**Title:** Targeting AXL and the DNA damage response pathway as a novel therapeutic strategy in melanoma

**Running title:** Dual inhibition of AXL and CHK1/2 in melanoma

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Abstract:

Receptor tyrosine kinase AXL is found upregulated in various types of cancer, including melanoma, and correlates with an aggressive cancer phenotype, inducing cell proliferation and epithelial-to-mesenchymal transition. Additionally, AXL has recently been linked to chemotherapy resistance and inhibition of AXL is found to increase DNA damage and reduce expression of DNA repair proteins. In light of this, we aimed to investigate if targeting AXL together with DNA damage response proteins would be therapeutically beneficial. Using melanoma cell lines, we observed that combined reduction of AXL and CHK1/CHK2 signaling decreased proliferation, deregulated cell cycle progression, increased apoptosis and reduced expression of DNA damage response proteins. Enhanced therapeutic effect of combined- as compared to mono-treatments was further observed in a patient-derived xenograft model and, of particular interest, when applying a three-dimensional ex vivo spheroid drug-sensitivity assay on tumor cells harvested directly from 27 patients with melanoma lymph node metastases.

Together, these results indicate that targeting AXL together with the DNA damage response pathway could be a promising treatment strategy in melanoma and that further investigations in patient groups lacking treatment alternatives should be pursued.

Keywords: AXL Receptor Protein Tyrosine Kinase, Targeted Molecular Therapy, DNA Damage, CHK1, CHK2, Melanoma
**Introduction:**

The incidence of melanoma is increasing worldwide (1). While the prognosis of early stage disease is very good, once the cancer progress survival drops dramatically, with over 20 000 melanoma-related deaths in Europe annually (2). Approximately 50% of all melanomas harbor activating BRAF mutations, with BRAF^{V600E} being the most prevalent. The development of BRAF^{V600E} inhibitors vemurafenib and dabrafenib has led to targeted treatment options for patients with these mutations. However, almost all patients develop resistance within a year, often due to reactivation of the MAPK pathway or other receptor tyrosine kinases independently of BRAF (3,4). Lately, immune checkpoint inhibitors, like monoclonal antibodies targeting PD-1 and CTLA-4, have shown promising therapeutic effects (5). Yet, only a portion of the patients respond, signifying the importance to identify alternative therapeutic strategies.

The receptor tyrosine kinase AXL; a 138 kDa single-pass transmembrane protein of the TYRO3, AXL, MERTK (TAM)-family, has been found overexpressed, both as mRNA and protein, in a wide range of cancers (6-8), including melanoma (9). AXL is reported to play a role in cancer progression, and has been shown to promote cell proliferation, migration, invasion and epithelial-to-mesenchymal transition (EMT) (10-13). Additionally, AXL is shown to mediate resistance to BRAF and MEK inhibitors (14,15), as well as immunotherapy (16). All the TAM-family members are activated by the vitamin K-dependent ligand Growth arrest-specific protein 6 (GAS6), with AXL having the highest affinity for the ligand (17). In addition, AXL can be activated independently of GAS6 through aggregation of the protein or by heterodimerization with non-TAM receptor tyrosine kinases (18). Activated AXL undergoes homodimerization and autophosphorylation to induce downstream effects that activate proteins involved in the PI3K, MAPK14 (p38/MAPK) and MAPK1 (ERK/MAPK) pathways (12,13,19).

Recently AXL expression was found to reduce the sensitivity to chemotherapies, as well as to PARP inhibitors (20-22). In ovarian cancer cell lines, an association between AXL and cisplatin resistance has been observed (23). Additionally, inhibited AXL expression has been found to induce DNA damage and reduce the expression of DNA damage repair proteins (21). Together, these data suggest a link between AXL and the DNA damage response (DDR) pathway. Central to the DDR are the serine/threonine specific kinases CHK1 and CHK2 that are activated by ATR or ATM, respectively, in response to single-stranded (ATR) or double-stranded (ATM) DNA-breaks. CHK1 and CHK2 transduce signals to effectors such as TP53 (p53), CDC25C, BRCA1 and RAD51, ultimately leading to DNA repair, cell cycle arrest and/or apoptosis (24).
In this study, we assessed how dual inhibition of AXL and CHK1/CHK2 altered proliferation, signal transduction, apoptosis and cell cycle distribution in melanomas. We discovered that targeting or inhibiting expression of AXL and CHK1/CHK2 in combination reduced cell proliferation and induced cell cycle arrest and apoptosis. We further showed that the combined treatment was superior to mono-treatment in a patient-derived xenograft (PDX) model and when analyzing drug sensitivity utilizing cells harvested directly from melanoma lymph node metastases in a 3D ex-vivo drug-efficacy assay. Together, these data suggest that dual targeting of AXL and DDR pathway is a promising treatment strategy for melanomas that should be further investigated in patients having developed resistance and where few treatment alternatives are available.

**Materials and methods:**

**Cell lines and patient material**

Melanoma cell lines were established from subcutaneous (Melmet 1) or lymph node (Melmet 5, FEMX-1 and HHMS) metastatic lesions of patients treated at the Norwegian Radium Hospital, Oslo University Hospital (25,26). WM115, WM902B, WM983 and WM1366 cells were a kind gift from Meenhard Herlyn, the Wistar cell line collection (Philadelphia, PA, USA). The melanoma cell lines MDA-MB-435 and MeWo were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were routinely checked for mycoplasma by PCR in-house. Melmet 1 and WM1366 cell lines were STR fingerprinted (April 2018) by Genetica Cell Line Testing (Burlington, NC, USA). The melanoma cells were grown in RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine (Lonza, Basel, Switzerland). Cells were maintained in a humidified incubator at 37°C and with 5% CO₂. All cells were used within 20 passages of thawing.

Melanoma lymph node metastases were obtained from patients operated at the Norwegian Radium Hospital, Oslo University Hospital. Patient material was collected with written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Norway Regional Committee for Medical and Health Research Ethics (approval number 2014/2208 and 2015/2434).

**Immunoblot, protein analysis and antibodies**

Protein extracts and immunobLOTS were performed as described (27), with the following exceptions: Proteins were lysed in a buffer containing 1% Triton X-100, 50mM Hepes (pH 7.4), 150mM NaCl, 1.5Mm MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na Pyruvate, 1mM Na₂VO₄ and 10% Glycerol, with addition of 10 µL/mL protease and phosphatase inhibitor cocktails (cOmplete Mini and PhosSTOP™, Roche, Mannheim, Germany). Antibodies used were: pAXL (#5724), AXL (#8661), pAKT (#9271), AKT (#9272), pERK (#9101), pp38 (#9211), p38 (#8690), pSRC (#12432), SRC (#2108), pp53
(#9284), p53 (#2524), CDKN1A (p21) (#2947), pCDC25C (#9528), CDC25C (#4688), pCHK1 (#2341), CHK1 (#2360), pCHK2 (#2661), CHK2 (#6334), (all diluted 1:1000, Cell Signaling, Boston, MA, USA), ERK2 (D2) (#sc-1647, 1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) and α-tubulin (DM1A) (#05-829, 1:50 000, Millipore, Burlington, MA, USA). Protein bands were visualized by SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and exposed in a Syngene G Box. If not otherwise specified, protein lysates were made from cells that had been subjected to 400 ng/mL GAS6 (R&D, Minneapolis, MN, USA) and 10 µg/mL Vitamin K (Sigma Aldrich, St. Louis, MO) for 60 minutes. USA Simple Western immunoassay was performed according to the manufacturer protocol and run on the Peggy Sue™ machine (ProteinSimple, San Jose, CA, USA). Antibodies used were AXL (1:100, #8661 Cell Signaling, Boston, MA, USA) and β-actin (1:300, #4967 Cell Signaling, Boston, MA, USA). Data was analyzed using the Compass Software (Protein Simple, San Jose, CA, USA).

Reagents

BGB324 (previously known as R428, first described in (28)) was a kind gift from BerGenBio (Bergen, Norway). AZD7762 (first described in (29), cat# S1532) and VE-822 (first described in (30,31), compound 45, see company website, cat# S7102, for updated structure) was purchased from Selleck Chemicals (Huston, TX, USA). Inhibitors, diluted in DMSO, were used at concentrations and time periods indicated, with controls receiving the same amount of DMSO as the treatment groups.

RNA interference

Cells were transfected with 100 nM siRNA using Lipofectamine® 2000 in Opti-MEM Media (Thermo Scientific, Waltham, MA, USA) according to manufacturers protocol using the following siRNAs targeting AXL: 3 unique 27mer siRNA duplexes (Cat: SR319445, Origene, Rockville, MD, USA) and ON-TARGETplus Human AXL siRNA (Cat: J-003104-13-0002, Dharmacon, Lafayette, CO, USA), CHK1: ON-TARGETplus Human CHEK1 siRNA (Cat: J-003255-10-0002 and J-003255-11-0002, Dharmacon, Lafayette, CO, USA), and CHK2: ON-TARGETplus Human CHEK2 siRNA (Cat: J-003256-17-0002 and J-003256-18-0002, Dharmacon, Lafayette, CO, USA). ON-TARGETplus Nontargeting Pool Control siRNA (Cat: D-001810-10-05, Dharmacon, Lafayette, CO, USA) was used as control. Cells were left for 48 hours before they were used in further experiments.

In vitro proliferation and Caspase-3/7 cleavage

For analyzing the effect on proliferation, cells were plated at 15-25% confluency in 96-well or 6-well culture plates and left overnight before treatment with drugs for 72 hours. Cell confluence was visualized by IncuCyte FLR or IncuCyte Zoom Kinetic Imaging System (Essen Bioscience, Ann Arbor, MI, USA) light scanning microscopes. For colony formation assays, 500 or 1000 cells were plated in 6-well culture plates overnight before drug-containing media was added. After 21 days, colonies were.
fixated with ice-cold methanol before being stained with 0.05% crystal violet and counted using the GelCount™ machine (Oxford Optronix, Abingdon, UK).

Caspase-3/7 cleavage was determined using the CellPlayer™ 96-well Caspase-3/7 reagent (Essen Bioscience Ann Arbor, MI, USA) according to manufacturer’s protocol. In brief, cells were plated to yield 10-20% confluency. The following day, drugs and 2.5 µM caspase-3/7 reagent was added. Caspase-3/7 cleavage, yielding fluorescent signals, was visualized by IncuCyte FLR or IncuCyte Zoom Kinetic Imaging System (Essen Bioscience, Ann Arbor, MI, USA) light scanning microscopes. Fluorescence was related to the confluence of the respective well at the respective time points.

**Flow cytometry**

Cells were plated at 30% confluency in 6-well plates overnight before incubation with BGB324 and/or AZD7762 for 24 hours. Control cells were treated with DMSO. Harvested cells were fixated in 70% ice-cold methanol and stored at -20ºC for at least 24 hours. Cells were then labeled with 2.4 µL/mL Hoechst 33258 (Sigma Aldrich, St. Louis, MO, USA) or 500µL propidium iodide Cycloscope™ Reagent (Cytognos, Salamanca, Spain) and incubated for 10 minutes shielded from light. H2AX staining was performed on fixed cells resuspended and blocked in detergent buffer (0.1% Nonidet P40 (Igepal CA-630), 6.5mM Na₂HPO₄, 1.5mM KH₂PO₄, 2.7mM KCL, 137mM NaCl, 0.5mM EDTA PH 7.5 with 4% nonfat milk) before primary incubation with γH2AX antibody (1:500, Abcam, Cambridge, UK) and secondary incubation with Alexa Flour® 647 antibody (1:500, Abcam, Cambridge, UK). Cells were labeled with 2.4 µL/mL Hoechst 33258 (Sigma Aldrich, St. Louis, MO, USA). Analysis was performed using the LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo® v10 software (Ashland, OR, USA).

**Invasion and migration assays**

To measure cell invasion, 50 µg matrigel (BD Biosciences, San Jose, CA, USA) was added to Falcon® Transparent PET Membrane 24-well 8.0 µm cell culture inserts (Corning, Corning, NY, USA). Newly split cells were incubated with 0.1 mCi/mL ³H-Thymidine (Nerliens Mezansky, Oslo, Norway) for 24 hours. Thereafter, 50 000 serum-starved ³H-Thymidine labeled cells/well were plated in the inserts, in RPMI-1640 media (Sigma Aldrich, St. Louis, MO, USA) containing drugs, but without serum. Five percent FBS was added to the bottom well in addition to drugs in the same concentration as the top well. Cells were harvested by scraping from the bottom and top of the matrigel with a cotton swab that was further inserted into tubes containing 4 mL Aquasafe 300 scintillation fluid (Zinsser Analytic, Frankfurt, Germany). The invasive ability was determined by comparing ³H-Thymidine-radioactivity as a measure of number of cells on the bottom of the matrigel membrane divided by the total radioactivity of cells from top and bottom of the membrane.

Migration was measured by plating 50 000 cells/well in 96-well culture plates and scratching the wells the following day by The WoundMaker™ 96-well pin block (Essen Biosciences, Ann Arbor, MI, USA).
USA) before adding drug. Cell migration was determined using the Incucyte FLR or Incuzoom Zoom Kinetic Imaging System (Essen Biosciences, Ann Arbor, MI, USA), that scan the cells every three hours, and with the respective software calculating cell confluence.

**Ex vivo drug sensitivity assay**

Melanoma lymph node metastases obtained following surgery were disaggregated for one hour by 125 units collagenase type 2 (Sigma Aldrich, St. Louis, MO, USA) and 2.5 mg/mL DNase (Sigma Aldrich, St. Louis, MO, USA). To remove aggregates and debris the cell suspensions were filtered through 100 μM filters (WVR, Radnor, PA, USA). If necessary, red blood cells were removed using ACK lysing buffer (Lonza, Basel, Switzerland). Live cells (15,000-20,000 per well) were seeded in Nunc™ 96-Well Polystyrene Round Bottom Microwell plates (Thermo Scientific, Waltham, MA, USA) in RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA) medium supplemented with 5% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin (all Lonza, Basel, Switzerland) and allowed to form three-dimensional spheroids. Drugs were added at indicated concentrations immediately after seeding and the cells incubated for 5 days before viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA), and analyzed by Fluoroscan Ascent Fl (Thermo Scientific, Waltham, MA, USA). The *ex-vivo* assay was performed once for each patient sample, with at least three technical replicates per condition.

**In vivo studies**

Eight week old female athymic (foxn1 nu) nude mice were injected subcutaneously with 2x10⁶ Melmet 1 cells in the right flank. When the tumors reached a volume of approximately 50 mm³ the mice were randomized into four groups containing 6-8 mice in each group. 50 mg/kg BGB324 diluted in 0.5% Hydroxypropyl Methylcellulose/0.1% Tween-80 was given twice daily by oral gavage and 25 mg/kg AZD7762 diluted in 11.3% (2-Hydroxypropyl)-β-cyclodextrin was given intravenously three times a week. Treatment duration was fourteen days. Groups not receiving BGB324 and/or AZD7762 were administered drug vehicles in the same manner as treatment groups. Treatment toxicity was monitored by weight loss measured twice daily on treatment and twice weekly off treatment. Mice with ≥15% reduced weight were euthanized. Tumor diameters were measured twice a week by digital calipers and tumor volume calculated by the formula 0.5 x length x width². In line with governmental regulations, the mice were euthanized when the tumors reached a diameter of 16 mm and/or a volume of 2000 mm³. *In vivo* data is presented as average tumor volume + standard error of the mean (SEM). All mice were bred at the Department of Comparative Medicine, The Norwegian Radium Hospital, housed in rooms with alternating light/dark cycles of 12 hours, had *ad libitum* access to food and water and were kept according to regulations of the Norwegian Animal Welfare Act. Animal experiments were approved by the Norwegian Animal Research Authority (FOTS approval number 8554).
Statistical analysis

All values represent data average + standard deviation (SD) or SEM. Statistical significance was determined by student two-tailed t-test when comparing two groups or one-way ANOVA when comparing three groups. Significance over various time points in the animal experiments was determined by area under the curve (AUC) analysis. The statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). P-values of less than 0.05 were considered significant and marked with asterisks, where p<0.05 = *, p<0.01 = ** and p<0.001 = ***. Synergism was calculated by the CalcuSyn Software (Biosoft, Cambridge, UK) using the Chou-Talalay CI method (32). Experiments were performed at least three times with at least three technical replicates in each experiment, if not otherwise specified. Immunoblots were performed at least twice with independent lysates.

Results:

Decreased expression or inhibition of AXL reduced proliferation and MAPK and PI3K signaling

Ten melanoma cell lines were first examined for AXL expression by Simple Western immunoassay (Supplementary Figure 1A). Of the three AXL expressing cell lines (Melmet 1, WM1366 and MeWo), the two with the highest expression (Melmet 1 and WM1366) were chosen for further studies. The impact of AXL on proliferation was investigated following transfection with two different short interfering RNAs (siRNA). As shown in Figure 1A, silencing AXL decreased proliferation and reduced colony formation as compared to scrambled siRNA control. The effect on proliferation was further confirmed following treatment with the specific small-molecular AXL inhibitor BGB324 (28) (Figure 1B). A BGB324 concentration of 2 μM was chosen as a higher dose (3 μM) drastically reduced proliferation, suggesting off-target effects at this dose (Supplementary Figure 1B). Due to the role of AXL in epithelial-to-mesenchymal transition (EMT) (33), we next investigated the effect of AXL inhibition on migration and invasion. As shown in Supplementary Figure 1C, treatment with BGB324 for 24 hours reduced migration and invasion in both cell lines.

To investigate the effect of targeting AXL on cell signaling, we first confirmed that GAS6 activates AXL, as demonstrated by increased Tyrosine 702 phosphorylation (Supplementary Figure 1D). This phosphorylation site is found responsible for the general activation of the protein (34). BGB324 reduced AXL activation in a dose dependent manner in both cell lines (Figure 1C). Of particular note, BGB324 increased the total AXL protein level, suggesting an attempt to rescue the reduced AXL signaling. Next, the impact of AXL inhibition on downstream signaling pathways was examined. As demonstrated in Figure 1C, BGB324 treatment in GAS6 stimulated cells decreased phosphorylation of
AKT, ERK and particularly SRC, but not p38. These effects were confirmed in siAXL transfected cells (Figure 1D).

Combined targeting of AXL and the DNA damage response pathway reduced viability and tumor growth in melanoma cell lines and patient-derived models.

The newly discovered link between AXL signaling and DNA damage response (DDR) (21,35) spurred us to investigate the effect of combined inhibition of AXL and the DDR. As shown in Figure 2A and 2B, co-treatment with BGB324 and the CHK1/2 inhibitor AZD7762 synergistically decreased proliferation in both Melmet 1 and WM1366 cells. The effect was validated using a three-dimensional (spheroid) drug efficacy assay in Melmet 1 cells (Figure 2C). To rule out the possibility of off-target effects, we treated the AXL negative cell line WM115 with BGB324 and/or AZD7762 in vitro and using the spheroid drug sensitivity assay and only observed reduced proliferation mediated by the AZD7762 treatment (Figure 2D), suggesting no off-target effects of the BGB324 treatment. Further, reduced proliferation was also observed in siAXL cells treated with AZD7762 compared to treated and untreated scrambled control transfected cells (Figure 2E). The transfected cells were more responsive to AZD7762 than untransfected cells (Figure 2A), possibly due to the added stress of the transfection.

To elucidate if the effect was dependent on either CHK1 or CHK2 signaling, we diminished CHK1 or CHK2 expression by siRNA before treating the cells with BGB324. Reduced expression of CHK1 or CHK2 resulted in slight to no change in proliferation compared to scrambled control transfected cells (Figure 3A and 3B). In both cell lines, siCHK1 transfected cells responded with decreased proliferation in combination with BGB324 compared to BGB324 treated and untreated control transfected cells. This was only significant in cells where CHK1 was completely eradicated (siCHK1 #1), indicating that even a low expression of CHK1 is enough to partly protect the cells from growth inhibition. There was also lower proliferation in siCHK2 transfected cells treated with BGB324 compared to BGB324 treatment alone, however only significant for one of the siRNA molecules (siCHK2 #1). Reducing expression of either CHK1 or CHK2 did not lead to as pronounced decrease in proliferation as AZD7762 treatment, neither alone nor in combination with BGB324, suggesting that signaling through both proteins must be abolished to maximize the response. To examine this hypothesis, we reduced the expression of both CHK1 and CHK2 and observed reduced proliferation in the siCHK1 and siCHK2 cells comparable to AZD7762 mono-treatment (Figure 3C and 3D). The proliferation of the combined siCHK1 and siCHK2 transfected cells was further reduced when the cells were treated with BGB324, yielding results in concordance with cells treated with BGB324 and AZD7762.
Further, we aimed to determine if reduced proliferation was only dependent on diminished activation of the CHK1/2 proteins or if similar effect could be observed when the activation of other DDR proteins was lowered. Thus, we inhibited signaling of ATR, mainly working upstream of CHK1, but also shown to activate CHK2 (36), using the ATR inhibitor VE-822 ((30,31), compound 45) in combination with BGB324 (Figure 3E). In both cell lines, combinatorial treatment with VE-822 and BGB324 significantly inhibited cell proliferation compared to each mono-treatment. This illustrates that other proteins in the DDR pathway also could be targeted together with AXL and cause reduced cell proliferation. Overall, these data demonstrate that inhibiting or reducing the expression of AXL in combination with CHK1 and CHK2 or other proteins in the DDR pathway result in decreased cell viability.

The observed effect on proliferation upon simultaneous targeting of AXL and the DDR encouraged us to examine if this could also reduce proliferation in patient samples. To this end, cells harvested directly from 27 melanoma lymph node metastases were treated with BGB324 and AZD7762 alone or in combination and analyzed for effect on viability using the ex vivo drug sensitivity assay. As shown in Figure 4A, the mean effects of the mono-treatments were slightly reduced compared to control, however these results were not significant. BGB324 and AZD7762 in combination, however, significantly decreased the viability compared to either mono-treatment. Of note, cells from three of the patient tumor samples showed increased viability when treated with AZD7762 alone, and in two of them, the viability was not reduced following combined treatment. Finally, the superior effect of the combined treatment was confirmed in the mouse Melmet 1 xenograft model (Figure 4B and 4C). Whereas the mono-treated mice displayed insignificant reductions in tumor volume, mice treated with the combination showed significantly decreased relative tumor volume and prolonged survival time compared to untreated controls or following mono-treatments. No significant weight loss was observed, indicating that the treatments were well tolerated (Supplementary Figure 2).

**Combined inhibition of AXL and CHK1/CHK2 leads to cell cycle arrest and increased apoptosis**

Due to the observed effects on proliferation and viability we aimed to investigate how reduced AXL and CHK1/2 activity alone and in combination affected cell cycle progression and apoptosis. As shown in Figure 5A and Supplementary Figure 3A, BGB324 treatment had no effect on cell cycle progression in any of the cell lines. AZD7762 treatment, on the other hand, slightly increased the S-phase fraction in Melmet 1 cells at both 24 and 48 hours post-treatment, but had minimal effect in WM1366 cells. Combining the two inhibitors, however, resulted in a considerable S phase arrest in Melmet 1 cells at 24 hours, and S phase and G2/M phase arrest at 48 hours. Co-treatment of WM1366 cells led to G2/M arrest at both 24 hours and 48 hours, whereas S-phase arrest was only observed after 48 hours.
In addition, in both cell lines a marked sub-G1-peak, suggesting apoptosis or necrosis, was observed (Supplementary Figure 3B) following combined treatment with BGB324 and AZD7762. To analyze if this reflected apoptosis, cleavage of CASP3 (caspase-3) and CASP7 (caspase-7) was examined using a kit yielding a fluorescent signal upon cleavage. As shown in Figure 5B, left panels, mono-treatments slightly increased cleavage of caspase-3 and -7, while this was significantly augmented following the combined treatment. Caspase-3 cleavage was further confirmed by western blot analysis (Figure 5B, right panels), demonstrating caspase-3 cleavage induced by AZD7762 and further increased in cells receiving the combined treatment. However, no caspase-3 cleavage was observed in BGB324 treated cells as examined by western blot. This is in contrast to the BGB324-induced cleavage of caspase-3 and -7 observed by the apoptosis assay, suggesting that caspase-7 cleavage plays a more prominent role following BGB324 treatment.

Further, we investigated the molecular effects of BGB324 and/or AZD7762 treatments by western blot analyses. As seen in Figure 5C, both compounds alone and in combination reduced the phosphorylation of AXL and increased the expression of total AXL. This is in agreement with previous reports demonstrating that AZD7762 may reduce AXL phosphorylation (37). Also in line with previous reports (38), AZD7762 increased phosphorylation of CHK1 and CHK2 in both cell lines, indicating activation of the DDR pathway. In addition, AZD7762 increased Serine 216 phosphorylation of CDC25C, a downstream effector of CHK1 and CHK2. While BGB324 treatment alone did not show any effect on CHK1 and CHK2 phosphorylation compared to control, CDC25C was greatly phosphorylated. Phosphorylation and total expression of CHK1, CHK2 and CDC25C was reduced in the combined treatment.

Previous reports have suggested that BGB324 induces activation and expression of H2AFX (H2AX) (21). This was not evident in our cell lines (Figure 5C). H2AX phosphorylation and expression was, however, observed in AZD7762 treated cells and further increased following combined treatment. The H2AX immunoblot results were verified by flow cytometry for WM1366 cells (Supplementary Figure 3C).

BGB324 alone had no effect on expression of the DDR proteins p53 or CDKN1A (p21<sup>WAF1/Cip1</sup>) in any of the two cell lines, whereas AZD7762 and the combination increased p53 protein levels as well as Serine 15 phosphorylation in Melmet 1 cells (p53 wild-type). Surprisingly, increased Serine 15 phosphorylation of p53 was also observed in the p53 mutated cell line WM1366 after treatment with AZD7762 alone and in combination with BGB324. In both cell lines, AZD7762 increased the expression of p21<sup>WAF1/Cip1</sup> and this was further augmented when the two inhibitors were combined. While both mono-treatments decreased PI3K and MAPK signaling, enhanced reduction when combined was only seen in PI3K signaling in Melmet 1 cells (Supplementary Figure 3D). These data were also observed in cells treated with BGB324 and/or VE-822 (Supplementary Figure 3E).
Importantly, VE-822 treatment reduced pAXL expression (Supplementary Figure 3E), which was also observed in AZD7762 treated cells (Figure 5C). Further, short (10 minutes) exposure to AZD7762 or VE-822 monotherapy did not reduce pAXL expression to the extent of BGB324 treatment (Supplementary Figure 3F).

Together, our data indicates that targeting AXL in combination with the DDR pathway reduces proliferation, leads to downregulation of DDR response proteins and ultimately results in apoptosis. Thus, targeting AXL together with the DDR could be a beneficial treatment option in melanoma.

**Discussion**

AXL has been observed overexpressed in various types of cancer and linked to aggressive tumor traits, poor prognosis and drug resistance (33,39). In melanoma, acquired resistance to MAPK inhibitors (14,40) and immunotherapy (16) has been associated with increased AXL expression, making AXL an interesting target to overcome treatment resistance. AXL has also emerged as a promising therapeutic strategy in other types of cancers, and currently the AXL inhibitor BGB324 is in phase I/II clinical trials alone or in combination with chemotherapy (NCT02488408), erlotinib (NCT02424617), pembrolizumab (NCT03184558 and NCT03184571) or dabrafenib and trametinib (NCT02872259).

In accordance with a previous report (41), AXL was found expressed in 30% of the examined melanoma cell lines, and reducing (42,43) or inhibiting (20) AXL expression modestly reduced proliferation, migration and invasion. Inhibition of AXL led to decreased AXL-Tyrosine 702 phosphorylation, indicating less activation of the protein (44). Furthermore, AXL has been found to activate the PI3K and MAPK pathways to induce pro-survival and proliferative signals (13). In accordance with this, we observed less proliferation and reduced phosphorylation of SRC, AKT and ERK upon diminished expression or inhibition of AXL. It has been shown that SRC activity is dependent on partnerships with receptor tyrosine kinases such as EGFR and PDGFR (45). These receptor tyrosine kinases are closely related to AXL and the substantial decrease in pSCR expression at even low levels of BGB324 treatment indicate that SRC activity may be dependent on AXL signaling as well. In contrast to what has been observed by others (46), no effect on p38/MAPK signaling was observed, potentially due to cell line or cancer type specific differences in p38/MAPK mediated stress signaling.

Recently, inhibition of AXL signaling was found to induce DNA damage (21,35) and it has also been proposed that AXL protect cancer cells from fork collapse (35), which is mediated by ATR/ATM-CHK1/2 signaling. In the current study, we neither observed activation of H2AX nor CHK1/2 following BGB324 treatment, suggesting that inhibiting AXL does not induce DNA damage in our melanoma cell lines. On the other hand, BGB324 led to increased inhibitory phosphorylation (Serine...
216) of CDC25C, implying cell cycle arrest. CHK1/2 signaling was not activated by BGB324 treatment, suggesting that CDC25C is inhibited independently of CHK1/2, for instance through phosphorylation by MARK3 (c-TAK1), p38/MAPK, CAMK2A, and PRKA (AMPK), as has been reported by others (47,48). Additionally, CHK1/2-independent phosphorylation of CDC25C-Serine 216 must also hold true for AZD7762 treated cells as this inhibitor blocks the downstream signaling of CHK1/2 by acting as an ATP competitor (29).

Because of a prior article describing effects of AXL on DDR (21), we speculated whether treatment with BGB324 in combination with a DDR inhibitor could be a beneficial therapeutic strategy in melanoma. In support of this, we found that targeting AXL together with CHK1 and CHK2 inhibited proliferation and viability in cell cultures, PDX models and patient material. Decreased proliferation was coupled with cell cycle deregulation and increased apoptosis. These data are in accordance with a previous finding showing that inhibition of AXL in combination with WEE1, a regulator of cell cycle progression downstream of CHK1/2, reduced tumor growth and increased apoptosis in small cell lung cancer cells (49). While knockdown of CHK1 or CHK2 resulted in reduced proliferation in combination with BGB324, the effect was not as pronounced as when inhibiting or reducing the expression of both CHK1 and CHK2. This suggests that redundancy, crosstalk and overlapping roles of CHK1 and CHK2 (50) protect the cells from growth inhibition when targeting only one of the proteins.

It has previously been shown that AZD7762 treatment reduces AXL phosphorylation (37), a finding in accordance with our results. A direct influence of AZD7762 on AXL phosphorylation might suggest that the inhibitory effect on proliferation when combining the two inhibitors solely is caused by decreased AXL activity. In a kinase screen of AZD7762, the drug also showed selectivity towards AXL, although it was ten times lower for AXL than CHK1/2 (29). To rule out the possibility of AZD7762 affecting AXL signaling, we diminished CHK1 or CHK2 expression, or treated cells with an ATR inhibitor (VE-822), in combination with BGB324. These experiments led to similar results as when using the AZD7762 and BGB324 inhibitors. Importantly, decreased pAXL expression was also observed in cells treated with VE-822, suggesting that there is some unknown mechanism of the DDR pathway that indirectly or directly targets AXL signaling. This interpretation is strengthened by the observation that AZD7762 or VE-822 did not reduce pAXL expression to that of BGB324 treated cells in a short (10 minutes) exposure to the drugs. These data demonstrates that the observed consequences of the combined treatment is not due to off-target effects of the AZD7762 inhibitor.

Surprisingly, in the scrambled transfected control cells, we observed lower proliferation when the cells were treated with AZD7762 (Figure 2E) compared to the same treatment in untransfected cells (Figure 2A). This effect was not observed in control transfected cells treated with BGB324 (Figure 3A). We do not know the reason for this, but it is shown that lipofectamine treatment increases DNA damage and induces cellular stress (51,52). Thus, we speculate that DNA damage and cellular stress produced...
by the transfection will sensitize the cells for the AZD7762 treatment hindering DDR and inducing cellular toxicity. Despite this, cellular proliferation was even further decreased after treatment with AZD7762 in combination with AXL knockdown.

We show here that while AZD7762 treatment resulted in activation and expression of DDR proteins such as CHK1, CHK2 and CDC25C, combined treatment with BGB324 diminished the expression of these proteins, implying that AXL facilitates the DDR. In line with this, AXL inhibition in combination with inhibitors of the DNA repair protein PARP or the cell cycle regulator WEE1 has shown to reduce the expression of DDR and DNA repair proteins (21,49). Further, previous reports have shown that accumulation of p53 and p21WAF1/Cip1 following DNA damage is associated with reduced expression of CHK1 (53), CHK2 (54) and CDC25C (55), which was also observed in this study. We do not know, however, if the accumulation of p53 and p21WAF1/Cip1 precedes the downregulation of DDR protein expression, or if the downregulation of these proteins promotes increased p53 and p21WAF1/Cip1 activation and/or expression. p53 and p21WAF1/Cip1 activation and/or expression play a role in triggering apoptosis, and in line with this, we observed that the combined inhibition of AXL and CHK1/2 led to apoptosis through cleavage of caspase-3 and-7. AZD7762 treatment caused a more pronounced increase in caspase-3 cleavage, as assessed by immunoblot, than BGB324 treatment, while the caspase-3 and -7 cleavage was approximately similar in the two mono-treatments as measured by the fluorescent reagent. This indicates that BGB324 activates caspase-7 to a larger degree than AZD7762 treatment.

The observed effects on cell viability upon combined AXL and CHK1/2 targeting in cell lines, was further verified using disaggregated cells from melanoma lymph node metastases in an \textit{ex vivo} drug efficacy assay. The added effect of the combined treatment relative to the mono-treatments was less pronounced in the \textit{ex vivo} assay, probably due to the presence of non-malignant cells in the lymph node metastases or by cells that do not express AXL. Despite this, the assay clearly distinguishes patient-derived tumor cells with different sensitivity to the applied drugs. Previously, we have confirmed platinum chemotherapy resistance in ovarian cancer patients (56), and recently we demonstrated concordance between response to the mutated BRAF inhibitor vemurafenib and \textit{BRAF/NRAS} mutation status when analyzing tumor cells from melanoma lymph node metastases in the \textit{ex vivo} assay (57). Together, these data show that the \textit{ex vivo} assay is able to reflect patient response to various drugs, and should be further evaluated as a supplement to guide treatment in patients having developed resistance against standard treatment regimes.

To conclude, AXL is shown to be upregulated in melanoma and its expression is associated with treatment resistance, making AXL an interesting target to overcome resistance to therapy. In this study, we investigated the effect of targeting AXL together with the DDR and found that this combination resulted in reduced cell proliferation and tumor growth. We show that dual inhibition of
AXL and the DDR result in cell cycle retention and increased apoptosis through downregulation of CHK1, CHK2 and CDC25C, suggesting that AXL facilitate the DDR. These data strongly suggest that targeting AXL together with the DDR may be a promising treatment strategy for melanoma and studies to further investigate this possibility is highly warranted.

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References:


Figure legends

Figure 1: Effects of diminished AXL expression or activity on cell proliferation and signaling.

A) Proliferation of Melmet 1 and WM1366 cells with siRNA-mediated silencing of AXL expression measured by IncuCyte Live imaging 72 hours after plating (n=3) (left panels) or by colony formation 21 days after plating (right panels). Colony formation shows an average of two independent experiments for Melmet 1 cells and three independent experiments for WM1366 cells. B) Melmet 1 and WM1366 cells treated with 2µM BGB324 (AXL inhibitor) reduced proliferation as measured by the IncuCyte Live imaging system (n=3). Representative immunoblot analysis of indicated proteins following C) treatment with indicated concentrations of BGB324 for 24 hours and D) siRNA-mediated AXL silencing. Control cells were treated with C) DMSO or D) scrambled siRNA. Immunoblots were performed at least twice with independent lysates. p<0.05 = *, p<0.01 = ** and p<0.001 = ***.
**Figure 2:** Inhibition of AXL and CHK1 and CHK2 signaling reduced proliferation in melanoma cell lines.

A) Dual treatment with 2 μM BGB324 and 1 μM AZD7762 reduced average proliferation in Melmet 1 and WM1366 melanoma cell lines. B) Combination index (CI) values as estimated by the Chou-Talalay method using average proliferation of indicated doses of BGB324 and AZD7762. CI < 1 indicates synergy. C) Proliferation of Melmet 1 cells treated with 2 μM BGB324 and/or 1 μM AZD7762 measured by the 3D spheroid assay correlates to what is observed in vitro. D) Proliferation measured by Incucyte Live imaging system (in vitro) or after 5 days using CellTiter-Glo® Luminescent assay (3D spheroid assay). Control cells were treated with DMSO. Experiments show an average of three biological replicates + SEM. p<0.05 = *, p<0.01 = **.

**Figure 3:** Treatment with BGB324 and siCHK1 and/or siCHK2 or the ATR inhibitor VE-822 reduced cell proliferation.

A) siRNA-mediated silencing of CHK1 (left panels) or CHK2 (right panels) before treatment with BGB324 reduced proliferation in melanoma cell lines. B) Immunoblot of CHK1 or CHK2 protein expression in cells transfected with siRNAs targeting either CHK1 (right panels) or CHK2 (left panels). C) Diminished expression of both CHK1 and CHK2 further reduced proliferation in combination with BGB324 treatment. D) Immunoblot of CHK1 and CHK2 expression in cells transfected with siCHK1 and siCHK2. E) Proliferation after drug addition of BGB324 and indicated doses of the ATR inhibitor VE-822. All proliferation data was measured by Incucyte Live imaging and the data shows average values relative to control cells calculated 72 hours after drug addition of at least three independent experiments + SEM. BGB324: 2 μM. Control cells were treated with DMSO. p<0.05 = *, p<0.01 = ** and p<0.001 = ***. Δ equals p value = 0.0512.

**Figure 4:** Dual inhibition of AXL and CHK1/2 reduced cell viability in patient tumor samples and inhibited tumor growth in vivo.

A) Lymph node metastases from melanoma patients were disaggregated, cells were plated as spheres and treated with 2 μM BGB324 and/or 2μM AZD7762 for five days. Cell viability was measured by CellTiter-Glo® and related to control samples treated with DMSO (n=27 patients). B) Tumor volume relative to the volume at day of treatment initiation of Melmet 1 xenografts treated with 50 mg/kg BGB324 twice daily and/or 25 mg/kg AZD7762 three times a week for two weeks. Controls were treated with drug vehicle(s). There were 6-8 mice per group. C) Kaplan-Meier survival curve showing
percentage mice in B) still alive as function of time. The experiment was terminated at day 62 and all mice still alive (n=9) were censored. p<0.05 = * and p<0.01 = **.

**Figure 5:** Combined treatment of BGB324 and AZD7762 leads to cell cycle arrest and apoptosis with reduced expression of cell cycle regulators.

A) Cell cycle distribution of Melmet 1 and WM1366 treated with BGB324 and/or AZD7762 for 24 or 48 hours measured by Hoechst 33258 incorporation and analyzed by Flow Cytometry. The data is shown as average of three independent experiments for 24 hours and two independent experiments for 48 hours + SEM. B) Average apoptosis measured by fluorescence staining of a caspase-3/-7 reagent by IncuCyte Live imaging. Fluorescent intensity was related to number of cells in each well and to control 72 hours after treatment with BGB324 and/or AZD7762 (left panels). Apoptosis experiments show an average of three biological experiments + SEM. Protein expression in total lysates of Melmet 1 and WM1366 cells treated with BGB324 and/or AZD7762 as shown by a representative immunoblot for proteins indicated (right panels). C) Protein expression in total lysates of Melmet 1 and WM1366 cells treated with BGB324 and/or AZD7762 for 24 hours as shown by a representative immunoblot for proteins indicated. Immunoblots were performed at least twice with independent lysates. In all experiments, control cells were treated with DMSO. Concentration of BGB324: 2 μM and AZD7762: 1 μM. p<0.05 = *, p<0.01 = ** and p<0.001 = ***.
Figure 1

(A) Melmet 1

Proliferation, % of control

0 50 100 150
siSCR siAXL#1 siAXL#2

WM1366

Proliferation, % of control

0 50 100 150
siSCR siAXL#1 siAXL#2

(B) Melmet 1

Proliferation, % confluence

0 20 40 60 80 100 120
0 12 24 36 48 60 72
CONTROL BGB324 2µM

WM1366

Proliferation, % confluence

0 20 40 60 80 100 120
0 12 24 36 48 60 72
CONTROL BGB324 2µM

(C) Melmet 1

# of colonies, % of control

siSCR siAXL#1 siAXL#2

WM1366

# of colonies, % of control

siSCR siAXL#1 siAXL#2

(D) Melmet 1 WM1366

BGB324, µM

0 0.1 1 2

pAXL(Tyr702) AXL pAKT(Ser473) AKT pERK(Thr202/Tyr204) ERK p-p38(Tyr180/Tyr182) p38 pSRC(Tyr416) SRC α-tubulin

AXL pAKT(Ser473) AKT pERK(Thr202/Tyr204) ERK p-p38(Tyr180/Tyr182) p38 pSRC(Tyr416) SRC α-tubulin

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**Figure 2**

A) Melmet 1

![Graph showing proliferation of Melmet 1 cells in different conditions: CONTROL, BGB324, AZD7762, BGB324 + AZD7762.](image)

B) WM1366

![Graph showing C.I. (Combination Index) values for WM1366 cells in different conditions: CONTROL, BGB324, AZD7762, BGB324 + AZD7762.](image)

C) Melmet 1

![Graph showing proliferation of Melmet 1 -3D spheroids in different conditions: CONTROL, BGB324, AZD7762, BGB324 + AZD7762.](image)

D) WM115

![Graph showing proliferation of WM115 -monolayer in different conditions: CONTROL, BGB324, AZD7762, BGB324 + AZD7762.](image)

E) WM1366

![Graph showing proliferation of WM1366 -3D spheroids in different conditions: CONTROL, BGB324, AZD7762, BGB324 + AZD7762.](image)
Figure 3

A) Proliferation, % of control

B) Western Blot

C) Proliferation, % of control

D) Western Blot
Figure 4

A) Relative tumor volume

B) Treatment duration

C) Survival, %

CTRL  BGB324  AZD7762  BGB324 + AZD7762

Cell viability, % of control

0 5 10 15 20

0 20 40 60

0 50 100 150 200 250

0 10 20 30

0 5 10 15

0 20 30

0 20 40 60

Days

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Figure 5

A) % cells

B) Apoptosis, fold change

C) Western Blot

- BGB324
- AZD7762

* KTRL
- BGB324
- AZD7762
- BGB324 + AZD7762

- Melmet 1
- WM1366

- 0
- 20
- 40
- 60
- 80
- 100

- pp53(Ser15)
- p53
- p21WAF1/Cip1
- α-tubulin

- caspase-3
- caspase-3 cleaved
- α-tubulin

- pAXL(Tyr702)
- AXL
- pCHK1(Ser345)
- CHK1
- pCHK2(Tyr68)
- CHK2
- pCDC25C(Ser216)
- CDC25C
- pp53(Ser15)
- p53
- p21WAF1/Cip1
- pH2AX(Ser139)
- H2AX
- α-tubulin
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