Targeting CHK1 inhibits cell proliferation in FLT3-ITD positive acute myeloid leukemia

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CHK1 Ser/Thr kinase, a well characterized regulator of DNA damage response, is also involved in normal cell cycle progression. In this study, we investigate how CHK1 participates to proliferation of acute myeloid leukemia cells expressing the mutated FLT3-ITD tyrosine kinase receptor. Pharmacological inhibition of CHK1 as well as its shRNA mediated down regulation reduced the proliferation rate of FLT-ITD expressing leukemic cell lines in a cytostatic manner. Flow cytometry analysis revealed no accumulation in a specific phase of the cell cycle upon CHK1 inhibition. Accordingly, lentiviral-mediated CHK1 overexpression increased the proliferation rate of FLT-ITD expressing cells, as judged by cell viability and [3H] thymidine incorporation experiments. By contrast, expression of a ser280 mutant did not, suggesting that phosphorylation of this residue is an important determinant of CHK1 proliferative function.

Clonogenic growth of primary leukemic cells from patients in semi-solid medium was reduced upon CHK1 inhibition, confirming the data obtained with leukemic established cell lines. Surprisingly, 3 out of 4 CHK1 inhibitory compounds tested in this study were also potent inhibitors of the FLT3-ITD tyrosine kinase receptor. Altogether, these data identify CHK1 as a regulator of FLT3-ITD-positive leukemic cells proliferation, and they open interesting perspectives in terms of new therapeutic strategies for these pathologies.

1. Introduction

CHK1 Ser/Thr kinase is a key regulator of cell cycle progression, through its participation in cellular responses to DNA damage or other types of stress. P53, CDC25C, CDC25A, or Wee1 phosphorylation by CHK1 usually leads to Cyclin Dependent Kinases (CDK) inhibition and subsequent cell cycle arrest [1]. In addition to its role in response to DNA damage, CHK1 also displays important functions in unperturbed cell cycle progression, notably during DNA replication or mitosis completion [2–6].

Regarding its essential and multiple functions, CHK1 must be finely regulated to ensure its adequate activity. The major mechanism of CHK1 regulation consists in its phosphorylation by ATR on Ser 345 and 317 residues, which leads to catalytic activation and is currently considered as a hallmark of CHK1 activity level. In addition, autophosphorylation on Serine 296 also participates to the activation process [7], and phosphorylations catalyzed by CDK1 and CDK2 on Serine 286 and Serine 301 were reported to influence CHK1 functions at the G2/M transition and in response to DNA damage [8,9]. Although its subcellular localization has not been extensively questioned, interesting studies in a PTEN−/− model pointed to the phosphorylation of CHK1 on Ser 280 by the Akt/PI3K kinase [10,11], leading to retention of the protein in the cytoplasmic compartment of these cells. The occurrence of this process in other biological models has been recently questioned [12,13].

Acute myeloid leukemia (AML) is a group of myeloid malignancies characterized by a differentiation blockade occurring at different stages of the hematopoietic process, and by excessive proliferation of leukemic cells. Mutations of the FLT3 tyrosine kinase receptor are found in 25–30% of patients, the FLT3-ITD (Internal Tandem Duplication) being the most commonly detected [14].

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FLT3-ITD expression participates to increased proliferation rates and cell death resistance, due to constitutive activation of the receptor and of various downstream signaling pathways, including the PI3K/Akt signaling module and the STAT5 transcription factor [15–19]. Pim1 Ser/Thr kinase is one of the important transcriptional targets of STAT5 in these leukemic cells, and the role of Pim1 and Pim2 in the oncogenic process mediated by FLT3 has been documented [20,21]. The importance and the status of CHK1 in AML have been poorly investigated up to now [22,23]. We recently described constitutive activation of this kinase in these pathologies, especially in patients with complex karyotype and poor prognosis [24,25], and we proposed that CHK1 inhibition may sensitize leukemic cells to genotoxic treatments [26]. More recently, we described that Pim1/2 phosphorylate CHK1 on Serine 280 in leukemic cells, and that this event facilitates CHK1-dependent cell cycle arrest and cell death resistance in response to genotoxic agents [13].

We show here that CHK1 also governs the normal proliferation of FLT3-ITD leukemic cells, independently of its role in checkpoint establishment, and that Serine 280 phosphorylation is important for this function. Consequences in terms of therapeutic targeting in these pathologies are discussed.

2. Materials and methods

2.1. Pharmacological inhibitors and antibodies

The FLT-3 inhibitor III was purchased from Calbiochem (San Diego, CA, USA). CHK1 inhibitor SCH-900776 was from Active Biochem (Maplewood, NJ, USA), AZD7762 was a kind gift from AstraZeneca to CD, UCN-01 was provided by the National Cancer Institute, and CHIR-124 was purchased from Selleckbio (Selleck Chemicals, USA).

The antibodies used were: monoclonal anti-CHK1 (G4), polyclonal anti-Flt3/Flt2 (C-20) (Santa Cruz, CA, USA), polyclonal anti-phospho-Ser280-CHK1 from Abgent (San Diego, CA, USA), polyclonal anti-phospho-Tyr591-Flt3 and anti-STAT5 from Cell Signaling Technology (Beverly, MA, USA), monoclonal anti-phospho-STAT5 (Tyr694/699) from Millipore (Billerica, MA, USA), and monoclonal anti-tubulin from Sigma (Saint Louis, MO, USA).

2.2. Cell lines and culture conditions

Human myeloid leukemia cell lines MV4–11 and K562 (DSMZ, Braunschweig, Germany) MOLM-14 (kindly provided by Martin Carroll, University of Pennsylvania, Philadelphia, PA, USA) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Murine FLT3-ITD-expressing Ba/F3 cells were cultured in MEMα medium supplemented with 10% FBS and 2 mM l-glutamine. All cells were grown in the presence of 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37 °C and 5% CO₂.

2.3. Patient samples

Leukemic blasts from patients with diagnosed AML have been obtained after informed consent and stored at the HIMIP collection. According to the French law, HIMIP collection has been declared to the Ministry of Higher Education and Research (DC 2008-307 collection 1) and obtained a transfer agreement (AC 2008-129) after approbation by the “Comité de Protection des Personnes Sud-Ouest et Outremer II” (ethical committee). Clinical and biological annotations of the samples have been declared to the CNIL (Comité National Informatique et Libertés i.e., Data processing and Liberties National Committee). Frozen cells were thawed in IMDM medium with 20% FBS and immediately processed for clonogenic assays (see “Section 2.4”).

2.4. Clonogenic assays

Primary cells from patients were thawed and adjusted to 1 × 10⁵ cells/ml final concentration and grown in H4230 Stem Cell Technologies methyl cellulose medium (Stem Cell Technologies, Vancouver, BC) supplemented with 10% SDF7-CM as a stimulant [27], and increasing concentrations of SCH900776 (0, 250 and 500 nM). The cells were then plated in 35-mm Petri dishes in duplicate and allowed to incubate for 7 days in a humidified CO₂ incubator (5% CO₂, 37 °C). At day 7, the leukemic colonies (more than 20 cells) and clusters (more than 5 cells) were scored under an inverted microscope.

2.5. Proliferation assay

Measurements were made according to manufacturer’s instructions (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). Briefly, 20 μl of MTS reagent was added directly to the wells with 100 μl cell suspension was incubated at 37 °C for 3 h. Absorbance was measured at 490 nm (Mithras LB 940, Berthold technologies). Background absorbance was first subtracted using a set of wells containing medium only, then the OD value was shown.

2.6. Lentiviral infections

We generated Chk1 Ser 280 mutated to Ala by using site directed mutagenesis with forward primer 5’ CCCCCAGTCACCTGAGGTGGTGTGTC 3’ and reverse primer 5’ GACACACCACTGCAGTGAGTGGG 3’. To generate lentiviral vectors expressing CHK1 proteins, WT or mutated S280A HA-CHK1 sequences were cloned into the pTRip-TAL-Ires-GFP lentiviral vector. We used 293T packaging cells, co-transfected with lentiviral protein (GAG, POL, and ENV) encoding plasmids, and plasmids containing GFP (as a control), CHK1 or CHK1 S280A genes, separately. Supernatants containing lentivirus were collected 48 h after transfection, then during 3 consecutive days, and stored at −80 °C. Same approach was used for shRNA particles production (TRCN0000039855NM_001274.2-13785s1c1TRC1, Sigma). Murine FLT3-ITD-expressing BA/F3 cells were plated at 5 × 10⁵ cells in 200 μl in serum-free medium and 5 μl of lentiviral supernatant was added during 3 h. Cells were then grown in 10% FBS medium. The percentage of infected cells was then determined by flow cytometry GFP detection, allowing subsequent GFP-positive cell sorting.

2.7. [3H]Thymidine proliferation assay

5 × 10⁴ FL3-ITD-expressing BA/F3 cells were incubated 48 h in 100 μl of 5% FCS supplemented MEM, and then pulsed for 6 h with [3H]thymidine (1 μCi, [37 kBq]). The amount of radioactivity incorporated to neo-synthetized DNA was then determined after trichloracetic acid precipitation.

2.8. Cell viability assay

2.5 × 10⁴ FL3-ITD-expressing BA/F3 cells were plated in 100 μl of 5% FCS supplemented MEM during 48 h. The UptiBlueTM viable cell counting reagent (Interchim, Montlucon, France) was then added during 3 h according to manufacturer’s instructions, and the fluorescence was measured using a typhoon 8600 scanner.

2.9. Western blot analysis

Cells (2 × 10⁶) were usually in 100 μl of Laemmli sample buffer, sonicated for 7 s, and boiled for 3 min. Proteins were then resolved on 4–12% gradient SDS-PAGE and transferred to nitrocellulose membrane. Saturation of the membrane was done for
1 h in TBS with Tween 0.1% (TBS-T) containing 5% nonfat milk or 5% bovine serum albumin (BSA). Membranes were blotted with proper antibodies overnight at 4 °C or for 1 h at room temperature, washed thrice with TBS-T, and incubated for 30 min with HRP-conjugated secondary antibody (Promega). After three additional washes, detection was achieved with Supersignal Westpico chemiluminescent substrate (Perkin Elmer, Shelton, CT).

2.10. Apoptosis and cell cycle analysis

Cells were harvested and fixed in ice-cold 70% ethanol, permeabilized with PBS-0.25% Triton X-100, and stained with propidium iodide. Data were collected on a BD FACS Calibur cytometer and analyzed using the Cell Quest (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Treestar, Ashland, USA). Apoptotic cells were detected with Annexin V-FITC detection kit from BD PharMingen (San Diego, CA, USA) according to the manufacturer instructions.

3. Results

3.1. CHK1 inhibition reduces proliferation of FLT3-ITD cells

We used MV4-11 as a model of human AML cell line expressing the FLT3-ITD receptor. When exponentially growing MV4-11 cells were treated with either of the 4 different CHK1 inhibitors, we observed a dose- and time-dependent inhibition of cell proliferation (Fig. 1). As a comparison, the effect of FLT3 inhibition on MV4-11 cells proliferation is shown in Fig. 1A. By contrast, the FLT3-ITD negative and BCR-ABL positive leukemic cell line K562 was insensitive to the CHK1 inhibitors SCH900776 and AZD7762, as well as to the FLT3 inhibitor (Supplementary Fig. 1). These data suggest that CHK1 participates to normal proliferation of leukemic cells expressing FLT3-ITD.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.08.020.

To control the efficacy of CHK1 inhibitors, we followed phosphorylation of Ser 345, a hallmark of CHK1 inhibition. Indeed, phosphorylation of this residue is currently used as a marker of ATR-dependent activation, but it was also reported that pharmacological inhibition of CHK1 leads to Ser345 phosphorylation, due to regulatory loop involving PP2A phosphatase inhibition or DNA damage induction [28]. As observed in Supplementary Fig. 2, SCH900776, and to a lesser extent UCN-01, CHIR-124, and AZD7762 induced CHK1 Ser 345 phosphorylation at concentrations inhibiting cell proliferation, confirming their efficacy in this model.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.08.020.

In order to confirm these data, we performed shRNA-mediated down-regulation of CHK1 in MV4-11 and MOLM-14 cells. As shown in Fig. 2, this significantly reduced cell proliferation in both cell lines, confirming the data obtained with pharmacological inhibitors. Altogether these data demonstrate that CHK1 is an important actor of cell proliferation in FLT3-ITD leukemic cells.

We then questioned the specificity of these inhibitors by determining their impact on FLT3-ITD-dependent signaling pathways. Surprisingly, we observed that AZD7762, UCN-01 and CHIR-124 triggered FLT3-ITD tyr 591 dephosphorylation, as well as a drop in STAT5 activation levels (Fig. 3B–D). These data suggest either direct inhibition of FLT3-ITD by these drugs, or the existence of a signaling feedback loop between CHK1 and FLT3. However, the fact that SCH900776 efficiently inhibited CHK1 without any impact on
Fig. 2. CHK1 knock-down impairs leukemic cell proliferation. MTS proliferation assay of MV4-11 (A) and Molm14 (B) leukemic cells transduced with shRNA control or against CHK1. CHK1 protein levels were determined by Western blot (lower panels). Briefly, 48 h after CHK1 knock-down with shRNA, leukemic cells were incubated for additional 24 h on growing conditions. Data shown are representative of 2 independent experiments.

FLT3-ITD or STAT5 activity (Fig. 3A) strongly argues for the first hypothesis. Altogether these data indicate that AZD7762, UCN-01 and CHIR-124 are also potent inhibitors of the FLT3-ITD tyrosine kinase receptor. In the next experiments, in order to specifically question CHK1 functions, we essentially used SCH900776 at a concentration of 500 nM, which does not affect FLT3 activity but completely blocks MV4-11 cells proliferation (Fig. 3).

As a control, similar experiments were performed in a murine Ba/F3 cell line expressing human FLT3-ITD. SCH900776 reduced proliferation of these cells (Supplementary Fig. 3) at concentrations

Fig. 3. Specificity of Chk1 inhibitors in MV4-11 leukemic cells. Exponentially growing MV4-11 cells were treated for 2 h with Chk1 inhibitors at different dose as described in Fig. 1, then p-Flt3 and Flt3, p-Stat5 and Stat5, were detected by western blot. Tubulin was used as loading control. (A) Cells were treated with SCH900776 (250 nM and 500 nM); (B) cells were treated with Flt3 inhibitor III (50 nM) or AZD7765 (0.5 nM); (C) cells were treated with UCN01 (50 nM, 100 nM and 300 nM); (D) cells were treated with CHIR124 (50 nM, 100 nM and 300 nM). Data are representative of three independent experiments.
inhibiting CHK1 activity (Supplementary Fig. 4) without modifying FLT3-ITD/STAT5 signaling pathway (Supplementary Fig. 5).

Supplementary Figs. S3–S5 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.08.020.

3.2. Impact of CHK1 inhibitors on cell cycle progression and cell death in leukemic cell lines

We then investigated how CHK1 inhibition may affect FLT3-ITD-positive cells proliferation. First, Annexin V labeling analysis indicated that SCH900776 did not significantly trigger apoptotic cell death in FLT3-ITD positive cells (data not shown) at a concentration inhibiting cell proliferation. To further precise the mechanisms of this inhibition, we then investigated the impact of CHK1 inhibition on cell cycle progression. As shown in Fig. 4, treatment with SCH900776 did not dramatically modify the cell cycle profile of MV4-11 cells, with a moderate accumulation in G1 and a corresponding decrease of the G2/M phase. Since proliferation was completely blocked at this concentration (see Fig. 1), this suggests that cell proliferation arrest occurred at different phases. As a control, the FLT3 inhibitor induced a clear G1 accumulation and an S phase depletion of MV4-11 cells (Fig. 4C), as did AZD7762 (Fig. 4D), in good accordance with its effect on FLT3-ITD activity.

Altogether, these data argue for the occurrence of a cytostatic effect of CHK1 inhibition in FLT3-ITD AML cells.

3.3. Impact of CHK1 inhibitors on clonogenicity of primary leukemic cells from patients

We then investigated the impact of CHK1 inhibition on the clonogenic potential of 3 samples from FLT3-ITD positive AML patients. Treatment with SCH900776 dramatically decreased the clonogenic growth of leukemic progenitor cells (Fig. 5), at the same concentrations found to inhibit cell lines proliferation, further underlying CHK1 as a key regulator of FLT3-ITD-dependent proliferation. We did not detect significant cell death in the bulk of cells treated with this inhibitor (data not shown), suggesting that clonogenicity reduction occurs through proliferation inhibition, as observed for the MV4-11 cell line (Fig. 4).

3.4. CHK1 overexpression increases proliferation of FLT3-ITD cells

Since CHK1 inhibition induced leukemic cells proliferation arrest, we reasoned that overexpressing CHK1 may have an opposite positive effect on proliferation of FLT3-ITD expressing cells. To test this hypothesis, we thus expressed CHK1 through lentiviral infection in FLT3-ITD-positive Ba/F3 cells. Exogenous CHK1 was expressed at moderate level, equivalent to the endogenous protein (Supplementary Fig. 6). As shown in Fig. 6, exogenous CHK1 expression significantly improved the viability of these cells as compared to control (Fig. 6A), without modifying their basal apoptotic level (not shown). [3H]Thymidine incorporation experiments indicate
that DNA replication rate was significantly augmented (Fig. 6B) by CHK1 overexpression, suggesting that moderate increase in CHK1 protein level accelerates cell proliferation. These data appear in good agreement with the pharmacological inhibition experiments shown in Fig. 1.

Supplementary Fig. S6 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.08.020.

3.5. Ser280 phosphorylation of CHK1 is important for its implication in leukemic cells proliferation

In a recent work, we described phosphorylation of CHK1 at S280 by the Ser/Thr kinase Pim downstream of FLT3-ITD, and the impact of this phosphorylation on the DNA damage response elicited by CHK1 [13]. In consequence, we tested if Serine 280 phosphorylation is also necessary for the CHK1 proliferative

![Fig. 5. Impact of CHK1 inhibition on the clonogenic capacities of primary cells from FLT3/ITD-positive patients. Primary cells from patients were thawed as described in Section 2, and plated at a concentration of 1 x 10^5 cells/ml in duplicate, in the presence (250 nM and 500 nM) or absence of SCH900776. The number of colonies was scored at day 7. Results are expressed as percentage of control (untreated). The characteristics of these primary samples are as follows:

Patient 1: FAB4, bone marrow, intermediate risk, 92% blasts.
Patient 2: FAB4, blood, intermediate risk, 80% blasts.
Patient 3: FAB2, bone marrow, high risk, 61% blasts.

![Fig. 6. Ser 280 phosphorylation is important for CHK1-dependent proliferation of FLT3-ITD positive cells. Ba/F3 FLT3-ITD cells expressing either GFP as a control, wild type CHK1, or CHK1 S280A, were generated by lentiviral infection as described in the “Section 2”. Control, CHK1-WT and CHK1 S280A cells were plated in triplicate at 10^4 (proliferation) or 2.5 x 10^3 (viability) in 100 μl of 10% FCS supplemented medium during 48 h. Cell viability was measured by UptiBlueTM viable cell counting (A) and proliferation was determined by [3H]Thymidine incorporation (1 μCi, [37 kBq]) (B) and results are expressed in beats per minute (bpm). Vertical bars indicate standard deviations. Comparisons were operated by Student t-test (ns = not significant and *p < 0.05; **p < 0.01).]
function described here. First, exogenously expressed wild type CHK1 was phosphorylated on Ser 280, and pharmacological inhibition of FLT3 suppressed this phosphorylation (not shown). In these experiments performed in Ba/F3 FLT3-ITD expressing cells, both wild type and Serine 280 mutated CHK1 (S280A, unphosphorylated mutant) were expressed at similar levels (Supplementary Fig. 5). By contrast with wild type CHK1, expression of this mutant did not increase the proliferation rate of these cells (Fig. 6B), demonstrating that Ser 280 phosphorylation is a key determinant of CHK1-dependent proliferation downstream of FLT3-ITD.

4. Discussion

In this work, we demonstrate that Chk1 is an important mediator of leukemic cells proliferation down-stream of the oncogenic tyrosine kinase receptor FLT3-ITD, independently of its DNA damage response function. The involvement of Chk1 in normal proliferation of different cell types has been described, and various mechanisms occurring at different phases of the cell cycle and supporting this function have been proposed [2–6]. Our cell cycle analysis in response to pharmacological inhibition of CHK1 in MV4-11 cells appears in good agreement with these data, since we did not observe massive accumulation in any specific phase of the cell cycle. Intriguingly, according to these results, cell proliferation arrest also occurred in G1, suggesting the existence of CHK1 functions in this phase. Further studies are needed to establish which substrates of CHK1 regulate cell proliferation of leukemic cells.

The status of CHK1 expression and activation was poorly investigated in AML. In a previous work, we described constitutive phosphorylation of Ser 345 and Ser 317 activating residues in AML with complex karyotype, and we linked this phosphorylation state with higher sensitivity to CHK1 inhibition [25,26]. The origin of this phosphorylation remains unclear and did not correlate with constitutive DNA damage in these cells. Our present results suggest that Ser 345 and Ser 317 phosphorylation levels are not sufficient markers to estimate the activation state of CHK1 in cancer cells. Taking into account other modifications of the protein, like Ser 296 autophosphorylation or Ser 280 regulatory phosphorylation would be more relevant and probably facilitate its identification as a therapeutic target in AML and in other cancers.

We recently described Ser 280 phosphorylation of CHK1 by Pim1/2 in FLT3-ITD positive AML cells, and the importance of this event for resistance to DNA damaging agents [13]. Our present results indicate that this regulation of CHK1 by Pim is also important for its proliferative function. Pim kinases are frequently over-expressed in AML [29], independently of FLT3 mutational status, and recent studies point to the importance of Pim2 in the regulation of the mTOR pathway in these pathologies [30,31]. Pim1/2 are constitutively active kinases and their activity supports in vitro and in vivo tumor cell growth and survival through multiple substrates, including cell cycle and apoptosis regulators [32]. In light of this, many reports provide new insights into their oncogenic action, and support the development of Pim specific inhibitors as anti-cancer agents to increase the efficacy of conventional therapies. Interestingly, a recently described pharmacological Pim inhibitor was also characterized as a potent FLT3-ITD inhibitor, with a potential clinical interest linked to this double specificity characteristic [31].

This is reminiscent of our present observation that three independent CHK1 inhibitors also efficiently inhibit FLT3 receptor activity in leukemia cells. In fact, although these results sound surprising, IC50s for FLT3 inhibition compatible with these data had been measured in previous studies describing these compounds (for a review on CHK1 inhibitors see [32]). A consequence of these observations is that such molecules could be considered as possible double specificity inhibitors aimed at targeting both the FLT3 receptor activity and the Ser/Thr kinase CHK1.

CHK1 is an essential regulator of DNA damage checkpoint and of resistance to genotoxic agents, and is considered to this respect as an interesting target in many cancers in association with DNA damaging drugs. Several pharmacological inhibitors against this kinase have been developed and tested in clinical trials, including SCH 900776 in AML. Here we also identify CHK1 as an important player of the FLT3-ITD- and Pim-dependent proliferation of leukemic cells, suggesting that its inhibition or that of upstream regulators could be considered for more personalized treatment of these pathologies independently of the association with DNA damaging agents. Considering together our different studies, we propose to consider CHK1 inhibition in association with conventional chemotherapy (daunorubicin/ara-cytin) as a preferential protocol in complex karyotype and FLT3-ITD AML subgroups, and single CHK1 inhibition as an interesting alternative in this latter category.

This opens the interesting question of how the cellular signaling landscape may influence the functionality of this kinase, and specially its relative importance for unperturbed cell cycle progression. To this respect, our data obtained with the BCR-ABL positive leukemic cell line K562 are interesting, since they suggest that CHK1 function in cell proliferation may differ depending on the cell environment. Indeed, CHK1 inhibition did not significantly affect the proliferation process in this model, although the two models (MV4-11 and K562) are characterized by the expression of an oncogenic tyrosine kinase leading to the activation of similar signaling networks. This further reinforces the idea that careful and multi-parameter characterization of CHK1 will be necessary to establish its potential therapeutic target status in different oncogenic contexts.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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