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Blockade of NFκB activity by Sunitinib increases cell death in Bortezomib-treated endometrial carcinoma cells

Anabel Sorolla^{a,1}, Andrée Yeramian^{a,1}, Joan Valls^b, Xavier Dolcet^a, Laura Bergadà^a, Antoni Llobart-Cussac^c, Rosa Maria Martí^{d,2}, Xavier Matias-Guiu^{a,*,2}

^aPathology Group, Department of Pathology and Molecular Genetics, Hospital Universitari Arnau de Vilanova, University of Lleida, IRB-Lleida, 80 Avinguda Alcalde Rovira Roure, 25198 Lleida, Spain

^bBiostatistics Unit, Hospital Universitari Arnau de Vilanova, University of Lleida, IRB-Lleida, 80 Avinguda Alcalde Rovira Roure, 25198 Lleida, Spain

^cDepartment of Oncology, Hospital Universitari Arnau de Vilanova, University of Lleida, IRB-Lleida, 80 Avinguda Alcalde Rovira Roure, 25198 Lleida, Spain

^dDepartment of Dermatology, Hospital Universitari Arnau de Vilanova, University of Lleida, IRB-Lleida, 80 Avinguda Alcalde Rovira Roure, 25198 Lleida, Spain

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ABSTRACT

Endometrial carcinoma is one of the most common malignancies in the female genital tract, usually treated by surgery and radiotherapy. Chemotherapy is used when endometrial carcinoma is associated with widespread metastasis or when the tumor recurs after radiation therapy. In the present study, we demonstrate that the tyrosine kinase receptor inhibitor Sunitinib reduces cell viability, proliferation, clonogenicity and induces apoptotic cell death in endometrial carcinoma cell lines, which is not due to its action through the most known targets like VEGFR, nor through EGFR as demonstrated in this work. Interestingly, Sunitinib reduces NFκB transcriptional activity either at basal level or activation by EGF or TNF-α. We observed that Sunitinib was able to inhibit the Bortezomib-induced NFκB transcriptional activity which correlates with a decrease of the phosphorylated levels of IKKα and β, p65 and IκBα. We evaluated the nature of the interaction between Sunitinib and Bortezomib by the dose effect method and identified a synergistic effect (combination index < 1). Analogously, silencing of p65 expression by lentiviral-mediated short-hairpin RNA delivery in Bortezomib treated cells leads to a strongly increased sensitivity to Bortezomib apoptotic cell death. Altogether our results suggest that the combination of Sunitinib and Bortezomib could be considered a promising treatment for endometrial carcinoma after failure of surgery and radiation.

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Abbreviations: TNF, tumor necrosis factor; shRNA, short hairpin RNA; CI, combination index; RTK, receptor tyrosine kinase.

* Corresponding author. Tel.: +34 973 70 53 12; fax: +34 973 70 22 01.

E-mail addresses: anabel.sorolla@gmail.com (A. Sorolla), andree.yeramian@cmb.udl.cat (A. Yeramian), jvalls@irbllleida.cat (J. Valls), dolcet@cmb.udl.cat (X. Dolcet), laura.bergada46@hotmail.com (L. Bergadà), allombart1@yahoo.com (A. Llobart-Cussac), marti@medicina.udl.cat (R.M. Martí), fjmatiaguui.lleida.ics@gencat.cat (X. Matias-Guiu).

¹ AS and AY are first authors.

² RMM and XM are senior authors.

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1. Introduction

Endometrial carcinoma is one of the most common malignancies in the female genital tract, responsible for one third of deaths related to gynecologic cancers (Jemal et al., 2008). The early stage of endometrial carcinomas are treated by surgery and radiation and the advanced stage has a very poor prognosis (Wolfson et al., 1992). During the past years, many efforts have been undertaken to understand the biology of endometrial carcinoma. Endometrial carcinoma can be divided into two clinicopathological variants, type I and type II (Bokhman et al., 1998), endometrioid and non-endometrioid endometrial carcinoma. Despite this knowledge, the pharmacological treatment of advanced endometrial carcinoma still remains treated with progesterone (Kauppila, 1984) and the combined regimen of doxorubicin and cisplatin (Dizon, 2010; Lovecchio et al., 1984). Often, treatments fail due to the emergence of chemoresistance and new other therapeutic options are very limited (Dizon, 2010). Therefore, further investigations are needed to better understand the signal transduction pathways dysregulated in endometrial carcinoma. This will lead to the identification of novel therapeutic targets that could improve patient's prognosis and treatment outcomes.

It is well known that the NF κ B pathway is constitutively activated in many cancers (Karin et al., 2002; Sosman and Puzanov, 2006), including endometrial carcinoma (Pallares et al., 2004). This pathway controls several critical processes such as cell growth, differentiation and apoptosis (Karin et al., 2002) and its dysregulation can induce cell transformation. NF κ B inhibitors can act on different levels, for example by inhibiting IKKs (Tiedemann et al., 2009) or preventing the degradation of I κ B α by impairing the ubiquitin proteasome pathway. A well described inhibitor of the NF κ B pathway is Bortezomib (Velcade[®], also known as PS-341; Millennium Pharmaceuticals, Cambridge, MA), a dipeptidyl boronic acid that reversibly inhibits the chymotrypsin like activity of the proteasome and is a well described drug for treating multiple myeloma (Hideshima et al., 2001; Orlowski et al., 2002; Raab et al., 2009). The ability of proteasome inhibitors to inhibit the NF κ B pathway is controversial and conflicting results have been reported for Bortezomib. While proteasome inhibitors inhibit the NF κ B pathway in some cancer types, the opposite effect has been demonstrated in various tumors, including endometrial carcinoma (Dolcet et al., 2006), multiple myeloma (Hideshima et al., 2009), gastrointestinal stromal tumor (Bauer et al., 2010) and in a colon adenocarcinoma cell line (Nemeth et al., 2004), despite showing cytotoxic effects.

A recent study by Miller et al. (2010) revealed a list of known compounds capable of inhibiting NF κ B activity, highlighting different means of blocking this pathway. One of the drugs tested was Sunitinib. Sunitinib (SU11248; Sutent[®], Pfizer, New York, NY) is an inhibitor of multiple tyrosine kinase receptors that potently inhibits c-kit, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and to a lesser extent EGFR (Chow and Eckhardt, 2007; Mendel et al., 2003). Recently, Sunitinib maleate has been approved by the FDA for the treatment of renal cell carcinoma and gastrointestinal stromal tumors (Atkins

et al., 2006). Moreover, Sunitinib is currently being tested in two different phase II studies of patients with recurrent and metastatic endometrial carcinoma, showing partial responses and stable disease in some cases (Welch et al.; Correa et al., both unpublished).

A priori, the use of Sunitinib in endometrial carcinoma is rational due to the presence of several tyrosine kinase receptors and their ligands. Examples are both c-kit receptor and its ligand, stem cell factor (SCF), which are expressed in endometrial carcinoma (Elmore et al., 2001; Inoue et al., 1994; Scobie et al., 2003; Slomovitz et al., 2004). VEGF showed high expression in endometrial tumor cells in comparison with normal endometria and the receptors VEGFR1, VEGFR-2 and VEGFR-3 have been shown to be expressed in the surrounding endothelial cells (Guidi et al., 1996; Slomovitz et al., 2004; Yokoyama et al., 2003). As for the receptor PDGFR α , its expression has been demonstrated in endometrial carcinoma (Slomovitz et al., 2004). Finally, EGF receptor was also found to be expressed in this cancer (Niikura et al., 1995).

We have previously demonstrated a synergistic interaction between Bortezomib and Sunitinib in melanoma (Yeramiyan et al., 2011). In the present study, we first investigated the effects of Sunitinib in endometrial carcinoma cell lines and found that Sunitinib treatment leads to a decrease in proliferation and apoptotic cell death. Moreover, Sunitinib inhibited the NF κ B pathway, which was induced by different known stimulus such as TNF- α and EGF and even the induction of NF κ B by Bortezomib. Furthermore, we observed a synergistic interaction between Sunitinib and Bortezomib (CI < 1) and postulate that this is mediated by the ability of Sunitinib to block the NF κ B pathway via the desphosphorylation of p65.

Our principal goal was to determine if the combined treatment of Sunitinib and Bortezomib could result in a more effective treatment for endometrial carcinoma. In particular, we investigated the ability of Sunitinib to inhibit the NF κ B pathway induced by Bortezomib, which was found to enhance the cell cytotoxicity. The results of this endeavor could provide essential information for the design of future preclinical and clinical studies in endometrial carcinoma.

2. Materials and methods

2.1. Cell cultures and treatment procedures

The Ishikawa 3-H-12 cell line (IK) was obtained from the American Type Culture Collection (Manassas, VA). RL-95 and Hec-1A cells were provided by Dr. Reventos (Hospital Vall d'Hebron, Barcelona). These endometrial carcinoma cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco, Barcelona, Spain), 20 mmol/L of L-glutamine and antibiotics at 37 °C and 5% CO₂. The tyrosine kinase inhibitor Sunitinib and the proteasome inhibitor Bortezomib were dissolved in water at final concentrations of 25 mM and 2.6 mM respectively, and added to the complete media to the appropriate final concentrations. All treatments were carried out in complete media, performed in triplicate, and repeated at least three times.

2.2. Chemical reagents and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RNase A, propidium iodide, monoclonal antibody to tubulin and EGF were obtained from Sigma (St Louis, MO). The proteasome inhibitor Bortezomib was manufactured by Millennium Pharmaceuticals. The tyrosine inhibitor Sunitinib was kindly provided by Pfizer Inc. The EGFR inhibitor AG 1478 was from Merck (Darmstadt, Germany). Antibodies against cleaved caspase 3, PDGFR α , PDGFR β , VEGFR2, EGFR, EGFR-phosphotyrosine 1068, p65-phosphoserine 536, I κ B α -phosphoserine 32, phospho-p44/42 MAPK (ERK 1/2) and IKK α -phosphoserine 180/IKK β -phosphoserine 181 were manufactured by Cell Signaling (Beverly, MA, USA). Dako (Glostrup, Denmark) antibodies against c-kit and Santa Cruz Biotechnology (Santa Cruz, CA, USA) antibodies against p65 were used.

2.3. Determination of cell viability and clonogenicity

Cell viability was determined by MTT assays. Endometrial carcinoma cell lines were plated on M96 well plates. After the corresponding treatments, cells were incubated for 45 min with 0.5 mg/mL of MTT reagent and lysed with DMSO. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmond, CA, U.S.A.). For clonogenic assays, 10^3 Ishikawa, $3 \cdot 10^3$ Hec-1-A and $3 \cdot 10^3$ RL-95-2 cells were seeded in 2 ml culture medium per well (6 well plate), left untreated or treated every two days with 0.1 μ M, 0.2 μ M, 0.5 μ M and 1 μ M of Sunitinib and Bortezomib 0.5 nM, 1 nM and 2 nM. After 14 days of culture, colonies were visualized by MTT.

2.3.1. Determination of apoptosis

Apoptotic cells were identified by nuclear staining with bis-benzimide fluorescent dye (Hoechst 33258) that was added to the culture medium at a final concentration of 5 μ g/mL. Apoptotic nuclei were counted using a fluorescence microscope.

2.4. Western blot analysis

Endometrial carcinoma cell lines were washed twice with cold PBS and lysed with lysis buffer (2% SDS, 125 mM Tris–HCl pH 6.8). Protein concentrations were determined with the Protein Assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Non-specific binding was blocked by incubation with TBST (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4 °C. The signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

2.5. Lentiviral production and infection

In order to downregulate the protein expression, we infected endometrial carcinoma cells with lentivirus carrying for the shRNA of interest. Oligonucleotides to produce plasmid-based shRNA were cloned into the FSV vector using AgeI–BamHI restriction sites. shRNA target sequence to p65 was 5'-ACACTGCCGAGCTCAAGATCT-3'. To produce infective

lentiviral particles, 293T cells were co-transfected by PEI method with the virion packaging elements (VSV-G and D8.9) and the FSV on 293T human embryonic kidney. Supernatants were collected after 3 days, concentrated by centrifugation through a filter column of 100 kDa (VWR International LLC, West Chester, PE, USA) for 1 h at 4000 rpm. Cells were incubated overnight in the presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for at least 72 h to allow endogenous protein knockdown.

2.6. Detection of changes in cell cycle profiles

Changes in cell cycle profile after drug treatments were determined by propidium iodide (PI) staining and flow cytometry. Following treatment, approximately $1 \cdot 10^6$ cells were fixed in 70% ethanol for at least 1 h on ice. The cells were then resuspended in 2 mL of cell cycle buffer (20 μ g/mL propidium iodide, in PBS containing 0.1% Triton X-100 and 50 μ g/mL RNase A) for 1 h at 37 °C. PI fluorescence emission was measured using a FACSCantoII (BD Biosciences, San Jose, CA, U.S.A.), and cell cycle distribution was analyzed with ModFIT LT software (Verity Software House, Topsham, ME).

2.7. Luciferase assay

To determine changes in NF κ B transcriptional activity after different stimuli we performed a luciferase report assay. Endometrial carcinoma cell lines were plated in M24 multiwell plates and transfected using Lipofectamine 2000 following the manufacturer's instructions, with the reporter NF κ B-LUC construct together with a plasmid encoding β -galactosidase. After 16 h, cells were treated as indicated in each experiment, lysed with 60 μ L of lysis buffer (25 mmols/L glycylglycine, pH 7.8, 15 mmols/L Mg $_2$ SO $_4$, 1% Triton X-100, 5 mmols/L EGTA) and rocket on ice for 15 min. 25 μ L of lysates were transferred to M96 multiwell plates and 25 μ L of luciferase assay buffer was added to a final concentration of (25 mmol/L glycylglycine, 15 mmol/L KHPO $_4$, pH 7.8, 15 mmols/L Mg $_2$ SO $_4$, 1% Triton X-100, 5 mmols/L EGTA, 1 mmol/L dithiothreitol containing 2 mmol/L ATPm 100 mmol/L acetyl-coenzyme A, and 100 mmols/L luciferine). Luciferase was measured using a microplate luminometer. Subsequently, 60 μ L of $2 \times \beta$ -galactosidase buffer (200 mmol/L NaPO $_4$, 20 mmol/L KCl, 2 mmol/L Mg $_2$ SO $_4$, 4 mg/mL o-nitrophenyl β -D-galactopyranoside) were added to each well and β -galactosidase activity was measured on a microplate reader at 415 nm.

2.8. Analysis of combined drug effects

The combining effect of Sunitinib and Bortezomib was assessed by the median effect method proposed by Chou and Talaly (1977) using the Calcsyn software (Biosoft, Oxford, United Kingdom). Bortezomib and Sunitinib were administered at the fixed ratio of 1/1000. This method determines the combination index (CI) between two drugs. CI above 1 indicates antagonism and CI below 1 indicates synergistic interaction between two drugs.

3. Results

3.1. Sunitinib decreases the *in vitro* growth of endometrial carcinoma cell lines

Antitumoral effects of Sunitinib in endometrial carcinoma were determined by exposing the three endometrial carcinoma cell lines, Ishikawa (IK), RL-95-2 and Hec-1A to different concentrations of Sunitinib (0–40 $\mu\text{mol/L}$) for 24 h, 48 h and 72 h. (Figure 1a). The three endometrial carcinoma cells lines showed to be sensitive to Sunitinib, displaying a post-treatment viability of 39.8% (IK), 52.9% (RL-95-2) and 72.1% (Hec-1A) with 5 $\mu\text{mol/L}$ of Sunitinib at 72 h. IC₅₀ of Sunitinib at 72 h are 5.49 $\mu\text{mol/L}$ (IK), 7.07 $\mu\text{mol/L}$ (RL-95-2) and 6.63 $\mu\text{mol/L}$ (Hec-1A). To assess changes in cell cycle profile after treatment, Sunitinib was administrated to IK, RL-95-2 and Hec-1A cells with a concentration of 5 $\mu\text{mol/L}$ for 72 h. A decrease in S phase and an increase in subG1 phase was observed for all the cell lines (Figure 1b). Moreover, apoptotic cell death was observed after 72 h of Sunitinib treatment in IK, RL-95-2 and Hec-1A cells. (Figure 1c), demonstrated by the cleavage of the executioner caspase 3. Interestingly, very low Sunitinib doses induced a reduction in the number of colonies in the IK, RL-95-2 and Hec-1A cells, supporting the cytotoxic effect of Sunitinib at more physiological concentrations (Figure 1d).

3.2. Sunitinib does not act through the most known targets in endometrial carcinoma cell lines

To determine the existence of a possible target of Sunitinib in endometrial carcinoma, the expression of various known tyrosine kinase receptors, targets of Sunitinib, were analyzed by Western Blot. As shown in Figure 2a, the expression of c-kit, PDGFR α , PDGFR β , VEGFR2 and EGFR was measured for IK, RL-95-2 and Hec-1A cells. c-kit was found to be expressed only in IK cells and EGFR in all three endometrial carcinoma cell lines. In contrast, PDGFR α , PDGFR β or VEGFR2 could not be detected in any of the cell lines. As the only shared RTK among all cell lines was EGFR and they were shown to display a similar sensitivity to Sunitinib, we next analyzed the tyrosine phosphorylation of EGFR in IK, RL-95-2 and Hec-1A cell lines after Sunitinib treatment. Stimulation with EGF induced a phosphorylation of EGFR. Interestingly, this phosphorylation was not abolished when Sunitinib (at 1 and 10 $\mu\text{mol/L}$) was added for 1 h before EGF stimulation. Moreover, treatment with AG1478, a selective inhibitor of EGFR did not have any effect in cell viability (Supplementary figure 1). With these results, we conclude that the antitumoral effect of Sunitinib is not by inhibition of any of the tyrosine kinase receptors.

3.3. Sunitinib blocks both basal and induced NF κ B pathway

Sunitinib has a pleiotropic mechanism of action. Here, we observe that Sunitinib has no effect neither in MAPK nor in Akt pathway in Ishikawa cells. As shown in Figure 3a, Sunitinib at 1 μM or 10 μM is able to reduce the phosphorylation of ERK but not the phosphorylation of Akt, in the residues

needed for its fully activation (serine 473 and threonine 308). In a parallel experiment, we wanted to check the effects of Sunitinib on NF κ B pathway. The ability of Sunitinib to modify the activation of the NF κ B pathway was assessed by luciferase reporter assays and by Western Blot. Sunitinib alone, at 1 $\mu\text{mol/L}$ or 10 $\mu\text{mol/L}$, reduced basal NF κ B transcriptional activity by 48% and 78% respectively (Figure 3b and c). Sunitinib also decreased the basal phosphorylation of four components of the canonical/alternative NF κ B pathway, namely IKK α and IKK β , p65 and I κ B α . (Figure 3d). In a further experiment, cell lines were treated with different putative NF κ B activators such as EGF and TNF in the presence or absence of Sunitinib at 1 $\mu\text{mol/L}$. As shown in Figure 3b, EGF and TNF were potent inducers of NF κ B pathway. Interestingly, Sunitinib treatment resulted in a reduced induction of the NF κ B pathway activity, regardless of the used ligand. Furthermore, as described in a previous report, Bortezomib activates the NF κ B pathway in endometrial carcinoma (Dolcet et al., 2006), which could be prevented by the joined treatment with Sunitinib (Figure 3c). Concordant results were observed when testing the phosphorylation status of NF κ B proteins. (Figure 3d). A loss of phosphorylation of IKK α and β , p65 and I κ B α was observed in IK cells when Sunitinib was combined with Bortezomib at all examined timepoints, demonstrating the ability of Sunitinib to block Bortezomib-induced NF κ B pathway.

3.4. The combined treatment with Sunitinib and Bortezomib results in a synergistic increase of cell death

Motivated by the results described above, we investigated if Sunitinib enhances the growth inhibitory effects of Bortezomib. To address this point, we carried out a median dose effect analysis, which was determined using the MTT assay. The concentrations used for Bortezomib and Sunitinib were 1–20 nmol/L and 1–20 $\mu\text{mol/L}$ respectively in IK and RL-95-2 cells and 5–40 nmol/L and 5–40 $\mu\text{mol/L}$ in Hec-1A cells. The drugs were used alone or in combination with a fixed ratio of 1/1000. A clear decrease in cell viability was observed when both drugs were combined (Figure 4a), which indicates a relevant interaction. CI values in respect to the fraction of cells affected by Sunitinib + Bortezomib are plotted in Figure 4b. In all three cell lines analyzed, CI values are generally significantly below one, indicating a synergistic interaction of Sunitinib and Bortezomib of inducing cell death. In a similar experiment, using clonogenic assays, we observed a synergistic effect between both drugs administered at very low concentrations (Figure 4c). In addition, we added a table (Table 1) showing the IC₅₀ corresponding to each drug separately and to the combination. These results clearly evidence the synergistic effect observed.

3.5. The blockade of the canonical NF κ B pathway by p65 shRNA potentiates the cytotoxic effects of Bortezomib, mimicking the effects of Sunitinib in NF κ B pathway

Afterwards, we decided to check if the synergism between Sunitinib and Bortezomib in endometrial carcinoma could be related to the inhibition of NF κ B pathway by Sunitinib. To confirm such hypothesis, we blocked NF κ B pathway by shRNA against p65, as simulating the effects of Sunitinib on NF κ B

Figure 1 – Sunitinib decreases the in vitro growth of endometrial carcinoma cell lines. (a) Three endometrial carcinoma cell lines (IK, RL-95-2 and Hec-1-A) were treated with Sunitinib (0–1–5–10–20–30–40 $\mu\text{mol/L}$) for 24, 48 and 72 h, and cell viability was assessed by MTT assay. (b) The three endometrial carcinoma cell lines were treated with 5 $\mu\text{mol/L}$ of Sunitinib for 72 h or left untreated, after which propidium iodide staining was done in order to check the DNA content. (c) Endometrial carcinoma cells were treated with Sunitinib at 5 $\mu\text{mol/L}$ for 24, 48 and 72 h. Then, cell lysates were subjected to Western Blot so as to determine caspase-3 processing. Tubulin was used as a loading control. (d) IK, RL-95-2 and Hec-1-A cells were cultured for 14 days, with 0.1, 0.2, 0.5 and 1 $\mu\text{mol/L}$ of Sunitinib or without treatment. Sunitinib was added to the media every two days. After 14 days, colonies were stained with MTT, fixed and counted. The graphs on the right show the number of colonies obtained for each condition in IK, RL-95-2 and Hec-1A cell lines.

Figure 2 – Sunitinib does not act through the most known targets in endometrial carcinoma cell lines. (a) Whole cell lysates of the three endometrial carcinoma cell lines (IK, RL-95-2 and Hec-1A) and the corresponding positive controls were analyzed by Western Blot for c-Kit, PDGFR α , PDGFR β , VEGFR2 and EGFR expression. The positive control for c-Kit, PDGFR α and PDGFR β consisted of M16 cell lysates, for VEGFR2 M28 cell lysates and for EGFR HeLa cell lysates, as previously described (Yeramian et al.). Tubulin was used as a loading control. **(b)** IK, RL-95-2 and Hec-1A cell lines were incubated with 1 and 10 μ M of Sunitinib for 1 h with or without the presence of 50 ng/mL EGF for 15 min. Subsequently, tyrosine receptor phosphorylation was assessed using a phospho-EGFR antibody.

pathway. Then, cells subjected to p65 knockdown were exposed to Bortezomib. Lentiviral delivery of shRNA against p65 selectively blocked the expression of the p65 gene product, its phosphorylation (Figure 5b) and inhibited NF κ B transcriptional activity (Figure 5a). Cells with shRNA mediated downregulation of p65 displayed an increase of apoptosis when treated with Bortezomib at 10 nmol/L and 25 nmol/L as shown by caspase-3 processing (Figure 5b). Similar results were obtained quantitatively by flow cytometry analysis (Figure 5c) and Hoechst staining (Figure 5d). After treatment with 10 nmols/L Bortezomib, we observed a considerable increase of the sub-G1 fraction, hallmark of cell death, in cells where p65 was downregulated when compared to the other conditions. Also when analyzed with Hoechst staining, p65 downregulated cells displayed a net increase in apoptotic cell death compared to the remaining conditions. Moreover, the ability to form colonies was considerably diminished in the p65 downregulated cells after 2 nmol/L of Bortezomib treatment. Altogether, these results indicate that the inhibition of NF κ B pathway by Sunitinib could mediate the synergy between Sunitinib and Bortezomib in endometrial carcinoma.

4. Discussion

Herein, we first demonstrate the ability of Sunitinib to reduce cell viability, cell proliferation and apoptosis in endometrial

carcinoma. This is in agreement with earlier reports that described similar cytotoxic effects of Sunitinib in other cancer types (Ikezoe et al., 2006a, 2006b; Jeong et al., 2011; Yeramian et al., 2011). Moreover, we show that Sunitinib has an antitumor effect at closer concentrations to those existing in human plasma, ranging from 0.1 μ M to 1 μ M in the clonogenic assay, demonstrating the antiproliferative ability of the drug at more physiological conditions.

We further tested the expression of multiple Sunitinib targets receptors in endometrial carcinoma cell lines. The examined receptors were c-kit, VEGFR2, PDGFR and EGFR, which have been previously found to be expressed in endometrial carcinoma and which are inhibited by Sunitinib with high efficacy, with the lowest IC₅₀ (Chow and Eckhardt, 2007). All three endometrial carcinoma cell lines were sensitive to Sunitinib and EGFR was the only tested target receptor expressed in all three cell lines. We therefore investigated the phosphorylated status of the EGF receptor. Interestingly, Sunitinib was not able to inhibit the EGF-induced phosphorylation. Moreover, selective inhibition of EGFR by AG1478 did not reduce the viability of the endometrial carcinoma cells. These results suggest that Sunitinib acts inhibiting tumor cell growth independently of EGFR and therefore, independently of the most known targets of Sunitinib.

Little is known about the survival signaling pathways inhibited by Sunitinib. Some authors point out that the mechanism of action of Sunitinib goes through inhibition of the

Figure 3 – Sunitinib blocks both basal and induced NFκB pathway by several stimulus. (a) IK cells were treated 1 h with Sunitinib at 1 μM and 10 μM and additionally with EGF at 50 ng/mL for 15 min. This was used as a positive control. After the treatments, cell lysates were obtained and subjected to Western Blot for the detection of P-ERK p42/44, P-Akt serine 473 and P-Akt threonine 308. Tubulin was used as a loading control. (b) IK cells were transfected with plasmids encoding for NFκB-LUC and β-galactosidase constructs, using Lipofectamine. 24 h posttransfection, cells were treated with 5 ng/mL of EGF and 50 ng/mL of TNF alone or in combination with 1 μmol/L of Sunitinib or left untreated for 16 h. After treatments, luciferase reporter assay was performed. The graphic shows NFκB transcriptional activity expressed in relative luciferase units, normalized with β-galactosidase activity. (c) NFκB transcriptional activity in IK cells after being treated for 16 h with 10 nmol/L of Bortezomib, 50 ng/mL of TNF, 10 μmol/L of Sunitinib and the combination treatment of Sunitinib and Bortezomib. (d) IK cell line was treated with 10 μmol/L of Sunitinib, 10 nmol/L of Bortezomib and with the combined treatment. Then, total protein lysates were extracted at 0, 12, 24 and 48 h after the treatments and were subjected to Western Blot for the expression of phospho-IKKα and IKKβ, phospho-p65 and phospho-IκBα. Bands from Western Blot were quantified and the relative intensities calculated for the untreated condition and the remaining conditions are shown in the graphs. Tubulin is shown as the loading control.

Akt/mTOR pathway (Ikezoe et al., 2006a, 2006b; Yeramian et al., 2011). Our study demonstrates that Sunitinib has no effect in MAPK nor in Akt pathways but in NFκB pathway in endometrial carcinoma cells. Of note, this is the first study indicating that the NFκB pathway is a direct target of Sunitinib in an in vitro cancer model. Our results are in accordance with one report stating that Sunitinib, as well as two further tyrosine kinase inhibitors, could have an inhibitory effect on NFκB signaling (Miller et al., 2010). It is known that NFκB pathway can be activated by numerous stimuli, such as growth

Figure 4 – The combined treatment with Sunitinib and Bortezomib results in a synergistic increase of cell death. (a) Cell viability (%) of IK, RL-95-2 and Hec-1A cells treated with Sunitinib, Bortezomib or both, was examined using the MTT assay at 24 h posttreatment. Each point represents the mean of the three independent experiments ($n = 3$), performed in triplicate. Error bars, using the standard error of the mean, are shown. Both drug doses are indicated. (b) Combination index obtained for different fractions affected. Dotted lines indicate $CI \pm$ standard deviation, which are computed using Monte Carlo simulation. (c) Clonogenic assays of IK and Hec-1A cell lines after the treatments of Sunitinib, Bortezomib alone or combined. The graph below the figure show the number of colonies obtained for each condition in IK and Hec-1A cells.

Figure 5 – The blockade of the canonical NF κ B pathway potentiates the cytotoxic effects of Bortezomib. (a) IK cells were infected with the lentiviral vector carrying shRNA for p65. At Seventy-two hours postinfection, cell lysates were analyzed by luciferase reporter assay. (b) After the infection, IK cells were treated for 24 h with 10 and 25 nmol/L of Bortezomib or maintained untreated. Then, cell lysates were subjected to Western Blot analysis for the detection of p65, phospho-p65 and caspase-3 processing. Tubulin was used as a loading control. (c) IK cell line was treated for 24 h with Bortezomib 10 nmol/L at 72 h postinfection. Then, cell cycle profile was determined by flow cytometry using propidium iodide staining. (d) 72 h postinfection and after 24 h of treatment with Bortezomib 10 nmol/L, cells were stained with Hoechst. The graph on the right shows the percentage of cells displaying apoptotic nuclei in each condition. (e) IK cells were infected with lentivirus carrying shRNA against p65 and treated with Bortezomib 2 nM for 14 days. After that, colonies were stained, fixed and counted. The graph on the right shows the number of colonies observed in all the conditions.

factors (Aggarwal, 2004), ionizing radiation (Aggarwal, 2004; Dritschilo, 1999) and even by chemotherapeutic drugs (Aggarwal, 2004). Examples of chemotherapeutic drugs are Temozolomide (Amiri et al., 2004), Etoposide (Basu et al., 1998), Vorinostat (Dai et al., 2005) or proteasome inhibitors

(Bauer et al., 2010; Dolcet et al., 2006; Hideshima et al., 2009). In this line, numerous attempts have been done in order to reduce this induced NF κ B hyper-activation, finally conducting to a chemosensitization (Amiri et al., 2004); (Dai et al., 2005) or a radiosensitization (Goel et al., 2006; Munshi et al., 2004)

Table 1 – The combined treatment of Sunitinib and Bortezomib results in a synergistic increase of cell death according to the IC₅₀ values. Dose to reach a median effect (Dm), in nmol/L, and confidence interval (95%) for each drug and combination in IK, RL-95-2 and Hec-1A cell lines. Dm values (also known as IC50 values) are interpreted as the needed doses to reach a 50% of cell death.

		IK		Hec-1A		RL-92-5	
		Dm	CI 95%	Dm	CI 95%	Dm	CI 95%
Individual effect	Bortezomib	41.07	(1.23; 1361)	268.98	(23.96; 3019.7)	1228.3	(0.49; 3,076,600)
	Sunitinib	19638	(3740; 103,100)	7825.67	(885.97; 6912)	59,957	(21817; 164,770)
Combination	Bortezomib	8.8	(0.51; 515.93)	4.73	(1.03; 1039.19)	17.22	(4.32; 68.56)
	Sunitinib	8889.6	(163.16; 153,170)	4739.65	(21.61; 21,617)	17,222	(4325.62; 68,565)

effect. We demonstrate that Sunitinib inhibits both basal and induced NFκB transcriptional activity by known molecules such as EGF, TNF and Bortezomib. Although Sunitinib does not directly act through EGFR, it could be that it modifies some proteins associated with this receptor. This may explain the observation that Sunitinib can reduce the EGF-induced NFκB activity. We further observed that Sunitinib attenuated Bortezomib-induced phosphorylation of IKKα and β, p65 and IκBα. In addition, we found a synergistic effect when combining Sunitinib, our proposed inhibitor of NFκB signaling, with Bortezomib, a potent inducer of the NFκB pathway in endometrial carcinoma cells.

Regarding statistical issues, here we used the Calcsyn software and we obtained a combination index lower than 1, suggesting an evident synergistic effect. To undertake the analyses, we decided to perform our experiments in a constant-ratio drug combinations framework (1/1000) as recommended in a recent review (Chou, 2010) and supported by previous reports in the literature (Damaraju et al., 2007; Singh et al., 2011; Wozniak et al., 2010; Yeramian et al., 2011). Moreover, a limitation of our work may arise concerning to this point, given that a non-constant ratio for drug combination could have been used in addition to other possible mechanism-specific equations for assessing synergy. Nevertheless, our results are clear and proved the synergistic interaction between Sunitinib and Bortezomib.

The observed synergy is in line with reports of our group (Yeramian et al., 2011) and others, which previously described a synergistic effect of Bortezomib and other first-generation tyrosine kinase receptors inhibitors, such as Sorafenib (Yu et al., 2006) or Imatinib mesylate (Hu et al., 2009).

In order to confer to NFκB inhibition by Sunitinib the enhancement of cell death obtained in Bortezomib-treated cells, we downregulated p65 by shRNA, mimicking Sunitinib treatment. As expected, shRNA-mediated downregulation of p65 also reduced NFκB transcriptional activity and induced apoptotic cell death in Ishikawa cells. In accordance with Sunitinib treatment, also p65 downregulated cells have a considerably increase of apoptotic cell death and a diminution in the clonogenic ability when treated with Bortezomib. Similarly, it has been described that the pharmacologic inhibition of Ikkβ significantly enhanced Bortezomib-induced cell death due to the inability of Bortezomib of blocking constitutive NFκB pathway (Hideshima et al., 2009). Altogether these results suggest that the potential mechanism responsible for the synergy observed between Sunitinib and Bortezomib goes through the NFκB pathway. It is well known that NFκB

is a survival pathway. Although Bortezomib induces cell death in endometrial carcinoma, it is not done by reducing NFκB activity. Alternative mechanisms have been proposed for Bortezomib-induced cell killing like the up-regulation of BH3 only proteins such as Noxa (Fernández et al., 2005), Puma (Zhu et al., 2005) or the induction of endoplasmatic reticulum stress (Nawrocki et al., 2005). In contrast, Bortezomib activates NFκB activity and therefore could induce cell survival even though the net balance conducts to cell death. By the addition of Sunitinib, the possible NFκB-induced survival effects are prevented resulting in an enhancement of cell toxicity.

4.1. Conclusions

In conclusion, the current study shows that Sunitinib can reduce cell growth independently of the most known targets of Sunitinib. Moreover, Sunitinib inhibits the basal activity of the NFκB pathway, which is induced by several known stimuli, including Bortezomib. Interestingly, Sunitinib mediated downregulation of p65 sensitizes endometrial carcinoma cells to the cytotoxic effects of Bortezomib. There is no optimal treatment for endometrial carcinoma patients with metastasis and recurrence after radiation, since current chemotherapeutic strategies are associated with low frequencies of complete response. Based in our results we suggest the combination of Sunitinib and Bortezomib in advanced stage endometrial carcinoma patients.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molonc.2012.06.006>.

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