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## Kinome expression profiling to target new therapeutic avenues in multiple myeloma

Hugues de Bussac,<sup>1</sup> Angélique Bruyer,<sup>1</sup> Michel Jourdan,<sup>1</sup> Anke Maes,<sup>2</sup> Nicolas Robert,<sup>3</sup> Claire Gourzones,<sup>1</sup> Laure Vincent,<sup>4</sup> Anja Seckinger,<sup>5,6</sup> Guillaume Cartron,<sup>4,7,8</sup> Dirk Hose,<sup>5,6</sup> Elke De Bruyne,<sup>2</sup> Alboukadel Kassambara,<sup>1</sup> Philippe Pasero<sup>1</sup> and Jérôme Moreaux<sup>1,3,8</sup>

<sup>1</sup>IGH, CNRS, Université de Montpellier, Montpellier, France; <sup>2</sup>Department of Hematology and Immunology, Myeloma Center Brussels, Vrije Universiteit Brussel, Brussels, Belgium; <sup>3</sup>CHU Montpellier, Laboratory for Monitoring Innovative Therapies, Department of Biological Hematology, Montpellier, France; <sup>4</sup>CHU Montpellier, Department of Clinical Hematology, Montpellier, France; <sup>5</sup>Medizinische Klinik und Poliklinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany; <sup>6</sup>Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany; <sup>7</sup>Université de Montpellier, UMR CNRS 5235, Montpellier, France and <sup>8</sup> Université de Montpellier, UFR de Médecine, Montpellier, France

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### ABSTRACT

Multiple myeloma (MM) account for approximately 10% of hematological malignancies and is the second most common hematological disorder. Kinases inhibitors are widely used and their efficiency for the treatment of cancers has been demonstrated. Here, in order to identify kinases of potential therapeutic interest for the treatment of MM, we investigated the prognostic impact of the kinome expression profile in large cohorts of patients. We identified 36 kinome-related genes significantly linked with a prognostic value to MM, and built a kinome index based on their expression. The Kinome Index (KI) is linked to prognosis, proliferation, differentiation, and relapse in MM. We then tested inhibitors targeting seven of the identified protein kinases (PBK, SRPK1, CDC7-DBF4, MELK, CHK1, PLK4, MPS1/TTK) in human myeloma cell lines. All tested inhibitors significantly reduced the viability of myeloma cell lines, and we confirmed the potential clinical interest of three of them on primary myeloma cells from patients. In addition, we demonstrated their ability to potentialize the toxicity of conventional treatments, including Melphalan and Lenalidomide. This highlights their potential beneficial effect in myeloma therapy. Three kinases inhibitors (CHK1i, MELKi and PBKi) overcome resistance to Lenalidomide, while CHK1, PBK and DBF4 inhibitors re-sensitize Melphalan resistant cell line to this conventional therapeutic agent. Altogether, we demonstrate that kinase inhibitors could be of therapeutic interest especially in high-risk myeloma patients defined by the KI. CHEK1, MELK, PLK4, SRPK1, CDC7-DBF4, MPS1/TTK and PBK inhibitors could represent new treatment options either alone or in combination with Melphalan or IMiD for refractory/relapsing myeloma patients.

### Correspondence:

JEROME MOREAUX,  
jerome.moreaux@igh.cnrs.fr

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### Introduction

MM is the second most common hematological disorder,<sup>1</sup> and is characterized by the clonal accumulation of malignant plasma cells in the bone marrow.<sup>2</sup> MM is a genetically and clinically heterogeneous disease and genome sequencing studies have recently revealed considerable heterogeneity and genomic instability, a complex mutational landscape and a branching pattern of clonal evolution.<sup>3,4</sup>

Novel agents have been developed in MM including the proteasome inhibitors bortezomib and carfilzomib, and the immunomodulatory drugs thalidomide, Lenalidomide and pomalidomide.<sup>5</sup> However, patients invariably relapse after multiple lines of treatment, with shortened intervals in between relapses, and finally

become resistant to any treatment, resulting in loss of clinical control over the disease. It thus remains an unmet need for new therapeutic approaches to improve treatment of MM patients.

Protein kinases are key actors in various cancers where they are involved in proliferation, survival, migration but also drug resistance.<sup>6</sup> Protein kinases have been a potent source of targets for cancer treatment with inhibitors already approved or in clinical evaluation in numbers of malignancies. Kinases represent interesting druggable targets in MM. Indeed, whereas major signaling pathways have been studied in myeloma, they only represent a small proportion of the whole kinome.<sup>7</sup>

In a first study, Tiedemann and colleagues<sup>8</sup> used a high-throughput systematic RNA interference approach to investigate kinome expression in human myeloma cell lines (HMCL) and identified potential new targets for MM therapy. Here, we investigated the kinome expression profiling in large cohorts of MM patients to identify key targets and new synergistic combinations with conventional treatment. We used a list of kinases or kinase-related genes<sup>9</sup> and investigated the prognostic impact of the kinome expression profile in MM. We identified 36 kinases significantly involved in patient's outcome in three independent cohorts and further analyzed the potential impact of selected available kinases inhibitors in HMCL and primary human myeloma cells. We thus provide a list of protein kinases representing potent therapeutic targets for high-risk MM patients and propose new synergistic combinations of kinase inhibitors and conventional MM treatment.

## Methods

### Gene expression profiling and statistical analyses

We used the gene expression profiling (GEP) from three independent cohorts constituted of MM cells (MMC) purified from untreated patients: the Heidelberg-Montpellier cohort of 206 patients (ArrayExpress public database under accession number E-MTAB-362)<sup>10,11</sup> the UAMS-TT2 cohort of 345 patients from the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR, USA; accession number GSE2658),<sup>12</sup> and the UAMS-TT3 cohort of 158 patients (E-TABM-11,<sup>38</sup> accession number GSE4583).<sup>13</sup> Gene expression data were normalized with the MAS5 algorithm and processing of the data was performed using the webtool *genomicscape* (<http://www.genomicscape.com>).<sup>14</sup> *STRING* webtool (<https://string-db.org>) was used to evaluate interconnections between genes and analyzed the enriched pathways. Cluster (v2.11) and Tree View were used to visualize gene expression data.<sup>15</sup> Univariate and multivariate analysis of genes prognostic for patients' survival was performed using the Cox proportional hazard model.

### Multiple myeloma cell lines

HMCL AMO-1 and OPM2 were purchased from DSMZ (Braunschweig, Germany), XG1 and XG21 were obtained as described.<sup>16</sup> HMCL were cultured in RPMI 1640 medium, 10% foetal calf serum (FCS) (control medium). For XG - IL-6 dependent HMCL, 2ng/mL IL-6 was added. Cells were cultured in 96-well flat-bottom microtiter plates in the presence of a concentration range of selected compounds: AZD7762/CHK1i and OTSSP167/MELKi (Selleck, euromedex), HITOPK032/PBK1i, XL413/CDC7-DBF4i, SRPIN340/SRPK1i (Sigma), AZ3146/MPS1i, Centrinone B/PLK4i (Tocris). Cell Titer Glo Luminescent Assay

(Promega, Madison, WI, USA) was used to assess cell viability, and the 50% inhibition (IC50) was determined using GraphPad Prism software (<http://www.graphpad.com/scientific-software/prism/>).

The 5T33vv cells originated spontaneously in aging C57BL/KaLwRij mice and have since been propagated *in vivo* by intravenous transfer of the diseased marrow in young syngeneic mice.<sup>17</sup>

### Primary multiple myeloma cells

Bone marrow of patients presenting with previously untreated MM (n=5) at the University Hospital of Montpellier was obtained after patients' written informed consent in accordance with the Declaration of Helsinki and agreement of the Institutional Review Board and the Montpellier University Hospital Centre for Biological Resources (DC-2008-417). Primary myeloma cells of patients were cultured with or without graded concentrations of selected inhibitors and MMC cytotoxicity was evaluated using anti-CD138-Phycoerythrin monoclonal antibody (clone B-A38) and CD38-Allophycocyanin (clone-LS198-4-3) (Beckman-Coulter) as described.<sup>11</sup> In each culture group, viability (trypan blue) and cell counts were assayed and the percentage of CD138+ viable myeloma cells was determined by flow cytometry.

Additional information concerning the methodology are included in the *Online Supplementary Materials and Methods*.

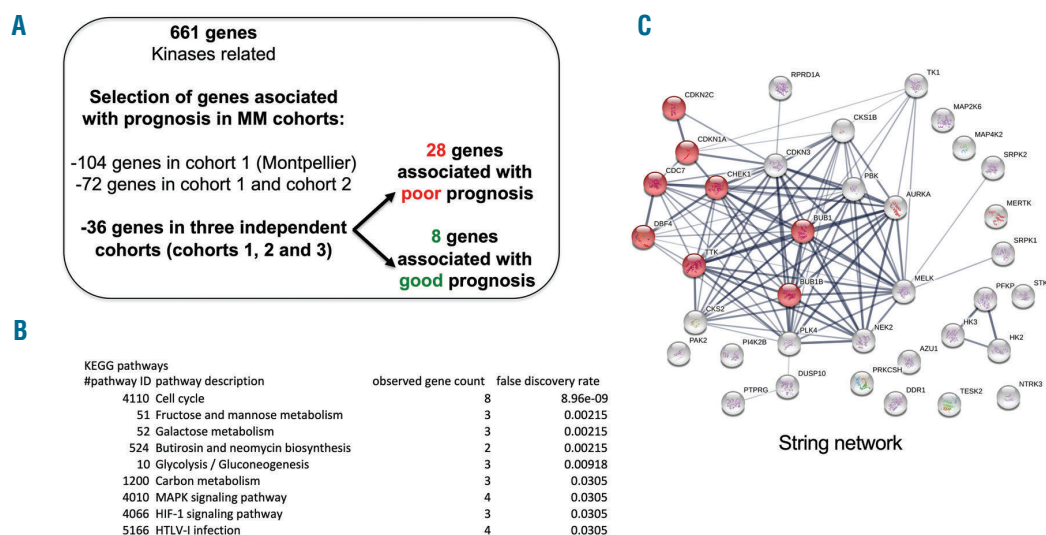
## Results

### Identification of 36 kinome-related targets linked to prognosis in three independent MM cohorts

Considering the crucial role played by protein kinases in pathologies, including MM, we first aimed to identify kinome-related genes associated with prognostic value in MM. A list of 661 genes extracted from the literature, representing 661 kinases or kinase-related genes<sup>9</sup> (*Online Supplementary Table S1*) were thus tested for their prognostic value in the Heidelberg-Montpellier cohort (n=206) using the Maxstat algorithm.<sup>10,11</sup> Among the 661 genes investigated, the expression of 104 demonstrated a significant prognostic value after Benjamini Hochberg multiple testing correction. We searched to validate the prognostic value of the 104 selected kinases in two other independent cohorts of newly diagnosed patients (UAMS-TT212 and UAMS-TT313) and defined a final list of 36 kinases with significant prognostic value in the three cohorts (Figure 1A and *Online Supplementary Table S2*). Among the 36 kinase or kinase-related genes identified, eight of them were associated with a favorable prognosis (*AZU1; CDKN1A; DDR1; HK3; MAP4K2; MERTK; PRKCSH; TESK2*), while 28 demonstrated a poor prognostic value (*AURKA; BUB1; BUB1B; CDC7; CDKN2C; CDKN3; CHEK1; CKS1B; CKS2; DBF4; DUSP10; HK2; PI4K2B; MAP2K6; MELK; NEK2; NTRK3; PAK2; PBK; PFKP; PLK4; PTPRG; RPRD1A; SRPK1; SRPK2; STK39; TK1; TTK*).

Analysis of their involvement in cellular physiology highlighted the cell cycle as the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Figure 1B), and string network of the 36 genes showed highly interconnected proteins particularly for those with a role in cell cycle (Figure 1C).

Hierarchical clustering underlined a spread expression of the genes among MM patients, except for a cluster composed of 14 kinases related to proliferation/mitosis (*CDKN2C; CDC7; CDKN3; BUB1B; MELK; BUB1; AURKA; NEK2; PBK; TTK; CHEK1; PLK4; CKS1B* and



**Figure 1. Identification of 36 kinome related probe sets linked to prognosis in three independent cohorts of newly diagnosed multiple myeloma patients.** (A) Workflow analysis used to identify kinases with gene expression associated with a prognostic value in multiple myeloma (MM). Cohort 1: HM-Montpellier cohort, Cohort 2: UAMS-TT2, Cohort 3: UAMS-TT3. Poor prognosis means that high gene expression is associated with a significant negative outcome, while good prognosis means that high gene expression is linked to a better outcome (B) Reactome molecular signatures significantly enriched in the kinases related to a poor outcome in MM (C) String network of the 36 identified kinases. Red color represents cell cycle related kinases.

*TK1*), which exhibited a specific pattern of overexpression in a subgroup of patients (*Online Supplementary Figure S1*). Interestingly 10 of these 14 kinases are part of the CIN-SARC signature, associated with chromosomal instability in many cancer types including multiple myeloma.<sup>19</sup>

### Building a Kinome Index (KI) linked to the patient's outcome

We next combined the prognostic information of the 36 identified kinases in a GEP-based a KI. This KI is the sum of the standardized expression value of the 28 kinase genes associated with a poor prognostic value minus the sum of the standardized expression value of the eight genes associated with a favorable prognosis (*Online Supplementary Figure S2*). Maxstat algorithm segregated the HM cohort into two groups with 31% of the patients with a  $KI > 2.1$  and 69% of the patients with a  $KI \leq 2.1$  with a maximum difference in overall survival (OS) (Figure 2A). Patients with  $KI > 2.1$  have a median OS of 50.6 months *versus* not reached for patients with  $KI \leq 2.1$  ( $P = 1.7E-05$ ) and a median event free survival (EFS) of 20.1 months *versus* 40.6 months ( $P = 4.5E-05$ ) in the HM cohort (Figure 2B). The prognostic value of the KI was validated in the two additional independent UAMS-TT2 and TT3 cohorts for OS and EFS (*Online Supplementary Figure S3*).

KI is significantly higher in the proliferation (PR) and MAF MM molecular subgroups<sup>20</sup> known to be associated with a poor outcome ( $P < 8E-18$ ). Furthermore, higher KI was associated with the proliferating stages of B-cell to plasma-cell differentiation including activated B cells, pre-plasmablasts and plasmablasts compared to non-proliferating memory B cells and mature plasma cells (Figure 2D). This observation corroborates the association of the 36 kinases to cell cycle (Figure 1B) and the PR subgroup (Figure 2C), as well as the well-known association of kinase activation with proliferation. In addition, KI values increased with disease progression from normal bone marrow plasma cells (BMPC) to MM cells with a homoge-

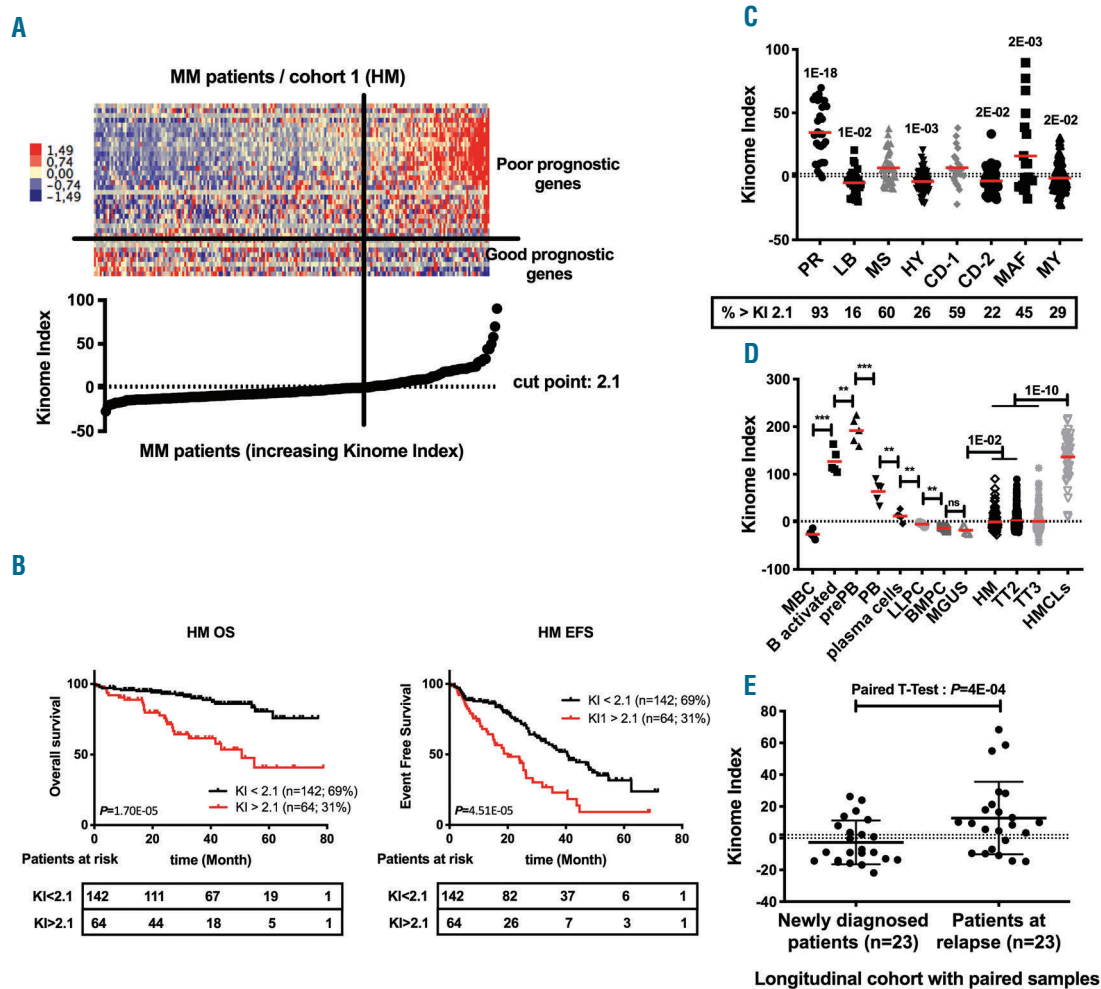
neous index between the different cohorts tested (HM, TT2 and TT3) and HMCL ( $P < 0.01$ ) (Figure 2D). Finally, we tested the KI in a cohort of 23 patients with paired samples at diagnosis and relapse, and identified a significant increase of the KI at relapse ( $P = 4E-04$ ) (Figure 2E). Altogether these observations further highlight that the selected kinases comprising markers of genomic instability,<sup>19</sup> could represent new potential therapeutic targets for high-risk MM patients.

### KI kinases' inhibition leads to MM cell death *in vitro*

According to our *in silico* analysis, the 36 genes demonstrated an outstanding connection with MM pathophysiology and prognosis. Thus, we next assessed selected kinases of interest for their individual therapeutic potential on MM cells using specific inhibitors. For that purpose we first excluded the eight genes associated with favorable prognosis, and analysed the 28 remaining kinases for their link with MM in the literature. Three genes whose connections with MM have already been widely studied (more than five references identified in PubMed) (*CKS1B*<sup>21</sup>; *AURKA*<sup>22</sup>; *CDKN2C*)<sup>23</sup> were then also excluded, and we finally selected the seven kinases (*PBK*; *CHEK1*; *MPS1/TTK*; *CDC7-DBF4*; *MELK*; *PLK4*; *SRPK1*) that had commercially available specific inhibitors at the time of the study (Figure 3A). It has to be noted that all selected kinases are involved in the mitotic checkpoint (*PBK*; *MPS1/TTK*; *MELK*; *PLK4*) or replicative stress response (*CHK1*; *CDC7-DBF4*; *SRPK1*), and the expression of all the selected kinases is individually correlated to high-risk KI-defined MM subgroup (*Online Supplementary Figure S4*).

Then we assessed the kinase inhibitors for their potential anti-myeloma effect on four HMCL (AMO-1, OPM2, XG-1 and XG-21). Remarkably all tested drugs led to a significant decrease in HMCL viability and cell growth, with an IC50 indicated in Figure 3B and *Online Supplementary Figure S5*. We next investigated how the tested drugs impact cell death in the AMO1 HMCL using two drugs





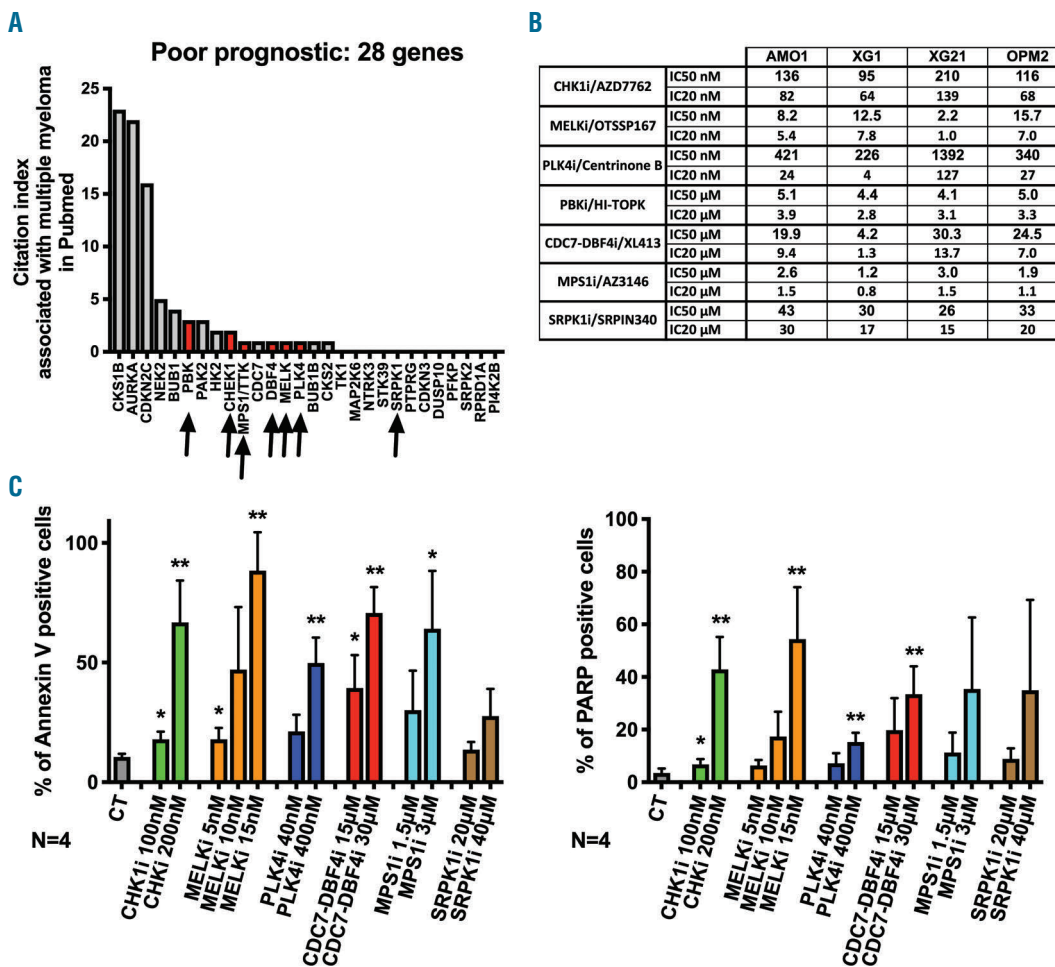
**Figure 2. Prognostic value of the Kinome Index in multiple myeloma.** (A) Clustergram in the 206 HM cohort's patients (206 patients) of the 36 genes signal used to build the Kinome Index (KI). Signals are displayed from low (deep blue) to high (deep red) expression. (B) Patients of the HM cohort were ranked according to increased KI and a maximum difference in OS was obtained with KI of 2.1 splitting patients into high-risk (31%) and low-risk (69%) groups (OS and EFS). (C) The KI was computed for MMC of patients belonging to the subgroups of the University of Arkansas for Medical Science (UAMS) molecular classification of MM, using UAMS-TT2 cohort. CD1: cyclin D1 and cyclin D3; CD2: cyclin D1 and cyclin D3; HY: hyperdiploid; LB: low bone disease; MF: c-MAF and MAFB; MS: MMSET; MY: myeloid; PR: proliferation; D) KI is increased in Pre-plasmablasts characterized by high proliferation during normal B- to PC-differentiation. MBC: memory B cells (n=5); prePB: pre-plasmablast (n=5); PB: plasmablast (n=5); LLPC: long live plasma cells (n=5); BMPC: bone marrow plasma cells (n=5); HM MM cohort (n=206); TT2 MM cohort (n=345); TT3 MM cohort (n=158); HMCL: human myeloma cell lines (n=44). (E) KI is significantly higher at relapse compared to diagnosis in a cohort of 23 paired patient's samples (paired T-Test).  $P$ -value: \* $<0.05$ ; \*\* $<0.01$ ; \*\*\* $<0.001$ .

concentrations around the calculated IC50. As shown in Figure 3C, all drugs induced apoptosis as measured by the dramatic increase of annexin V and cleaved PARP staining following treatment. Interestingly, this effect was not observed at the lower concentration used, thus confirming our previous observation of a dose-dependent efficacy of the drugs. We then tested the ability of the kinase inhibitors to perturb cell-cycle progression. CHK1i, MELKi and CDC7-DBF4i are associated with a significant blockade of MM cells in S phase, while PLK4i and MPS1i induced a significant accumulation in G0/G1 in AMO1 HMCL (*Online Supplementary Figure S6A-B*). Thus, the different inhibitors tested here induced both apoptosis and deregulate MM cell proliferation. We also investigated the effect of phosphatase receptor type  $\gamma$  (PTPRG) depletion using siRNA. PTPRG was shown to be spiked and mutated in MM.<sup>24</sup> Depletion of PTPRG results in a significant

decrease in MM cell growth together with apoptosis induction (*Online Supplementary Figure S15*).

Next, we focused on the three inhibitors that induced MM cells toxicity at nanomolar concentration (CHK1i; MELKi; PLK4i) to validate their therapeutic interest using primary MM cells from patients co-cultured with their bone marrow microenvironment. Remarkably, all three tested drugs significantly reduced the number of tumor cells without toxicity for the bone marrow microenvironment (Figure 4A and *Online Supplementary Figure S6C-E*).

In addition, in order to demonstrate the capability of preclinical studies for the three selected inhibitors, we tested them in 5T33v cells, a murine model of MM.<sup>17</sup> As shown in Figure 4B, CHK1i and MELKi demonstrated similar efficiency while PLK4i was less effective in influencing 5T33v cell viability compared to human myeloma cells.



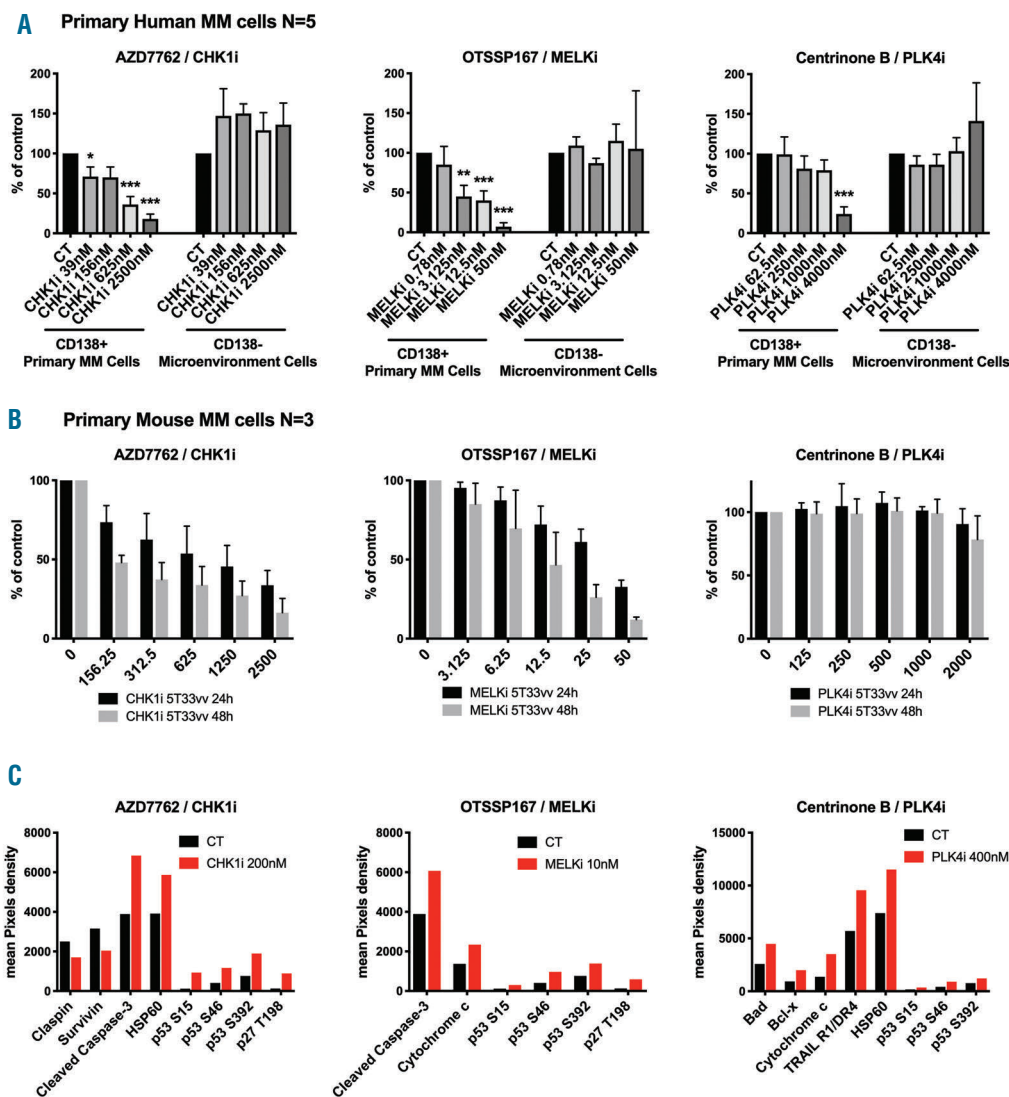
**Figure 3. Selected kinases inhibition induces human myeloma cell toxicity.** (A) Selection of seven kinases for biological investigations based on citation report in Pubmed and the availability of inhibitors. (B) IC50 of the different drugs in four human myeloma cell lines (HMCL), and calculated IC20 for the AMO1 HMCL; (C) Kinase inhibitors induce apoptosis (annexin V and PARP cleavage) in AMO1 MM cell line at concentrations close to the calculated IC20 and IC50. Annexin, and PARP cleavage, were monitored by flow cytometry after four days of treatments. Results are representative of four independent experiments. Statistical significance was tested using a Student T-Test for pairs. *P*-value: \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001.

Finally, using a proteome array we examined the pathways involved in apoptosis and cell cycle following treatments in AMO1 cells and in OPM2 cells that are p53 mutated.<sup>25</sup> For all three tested treatments we observed in AMO1, but as expected not in OPM2, an increased p53 phosphorylation on S15 (DNA damage response), S46 (apoptosis) and S392 (growth inhibition) (Figure 4C and *Online Supplementary Figure S7*). Other apoptotic markers including caspase 3 cleavage, p27, cytochrome C, HSP60, TRAIL, BAD and BCL-X were also induced. Upon CHK1i treatment in AMO1, we also observed a decrease in Claspin and Survivin levels, two proteins involved in cell cycle and replication that have been linked to the CHK1 pathway. Indeed Claspin is a co-activator of CHK1,<sup>26,27</sup> whereas Survivin degradation depends on the XAF1/XIAP1<sup>28</sup> a pro-apoptotic complex involved in CHK1 degradation.<sup>29</sup> Those effects were not observed in OPM2 cells although we observed an increase of the pro-apoptotic proteins Diablo and FADD and a decreased in the proliferation related proteins TOR and P70 S6 kinases.<sup>30</sup> Heterogeneity of the cell lines regarding the p53 status

could explain these differences. However, in both tested cell lines anti-, and pro-apoptotic signals were deregulated. Altogether, these data demonstrate the pro-apoptotic and anti-proliferative effects of these three molecules in MM cells and highlight the potential of these kinases as new therapeutic targets in high-risk MM patients.

**Conventional MM therapies are potentiated by selected kinase inhibitors**

We then investigated the therapeutic interest of combining these kinase inhibitors with therapeutic drugs commonly used in MM (e.g. Melphalan, Lenalidomide, Velcade). Combining sub-lethal IC20 for all the kinase inhibitors with increasing concentrations of standard agents allowed us to identify a significant potentiation of Melphalan toxicity by CHK1, MELK, PBK and CDC7-DBF4 inhibitors in at least two out of the four HMCL investigated. However, no significant effect on the calculated IC50 was noticed for the co-treatment of Melphalan with PLK4, MPS1 and SRPK1 inhibitors with a potential calculated antagonism of the two molecules (Figure 5A

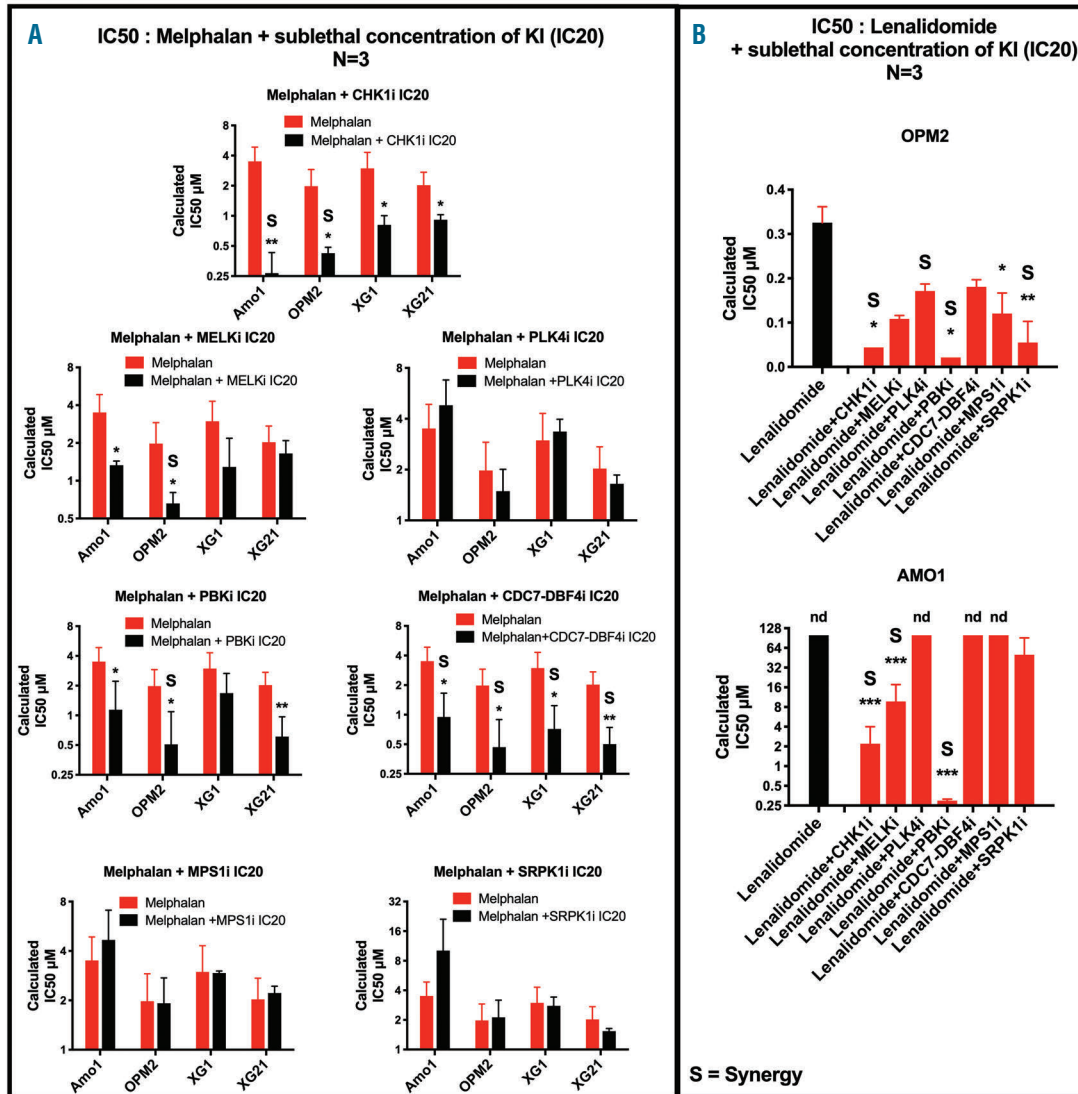


**Figure 4. Selected kinases inhibition induces human primary multiple myeloma (MM) cell death and toxicity on 5TMM murine cells.** A) Mononuclear cells from five patients with MM were treated or not with CHK1i, MELKi and PLK4i. At day 4 of culture, the viability and total cell counts were assessed and the percentage of CD138+ viable plasma cells and bone marrow non-myeloma cells were determined by flow cytometry. Results are median values of the numbers of myeloma cells in the culture wells. Results were compared with a Student T-Test for pairs. B) Murine myeloma cell (5T33vv) viability was monitored by CTEG after 24 and 48 hours treatment with CHK1i, MELKi and PLK4i. Results are representative of three independent experiments C) Apoptosis and Signaling pathways targeted by CHK1i, MELKi and PLK4i. Proteins accumulations were monitored after 48h treatment on AMO1 human myeloma cell lines (HMCL) using proteome profiler array. Relative amount was calculated as the mean of pixel density.  $P$ -value: \* $<0.05$ ; \*\* $<0.01$ ; \*\*\* $<0.001$ .

and *Online Supplementary Figure S8A*). For the immunomodulatory agent Lenalidomide, no significant effect was observed with the tested combinations in two Lenalidomide resistant HMCL: XG1 and XG21. However, the effect of Lenalidomide was significantly potentialized in two other HMCL (AMO1 and OPM2) in combination with the CHK1, MELK or PBK inhibitors. Remarkably, addition of CHK1i, MELKi or PLK4i could overcome Lenalidomide resistance of the AMO1 cell line (Figure 5B and *Online Supplementary Figure S8B*). Conversely, we could not observe any synergy or even additivity for the co-treatment with Velcade, regardless of the cell line tested or the kinase inhibitor used (*Online Supplementary Figure S9A*). Altogether these results demonstrate the therapeutic

interest of CHK1i, MELKi, CDC7-DBF4i and PBKi in combination with Melphalan and IMiDs in MM (*Online Supplementary Figure S9B*).

To characterize the mechanisms involved, we monitored apoptosis after co-treatments of kinases inhibitors with Melphalan or Lenalidomide in AMO1 and OPM2 cells. A sub-lethal dose of Melphalan or Lenalidomide was used in combination with the calculated IC<sub>20</sub> of the kinase inhibitors. CHK1i, MELKi and CDC7-DBF4i increased cell death via apoptosis when cells were co-treated with Melphalan or Lenalidomide. In addition, PLK4i co-treatment only potentialized cell death with Lenalidomide (Figure 6A and *Online Supplementary Figure S10A*). As expected from cell growth analyses, SRPK1i and



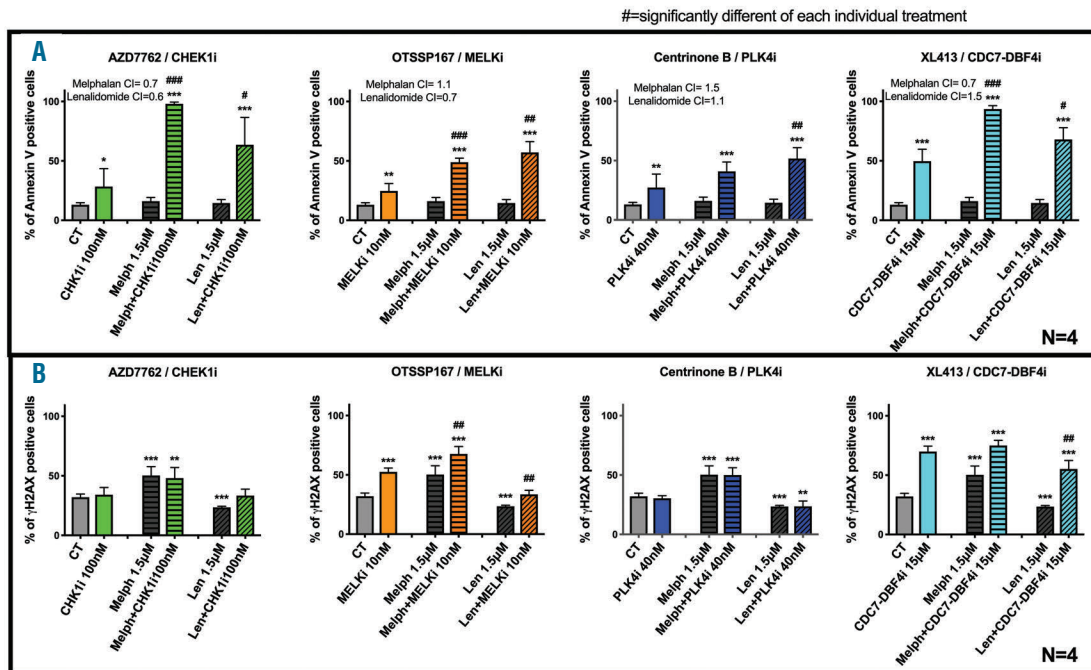
**Figure 5. Kinase inhibitors enhance the sensitivity of multiple myeloma cells to conventional treatments.** Human myeloma cell lines (HMCL) were cultured for four days in 96-well flat-bottom microtiter plates in RPMI 1640 medium, 10% fetal calf serum, 2 ng/mL IL-6 culture medium (control) and graded Melphalan concentrations (A) or Lenalidomide concentrations (B) in presence or absence of IC20 of CHK1i, MELKi, PBKi, CDC7-DBF4i, SRPKi, MPS1i and PLK4i. IC50 were calculated after viability assessment by CellTiter-Glo® Luminescent Cell Viability Assay. Results are representative of three independent experiments. P-value: \* $<0.05$ ; \*\* $<0.01$ ; \*\*\* $<0.001$ . S: significant synergy calculated by the method of Chou and Talalay.

MPS1i did not increase cell death (*Online Supplementary Figure S9C and S10A*). Next, we monitored DNA damage by measuring levels of the DNA double-strand break (DSB) marker  $\gamma$ H2AX after the different co-treatments. As expected, Melphalan treatment alone, even at the sublethal dose, increased the level of  $\gamma$ H2AX, while Lenalidomide did not demonstrate any effect (*Figure 6B and Online Supplementary Figure S10B*). However, among all the combinations tested, only MELKi significantly potentiated Melphalan-induced DNA damage in AMO1 but not in OPM2 cells. Interestingly MELKi, CDC7-DBF4i and SRPK1i alone induced DSB as monitored by  $\gamma$ H2AX levels (*Figure 6B and Online Supplementary Figure S9D*) although it should be noted that high concentrations of the CHK1 inhibitor AZD7762 or MELK inhibitor

OTSSP167 induced early DSB that progressively decrease as monitored by measuring  $\gamma$ H2AX in AMO1 after 24 and 48 hours of treatment (*Online Supplementary Figure S11*). Thus, the significant potentiation of Melphalan and Lenalidomide toxicity by CHK1i, MELKi, CDC7-DBF4i and SRPK1i appears to be due to an increased induction of apoptosis, and not to an increase of DNA damage or cell cycle deregulation (*Online Supplementary Figure S12*).

According to these results, we investigated the therapeutic interest of kinases inhibitors to overcome Melphalan resistance using Melphalan resistant (Mres) XG7 and XG2 cell lines (*Figure 7A and Online Supplementary Figure S13A*). Interestingly, while no clear differences could be observed for the IC50 of MELKi, CHK1i, PBKi and MPS1i in the Mres and sensitive (WT)





**Figure 6. Conventional multiple myeloma therapies are potentiated by selected kinase inhibitors.** Co-treatment with selected kinase inhibitors at IC20 and Melphalan or Lenalidomide. (A) Apoptosis induction was analyzed using Annexin V APC staining by flow cytometry. (B) DNA damage induction was analyzed measuring  $\gamma$ H2AX levels; Results are representative of four independent experiments. CI: calculated combination index. Statistical significance was tested using a Student T-Test for pairs.  $P$ -value: \* $<0.05$ ; \*\* $<0.01$ ; \*\*\* $<0.001$ . #Significantly different of each individual treatment.

cell lines, PLK4i and CDC7-DBF4i demonstrated a significantly higher toxicity in the XG7 Mres cell line (Figure 7B) but not in XG2 Mres HMCL (*Online Supplementary Figure S13B*). Sublethal IC20 of CHK1i, PBKi and CDC7-DBF4i overcame Melphalan resistance of both cell lines tested (Figure 7C and *Online Supplementary Figure S13C*), while the other inhibitors tested did not show a significant effect. It should however be underlined that the inhibitors alone are active on both resistant and sensitive cell lines as shown in Figure 7B and *Online Supplementary Figure S13B*. Thus, our results highlight the therapeutic interest of CHK1i, MELKi, CDC7-DBF4i and SRPK1i used alone or in combination with conventional therapies, even in case of acquired resistance.

## Discussion

Here we identified 36 kinases associated with a prognostic value in three independent cohorts of MM patients, allowing the creation of a kinase-related gene expression profile (GEP) risk score KI. Among them, CHK1, CDC7-DBF4, and MELK were identified as being of therapeutic interest in MM.<sup>31-33</sup> PLK4, SRPK1, MPS1/TTK and PBK represent new therapeutic targets in MM. Using inhibitors of these seven kinases, we validated their therapeutic interest to target MM cells alone or in combination with conventional therapies. In addition, we also highlighted a list of protein kinases for which no inhibitor is currently available and which represent promising new therapeutic targets at least in MM.

Our approach differs from a previous study exploiting a

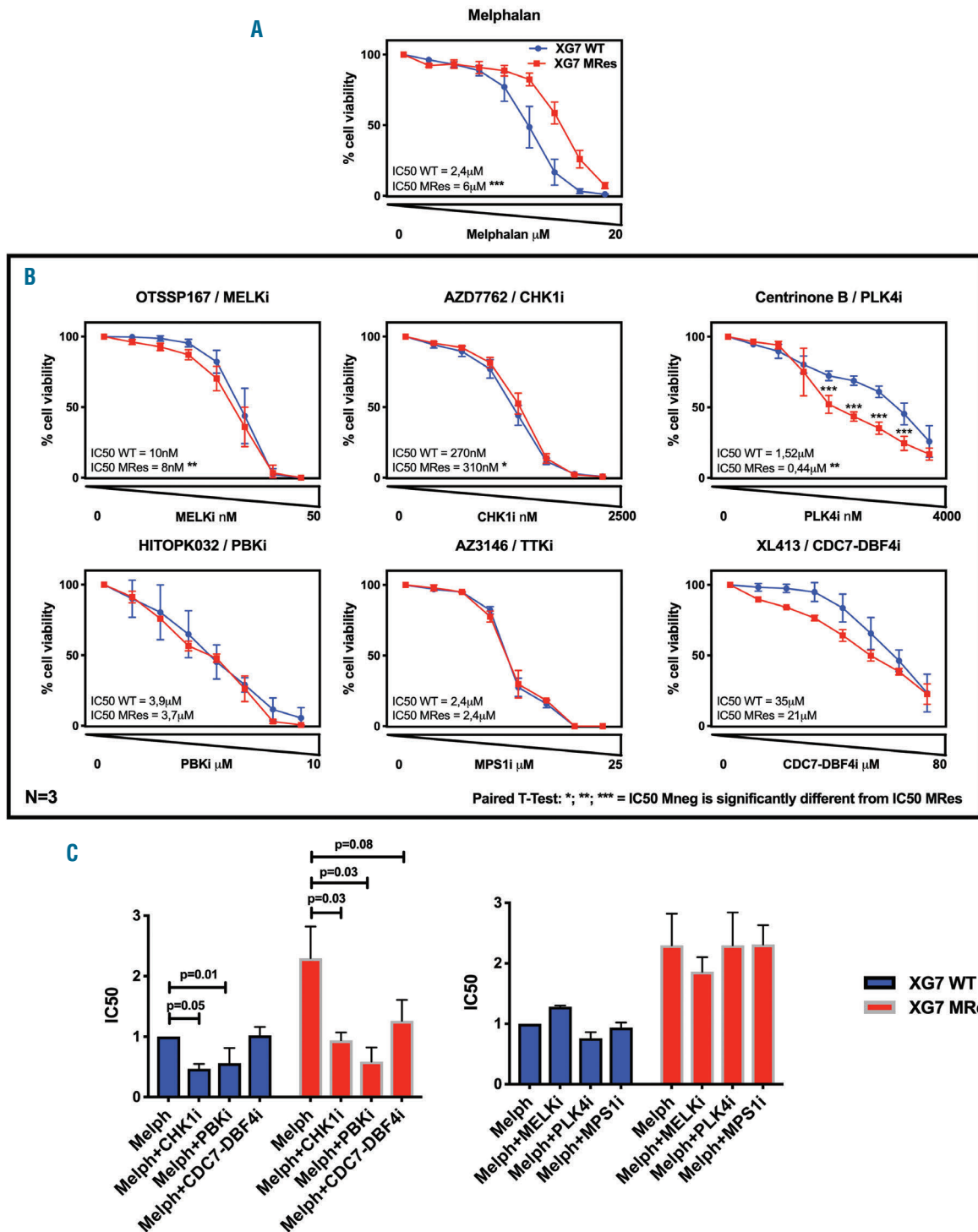
RNAi library to target the human kinome in six myeloma cell lines.<sup>8</sup> Surprisingly, only one kinase, AURKA, was selected in both studies. This discrepancy could reflect the fact that our study relies on the analysis of primary MM cells from patients and not on HMCL as in previous studies. Since a large number of kinase (135/661) are differentially expressed between primary MM cells and HMCL (*Online Supplementary Table S3*), we believe that our study provides a relevant analysis of the protein kinases important for the survival of MM cells.

Our KI is strikingly enriched in kinases involved in the progression through mitosis (PBK, PLK4, MELK, MPS1) and in the replication stress response (CHK1, CDC7-DBF4, SRPK1). These kinases are also enriched in proliferation<sup>34</sup> and proliferation GEP-based signatures, which represent also powerful risk factors in MM.<sup>10,35</sup> The 36 genes of the KI only have a limited overlap with these signatures indicating that KI does not simply reflect a higher cell proliferation index.

Among the inhibitors against targets validated here (CHK1, MELK, PLK4, SRPK1, CDC7-DBF4, MPS1/TTK and PBK), the CHK1 inhibitor AZD7762 was of particular interest due to its ability to act alone or in combination with other drugs. Our results differ from two earlier studies reporting a limited toxicity of AZD7762 on HMCL at doses equivalent of our calculated IC50, but at high Melphalan concentration, when combined with this drug.<sup>31,36</sup> These discrepancies could reflect differences in culture conditions, as in our hands, the drug sensitivity of HMCL depended exquisitely on the confluency status at seeding and on the treatment protocol. Furthermore, we validated the therapeutic interest of CHK1i using primary

MM cells from patients co-cultured with their bone marrow microenvironment, without detecting significant toxicity on non-myeloma cells. Our observations greatly implement the previous studies, either on the activity of the molecule alone, in combination with Melphalan and IMiD, or to overcome MM drug resistance.

The maternal embryonic leucine zipper kinase (MELK) inhibitor OTSSP167 also demonstrated therapeutic interest. MELK is linked to multiple solid cancer types,<sup>37</sup> and recently two groups showed the potential of this inhibitor in MM.<sup>53,58</sup> In addition to their work, we demonstrated the synergy between OTSSP167 with Melphalan and



**Figure 7. Kinase inhibitors overcome resistance of Melphalan resistant multiple myeloma cells.** (A) Dose response curves of XG7 WT and XG7 MRes cell lines. (B) XG7 WT and XG7 MRes HMCL were cultured for 4 days in 96-well flat-bottom microtiter plates in RPMI 1640 medium, 10% fetal calf serum, 2 ng/mL IL-6 culture medium (control) and graded Melphalan concentrations and selected kinase inhibitors at IC20. At day 4 of culture, the viability was assessed by CellTiter-Glo® Luminescent Cell Viability Assay. Data are mean values  $\pm$ SD of three independent experiments. P-value: \* $<0.05$ ; \*\* $<0.01$ ; \*\*\* $<0.001$  using a student T-Test for pairs. Mres: Melphalan resistant; SD: standard deviation. WT: wild-type.

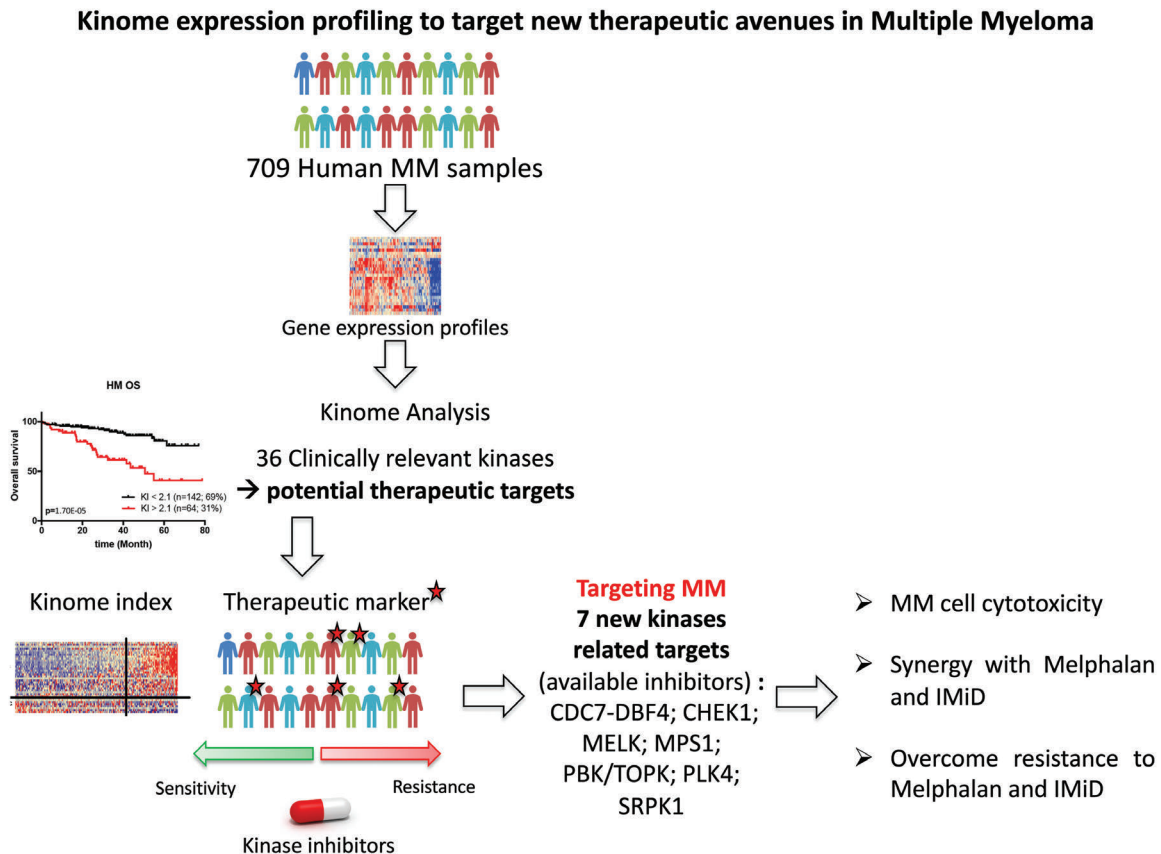
Lenalidomide and its interest to overcome Melphalan drug resistance. Interestingly, OTSSP167 off-targets' BUB1 and TTK/MPS1<sup>39</sup> are also part of our 36 selected kinases, which further highlight the potential of this inhibitor to target MM cells.

Our study represents the first attempt to investigate the therapeutic potential of PLK4, CDC7-DBF4, MPS1, PBK and SRPK1 inhibitors in MM, even though their effect on other cancer cell types has already been established.<sup>40-44</sup> All inhibitors did not demonstrate comparable effects, but they all showed MM cell toxicity when used alone. Furthermore, the toxicity of PLK4i was validated on primary MM cells, and synergy in MM apoptosis induction was also identified for PLK4i and CDC7-DBF4i when combined with Melphalan and Lenalidomide.

Remarkably, all the tested inhibitors (CHK1i, MELKi, PLK4i, SRPK1i, CDC7-DBF4i, MPS1/TTKi and PBKi) demonstrated anti myeloma activity by reducing viability and inducing cellular death of MM cells. Interestingly, a significant correlation between the KI and response to PLK4i was identified (*Online Supplementary Figure S15*). The analysis of the potential mechanisms involved revealed that both cell cycle arrest and apoptosis contributed to the observed phenotype. Both intrinsic and extrinsic apoptosis pathways were involved for AZD7762, OTSSP167 and Centrinone B. Interestingly, these three

inhibitors induced p53 pathway in AMO1, although we believe that the effect of these molecules is not exclusively p53 dependent since they similarly demonstrated significant toxicity in p53 proficient (XG1, OPM2) or p53 deficient (XG21, AMO1) MM cell lines. Though, considering AZD7762, this observation is surprising since several studies noted that CHK1 inhibitors were particularly toxic for p53-deficient cells<sup>45</sup> probably *via* the simultaneous abrogation of the G2 (CHK1) and G1 (p53) checkpoints, and initiation of mitotic catastrophe.<sup>31</sup> However, CHK1 can also suppress death pathways and therefore inhibition of CHK1 can reactivate apoptosis in a p53-independent fashion via caspase 2 activation, mitochondrial outer membrane permeabilization and cytochrome C release.<sup>46</sup> As cytochrome C induction was observed for the three inhibitors tested, this last mechanism could explain the p53-independent effect, which implements considerably its therapeutic interest in MM, where p53 status is highly linked to prognosis.

Here, we demonstrated that low doses of CHK1, MELK, PBK and CDC7-DBF4 inhibitors were able to synergize or even reverse Melphalan resistance. This is very important considering that virtually all MM patients eventually relapse and develop drug resistance. These kinases have all been shown to decrease DNA damage tolerance,<sup>47-50</sup> which could explain this observation. Similarly, CHK1, MELK and



**Figure 8. Kinome expression profiling to define new therapeutic targets in multiple myeloma.** The prognostic impact of the kinome expression was challenged in three independent cohorts of newly-diagnosed multiple myeloma (MM) patients representing 709 patients. 36 clinically relevant genes were selected as potential therapeutic targets, and were used to create a Kinase Index (KI) with a strong prognostic value. Among the 36 selected kinases, we validated seven kinases as new therapeutic targets in MM, as their related inhibitors presented therapeutic interest in MM for personalized treatments.



PBK inhibitors could overcome Lenalidomide resistance. Even if these observations are promising, additional *in vivo* experiments are needed to confirm the potential and elucidate the mechanistic roles of these kinases in Lenalidomide and Melphalan resistance reversion.

The development of the KI could be used to identify high-risk patients that could benefit from treatment with selected kinases inhibitors. Developing the KI, we also identified kinases that have already been linked to MM pathophysiology including CKS1B,<sup>21</sup> AURKA,<sup>22</sup> CDKN2C,<sup>23</sup> NEK2<sup>51</sup> and BUB1B.<sup>52</sup> In addition, we also identified a number of kinases (PAK2, HK2, CDC7, BUB1, CKS2, TK1, MAP2K6, NTRK3, STK39, PTPRG, CDKN3, DUSP10, FFKP, SRPK2, RPRD1A, PI4K2B) without a clear or documented connection with MM, but which are considered as potential targets in other cancers. According to the high degree of heterogeneity of the disease, we look forward to the development of new inhibitors targeting these kinases,

which could be of therapeutic interest in MM.

To date, no kinase inhibitors have received the approval of the Food and Drug Administration for the treatment of MM.<sup>7</sup> Our study demonstrates that kinase targeting could be of therapeutic interest, especially in high-risk MM patients defined by the KI. Since this index significantly increases at relapse compared to newly diagnosed patients, CHK1, MELK, PLK4, SRPK1, CDC7-DBF4, MPS1/TTK and PBK inhibitors could represent new treatment options alone or in combination with Melphalan or IMiD for refractory/relapsing MM patients.

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