Research Paper

A screening-based approach identifies cell cycle regulators AURKA, CHK1 and PLK1 as targetable regulators of chondrosarcoma cell survival

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ABSTRACT

Chondrosarcomas are malignant cartilage tumors that are relatively resistant towards conventional therapeutic approaches. Kinase inhibitors have been investigated and shown successful for several different cancer types. In this study we aimed at identifying kinase inhibitors that inhibit the survival of chondrosarcoma cells and thereby serve as new potential therapeutic strategies to treat chondrosarcoma patients.

An siRNA screen targeting 779 different kinases was conducted in JJ012 chondrosarcoma cells in parallel with a compound screen consisting of 273 kinase inhibitors in JJ012, SW1353 and CH2879 chondrosarcoma cell lines. AURKA, CHK1 and PLK1 were identified as most promising targets and validated further in a more comprehensive panel of chondrosarcoma cell lines. Dose response curves were performed using tyrosine kinase inhibitors: MK-5108 (AURKA), LY2603618 (CHK1) and Volasertib (PLK1) using viability assays and cell cycle analysis. Apoptosis was measured at 24 h after treatment using a caspase 3/7 assay. Finally, chondrosarcoma patient samples (N = 34) were used to examine the correlation between AURKA, CHK1 and PLK1 RNA expression and documented patient survival.

Dose dependent decreases in viability were observed in chondrosarcoma cell lines after treatment with MK-5108, LY2603618 and volasertib, with cell lines showing highest sensitivity to PLK1 inhibition. In addition increased sensitivity to conventional chemotherapy was observed after CHK1 inhibition in a subset of the cell lines. Interestingly, whereas AURKA and CHK1 were both expressed in chondrosarcoma patient samples, PLK1 expression was found to be low compared to normal cartilage. Analysis of patient samples revealed that high CHK1 RNA expression correlated with a worse overall survival.

AURKA, CHK1 and PLK1 are identified as important survival genes in chondrosarcoma cell lines. Although further research is needed to validate these findings, inhibiting CHK1 seems to be the most promising potential therapeutic target for patients with chondrosarcoma.

1. Introduction

Chondrosarcomas account for 20% of primary bone tumors and are characterized by malignant cartilage producing cells [1]. Depending on the morphology and the location, chondrosarcoma can be subdivided into conventional chondrosarcoma and more rare subtypes: dedifferentiated chondrosarcoma, mesenchymal chondrosarcoma, clear cell chondrosarcoma and periosteal chondrosarcoma. Conventional chondrosarcoma accounts for 85% of all chondrosarcoma cases [1] and is further classified into central chondrosarcoma, located in the medulla of the bone, and peripheral chondrosarcoma, found at the surface of the bone [2]. These two different conventional chondrosarcoma subtypes show the same histological features: however a distinct molecular background is observed [1]. Conventional chondrosarcoma is classified into three different grades, which is the most important prognostic factor. Atypical cartilaginous tumors (ACT)/chondrosarcoma grade I show a low cellularity, a large amount of cartilage matrix, rarely metastasize, and have a relatively good prognosis. Grade II and grade III chondrosarcomas behave more aggressive and show a more cellular histology with reduced cartilage matrix and a poor prognosis. Patients
with grade II chondrosarcomas show 64% overall survival and patients with grade III chondrosarcomas show a very poor 10 years overall survival of only 29% [1,2]. As chondrosarcomas are resistant to conventional radio- and chemotherapy, the only treatment option to date for patients with chondrosarcoma is surgical removal. This is a major problem especially for patients with tumors in inoperable locations and patients with metastatic disease [2].

Protein kinases are important for cellular processes and are often found deregulated in cancer [3]. Kinome profiling in chondrosarcoma cell cultures previously revealed that the AKT, Src and Ras/Raf/MEK pathways were most active in chondrosarcoma [4]. In addition, Src kinases were shown to be important for chemoresistance, as shown by sensitization for doxorubicin upon inhibition with dasatinib, as well as a high expression of Src kinase family members in chondrosarcoma patient tissues [5]. Using phospho-RTK arrays the phosphorylation status of 42 RTKs was investigated in chondrosarcoma cell lines, which led to the observation of a heterogeneous RTK activation pattern in these cells. P-S6 activation was found in 69% of conventional

Fig. 1. siRNA screen and compound screen identify PLK1, AURKA and CHK1 as potentially important kinases for survival of chondrosarcoma cells. A. Set-up of siRNA screen. Primary screening was performed on 779 SMARTpool siRNAs targeting kinases and kinase related genes. The secondary screen was performed in J012 and CH2879 cells and consisted of 35 SMARTpool siRNAs identified in the primary screen (decreased cell proliferation below 20% compared to mock conditions). Deconvolution consisted of 4 separate siRNAs and the SMARTpool targeting 9 different genes. B. Hoechst area as a percentage to mock for J012 cells. Each dot represents one SMARTpool targeting one kinase or kinase related gene. Duplicates are shown for each gene and only when both screens showed a percentage below 20% it was considered as a hit. C. Kinases that showed cell killing in both J012 and CH2879 were selected for deconvolution (AURKA, CHK1, CNKSR1, COPB2, EPHA6, IRAK3, STK39, TRAT1, PLK1). D. Deconvolution results in J012 and CH2879 cells showing that AURKA, CHK1, COPB2, CNKSR1 and PLK1 are important for cell survival in both cell lines. E. Compound screen results in J012, CH2879 and SW1353 showing 35 hits in common in the top 50 compounds in each cell line. In addition, 8 compounds were found in J012 and CH2879, 6 in J012 and SW1353 and 2 in CH2879 and SW1353. F. Compounds that were identified in all three or two out of three cell lines were selected and showed that Aurora kinase, PI3K-mTOR, mTOR, PLK, CDK and multi-target comprised the largest groups. In addition, compounds targeting c-MET, ALK, SRC, SYK, JAK, IKK and CHK were identified.
chondrosarcoma and 44% of dedifferentiated chondrosarcoma indicating that the downstream PI3K/mTOR pathway might be an important therapeutic target [6]. Furthermore, we previously showed a role for mTORC1 and C2 as an important regulator of chondrosarcoma metabolism [7].

To further unravel the role of kinases in chondrosarcoma we chose a screening-based approach using siRNAs targeting 779 different kinases and kinase related genes. In addition a compound screen was performed consisting of 273 compounds targeting kinases implicated in survival pathways often deregulated in cancer. By comparing the hits we aim at identifying kinase regulated pathways that are important for chondrosarcoma survival.

2. Material and methods

2.1. Cell culture

Conventional chondrosarcoma cell lines SW1353 (ATCC), JJ012 [8], CH2879 [9], CH3573 [10] and L835, and dedifferentiated chondrosarcoma cell lines L3252B, L2975 [11] and NDCS1 [12] were cultured in RPMI-1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 10 or 20% Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Mesenchymal chondrosarcoma cell line MCS170 [13] was cultured in IMDM medium (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 15% fetal Calf Serum. All cell lines were cultured at 37 °C in a humidified incubator (5% CO₂). Identity of cell lines was confirmed using the Cell ID Gene Print 10 system (Promega Benelux BV, Leiden, The Netherlands) before and after completion of the experiments. Mycoplasma tests were performed on a regular basis.

2.2. Compounds

MK-5108 (S2770), LY2603618 (S2626), Volasertib (S2235) and ABT-737 (S1002) (positive control apoptosis assay) were purchased from Selleckchem and dissolved in DMSO to a working stock of 10 mM according to the manufacturer's instructions. Z-VAD-FMK was obtained from BD biosciences (550377). Doxorubicin and Cisplatin were obtained according to the manufacturer's instructions. Z-VAD-FMK was obtained from BD biosciences (550377). Doxorubicin and Cisplatin were obtained in a solution of 0.9% NaCl from the inhouse pharmacy of the Leiden University Medical Centre.

2.3. siRNA screen

To identify critical genes for chondrosarcoma cell survival, a focused targeted siRNA screen was performed on the JJ012 central chondrosarcoma cell line targeting kinases and kinase related genes (Dharmacon, GE life sciences, Landsmeer, the Netherlands, G-003505). Hits were selected for further validation, when both duplicates showed a reduction in cell numbers of 80% or more. A secondary screen including 35 most promising hits was performed in JJ012 and CH2879 chondrosarcoma cell lines (Fig. 1A). Reverse transfection was performed using SMARTpools of 4 different siRNAs targeting the same gene in a final concentration of 50 nM DharmaFECT 3 (Thermo Fisher Scientific Inc. Waltham, MA USA, T-2003) was used as a transfection reagent according to the manufacturer's instructions. Deconvolution confirmation screens, including 9 hits identified in both cell lines were performed on JJ012 and CH2879 cells where each of the four individual siRNAs was transfected separately. A gene was considered as a hit when three out of four individual siRNAs mimicked the SMARTpool in both cell lines or when one cell line showed at least three out of four the other at least two out of four siRNAs mimicking the SMARTpool. Mock (no siRNA), GFP, and GAPDH siRNAs were used as a negative control and KIF11 siRNA as a positive control. Transfection was performed using 7000 cells/well for JJ012 and 10,000 cells/well for CH2879 cells in μ-clear 96 well black clear bottom plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands). 24 h after transfection the medium was replaced with medium containing either 1 μM doxorubicin, 5 μM cisplatin or PBS and after five days cells were fixed with formalin and stained with Hoechst. Imaging was performed using a BD-pathway microscope. To quantify the amount of nuclei the total Hoechst area was determined using Image Pro analyzer software and normalized to mock treated cells as described previously [14].

2.4. Compound screen

A compound screen was performed in JJ012, SW1353 and CH2879 cells using a kinase library from Selleckchem (2014, L1200) containing 273 compounds targeting different pathways. SW1353 and JJ012 were plated at an optimal density of 5000 cells/well and CH2879 cells were plated at a density of 7000 cells/well. The screen was performed in duplicate in μ-clear 96 well black clear bottom plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands). After overnight attachment of the cells, compounds were added in a concentration of 1 μM as single treatment or in combination with 0.05 μM doxorubicin or 0.8 μM cisplatin. A high concentration of doxorubicin (5 μM) was used as a positive control. After 72 h of incubation cell viability was assessed using Presto Blue viability reagent (see next paragraph).

2.5. Viability assay

Optimal cell amounts for each cell line were seeded in triplicate in 96-well plates. After 24 h, increasing concentrations from 0 to 1000 nM of MK-5108 and Volasertib or 0–1250 nM LY2603618 were added to the appropriate wells and cells were incubated for an additional 72 h. After the incubation period, a Presto Blue assay (Thermo Fisher Scientific Inc. Waltham, MA USA, A13262) was carried out according to the manufacturer's instructions. After 1 h, viability results were measured by fluorescence at 590 nm on a fluorometer (Victor3V, 1420 multilabel counter, Perkin-Elmer, Groningen, the Netherlands). All experiments were performed in triplicate at least 3 times.

2.6. RNA isolation and quantitative real time PCR

RNA was isolated from fresh frozen tissue of 34 conventional chondrosarcoma primary tumor tissues and six cartilage control tissues: three growth plates, and three articular cartilage tissues (Supplementary Table 1). RNA was isolated using TRIzol (Invitrogen, Carlsbad CA) followed by RNA clean up using the RNasey mini kit (Qiagen) according to the manufacturer's instructions. Samples were handled as approved by the LUMC ethical board (B17.021). AURKA, CHK1 and PLK1 expression was normalized towards housekeeping genes PPIA and CPSF6 as previously described [15]. Primer pairs are described in supplementary Table 2.

2.7. Cell cycle analysis

Optimal cell amounts for each cell line were seeded in 6-well plates and allowed to attach overnight. Cells were treated with ICG50 concentrations of LY2603618, MK-5108 or Volasertib. After 24 h, cells were stained with Solution 18 Ao-DAPI (Catalog no. 910-3018, Chemometec, Denmark) according to the manufacturer's instructions and cells were counted using an automated cell analyzer (NucleoCounter NC-250, Chemometec, Denmark). Remaining cells were centrifuged for 5 min at 500 g at 4 °C. Supernatant was removed and the cells were washed with PBS (B. BraunMelsungen AG, Melsungen, Germany). Methanol fixation was carried out, after which cells were washed with PBS/Tw 0.05%. The samples were centrifuged using the same settings and were then washed with PBA/Tw 0.05% (PBS/1.0% BSA/Tw 0.05%). After the final centrifugation of the cells, cells were stained with10 μM DAPI in PBA/Tw 0.05%. The cells were stored at 4 °C and analysis was carried out next day using the NC-250 nucleoCounter. Results were analyzed using, Winlist 3D and ModFit LT.
software (Verity software house). Five biological replicates were included for each sample.

2.8. Apoptosis assay

The caspase glo 3/7 assay (Promega, Madison, WI, USA) was used to detect apoptosis, according to the manufacturer's instructions. Cells were plated in a white 96-well plate and treated the next day with IC50 concentrations (obtained from dose response curves) of MK-S108, LY2603618, Volasertib for 24 h. ABT-737 and Doxorubicin were added to the cells as a positive control. Z-vad-FMK, a caspase inhibitor was used as a positive control. After treatment period, caspase glo 3/7 assay was added to cells, which were incubated for an additional 30 min at room temperature. Caspase activity was measured by luminescence using a luminometer (Victor3V, 1420 multilabel counter, Perkin-Elmer) according to the manufacturer's instructions. Experiments were performed three times in duplicate.

2.9. Western blotting

Protein expression of Chk1 (Cell signaling technology #2360) and p-Chk1(S345) (Cell signaling technology, #2348) was determined in JJ012, SW1353 and CH2879 in control conditions and after treatment for 2 or 24 h with IC50 concentrations of LY2603618 (JJ012 1 µM, SW1353 441 nM, CH2879 449 nM). In addition PARP cleavage (Cell signaling technology #9532) was assessed after treatment of JJ012, SW1353 and CH2879 for 2 or 24 h with IC50 concentrations of MK-S108, LY2603618 or Volasertib (MK-S108, SW1353: 1 µM, JJ012: 513 nM, CH2879: 847 nM, Volasertib: SW1353: 34 nM, JJ012 11 nM, CH2879: 24 nM, LY2603618 as described above). Lysates were obtained of cells grown until 70% confluence using hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor (Roche #11697498001) and phosphSTOP (Roche #04906837001) as previously described [4]. Expression of gapdh (Cell signaling technology #5174) was determined as a loading control. A total of 10 µg was loaded on the gel for each sample and blocking was performed using 5% milk. Primary antibodies were diluted in 5% BSA (bovine serum albumin) and incubated overnight. Blotting was performed on PVDF membranes and detection was done using enhanced chemo-luminescence (west Pico Plus chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA) followed by visualization using the ChemiDoc imaging system of BioRad.

2.10. Statistical analysis

Dose response curves and IC50 values were determined using Prism 7 GraphPad software. Statistically significant differences were assessed by performing a 2-way ANOVA test, correcting for multiple comparisons using Turkey's test. To assess synergy access over Bliss was calculated and percentages above 12 were considered as synergistic [16,17]. Overall survival was determined using SPSS software by performing a Kaplan Meyer analysis and assessing significance using a Mantel Cox Log Rank test.

3. Results

3.1. siRNA screen identifies PLK1, AURKA, COPB2, CHEK1, and CNKSR1 as most important survival genes in chondrosarcoma cells

A siRNA screen targeting 779 kinases and kinase related genes identified 35 genes that decreased survival of JJ012 chondrosarcoma cells more than 80% upon inhibition in both duplicate measurements (see Fig. 1A and B and supplementary Table 3). No siRNAs were identified that could sensitize JJ012 cells to doxorubicin or cisplatin. Transfection with control GFP siRNAs led to a slight reduction in cell amounts compared to mock conditions in most plates, however silencing of GAPDH led to a large reduction in cell amounts, indicating that this cannot be used as a control for CS siRNA screens (supplementary figure 1). Knock down of KIF11 led to a strong reduction in cell amounts, indicating successful knock down in all plates except plate 7 which was treated with doxorubicin or cisplatin. This did not influence the hit selection process. A second validation screen was performed in JJ012 and CH2879 cell lines including the 35 most promising siRNAs to select genes important for both cell lines. This reduced the number of candidates to eight that were found in both JJ012 and CH2879 cells (Fig. 1C). The remaining 19 targets, from the first JJ012 screen, could not be reproduced/identified in JJ012 and were absent as well in CH2879 cells. The eight hits identified in both JJ012 and CH2879 were selected for deconvolution and PLK1, AURKA, CHK1, COPB2 and CNKSR1 were confirmed (Fig. 1D).

3.2. Compound screening identifies cell cycle regulators as most promising targets in chondrosarcoma cell lines

In parallel with the siRNA screen a compound screening including 273 compounds targeting kinases and kinase related pathways was performed in three different chondrosarcoma cell lines. The top 50 compounds, that showed the highest reduction in cell numbers as compared to DMSO treated controls, of each cell line were compared and 34 compounds were found to be effective in all three cell lines (Fig. 1E), while in total 48 compounds were shared between at least 2 cell lines. The percentage of compounds targeting a specific pathway as compared to the total of 48 hit compounds are represented in Fig. 1F. Inhibitors of cell cycle regulators Aurora kinases (13/16), Polo like kinases (5/5) and Cyclin dependent kinases (4/12) represent a substantial portion (Fig. 1F), confirming the findings of the siRNA screen. Also, inhibitors of the PI3K and mTOR pathway (12/25) are one of the major hits. Furthermore, inhibitors of cMET (2/13), ALK (1/2), SRC (1/3), SYK (1/5), JAK (1/3), IKK (1/2) and CHK (1/3) are also represented (see supplementary Table 4 for a list of selected compounds). Similar to the siRNA screen, no pathways were identified that upon inhibition clearly sensitized for either doxorubicin or cisplatin (supplementary Table 5).

Based on the overlap between the siRNA and the kinase inhibitor screen we chose to continue with PLK1, AURKA and CHK1 as most promising targets. In addition, previous studies already showed that mTOR, CDK and Src are important therapeutic targets for patients or subsets of patients with chondrosarcoma [4,18,19].

3.3. Inhibition of AURKA, CHK1 and PLK1 in chondrosarcoma cells results in a dose dependent decrease in viability, which is not related to RNA expression levels

Dose dependent decreases in viability were observed when different chondrosarcoma cell lines were treated with inhibitors for AURKA (MK-S108), CHK1 (LY2603618) or PLK1 (Volasertib) (Fig. 2A). Lowest IC50 values were obtained when cell lines were treated with PLK1 inhibitor volasertib, however treatment with CHK1 inhibitor LY2603618 also led to low IC50 values (see Table 1). These values have previously been shown as clinically achievable in human plasma in phase I and phase II trials [20–22]. L835, L3252 and MCS170, which are slower growing TP53 wildtype cell lines showed higher IC50 values for all compounds compared to the other faster growing cell lines. RNA expression analysis showed a variable expression pattern of AURKA, CHK1 and PLK1 across the cell lines, with highest expression of all three in CH2879 (Fig. 2B). Only few cell lines responded to inhibition of AURKA, which was not correlated to the level of AURKA expression. Likewise, no correlation was observed between sensitivity to CHK1 inhibition and CHK1 expression levels. Interestingly, of the three lines showing low PLK1 expression, L835 and MCS-170 showed a poor response to PLK1 inhibition, whereas L2975 exhibited a response, which although PLK1 expression was lower, still resembled the response patterns of high PLK1 expressors, but never reached 0% cell viability. Combination
treatment with doxorubicin or cisplatin and MK-5108 and LY2603618 was performed in three conventional chondrosarcoma cell lines and results showed a synergistic effect between doxorubicin and cisplatin with the CHK1 inhibitor LY2603618 in JJ012 and SW1353 cell lines as shown by more than 12% increase in Excess over Bliss score (Fig. 2C). This shows that chondrosarcoma cells are more sensitive towards treatment with chemotherapy when CHK1 is inhibited. Increased phosphorylation of CHK1 on Ser345 was observed after treatment with LY2603618 in JJ012, SW1353 and CH2879, indicating that inhibiting CHK1 under these conditions leads to activation of ATM/ATR (Fig. 2D). No basic CHK1 phosphorylation was observed on Ser345.

3.4. Cell cycle analysis reveals a block in G2 after AURKA inhibition

Cell cycle analysis was carried out after 24 h of treatment with MK-5108, LY2603618 or Volasertib and showed that inhibition of AURKA with MK-5108 caused a block in G2/M in both JJ012 (p < 0.0001) and CH2879 (p < 0.0001) cell lines (Fig. 3A, Supplementary figure 2).
Treatment with CHK1 inhibitor LY2603618 did result in an increase in S-phase (p = 0.0003) and debris (p = 0.0004) in JJ012, but not in CH2879. Thus, while inhibiting AURKA clearly showed a G2/M arrest in both cell lines, only JJ012 showed a clear S-phase arrest after CHK1 inhibition. To assess whether this cell cycle arrest led to apoptotic cell death, caspase 3/7 dependent apoptosis was evaluated after 24 h of treatment with MK-5108, LY2603618 or Volasertib (Fig. 3B). Results were variable, but small increases were observed after treatment with MK-5108 or Volasertib in SW1353. PARP cleavage was assessed to determine general cell death and showed a small amount of PARP cleavage, especially in MK-5108 treated JJ012 and CH2879 cells, but no clear differences were observed (Fig. 3C). These results indicate that even though the cell cycle inhibitors successfully caused cell cycle arrest in both cell lines, this did not directly lead to an increase in cell death after 24 h.

3.5. High CHK1 RNA expression in chondrosarcoma tissue samples is correlated with a worse overall survival

Expression of AURKA, CHK1 and PLK1 was investigated in a panel of chondrosarcoma tissue samples and revealed that both AURKA and CHK1 showed higher RNA expression compared to cartilage in a subset of chondrosarcoma patients (Fig. 4A). PLK1 expression was lower compared to normal cartilage in all samples tested, but high in chondrosarcoma cell lines. Most samples that showed higher AURKA or CHK1 expression compared to normal cartilage were in the high-grade group, but this was not significantly different. Samples were divided into high and low CHK1 expression groups. The Kaplan-Meier survival analysis showed a significant decrease in overall survival for patients with high CHK1 expression compared to low CHK1 expression (p = 0.0001). This result suggests that high CHK1 expression in chondrosarcoma tissue samples is correlated with a worse overall survival.

**Table 1**

|                      | MK-5108         | LY2603618       | Volasertib      |
|----------------------|-----------------|-----------------|-----------------|
| JJ012                | >1000           | >1000           | >1000           |
| SW1353               | >1000           | 441.2 (386.6–502.8) | >1000           |
| CH2879               | >1000           | 847.6 (723.3–109)  | 449.3 (401–404.6)  |
| CH3573               | >1000           | 815.2 (730.2–918.4) | 8.3829 (7.083–9.809) |
| L835                 | >1000           | >1000           | >1000           |
| L2975                | 520.8 (440.5–631) | 826.1 (781.3–875.8) | 11.33 (9.109–13.92) |
| Ndsi                 | >1000           | 442.6 (420.5–465.8) | 3.166 (2.544–4.003) |
| L3252                | >1000           | >1000           | >1000           |
| MCS170               | >1000           | >1000           | >1000           |

**Fig. 3.** Cell cycle and cell death analysis after AURKA, CHK1 or PLK1 inhibition. A. Cell cycle analysis after 24 h of treatment with MK-5108, LY2603618 or Volasertib in JJ012 or CH2879 cells. Both cell lines show a decrease in G1 and an increase in G2 phase after treatment with MK-5108. In addition, CH2879 cells show a decrease in S phase after treatment with MK-5108. JJ012 cells show a decrease in G1 after inhibition with either MK-5108, LY2603618 or Volasertib and an increase in S-phase and debris after LY2603618 treatment. B. Apoptosis induction measured using the caspase-glo 3/7 kit in JJ012, CH2879 and SW1353 after treatment for 24 h with IC50 concentrations of MK5108, LY2603618 and Volasertib. Z-vad was added as a control. Only the positive control showed significant caspase induction in all cell lines. SW1353 showed significant upregulated caspase activity after treatment with MK-5108 and Volasertib compared to dms0 treated controls. For both cell cycle and apoptosis experiments mean values are shown of three experiments performed in duplicate. P-values were calculated using a 2way ANOVA test, correcting for multiple comparisons using Tukeys test. C. PARP cleavage assessed after 24 h of treatment using IC50 concentrations of MK-5108, LY2603618 and Volasertib. As a positive control CH2879 cells treated with the combination of ABT-737 and doxorubicin has been taken along.
into high and low expression based on expression compared to normal cartilage (>1=high expression, <1=low expression) and survival analysis was performed (Fig. 4B). No significant difference in survival was observed between low or high expression of AURKA (p = 0.131), but high CHK1 expression was significantly correlated with a worse overall survival compared to low CHK1 expression (p = 0.018). These results show that CHK1 expression is correlated towards a poor prognosis in chondrosarcoma patients.

4. Discussion

Chondrosarcoma patients suffer from limited treatment options due to relative chemo- and radio resistance. In this study we sought to identify new targetable pathways in chondrosarcoma cells by performing a kinase focused siRNA and compound screen. By comparing the hits that were identified in both screens we identified AURKA, PLK1 and CHK1 as important survival regulators in chondrosarcoma cells. AURKA, PLK1 and CHK1 are all involved in cell cycle regulation and often deregulated in cancer [23].

A subset of chondrosarcoma cell lines showed a dose dependent decrease in viability after inhibiting AURKA using MK-5108, which was not related towards AURKA RNA expression. A clear block in G2/M phase of the cell cycle was observed after 24 h of treatment with MK-
5108. We did not find any correlation between expression of AURKA and survival, in contrast to a study from 2012 by Liang et al. in which they showed a correlation between high AURKA expression and a worse overall survival in chondrosarcoma patients [28]. A clinical study including 6 chondrosarcoma patients investigating the efficacy of Aurora Kinase A inhibition using Alisertib showed a partial response in one patient with dedifferentiated chondrosarcoma [29]. However a phase III study in lymphoma was discontinued because of lack of response compared to the other study arm [30]. Different studies testing combination strategies show different adverse effects and maximum tolerated dose, which indicates that results might vary between tumor types and patients [21,31–33]. Recently AURKA inhibitors have been identified as synthetic lethal with defective RB1 [34]. In chondrosarcoma 33% of tumors show a defect in the RB1 pathway [35], meaning that in these tumors treatment with AURKA inhibitors might be good treatment option.

Chondrosarcoma cell lines were sensitive for inhibition of PLK1 using Volasertib, but no clear cell cycle effects were observed after 24 h of treatment. This is surprising because PLK1, just like AURKA, is important for entry in M-phase, although multiple other non-cell cycle related functions have been proposed as well, for example PLK1 can regulate mTORC1 activity [36,37]. This can influence the effects that we detect on the cell cycle. Expression of PLK1 in chondrosarcoma tissue samples was low, indicating that targeting PLK1 might not be a good therapeutic strategy for chondrosarcoma patients.

CHK1 is activated by ATM or ATR after the occurrence of DNA damage. Its activation will cause the cell to halt cell proliferation in S or G2 and allows the cell to repair DNA damage. When CHK1 is inhibited DNA damage will accumulate and the cell will die during mitosis. Like AURKA and PLK1, CHK1 is also overexpressed in different types of cancer. Like AURKA and PLK1, CHK1 is also overexpressed in different types of cancer. Using LY2603618 CHK1 was inhibited in a panel of chondrosarcoma cell lines, which resulted in an increased phosphorylation of CHK1 at position S345, which is in agreement with previous reports [38]. Possibly single CHK1 inhibition already leads to activation of the DNA damage response, and IC50 concentrations are not high enough to completely prevent phosphorylation by ATM/ATAK. A subset of chondrosarcoma cell lines was responding to the treatment, and an increase in S phase was observed in JJ012 cells, but not in CH2879 cells treated with LY2603618. In addition, JJ012 and SW1353 could be sensitized for chemotheraphy using CHK1 inhibition. Previous studies already showed the importance of CHK1 in Ewing, Osteo- and soft tissue sarcomas [39–41] and its use in single and combination treatment in pre-clinical models. In addition, we also found a correlation between overall survival and CHK1 RNA expression in chondrosarcoma patient tissues, indicating that more aggressive chondrosarcomas show higher expression of CHK1. Second generation CHK1 inhibitors, showing less toxicity compared to first generation inhibitors are currently tested in the clinic in combination with chemotherapy in advanced cancers [42–44].

Cells with defective P53 protein function have been shown to be more sensitive to inhibitors for CHK1 [45] as well as AURKA and PLK1 compared to cells with intact P53 [46,47]. Inactivation of P53 will lead to a compromised G1 checkpoint, which makes mutated cells more dependent on the G2 checkpoint to be able to repair DNA damage. In our study we do observe a difference in sensitivity between TP53 wildtype and TP53 mutant chondrosarcoma cell lines; cell lines with intact P53 are less sensitive to inhibition of AURKA, CHK1 or PLK1. However, these cells also grow slower compared to the other cell lines, which could confound the observed difference in response. Mutations in TP53 have been observed in 20% of chondrosarcomas [35], indicating that inhibitors of cell cycle regulators might be of interest especially for these patients.

In conclusion, we performed a kinase focused siRNA and compound screen and identified cell cycle regulators AURKA, CHK1 and PLK1 as interesting targetable proteins for follow up studies. RNA expression analysis revealed expression of AURKA and CHK1 in a subset of chondrosarcoma patients, while PLK1 expression was minimal, compared to normal cartilage tissue. In addition high CHK1 expression was correlated towards a decrease in survival time. Also inhibition of CHK1 could sensitize a subset of chondrosarcoma cells towards chemotherapy. Future studies should determine the role of cell cycle proteins in chondrosarcoma, however based on our results CHK1 seems to be a promising therapeutic candidate for patients with (TP53 mutated) chondrosarcoma, especially in combination strategies.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Supplementary materials

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