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Research Article

AXL as a modulator of sunitinib response in glioblastoma cell lines

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ABSTRACT

Receptor tyrosine kinase (RTK) targeted therapy has been explored for glioblastoma treatment. However, it is unclear which RTK inhibitors are the most effective and there are no predictive biomarkers available. We recently identified the RTK AXL as a putative target for the pan-RTK inhibitors cediranib and sunitinib, which are under clinical trials for glioblastoma patients. Here, we provide evidence that AXL activity can modulate sunitinib response in glioblastoma cell lines. We found that AXL knockdown conferred lower sensitivity to sunitinib by rescuing migratory defects and inhibiting apoptosis in cells expressing high AXL basal levels. Accordingly, over-activation of AXL by its ligand GAS6 rendered AXL positive glioblastoma cells more sensitive to sunitinib. AXL knockdown induced a cellular rewiring of several growth signaling pathways through activation of RTKs, such as EGFR, as well as intracellular pathways such as MAPK and AKT. The combination of sunitinib with a specific AKT inhibitor reverted the resistance of AXL-silenced cells to sunitinib. Together, our results suggest that sunitinib inhibits AXL and AXL activation status modulates therapy response of glioblastoma cells to sunitinib. Moreover, it indicates that combining sunitinib therapy with AKT pathway inhibitors could overcome sunitinib resistance.

Introduction

Glioblastoma is the most common form of primary brain tumor and one of the most lethal and challenging human malignancies [1–4]. Standard treatment consists in a combination of surgery, irradiation and temozolomide, which postpones progression and extends overall survival in 5 years from 2% up to 10%, but these tumors universally recur and unrelentingly result in patient death [4–8].

An increasing understanding of the molecular mechanisms underlying glioblastoma [9–11] has led to the identification of a number of promising therapeutic targets, including several members of the receptor tyrosine kinase (RTK) family [12–16]. Among the most interesting RTK targets are EGFR, PDGFRA and KIT, which have been intensively studied [17–20]. However, clinical trials with single-targeted agents that inhibit these molecules have shown only minimal therapeutic activity, with no significant

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prolongation of survival [21–28]. Therefore, it is currently believed that multi-kinase inhibitors simultaneously targeting several RTKs may yield greater clinical efficacy in selected glioblastoma patients [29–32].

AXL belongs to the TAM (Tyro3, AXL and Mer) receptor subfamily of RTKs that also includes Tyro3 and Mer [33]. The TAM receptors are characterized by a combination of two immunoglobin-like domains and dual fibronectin type III repeats in the extracellular region and a cytoplasmic kinase domain. The ligands for TAM receptors are Gas6 (growth arrest-specific 6) and protein S [34]. AXL was originally cloned from patients with chronic myelogenous leukemia and, when overexpressed, it exhibits transforming potential [33]. AXL overexpression has been reported in a variety of human cancers, being associated with tumor invasiveness and metastasis [35–38].

Regarding brain tumors, AXL has been implicated in gliomagenesis and chemoresistance [39]. Previous investigations found that AXL is constitutively phosphorylated in many glioma cell lines, murine xenograft tumors and primary patient tumor samples [40]. Immunohistochemical analysis of AXL and Gas6 demonstrated that co-expression of these proteins correlates with tumor recurrence and progression [41,42]. Furthermore, inhibition of AXL using *in vitro* and *in vivo* models resulted in a reduction of tumor growth, migration and invasion, as well as prolonged overall animal survival in xenografts containing dominant negative AXL [42]. Altogether the previous data has indicating that AXL targeted therapy may also diminish glioblastoma aggressiveness [43].

In a recent pre-clinical study performed by our group, the effectiveness of two pan-RTK inhibitors (sunitinib and cediranib) in glioblastoma cell lines was assessed and AXL was found as a common candidate target for both cediranib and sunitinib [31]. Other studies have also suggested AXL as target for sunitinib in other tumor types, such as in renal cell carcinoma [38,44]. Moreover, *de novo* activation of AXL was found in imatinib-treated gastrointestinal stromal tumors (GIST) and in Her-2 positive breast cancer cells treated with lapatinib and, in both cases, AXL was associated with therapy resistance [45,46]. Nevertheless, the role of this protein as a putative modulator of sunitinib therapy response is still in need of *in vitro* and *in vivo* validation.

Due to the importance of AXL in glioblastoma and its recent implication in RTK inhibitor responses, in the present study we aimed to validate AXL as a cediranib and sunitinib target and to determine whether it could act as a modulator of cediranib and sunitinib response in glioblastoma cell lines.

Materials and methods

Cell lines and cell culture

Eight immortalized glioblastoma cell lines were used: SW1088, SW1783, U-87 MG and A172 were obtained from ATCC (American Type Culture Collection), SNB-19 and GAMG were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures) and U251 and U373 were kindly provided by Professor Joseph Costello. All cell lines were maintained in DMEM-10 at 37 °C and 5% CO_2 as previously described [31]. Authentication of cell lines was performed by IdentiCell Laboratories (Department of Molecular Medicine (MOMA) at Aarhus University Hospital Skejby in Århus, Denmark), as described [31].

Generation of cell lines stably expressing short hairpin (sh) AXL

For the generation of cell lines stably expressing a short hairpin for AXL (shAXL), we used pRNA-U6.1/Neo vector containing a 19 bp shRNA. For AXL overexpression a complete AXL cDNA cloned into the pcDNA3 vector was used. Both vectors were kindly provided by Shuang-En Chuang from the National Health Research Institutes, Taiwan [47]. Electroporation with Neon Transfection System (Invitrogen, Life Technologies) was used to introduce the shAXL/AXL and the empty vectors into the cells, following the manufacturer's instructions. Twenty-four hours after transfection, stable transfectants were selected with 300–450 μg/ml of G418 (Sigma Aldrich) in complete DMEM medium.

Immunofluorescence analysis for AXL

For immunofluorescence analysis, the cells were plated in glass coverslips placed into 12-well plates at a density of 6×10^4 cells per well and allowed to adhere overnight. Then, the cells were fixed in cold methanol by 5 min at −20 °C. For block unspecific ligations the cells were incubated with a solution of PBS containing 10% FBS for 30 min at room temperature followed by incubation with a primary antibody against AXL (dilution 1:50; incubation ON at RT; AXL C-20 clone; Santa Cruz Biotechnology). The cells were then washed in a PBS solution with 0.5% FBS and incubated with a rabbit anti-goat antibody conjugated with TRITC (dilution 1:500, Life Technology) for 1 h at room temperature in the dark. Finally the cells were counterstained with 40,6-diamidino-2-phenylindole (DAPI) and images were obtained with the use of fluorescence microscopy (BX16; Olympus).

Western blot and human RTK arrays

To assess the effect of the drugs on the intracellular signaling pathways and RTKs, the cells were cultured in T25 culture flasks or six-well plates, allowed to grow to 85% confluence, serum starved for 2 h and then incubated with the drugs. When necessary the cells were stimulated with 400 ng/ml of Gas6 (human recombinant Gas6, R&D systems) for 15 min.

To assess apoptosis, the cells were incubated with increasing concentrations of sunitinib for 24 h. At the indicated time points, the cells were washed and scraped in cold PBS and lysed in buffer containing 50 mM Tris pH 7.6–8, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and 1/7 of protease cocktail inhibitors (Roche). Western blotting was performed using standard 10% SDS-PAGE, loading 20 μg of protein per lane. All the antibodies were used as recommended by the manufacturer and as previously described [31].

For detection of AXL activation we used a specific antibody to detect AXL phosphorylation, phospho-AXL (Tyr702) (Cell Signaling Technologies, D12B2). For calibration of the activation levels, an antibody to detect the total protein levels (AXL (C-20), Santa Cruz Biotechnology) was used. A specific antibody to detect EphA7 phosphorylation (Tyr791) (MyBiosource) was also used. Anti-GAPDH (Santa Cruz Biotechnology) was used as a loading control. All the other antibodies were used as previously described [31].

For the human RTK arrays, 500 μg of fresh protein lysates were incubated overnight at 4 °C with nitrocellulose membranes dotted with duplicated spots for 42 anti-RTK and control antibodies. Bound

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phospho-RTKs were incubated with a pan anti-phosphotyrosine-HRP antibody for 2 h at room temperature [31].

Antibodies were detected by chemiluminescence (Thermo Scientific Pierce ECL Western Blotting) using the ChemiDoc™ XRS+ System (Bio-Rad).

Drugs

All the drugs (cediranib, sunitinib, imatinib, selumetinib and MK2206) used in this work were obtained from Selleck Chemicals, USA and prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at −20 °C. To obtain an equal quantity of DMSO (1% final concentration) in each of the conditions studied, prior to the final dilution of the drugs to an appropriate concentration in DMEM medium, the drugs were first prepared as intermediate dilutions in DMSO [31].

Cell viability assay

To determine the concentration at which 50% of the cell growth is inhibited by drug treatment (IC_{50} concentration), cells were plated into 96-well plates at a density of 2×10^3 cells per well and allowed to adhere overnight in DMEM medium containing 10% FBS. Subsequently, the cells were treated with increasing concentrations of the drugs or with DMSO alone, both diluted in 0.5% FBS culture medium to a final concentration of 1% DMSO. When necessary the cells were incubated simultaneously with 400 ng/ml of Gas6, or with 2.5 μM of selumetinib or MK2206. After 72 h, cell viability was quantified using the Cell Titer96 Aqueous cell proliferation assay (Promega). The results were expressed as the mean percentage ± SD of viable cells relative to the DMSO alone (considered as 100% viability). The IC_{50} concentration was calculated by nonlinear regression analysis using GraphPad Prism software.

To assess the effect of a fixed concentration of the drug in cellular viability over time, the cells were plated onto 96-well plates at a density of 1×10^3 cells per well and allowed to adhere overnight in complete DMEM medium. Next, the viable cells were quantified using the Cell Titer96 Aqueous cell proliferation assay (Promega), and used for time point 0. Then, the cells were incubated with fixed concentrations of the drugs or with DMSO alone, both diluted in 0.5% FBS culture medium to a final concentration of 1% DMSO, over 24, 48 and 72 h. At the end of each

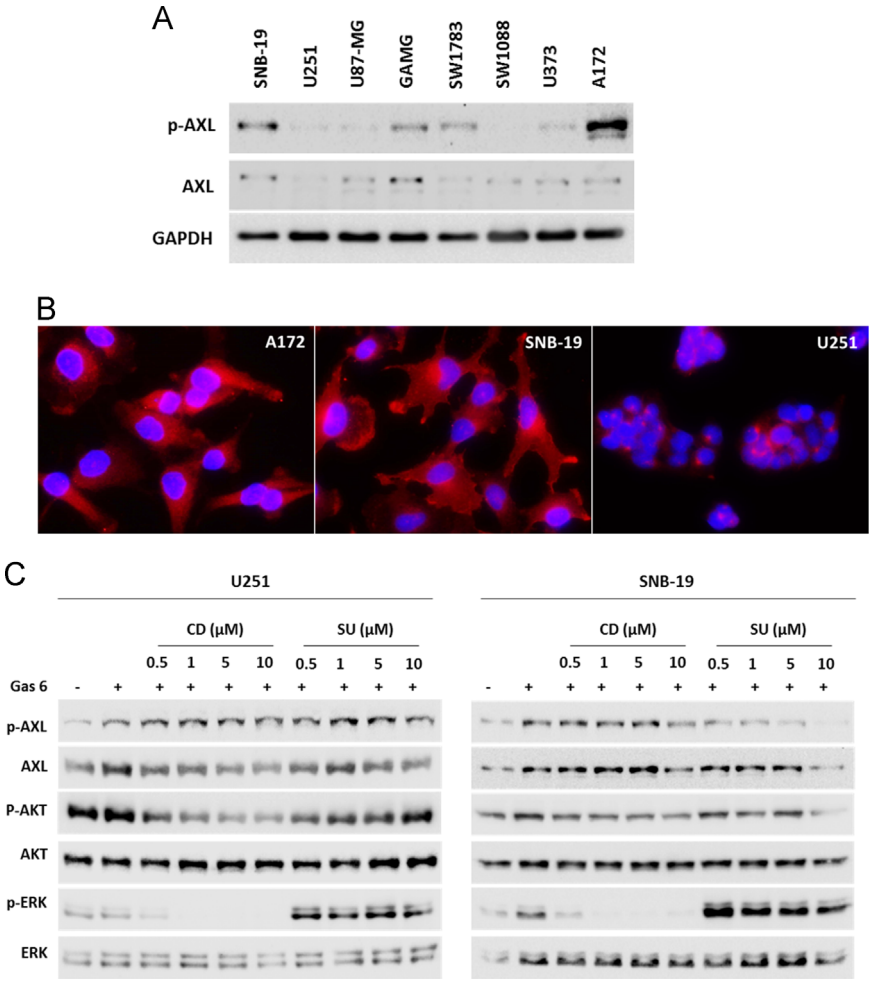

Fig. 1 – AXL is a target of cediranib and sunitinib therapy in glioblastoma cells. (A) Western blot analysis of AXL expression and activation (phosphorylated AXL: p-AXL) in eight glioblastoma cell lines. (B) Immunofluoresnce analysis of AXL expression in A172, SNB-19 and U251 cell lines. (C) U251 and SNB-19 cells were treated with increasing concentrations of cediranib (CD) and sunitinib (SU) for 2 h, and stimulated with 400 ng/ml of Gas6. The activation levels of AXL, MAPK (ERK) and AKT pathways were assessed by Western blot. GAPDH was used as a loading control.

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time point, cell viability was again assessed using the Cell Titer96 Aqueous cell proliferation assay. The results were calibrated to the starting viability (time 0 h, considered as 100% viability) and expressed as the mean ± SD. Both assays were performed in triplicate at least three times [31].

Wound healing migration assay

The cells were seeded on 6-well plates and cultured to at least 95% confluence. Monolayer cells were washed with PBS and scraped with a plastic 200 µl pipette tip. The cells were incubated with fixed concentrations of the drugs or with DMSO alone, both diluted in 0.5% FBS culture medium to a final concentration of 1% DMSO. When necessary the cells were incubated simultaneously with 400 ng/ml of Gas6 or with 2.5 µM of selumetinib or MK2206. The "wounded" areas were photographed by phase contrast microscopy at 0 and 48 h time points. The relative migration distance was calculated using the following formula: percentage of wound closure (%) = 100 (A − B)/A, where A is the width of cell wounds before incubation and B is the width of cell wounds after incubation. Results are expressed as the mean ± SD. The assay was performed in triplicate at least three times.

Statistical analysis

Single comparisons between the different conditions studied were made using the Student's *t* test and differences between groups were tested using two-way analysis of variance (ANOVA). Statistical analysis was done using Graph Pad Prism version 5. The level of significance in all the statistical analysis was set at $p < 0.05$.

Results

Inhibition of AXL signaling by cediranib and sunitinib

In order to determine the expression and phosphorylation levels of AXL in glioblastoma cell lines we performed western blot analysis with specific antibodies (Fig. 1A). Despite the weak expression of total AXL protein in all cell lines, we could confirm the presence of AXL activation in seven of the cell lines, being SW1088 the only one lacking AXL activity (Fig. 1A).

To further assess whether AXL is a cediranib and sunitinib target, we chose a cell line with high AXL phosphorylation levels (SNB-19) and a cell line with lower levels of AXL activation and expression (U251) (Fig. 1A and B). We found that in the U251 cell line, neither cediranib nor sunitinib were able to inhibit AXL activation upon ligand (Gas6) stimulation. In contrast, in SNB-19 cells, AXL phosphorylation was inhibited by both drugs (Fig. 1C). Specifically, we observed that only high doses of cediranib inhibited Gas6-induced AXL activation, while sunitinib was effective at lower doses. Regarding inhibition of intracellular signaling pathways, cediranib was effective in blocking ERK activation in both cell lines (Fig. 1C). In contrast, sunitinib was only able to inhibit AKT activation in SNB-19 cells at high doses when AXL inhibition was complete, suggesting

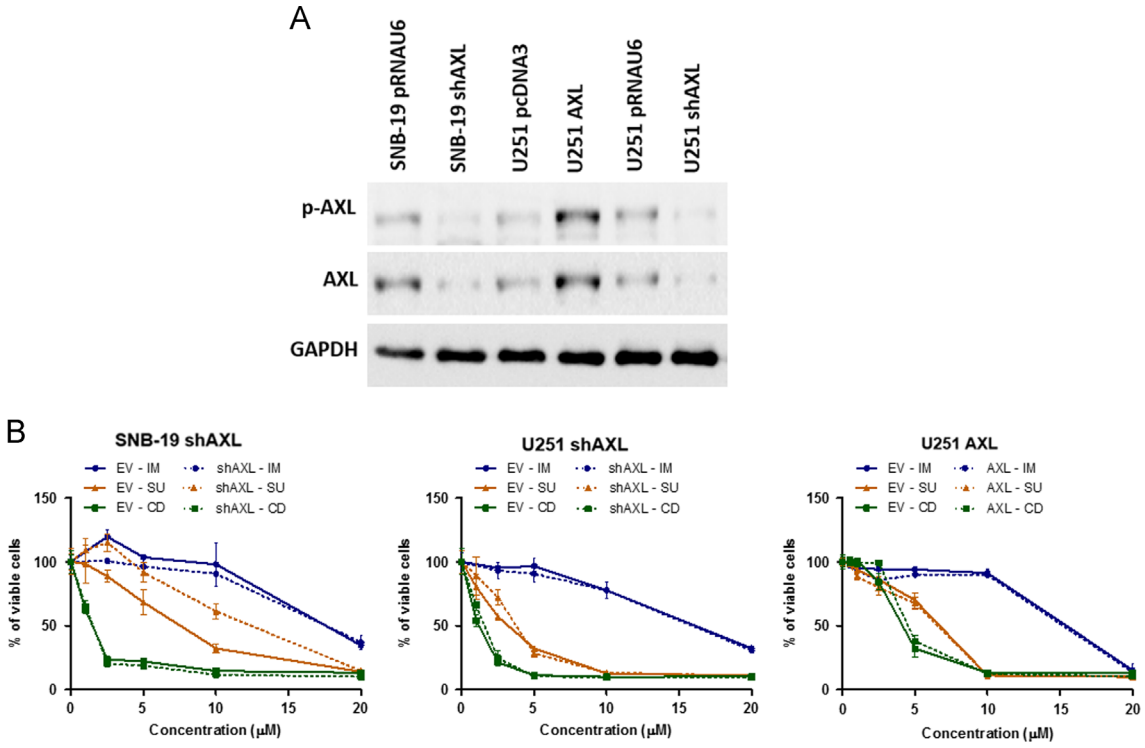

Fig. 2 – Role of AXL in the modulation of sunitinib, cediranib and imatinib therapy response in glioblastoma cells. (A) SNB-19 and U251 cells were stably transfected with a shRNA for AXL (shAXL), or full length AXL cDNA (AXL). The respective empty vectors (pRNAU6 or pcDNA3) were used as negative controls. Cell lysates were analyzed by Western blot for AXL expression. (B) IC_{50} values of transfected cells (shAXL, AXL and respective empty vectors: EV), were assessed for the three drugs (CD: cediranib; SU: sunitinib; IM: imatinib) by MTS after 72 h of treatment.

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that AKT could be dependent on AXL signaling in this cell line (Fig. 1C).

Role of AXL in cediranib and sunitinib therapy response

To determine whether AXL can modulate cediranib and sunitinib response in glioblastoma, we knocked down AXL in the U251 and SNB-19 cell lines, and overexpressed it in the U251 cell line (Fig. 2A). Following transfection of a short hairpin targeting AXL (shAXL), we observed a significant reduction of total and phosphorylated AXL protein levels when compared to the cells transfected with the empty vector (pRNAU6) in both cell lines (Fig. 2A). U251 cells successfully overexpressed AXL upon transfection with AXL cDNA when compared with cells transfected with the empty vector (pcDNA3) (Fig. 2A).

To analyze the effectiveness of cediranib and sunitinib in these AXL modulated cell lines, we determined the IC_{50} concentrations of each drug after cell transfection (Fig. 2B). Imatinib was used as negative control, since we have previously shown that this drug does not target AXL [20]. We found that in the U251 (low AXL activity) cell line, neither AXL inhibition nor AXL overexpression altered the sensitivity of the cells to any of the agents tested. In contrast, in the SNB-19 (high AXL activity) cell line, while no differences were observed for imatinib, as expected, nor to cediranib, a statistically significant ($p<0.05$) increase in the IC_{50} value was found for sunitinib on the shAXL cells (11.6 μM) when compared with the cells transfected with the empty vector (7.1 μM) (Fig. 2B).

To exclude the possibility of metabolic adaptation of the cells after transfection, we performed survival and migration assays at fixed doses of sunitinib (Fig. 3). AXL-silenced SNB-19 cells exhibited a survival advantage when treated over time with sunitinib compared to the empty vector control cells ($p<0.05$) (Fig. 3A). As expected, the treatment of SNB-19 cells with cediranib had no effect on viability (Fig. 3A). Also, no significant differences were observed in the survival of U251 transfected cells (empty vector *versus* AXL/shAXL cells) upon treatment with either drug over time.

Using a wound healing migration assay, we observed that both cediranib and sunitinib significantly reduced migration of both cell lines when compared to the DMSO control. In contrast, sunitinib no longer reduced migration in the AXL silenced SNB-19 cells compared to the DMSO control. Rather, we observed that shAXL transfected cells have a significantly higher migratory capability than

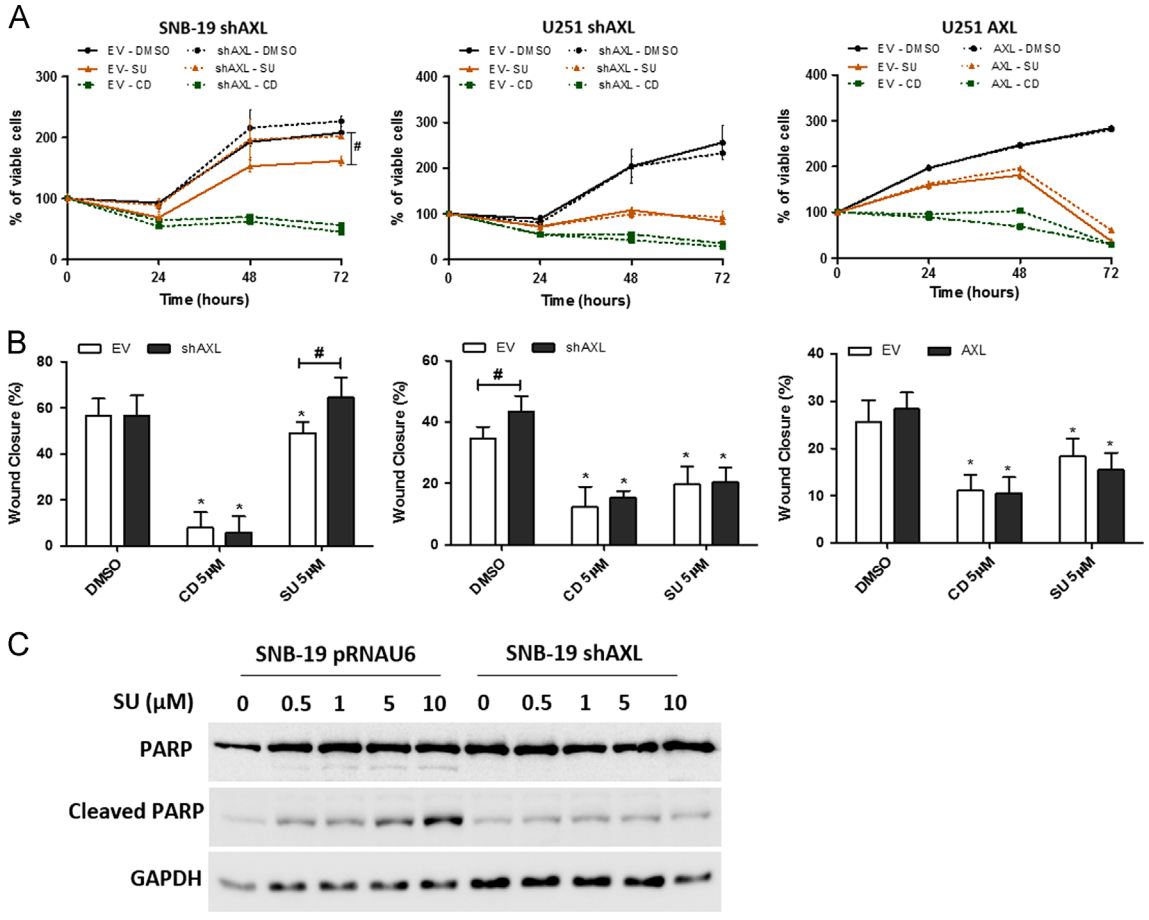

Fig. 3 – Role of AXL in glioma cells survival, migration and apoptosis after sunitinib treatment. (A) SNB-19 and U251 transfected cells were treated with 5 μM of cediranib (CD), 5 μM of sunitinib (SU) or DMSO as a control to assess cellular viability over time by MTS. Data is represented as the mean ± SD and differences in two-way analysis of variance (ANOVA) with a $p<0.05$ were considered statistically significant (#). (B) For migration assessment the cells were incubated for 48 h (*compared to DMSO and # compared to EV—empty vector using student's *t* test). (C) SNB-19 transfected cells were incubated with increasing concentrations of sunitinib (SU) for 24 h. Apoptosis was assessed by Western blot for PARP cleavage using two antibodies, one that detects total PARP and one that is specific for the cleaved form. GAPDH was used as a loading control.

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control cells after sunitinib treatment (Fig. 3B). Corroborating these results, we analyzed apoptosis by PARP cleavage and found that knockdown of AXL in SNB-19 cells inhibited apoptosis induced by sunitinib treatment when compared to the empty vector transfected cells (Fig. 3C).

These data indicate that AXL-inhibited cells are less sensitive to sunitinib treatment. To validate this, we performed the reverse experiment by analyzing whether AXL positive cells became more sensitive to sunitinib after receptor overactivation with its ligand (Fig. 4). For that, we selected the cell lines SNB-19 and A172 which had the highest basal AXL activation levels (Fig. 1A). By performing IC_{50} assays, we confirmed that the cells became more responsive to sunitinib following AXL activation with its ligand (Fig. 4A). Also in the wound healing migration assay, sunitinib had a greater impact on migration inhibition after Gas6 stimulation (Fig. 4B). However, the differences were not statistically significant in both assays ($p > 0.05$), suggesting that there are additional molecular mechanisms modulating sunitinib response.

Alterations of RTK signaling pathways upon AXL silencing

To interrogate whether AXL-inhibited cells can trigger the activation of alternative RTK signaling pathways to become less responsive to sunitinib, we determined the activation levels of other RTKs in the SNB-19 cells using phospho-arrays (Fig. 5A). We observed that upon sunitinib treatment, EphA7 phosphorylation was inhibited in the AXL-silenced cells when compared to the control cells. Furthermore, we confirmed that AXL is a target for sunitinib in the empty vector transfected cells, and that EGFR and EphA7 seemed to be overactivated after sunitinib treatment in these AXL positive cells. These phospho-array results were validated by Western blots of cell lysates (Fig. 5B), where we further found that EGFR is upregulated in AXL-silenced cells (Fig. 5B).

This switch in activated RTKs in response to AXL silencing could also drive a change in downstream intracellular signaling pathways. Thus, we performed Western blot analysis of cell lysates to assess activation of the AKT and MAPK pathways. We found that AXL knockdown resulted in higher phosphorylation levels of both AKT and ERK proteins, which was confirmed in two different lysates (Fig. 5C).

Combination of sunitinib with AKT and MAPK inhibitors

Based on our previous findings, we hypothesized that the combination of sunitinib with MAPK or AKT pathway inhibitors would enhance therapeutic inhibition, particularly in AXL-silenced cells. By combining sunitinib with selumetinib (MEK inhibitor), at a dose that effectively inhibited the targeted pathway (Fig. 5C), we found that selumetinib potentiated the effect of sunitinib. However, there was no synergistic effect in the shAXL cells, which were still more resistant to sunitinib comparatively to the empty vector cells (Fig. 5D). The AKT inhibitor MK2206 had a synergistic effect with sunitinib, and the two transfected cell lines (shAXL and the control) responded equally to the combination of sunitinib and MK2206 (both cell lines reached an IC_{50} of 2.9 μM).

Finally, using the wound healing assay, we observed that MK2206 was also able to partially revert the sunitinib-induced increase in migration in AXL-silenced cells (Fig. 5E).

Discussion

Targeted therapy with small-molecule compounds is changing the clinical practice for several advanced cancers [48,49]. Sunitinib malate (SU11248, Sutent by Pfizer) and cediranib (AZD2171, Recentin by AstraZeneca) are multi-target kinase drugs that inhibit PDGFR-α/ß,

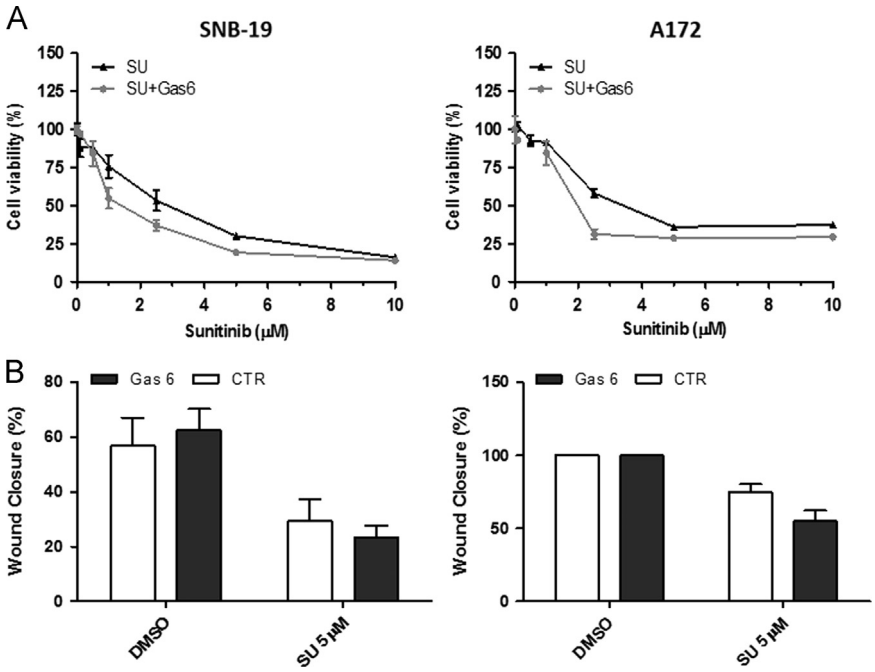

Fig. 4 – Effect of Gas6 stimulation on the sensitization of cells to sunitinib therapy. (A) SNB-19 and A172 cells were incubated with increasing concentrations of sunitinib (SU) simultaneously with stimulation by 400 ng/ml of Gas6 for 72 h. To assess the IC_{50} values, cellular viability was analyzed by MTS. (B) For migration assessment, SNB-19 and A172 cells were incubated for 48 h with 5 μM of sunitinib (SU) and with or without 400 ng/ml of Gas6.

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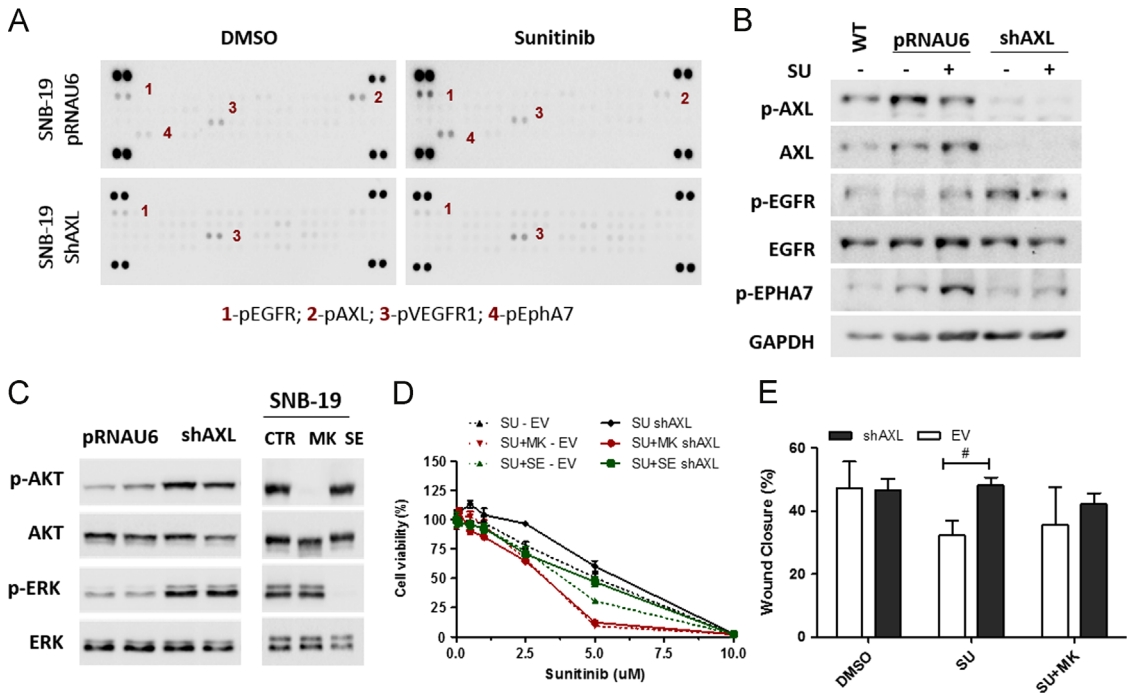

Fig. 5 – RTKs and intracellular pathway alterations in AXL negative cells. (A) Phospho-RTK arrays were performed for SNB-19 transfected cells before and after sunitinib treatment (2 h, 5 μM). Each RTK is represented in duplicate on the arrays (two spots side-by-side), and four pairs of phospho-tyrosine positive controls are located in the corners of each array. (B) Western blot validation of the arrays with specific antibodies to AXL, EGFR and EphA7 was performed on the same lysates. SNB-19 wild-type (WT) cells were also included as a control. GAPDH was used as a loading control. (C) Two independent lysates of the SNB-19 transfected cell line were analyzed by Western blot for the levels of MAPK (ERK) and AKT pathway activation. On the right, SNB-19 wild-type cell line was treated for 2 h with 2.5 μM of selumetinib (SE) or MK2206 (MK) to confirm their capability for inhibiting the MAPK and AKT pathways, respectively. (D) Combination studies were done in the SNB-19 transfected cell line with sunitinib and fixed concentrations of MK or SE (2.5 μM) over 72 h. To assess the IC_{50} values, the cellular viability was determined by MTS. (E) To migration quantification, the cells were incubated for 48 h with 5 μM of sunitinib (SU) and with or without 2.5 μM of Gas6.

VEGFR1-3, KIT, RET, FLT3, CSF1R, and VEGFR1-3, KIT, PDGFRα, respectively [50–52]. These therapeutic agents have shown interesting results in pre-clinical mouse glioma models, but failed to demonstrate benefit in progression free survival and overall survival in clinical trials [26,53–65]. A recent phase III clinical trial with cediranib showed that this drug failed to meet its primary end point of progression free survival prolongation, either as monotherapy or in combination with chemotherapy [26]. Hitherto, and in contrast to other tumor types [66], no predictive biomarkers for molecular targeted therapy response were yet identified in glioblastoma, hampering the design of efficient tailored therapies for these patients [30], and justifying to some extent the failure of the clinical trials until now.

In a previous work of our group, we reported that cediranib could also inhibit EGFR, EphA7, AXL, MET, EphB2, and sunitinib could target EphB2, ROR1 and AXL [31], besides the abovementioned "classical" targets. Other authors have recently identified AXL as target for sunitinib [67,68]. In the present work we aimed to validate AXL as a cediranib and sunitinib target in glioblastoma cells, using two glioblastoma cell lines with distinct AXL levels, namely U251 (low basal AXL levels) and SNB-19 (high basal AXL levels). Initially, we assessed AXL inhibition by western blot when cells were stimulated with the ligand GAS6. We showed that cediranib was able to inhibit AXL phosphorylation only at high doses in the SNB-19 cell line. Sunitinib strongly inhibited AXL in a dose-dependent manner and also inhibited the AKT pathway at high doses in the SNB-19 cell line. In accordance, modulation of AXL protein levels showed that the low levels of AXL activity could be associated with the observed response of SNB-19 cells to sunitinib therapy, but not to cediranib or imatinib. These results were not observed in U251 cell line with or without AXL expression. Several issues could explain these distinct drug responses. We have previously shown that the activation profile of RTKs in these two particular cell lines is very similar, however U251 is much more sensitive to sunitinib than SNB-19 [31]. This can in part explain the similarity in the sunitinib response after AXL modulation in U251 cells, since they already exhibit a highly sensitive response. Additionally, these findings can also suggest that sunitinib response is cell line-dependent and that AXL is not the only factor modulating the cells' responsiveness to this drug. Hence, the main predictive factor for the response to this drug remains to be discovered, as previously pointed out [56].

Moreover, our present work suggests that AXL could be a modulator of sunitinib response, at least for cell lines that present high endogenous levels of this RTK activation, since we observed an increased responsiveness to sunitinib in cells activated with AXL receptor ligand. A role for AXL in molecular targeted therapies modulation is not new and has been related with resistance. For example, *de novo* activation of AXL has been associated with imatinib, lapatinib treatment and mainly with resistance to EGFR

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inhibitors in several tumor models [45,46,69–72]. Interestingly, we found that AXL-silenced cells exhibited higher activation levels of the MAPK and AKT pathways and that some RTKs, such as EGFR, are overactivated upon AXL inhibition. Several preclinical and clinical studies have illustrated that deregulation of one signaling pathway can sometimes alleviate or bypass the "addiction" to another pathway [73,74]. Thus, to overcome the activation of intracellular signaling pathways that render resistance to RTK inhibitors, several combinations of drugs are currently being tested to target both ERK/MAPK and PI3K pathways, and their use in combination with other targeted therapies holds great promise [75–77]. Our *in vitro* studies showed that by combining sunitinib with AKT inhibitors the lower sensitivity of AXL-knockdown cells to sunitinib could be reverted. This suggests that this therapeutic strategy may be effective in glioblastoma patients, mainly for those with low AXL activity who are less likely to be responsive to sunitinib treatment alone.

In conclusion, we report that the levels of AXL activation could be one of the modulators of sunitinib response in glioblastoma patients. In addition, we showed that in the absence of AXL activation, the combination of sunitinib with specific inhibitors of the AKT pathway may overcome the eventual resistance phenotype of glioblastoma cells to sunitinib.

Acknowledgments

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