Enalapril and ASS inhibit tumor growth in a transgenic mouse model of islet cell tumors

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Abstract

Accumulating evidence suggests a role for angiotensin-converting enzymes involving the angiotensin II-receptor 1 (AT1-R) and the cyclooxygenase pathway in carcinogenesis. The effects of ASS and enalapril were assessed in vitro and in a transgenic mouse model of pancreatic neuroendocrine neoplasms (pNENs). The effects of enalapril and ASS on proliferation and expression of the AGTR1A and its target gene vascular endothelial growth factor (Vegfa) were assessed in the neuroendocrine cell line BON1. Rip1-Tag2 mice were treated daily with either 0.6 mg/kg bodyweight of enalapril i.p., 20 mg/kg bodyweight of ASS i.p., or a vehicle in a prevention (weeks 5–12) and a survival group (week 5 till death). Tumor surface, weight of pancreatic glands, immunostaining for AT1-R and nuclear factor kappa beta (NFKB), and mice survival were analyzed. In addition, sections from human specimens of 20 insulinomas, ten gastrinomas, and 12 non-functional pNENs were evaluated for AT1-R and NFKB (NFKB1) expression and grouped according to the current WHO classification. Proliferation was significantly inhibited by enalapril and ASS in BON1 cells, with the combination being the most effective. Treatment with enalapril and ASS led to significant downregulation of known target genes Vegf and Rela at RNA level. Tumor growth was significantly inhibited by enalapril and ASS in the prevention group displayed by a reduction of tumor size (84%/67%) and number (30%/45%). Furthermore, daily treatment with enalapril and ASS prolonged the overall median survival compared with vehicle-treated Rip1-Tag2 (107 days) mice by 9 and 17 days (P=0.016 and P=0.013). The AT1-R and the inflammatory transcription factor NFKB were abolished completely upon enalapril and ASS treatment. AT1-R and NFKB expressions were observed in 80% of human pNENs. Enalapril and ASS may provide an approach for chemoprevention and treatment of pNENs.

Key Words

► aspirin
► ACE inhibitors
► chemoprevention
► neuroendocrine tumors
► Rip1-Tag2 mouse model
Introduction

Pancreatic neuroendocrine neoplasms (pNENs) occur rarely with an annual incidence of 1/100,000 people, but their incidence is increasing steadily (Oberg & Eriksson 2005). pNENs present as either functional pNENs or non-functional pNENs (NF-pNENs). pNENs occur sporadically or can be associated with familial tumors such as multiple endocrine neoplasia type 1 (MEN1), von Hippel-Lindau (VHL) syndrome, or neurofibromatosis. The natural course of pNENs is highly variable. In general, a complete tumor resection improves survival in patients with pNENs. An aggressive surgical approach in patients with metastatic disease as part of a multimodal treatment often results in long-term survival (Metz & Jensen 2008). Survival is mainly determined by liver metastases, which can be targeted by several approaches such as surgery, chemoembolization, radiofrequency ablation, peptide radioreceptor therapy, or selective internal radiation therapy (Fendrich et al. 2006, Plockinger & Wiedenmann 2007). Biotherapy with somatostatin (SS) analogs is also frequently recommended for the treatment of metastatic disease achieving a tumor growth stabilization rather than inducing regression. Similar to SS analogs in midgut NENs, a tumorstatic effect may result in improved survival, but, at present, this has not been proven for pNENs (Plockinger & Wiedenmann 2007, Rajetta et al. 2009). Recently, the mTOR inhibitor everolimus and the multitargeted sunitinib have been shown to increase the overall survival in advanced neuroendocrine tumor patients in randomized controlled trials (Raymond et al. 2011, Yao et al. 2011).

The Rip1-Tag2 mouse model of pancreatic islet cell carcinoma offers a model of spontaneous multistep tumorigenesis. Transgenic mice express the oncogenic SV40 T antigen (Tag) under the control of the rat insulin gene promoter (RIP) and display different stages of tumor progression: onset of hyperproliferation, induction of angiogenesis, and formation of solid pNENs. Rip1-Tag2 mice succumb to insulinomas by the age of 14 weeks due to hypoglycemia. This multistage process suggests the sequential involvement of multiple genetic and epigenetic events in the progression from normal cells to tumors (Hanahan 1985, Folkman et al. 1989, Lopez & Hanahan 2002). As vascularization represents a key element in the progression of islet cell tumors, inhibition of tumor-induced angiogenesis offers a rational therapeutic approach. Vascular endothelium-derived growth factor A (VEGFA) and platelet-derived growth factor are essential promoters of adult angiogenesis in a physiological and pathological setting and are upregulated in tumors of Rip1-Tag2 mice (Inoue et al. 2002, Bergers & Benjamin 2003, Ferrara 2004). Recently, the authors have reported new therapeutic approaches with cyclopamine and the Smo antagonist LDE225 for the treatment of pNENs using the same transgenic mouse model of islet cell tumors as presented in this study. In addition, our recent chemoprevention study in a transgenic mouse model of pancreatic cancer has revealed a delay of pancreatic intraepithelial neoplasia progression and cancer formation upon treatment with enalapril and aspirin (Fendrich et al. 2010, 2011a,b). Increasing evidence suggests a role for angiotensin-converting enzymes (ACEs) in carcinogenesis in an angiotensin II–AT1-R axis-dependent manner, involving VEGF (Asano et al. 2004, De Paepe 2009, Miyajima et al. 2009).

Population-based studies have shown that ACE inhibitor usage can be associated with reduced cancer risks, although recent studies have provided conflicting data (Lever et al. 1998, Garcia Rodriguez & Gonzalez-Perez 2004, Gonzalez-Perez et al. 2004, Teo 2011). An improved outcome can be observed in certain tumors such as pancreatic (Nakai et al. 2010) and non-small lung cancer (Wilop et al. 2009) after ACE inhibition combined with chemotherapy. Similar protective effects have been reported for the cyclooxygenase (COX) enzyme inhibitor aspirin (ASS) (Kune et al. 1988, Thun et al. 1991). As in many other types of malignant neoplasms, COX2 (PTGS2) and nuclear factor kappa beta (NFKB (NFKB1)) are overexpressed in pancreatic carcinoma and in a subset of precursor lesions (Maitra et al. 2002, Sclabas et al. 2005). Consequently, COX2 and NFKB might represent potential target for chemoprevention with selective COX2 inhibitors (Kopp & Ghosh 1994, Amaya et al. 2004). A chemopreventive strategy for pNENs is completely unexplored so far.

In this study, we evaluated antitumorigenic properties of enalapril and ASS in vitro using the human neuroendocrine cell line BON1. The effects of these drugs on tumor growth and survival were also tested in a chemopreventive setting in a transgenic mouse model of islet cell tumors. Two major targets, which are known to mediate the antitumorigenic effects of enalapril and ASS, were assessed in a set of human insulinomas, gastrinomas, and NF-pNENs.

Materials and methods

Cell culture

BON1 pancreatic carcinoid cells were grown in RPMI (Biochrom, Invitrogen) supplemented with 10% FCS
(Biochrom, Berlin, Germany) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. Before treating, the cells were tested for mycoplasma infection using the QIAamp Viral RNA Mini Kit (Qiagen) for PCR.

**Proliferation assays**

For proliferation assay, BON1 cells were seeded into 96-well plates at a density of 5000 cells/well and 200 μl medium/well and were incubated overnight and, for RT-PCR, cells were seeded into 12-well-plates at a density of 80 000 cells/well and 2 ml medium/well. The following day, the cells were starved for another 24 h in a low-serum medium (RPMI with 0.5% FCS and 1% penicillin/streptomycin). Then, cells were treated with 100 μl low-serum medium and 100 μl vehicle for 24, 48, and 72 h. Vehicle concentration and combinations are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. Untreated cells served as controls. Proliferation assay was carried out after 24, 48, and 72 h. To determine viable cell mass, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide reagent (Thiazolyl Blue TetrazoliumBromid from Sigma) were added per well. Afterwards, cells in plates were incubated at 37 °C for 1 h. Optical density was read at an absorbance of 570/630 nm on a Wallac 1420 plate reader. All experiments were carried out in triplicate. For RT-PCR cells were treated until 48 h. Then, cells were lysed in the culture with 350 μl TRK buffer and whole RNA extraction was performed using RNA Kit PegLab. After RNA extraction, probes were transferred into cDNA with oligo-dT primer from Omniscript from Qiagen.

**RT-PCR**

All PCRs were carried out on a 7500 Real-Time PCR System (Applied Biosystems) over 40 cycles, with denaturation for 15 s at 95 °C and combined annealing/extension at 60 °C for 1 min, using the SYBR Green Master Mix from Applied Biosystems. Primer sequences for Vegf(Vegfa) were as follows: forward, 5’-CTC ACT TCC AGA AAC ACG ACA AA-3’ and reverse, 5’-CTG GTT GGA ACC GCC GTC ATC T-3’. For RELA, the most abundant from of NFKB, the primer sequence was as follows: forward, 5’-CCC GAC TTT GGT GGA TTA C-3’ and reverse, 5’-GCT CCG TCT CCA GGA GTG TGA-3’.

**Mice**

The generation of Rip1-Tag2 mice as a model of pancreatic islet cell carcinogenesis has been reported previously (Fendrich et al. 2011a). Mice used in this study were males and females of the Rip1-Tag2 transgenic mouse lineage bred in a C57Bl/6J background. All experiments were approved by the local committees for animal care and use. Animals were maintained in a climate-controlled environment at 22 °C, exposed to a 12 h light:12 h darkness cycle, fed on a standard laboratory chow diet and provided water ad libitum. For genotyping, genomic DNA was extracted from tail cuttings using the RedExtract-N-Amp Tissue PCR (PCR) Kit (Sigma–Aldrich). PCR was carried out for each animal, to test for the presence of Tag2. Primer sequences used were: 5’-GGA CAA ACC ACA ACT AGA ATG CAG-3’ and 5’-CAG AGC AGA ATT GTG GAG TGG-3’.

**Treatment**

The grouping and therapeutical algorithms are explained in Fig. 1. In the prevention group, animals were treated from 5 to 12 weeks of age and, in the survival group, from the fifth week of age until death defined by specific abort criteria in agreement with the standards for animal care. Rip1-Tag2 transgenic mice were randomly assigned to receive either i) aspirin (prevention OR survival group), ii) enalapril, or iii) vehicle. Aspirin and enalapril were dissolved in a sterile sodium chloride solution and administered to mice by an i.p. injection. Aspirin was administered once a day at a dose of 20 mg/kg bodyweight and enalapril was administered once a day at a dose of 0.6 mg/kg bodyweight, both intraperitoneally on a daily basis (Sakamoto et al. 2001). The dose of ASS is equivalent to a daily human dosage of 80–110 mg (Mahmoud et al. 1998). The equivalent human dosage of enalapril is unknown.

![Figure 1](http://erc.endocrinology-journals.org)

Study design of Rip1-Tag2 transgenic mice treated with enalapril and ASS.
Necropsy

After completion of drug treatment, mice from the prevention and survival groups were killed and the pancreas was removed, weighed, and inspected for grossly visible tumors and preserved in 10% formalin solution (Sigma–Aldrich) for histology. For evaluating a change in islet cell tumor growth, four sections from each mouse were analyzed. These sections were taken at a distance of 1, 10, 20, and 40 μm from the surface of the pancreas and stained with H&E. All the islets were marked and their surface calculated computerized.

Table 1  Clinical characteristics and immunostaining results for AT1-R and NFKB in 44 patients with functioning and non-functioning pNENs

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<th>IHC AT1 (positive/negative)</th>
<th>Enalapril (yes/no)</th>
<th>Aspirin (yes/no/ND)</th>
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M, metastases; IHC, immunohistochemistry; AT1-R, angiotensin II-receptor 1; NFKB, nuclear factor kappa beta; NF-pNEN, non-functional pancreatic endocrine neoplasm; PH, pancreatic head; PB, pancreatic body; PT, pancreatic tail; LNM, lymph node metastasis; LM, liver metastases; LN, lymph node, ND, no data.
Immunostaining

Formalin-fixed and paraffin-embedded archived tumor samples, the corresponding normal tissue, and murine pancreatic glands were stained as described previously (Fendrich et al. 2008). Primary and secondary antibodies including positive and negative controls are given in Supplementary Table 1 and Supplementary Fig. 1. Briefly, slides were heated to 60 °C for 1 h, deparaffinized using xylene, and hydrated by a graded series of ethanol washes. Antigen retrieval was accomplished by microwave heating in 10 mM sodium citrate buffer, pH 6.0, for 10 min. For immunohistochemistry, endogenous peroxidase activity was quenched by 10 min incubation in 3% H$_2$O$_2$. Nonspecific binding was blocked with 10% serum. Sections were then probed with primary antibodies overnight at 4 °C. For immunohistochemistry, bound antibodies were detected using the avidin–biotin complex (ABC) peroxidase method (ABC Elite Kit, Vector Labs, Burlingame, CA, USA). Final staining was developed using the Sigma FAST DAB peroxidase substrate kit (Sigma). The immunohistochemistry results were scored as described previously (Fendrich et al. 2007). The AT1 and NFKB staining was categorized by the fraction of positive cells (weak, <10%; medium, >10 and <50%; and high, >50%). Lymphocytes and endothelial cells as shown in Supplementary Fig. 1 served as an internal positive control.

Patient samples

The human samples were obtained from patients with pNENs, who were treated at the authors’ department. Serial sections of 20 insulinomas, 12 gastrinomas, and 12 NF-pNENs were confirmed by an experienced pathologist (A Ramaswamy) and subjected for further analyses. Clinical characteristics and WHO classification (Rindi 2010) are shown in Table 1. All patients gave their informed consent. The study followed the guidelines of the Local Ethics Committee.

Statistical analysis

Survival curves were calculated using the Kaplan–Meier method. Log-rank test was applied to identify significant differences. Non-categorical parameters were analyzed by the Mann–Whitney U-test. Comparisons of more than two groups were made by one-way ANOVA with post hoc Holm–Sidak analysis for pairwise comparisons and comparisons vs control and by Kruskal–Wallis one-way
ANOVA. *P* values <0.05 were considered statistically significant. Data were analyzed using the SPSS Software (version 14; SPSS, Inc.).

**Results**

**Tumor growth is inhibited by enalapril and ASS in vitro**

The proliferation of BON1 cells under the defined medium conditions was assessed by a standard MTT assay at 24, 48, and 72 after treatment with different doses of enalapril and ASS either alone or in combination and compared with different control groups (see also Fig. 2A, B and C). Enalapril as a single agent decreased proliferation in BON1 cells by 9–21%, which was significant after 48 and 72 h compared with non-treated and benzyl-treated cells as controls. ASS reduced the proliferation by 43, 59, and 68 h after 24, 48, and 72 h more significantly as a single agent and was only less effective than the combination with enalapril (see also Fig. 2A, B and C). RT-PCR revealed significant down-regulation of VEGF and RELA upon enalapril and ASS treatment, which are known mediators of antitumorigenic properties of enalapril and ASS (see Fig. 3) in cultured BON1 cells. The combination of enalapril and ASS showed no additional effect (data not shown).

**Chemoprevention with enalapril and ASS significantly reduces tumor growth**

We observed development of islet cell hyperplasia, angiogenic islets, and islet cell tumors in the Rip1-Tag2 mice cohort used for our study compared with WT control mice, as described previously. The median tumor size decreased significantly upon enalapril and ASS treatment in Rip1-Tag2 mice (prevention-group) by 84% for enalapril (2 572 226 vs 15 935 703 μm², *P*=0.002) and 67% for ASS (5 276 137 vs 15 935 703 μm², *P*=0.001). Accordingly, the treatment resulted in a reduction of the median tumor number by 30% (9 vs 6) for enalapril and 45% for ASS (9 vs 5). Upon treatment of enalapril and ASS, the pancreas/tumor weight was analogously reduced (0.12 and 0.10 vs 0.15 g; *P*=0.028 and *P*=0.015; see also Fig. 4). *In vivo* enalapril and ASS treatment led to a lower Ki-67 index (1 and 17%, *P*<0.0001 and *P*<0.0001) compared with mock-treated control Rip1-Tag2 mice (29%) in the prevention group, which confirmed the *in vitro* results in BON1 cells.

**Chemoprevention with enalapril and ASS led to loss of angiotensin II-receptor 1 and NFKB expression**

Enalapril-, ASS-, and vehicle-treated Rip1-Tag2 mice (*n*=3 each) were stained for AT1-R and NFKB. Angiotensin II-receptor 1 (AT1-R) expression could be observed in tumor cells of all vehicle-treated control mice, whereas all enalapril- and ASS-treated mice had no AT1-R-positive tumor cells. Accordingly, NFKB expression could be observed only in tumor cells of vehicle-treated control mice, whereas enalapril- and ASS-treated mice had no NFKB-positive tumor cells (see also Fig. 5).
Chemoprevention with enalapril and ASS prolongs survival

Analyzed mice were treated with either enalapril, ASS, or vehicle from week 5 until death. Based on survival data of each group, a Kaplan–Meier survival plot was calculated (see Fig. 6). Enalapril treatment extended survival compared with survival of vehicle-treated mice by 10 days (108 vs 118 days; \( P=0.022 \)) and ASS treatment prolonged the median survival by 16 days (108 vs 124 days; \( P=0.016 \)) (see also Fig. 6A and B). The enalapril- and ASS-treated mice revealed a significantly lower weight of the pancreas/tumors at autopsy than the vehicle-treated control (0.15 and 0.16 vs 0.18 g; \( P=0.043 \) and \( P=0.039 \)), reflecting the tumor growth inhibition.

Major reason of Rip1-Tag2 mice is hypoglycemia, which first impairs the behavior and finally leads to coma and death.

AGTR1 and NFKB expression in human sporadic and familial pNETs

To compare the AGTR1 and NFKB expression observed in the murine Rip1-Tag2 model with the situation in human sporadic and familial pNEN sections of insulinomas (\( n=20 \)) and gastrinomas (\( n=11 \)), and NF-pNENs (\( n=12 \)) were stained for AT1-R and NFKB expression. At IHC, 15 out of 20 insulinomas, six out of 11 gastrinomas, and all NF-pNENs revealed AT1-R expression. Accordingly, five out of eight insulinomas, three out of three gastrinomas, and four out of four NF-pNENs expressed NFKB (see also Fig. 7). Negative sections at IHC for AT1-R were found in one out of three patients who were on enalapril. Clinical characteristics and classification according to the current WHO classification of analyzed patients are summarized in Table 1.

Discussion

The mortality of pNENs ranges between 5 and 70% with a 5-year survival of 30–95% and depends on clinical features and the genetic background (Jensen et al. 2012). Chemopreventive strategies may be beneficial for high-risk individuals with a hereditary predisposition for pNENs such as MEN1, VHL, or neurofibromatosis type 2. Mortality in patients with MEN1 is mainly caused by pNENs and malignant NEN of the thymus (Bartsch et al. 2005, Fendrich et al. 2006, Jensen et al. 2006, Goudet et al. 2009, 2010, Waldmann et al. 2009). Therefore, potent chemoprevention strategies may help to reduce the overall pNEN-associated mortality, but no chemopreventive strategies are known for pNENs so far.

In this study, we show for the first time that enalapril and ASS inhibit proliferation of human BON1 cells as an in vitro model for pNENs. Downregulation of VEGF and RELA, the most abundant form of NFKB, upon enalapril and ASS treatment may be held accountable for the growth inhibition and presents a known mechanism in other
non-neuroendocrine tumor cell lines. NFKB orchestrates the expression of genes that encode key regulators of tumorigenesis, inflammation, and apoptosis and thus promotes tumor progression. Constitutive activation of NFKB is frequently observed in medullary thyroid cancer and NFKB protects pancreatic islet cells from tumor necrosis factor alpha-mediated cell death (Ludwig et al. 2001, Chang et al. 2003). Furthermore, activation of NFKB is a frequent event shared by differentiated and anaplastic thyroid cancer and first studies with NFKB inhibitors achieved promising results (Pacifico & Leonardi 2010). ACE inhibitors are known to reduce the expression of NFKB (Hernandez-Presa et al. 1998), while aspirin inhibits NFKB induction by decreasing the Ikk2 activity through binding to Ikk2 and thereby limits ATP binding. Tyrosine kinase and ERK1/2–MAP-kinase activation of the AT1-R induces VEGF expression, which mediates NFKB overexpression through the Akt pathway (Asano et al. 2004, Escobar et al. 2004, Hennessy et al. 2005). A similar mechanism was also described for the pancreatic adenocarcinoma (Arafat et al. 2007, Fendrich et al. 2010). Along with the literature, we could demonstrate in this study that enalapril and ASS significantly had an antiproliferative effect and reduced the target genes Vegf and Rela in vitro.

This observation was then confirmed by tumor growth inhibition in vivo with a decreased Ki-67 index after enalapril and ASS treatment in a transgenic mouse model of pNENs. Tumor growth inhibition was

Figure 5
Immunostaining for AT1-R, NFKB, and Ki-67 in the representative sections of islet cell tumors in Rip1-Tag2 mice. Sections were analyzed at a magnification of 10× and counterstained with hematoxylin. Brown staining indicates a positive signal of the primary antibody in endocrine cells of the different tumor types. NF-pNEN, non-functional pancreatic neuroendocrine neoplasm; NFKB, nuclear factor kappa beta; alpha-AT1, alpha-angiotensin II receptor 1.

Figure 6
Kaplan–Meier curves in (A) enalapril-treated and (B) ASS-treated Rip1-Tag2 mice. P values and estimated median survival were calculated by a log-rank test using ten mice per group.
accompanied by a significantly prolonged survival. Several groups have reported that blocking the AT1-R by angiotensin receptor blockers (ARBs) can lead to reduced tumor growth and metastasis in experimental models of solid tumors (Rivera et al. 2001, Miyajima et al. 2002, Uemura et al. 2006, Rodrigues-Ferreira et al. 2012). Several animal cancer models after pharmacological ACE inhibition or AT1-R depletion revealed antitumorigenic properties (Volpert et al. 1996, Kosaka et al. 2007, Vinson et al. 2012). These observations are further reinforced by genetic models with AT1-R depletion, revealing deeper insights into the mechanisms of antiangiogenesis and growth inhibition mediated by the AT1-R (Egami et al. 2003). Neo et al. (2010) demonstrated that during the development of colorectal liver metastases, AT1-R expression increases and could be reduced by captopril, which led to a reduction in the tumor volume. Most effects of the inhibition of AT1-R-driven angiogenesis are mediated through VEGF. The loss of AT1-R expression was also observed in enalapril- and ASS-treated animals compared with the vehicle control in this study. Parts of the antitumorigenic effect may be mediated by tumor-associated macrophages. A subpopulation of highly invasive breast cancer revealed a dramatic overexpression of AT1-R and raised the question of a therapeutical approach against breast cancer (Rhodes et al. 2009). The downregulation of AT1-R in islet cell tumors upon ACE inhibition may be mediated by the decreased angiotensin II levels, which are a consequence of the treatment with enalapril. This would be in the line with an induction of ATR1 in adrenal glands upon ACE inhibition, which was reported by Harrison-Bernard et al. (1999) in rats. Both the tissue-specific regulation of AT2-receptor isoforms (Sechi et al. 1996) and the complexity of multiple regulators challenge the theoretical explanation of the ablation of Atr1 in this study, which remains highly speculative.

These promising preclinical results have been challenged by a meta-analysis of randomized controlled trials, which showed a modestly higher individual risk for patients under ARBs (Sipahi et al. 2010). However, a meta-analysis of over 300 000 patients showed no increased risk for cancer, except in patients taking...
inhibitors of the ACEs in combination with ARBs (Bangalore et al. 2011).

Human pNENs showed robust expression of the ARB target ATR1 in the majority of analyzed pNENs in this study. This potentially points toward the AT1-R as a therapeutic target for AT1-R and ACE inhibitors for pNENs, especially in patients with an increased individual risk in a prophylactic setting. The loss of NFkB expression upon enalapril and ASS treatment in Rip1-Tag2 mice may be considered an additional potential effect.

This study found a significant antiproliferative effect of enalapril and ASS on islet cell tumors in vitro in a transgenic mouse model and revealed the target gene AT1-R to be expressed in the majority of human pNENs. Therefore, enalapril and/or ASS might be a valuable chemopreventive agent in patients with a high risk for pNENs that may attenuate the tumorigenic process rather than preventing it.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0175.

Declaration of interest
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