumor effects of cetuximab. HT-29 cells were cultured and treated with cetuximab (0.1 or 10 nM), human recombinant BDNF (10 ng/ml), or 10 nM cetuximab followed by 10 ng/ml BDNF, as described in **Materials and methods**. (A) Cetuximab at 10 nM produced a significant reduction in cell proliferation measured by cell counting, while treatment with cetuximab plus BDNF or BDNF alone led to an increase in proliferation. Data are mean ± SEM cell number (% of control); $n = 5$ independent experiments. (B) Cetuximab at 10 nM also reduced cell proliferation measured with MTT assays. BDNF had no effect by itself, but proliferation was increased in cells treated with cetuximab followed by BDNF. Data are mean ± SEM absorbance (the lowest value among replicates in the control group was taken as 100%); $n = 4$ independent experiments. (C) Cell survival assessed with a colony-forming assay was inhibited by cetuximab (10 nM), and this effect was completely blocked by BDNF. Treatment with BDNF alone had no effect. Data are mean ± SEM of the% surviving fraction (the lowest value among replicates in the control group was taken as 100%); $n = 4$ independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to controls.

in fact enhanced compared to controls, in cells treated with 10 nM cetuximab followed with BDNF ($P < 0.05$) or BDNF alone ($P < 0.001$). When the MTT assay was used as a measure of cell

proliferation (Fig. 3B), again cetuximab inhibited cell proliferation ($P < 0.001$), and this effect was completely reversed by BDNF. Again, proliferation was increased compared to controls in cells treated with cetuximab plus BDNF ($P < 0.001$), while BDNF alone had no effect. Cell survival measured with a colony formation assay was significantly reduced by BDNF ($P < 0.01$ compared to controls). BDNF by itself did not affect survival, but completely protected the cells from the effect of cetuximab (Fig. 3C). The results suggest that BDNF can promote CRC cell proliferation and rescue from the reduction in cell proliferation and survival produced by EGFR blockade.

3.4. TrkB inhibition synergistically enhances the antiproliferative effect of cetuximab in CRC cells

The previous experiments indicated that the antitumor effect of cetuximab on HT-29 CRC cells was associated with a reduction in BDNF and TrkB expression and blocked by the addition of exogenous BDNF. In the last experiment, we examined whether, conversely, blocking TrkB could potentiate the effect of cetuximab. Cells were treated with a low, ineffective dose of cetuximab (0.1 nM), the Trk inhibitor K252a (10 nM), BDNF (10 ng/ml), cetuximab combined with K252a, or cetuximab combined with both BDNF and K252a. Cell proliferation was measured with the MTT assay. The results are shown in Fig. 4. Neither cetuximab nor K252a affected proliferation when given alone. However, proliferation was reduced when the cells were treated with cetuximab combined with K252a ($P < 0.001$). Although K252a is not a selective TrkB inhibitor and acts also on other Trk receptors, the effect of cetuximab plus K252a was completely abolished by BDNF, which alone did not affect proliferation. This result suggests that TrkB inhibition can potentiate the inhibitory effect of cetuximab on CRC cell growth.

4. Discussion

Resistance to cetuximab in patients with metastatic CRC is currently thought to be associated primarily with mutant *KRAS* [1,2,16,17]. However, other mechanisms are likely to be important as well, since up to 65% of patients bearing wild-type tumors are resistant to anti-EGFR antibodies [16]. Other activating mutations in downstream effectors of EGFR signaling, such as *BRAF*, *NRAS*, and *PIK3CA* (which encodes a catalytic subunit of phosphatidylinositol 3-kinase, PI3 K) have been implicated [2–4,18,19].

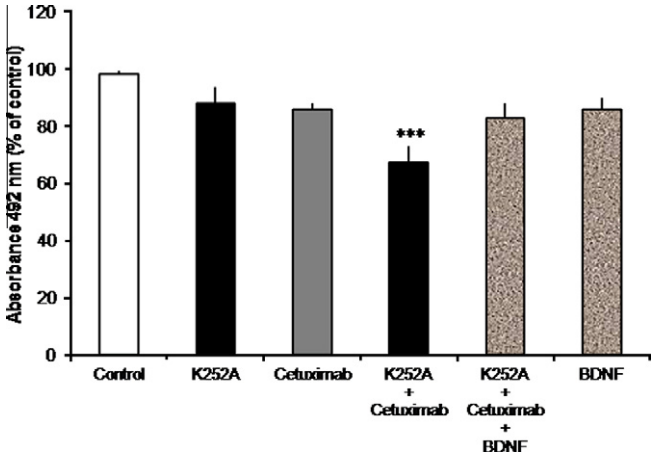

Fig. 4. The antiproliferative effect of cetuximab in CRC cells is synergistically enhanced by TrkB inhibition. HT-29 cells were cultured and treated with cetuximab (10 nM), the Trk inhibitor K252a (10 nM), BDNF (10 ng/ml), cetuximab combined with K252a, or cetuximab combined with both BDNF and K252a, as described in **Materials and methods**. Cell proliferation was measured with MTT assays. Data are mean ± SEM absorbance (the lowest value among replicates in the control group was taken as 100%); $n = 6$ independent experiments; $^{***}P < 0.001$ compared to controls.

Trk was originally described as an oncogene present in a human colon carcinoma [20]. However, neurotrophins and Trks have been for many years investigated mainly by neurobiologists as brain molecules involved in neuronal survival and synaptic plasticity [21], while their role in cancer has remained less explored. The function of neurotrophin/Trk signaling in cancer has been mostly characterized in neural tumors, particularly neuroblastoma. BDNF and TrkB are preferentially expressed in aggressive neuroblastomas and might stimulate tumor growth, invasion, and metastasis [reviewed in [8–10]]. BDNF/TrkB may also promote resistance to chemotherapy in neuroblastoma. Thus, treatment with BDNF protected neuroblastoma cells from etoposide toxicity *in vitro*, and high TrkB expression in experimental neuroblastoma in mice was associated with less sensitivity to etoposide in a xenograft mouse model [22].

In human CRC, TrkB has been found mutated on two different sites in its kinase domain, $TRKB^{T695I}$ and $TRKB^{D751N}$,[11,23], but experiments using rat epithelial cells and human tumor cell lines have failed to provide evidence for a gain-of-function effect of the mutations [23]. Still, it is possible that increased BDNF/TrkB signaling plays an important role in CRC. We have recently shown that BDNF and TrkB are expressed in CRC cell lines and sporadic CRC specimens, and BDNF levels were increased in tumor samples compared to non-neoplastic tissue. In addition BDNF/TrkB stimulation was associated with resistance to the antitumor effects of GRPR blockade in HT-29 CRC cells, through an EGFR-dependent mechanism [12]. Another recent study confirmed BDNF and TrkB expression in CRC and showed that BDNF stimulated cell proliferation and had an anti-apoptotic effect mediated through TrkB [13]. Moreover, high TrkB expression in tumors has recently been associated with poor prognosis in patients with CRC, and proliferation, migration, and invasion in CRC cells were inhibited by TrkB knockdown [24]. However, previous studies have not provided evidence that BDNF/TrkB can be a mechanism of resistance to anti-EGFR therapy in cancer. The present results provide early *in vitro* evidence suggesting that (1) the effects of cetuximab might require a reduction in BDNF and TrkB expression, (2) increased BDNF signaling might counteract the effects of cetuximab, and (3) combined targeting of EGFR and TrkB might increase sensitivity to anti-EGFR therapy in CRC cells.

Further experiments will be required to identify the intracellular signaling mechanisms underlying the functional interaction between cetuximab treatment and BDNF in CRC cells. EGFR and TrkB share common downstream effectors, including the Ras/extracellular signal-regulated protein kinase (ERK)/MAPK and the PI3 K/AKT cascades [9,21,25]. It is possible that increased BDNF/TrkB activity in CRC compensates for EGFR blockade by stimulating those shared intracellular protein kinase pathways.

Our findings raise the possibility that, under conditions in which BDNF/TrkB activity is up-regulated, as is likely the case in CRC [12,13,24], it may contribute to cetuximab resistance. *In vivo* studies aimed at investigating whether the combined targeting of EGFR and BDNF/TrkB might be a novel strategy to overcome cetuximab resistance in CRC are warranted.

Acknowledgments

This research was supported by the National Council for Scientific and Technological Development (CNPq; Grant number 303703/2009-1 to RR); the National Institute for Translational Medicine (INCT-TM); FAPERGS/CNPq Grant number 10/0044-3-PRONEX; the University Hospital Institutional Research Fund

(FIPE/HCPA); the South American Office for Anticancer Drug Development; and the Children's Cancer Institute (ICI-RS).

References

[1] C.S. Karapetis, S. Khambata-Ford, D.J. Jonker, C.J. O'Callaghan, D. Tu, N.C. Tebbutt, R.J. Simes, H. Chalchal, J.D. Shapiro, S. Robitaille, T.J. Price, L. Shepherd, H.J. Au, C. Langer, M.J. Moore, J.R. Zalcberg, K-ras mutations and benefit from cetuximab in advanced colorectal cancer, N. Engl. J. Med. 359 (2008) 1757–1765.

[2] A. Bardelli, S. Siena, Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer, J. Clin. Oncol. 28 (2010) 1254–1261.

[3] A. Bardelli, P.A. Jänne, The road to resistance. EGFR mutation and cetuximab, Nat. Med. 18 (2012) 199–200.

[4] L.A. Diaz Jr, R.T. Williams, J. Wu, I. Kinde, J.R. Hecht, J. Berlin, B. Allen, I. Bozic, J.G. Reiter, M.A. Nowak, K.W. Kinzler, K.S. Oliner, B. Vogelstein, The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers, Nature 486 (2012) 537–540.

[5] D.L. Wheeler, S. Huang, T.J. Kruser, M.M. Nechrebecki, E.A. Armstrong, S. Benavente, V. Gondi, K.T. Hsu, P.M. Harari, Mechanisms of acquired resistance to cetuximab: role of HER (ErbB) family members, Oncogene 27 (2008) 3944–3956.

[6] K. Yonesaka, K. Zejnullahu, I. Okamoto, T. Satoh, F. Cappuzzo, J. Souglakos, D. Ercan, A. Rogers, M. Roncalli, M. Takeda, Y. Fujisaka, J. Philips, T. Shimizu, O. Maenishi, Y. Cho, J. Sun, A. Destro, K. Taira, K. Takeda, T. Okabe, J. Swanson, H. Itoh, M. Takada, E. Lifshits, K. Okuno, J.A. Engelman, R.A. Shivdasani, K. Nishio, M. Fukuoka, M. Varella-Garcia, K. Nakagawa, P.A. Jänne, Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab, Sci. Transl. Med. 3 (2011). 99ra86.

[7] A. Bertotti, G. Migliardi, F. Galimi, F. Sassi, D. Torti, C. Isella, D. Corà, F. Di Nicolantonio, M. Buscarino, C. Petti, D. Ribero, N. Russolillo, A. Muratore, P. Massucco, A. Pisacane, L. Molinaro, E. Valtorta, A. Sartore-Bianchi, M. Risio, L. Capussotti, M. Gambacorta, S. Siena, E. Medico, A. Sapino, S. Marsoni, P.M. Comoglio, A. Bardelli, L. Trusolino, A molecularly annotated platform of patient-derived xenografts (''xenopatients'') identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer, Cancer Discov. 1 (2011) 508–523.

[8] C.J. Desmet, D.S. Peeper, The neurotrophic receptor TrkB: a drug target in anti-cancer therapy?, Cell Mol. Life Sci. 63 (2006) 755–759.

[9] C.J. Thiele, Z. Li, A.E. McKee, On Trk – the TrkB signal transduction pathway is an increasingly important target in cancer biology, Clin. Cancer Res. 15 (2009) 5962–5967.

[10] R. Roesler, C.B. de Farias, A.L. Abujamra, A.L. Brunetto, G. Schwartsmann, BDNF/TrkB signaling as an anti-tumor target, Expert Rev. Anticancer Ther. 11 (2011) 1473–1475.

[11] A. Bardelli, D.W. Parsons, N. Silliman, J. Ptak, S. Szabo, S. Saha, S. Markowitz, J.K. Willson, G. Parmigiani, K.W. Kinzler, B. Vogelstein, V.E. Velculescu, Mutational analysis of the tyrosine kinome in colorectal cancers, Science 300 (2003) 949.

[12] C.B. de Farias, D.B. Rosemberg, T.E. Heinen, P. Koehler-Santos, A.L. Abujamra, F. Kapczinski, A.L. Brunetto, P. Ashton-Prolla, L. Meurer, M.R. Bogo, D.C. Damin, G. Schwartsmann, R. Roesler, BDNF/TrkB content and interaction with gastrin-releasing peptide receptor blockade in colorectal cancer, Oncology 79 (2010) 430–439.

[13] H. Akil, A. Perraud, C. Mélin, M.O. Jauberteau, M. Mathonnet, Fine-tuning roles of endogenous brain-derived neurotrophic factor, TrkB and sortilin in colorectal cancer cell survival, PLoS One 6 (2011) e25097.

[14] D.G. Flores, C.B. de Farias, J. Leites, M.S. de Oliveira, R.C. Lima, A.S. Tamajusuku, L.P. Di Leone, L. Meurer, A.L. Brunetto, G. Schwartsmann, G. Lenz, R. Roesler, Gastrin-releasing peptide receptors regulate proliferation of C6 glioma cells through a phosphatidylinositol 3-kinase-dependent mechanism, Curr. Neurovasc. Res. 5 (2008) 99–105.

[15] C.B. de Farias, R.C. Lima, L.O. Lima, D.G. Flores, L. Meurer, A.L. Brunetto, G. Schwartsmann, R. Roesler, Stimulation of proliferation of U138-MG glioblastoma cells by gastrin-releasing peptide in combination with agents that enhance cAMP signaling, Oncology 75 (2008) 27–31.

[16] C.J. Allegra, J.M. Jessup, M.R. Somerfield, S.R. Hamilton, E.H. Hammond, D.F. Hayes, P.K. McAllister, R.F. Morton, R.L. Schilsky, American Society of Clinical Oncology provisional clinical opinion: testing for *KRAS* gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy, J. Clin. Oncol. 27 (2009) 2091–2096.

[17] S. Misale, R. Yaeger, S. Hobor, E. Scala, M. Janakiraman, D. Liska, E. Valtorta, R. Schiavo, M. Buscarino, G. Siravegna, K. Bencardino, A. Cercek, C.T. Chen, S. Veronese, C. Zanon, A. Sartore-Bianchi, M. Gambacorta, M. Gallicchio, E. Vakiani, V. Boscaro, E. Medico, M. Weiser, S. Siena, F. Di Nicolantonio, D. Solit, A. Bardelli, Emergence of *KRAS* mutations and acquired resistance to anti-EGFR therapy in colorectal cancer, Nature 486 (2012) 532–536.

[18] A. Sartore-Bianchi, M. Martini, F. Molinari, S. Veronese, M. Nichelatti, S. Artale, F. Di Nicolantonio, P. Saletti, S. De Dosso, L. Mazzucchelli, M. Frattini, S. Siena, A. Bardelli, *PIK3CA* mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies, Cancer Res. 69 (2009) 1851–1857.

[19] W. De Roock, B. Claes, D. Bernasconi, J. De Schutter, B. Biesmans, G. Fountzilas, K.T. Kalogeras, V. Kotoula, D. Papamichael, P. Laurent-Puig, F. Penault-Llorca, P. Rougier, B. Vincenzi, D. Santini, G. Tonini, F. Cappuzzo, M. Frattini, F. Molinari, P. Saletti, S. De Dosso, M. Martini, A. Bardelli, S. Siena, A. Sartore-Bianchi, J. Tabernero, T. Macarulla, F. Di Fiore, A.O. Gangloff, F. Ciardiello, P. Pfeiffer, C. Qvortrup, T.P. Hansen, E. Van Cutsem, H. Piessevaux, D. Lambrechts, M. Delorenzi, S. Tejpar, Effects of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis, Lancet Oncol. 11 (2010) 753–762.

[20] D. Martin-Zanca, G. Mitra, L.K. Long, M. Barbacid, Molecular characterization of the human trk oncogene, Cold Spring Harb. Symp. Quant. Biol. 51 (Pt 2) (1986) 983–992.

[21] E.J. Huang, L.F. Reichardt, Trk receptors: roles in neuronal signal transduction, Annu. Rev. Biochem. 72 (2003) 609–642.

[22] Z. Li, D.Y. Oh, K. Nakamura, C.J. Thiele, Perifosine-induced inhibition of akt attenuates brain-derived neurotrophic factor/TrkB-induced chemoresistance in neuroblastoma *in vivo*, Cancer 117 (2011) 5412–5422.

[23] T.R. Geiger, J.Y. Song, A. Rosado, D.S. Peeper, Functional characterization of human cancer-derived TRKB mutations, PLoS One 6 (2011) e16871.

[24] H. Fujikawa, K. Tanaka, Y. Toiyama, S. Saigusa, Y. Inoue, K. Uchida, M. Kusunoki, High TrkB expression levels are associated with poor prognosis and EMT induction in colorectal cancer cells, J. Gastroenterol. (2012), http://dx.doi.org/10.1007/s00535-012-0532-0.

[25] J.P. Spano, R. Fagard, J.C. Soria, O. Rixe, D. Khayat, G. Milano, Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives, Ann. Oncol. 16 (2005) 189–194.