Kinases and miRNAs in the pathogenesis of small B cell lymphomas
Wang, Miao

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 4

JNK is constitutively active in mantle cell lymphoma: cell cycle deregulation and polyploidy by JNK inhibitor SP600125

Miao Wang¹, Çiğdem Atayar¹,³, Stefano Rosati¹, Anneke Bosga-Bouwer², Philip Kluin¹, Lydia Visser¹

¹Department of Pathology & Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

² Department of Medical Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

³ Department of Pathology, Stichting Sazinon, Hoogeveen, The Netherlands

In preparation
Abstract:

Mantle cell lymphoma (MCL) is characterized by high genetic instability and a poor prognosis. Many blastoid variants are (hypo) tetraploid and have an even worse prognosis. We investigated the role of MAPK signaling in MCL. As compared to normal tonsil B cells, MCL cells showed activation of pan JNK and JNK2 in both a MAPK array and sandwich Elisa assay, whereas validation by immunohistochemistry showed overexpression of phospho-JNK in 30 of 37 MCL cases. These data suggest a constitutive activation in MCL. Inhibition of JNK with SP600125 resulted in growth arrest in all 4 MCL cell lines (Jeko-1, HBL-2, UPN-1, Granta-519), which could be reversed by the addition of CD40L and IL-4. Furthermore, SP600125 led to G2-M phase arrest on day 1 and a strikingly increased endoreduplication on day 2 and day 3, which was confirmed by karyotype analysis. G2-M arrest was associated with downregulation of EGR1. SP600125 induced polyploidy could be blocked by the BCL-2 inhibitor YC 137. These data suggest that constitutive JNK activity is necessary to promote proliferation and maintain diploidy in MCL and that disruption of this MAPK leads to a profound cell cycle deregulation and endoreduplication.
Introduction:

Cell signaling pathways involve changes of protein-protein interactions inside the cell induced by external signals to progress through the cell cycle and division. In B cells, deregulation of the B cell receptor (BCR) or CD40 signaling pathway can lead to B cell malignancies[1-4]. Extensive work by several groups has established that the Map kinases (MAPKs) pathway, which is an important downstream signaling target of BCR and CD40, plays critical roles in the pathogenesis of various hematological malignancies[5]. There are three major groups of MAPKs, the p38 Map kinase family (α/β/γ/δ) the extracellular signal-regulated kinase (Erk) family (Erk1/2), and the c-Jun NH2-terminal kinase (JNK) kinase family (JNK1-3). By analyzing different B cell lymphoma cell lines, Ogasawara [6] et al found constitutive activation of ERK and p38 MAPK but not of JNK. Furthermore, p38 kinase plays an important role in follicular lymphoma and its transformation[7]. In contrast, Gururajan [8] et al found that several human and murine B lymphoma cell lines constitutively express high levels of activated JNK. JNK-specific small interfering RNA (siRNA) inhibited the growth of B lymphomas by downregulating c-MYC and EGR1, which are essential for B-lymphoma survival and growth.

Mantle cell lymphoma (MCL) represents a Non Hodgkin’s Lymphoma (NHL) with a poor prognosis for which no effective treatment is available. Half of the blastoid variants with a high incidence of (hypo) tetraploidy have an even worse prognosis[9]. In addition to t (11; 14), secondary chromosome changes are very common, especially in the blastoid variants[10]. The mechanisms accounting for the resistance of these tumor cells to chemotherapeutic drugs and (hypo)tetraploid formation are poorly understood. Until now, the cell cycle and the DNA damage pathways are the most extensively studied signaling pathways in MCL cells[11]. Recently, new insights in the molecular pathogenesis of MCL have revealed many new potential targets, combining drugs that affect BCL-2, proteasome biology and the PI3K-AKT-mTOR pathway[12]. Although JNKs have been studied in many solid cancers and are potential targets for therapy[13], the knowledge in lymphomas and in particular MCL is still very limited.

In this study, we found high JNK kinase activity in mantle cell lymphoma. Disruption of JNK activity leads to major disturbances in cell cycle progression and a high level of endoreduplication and polyploidy.

Materials and methods:

Cell lines and patient materials

Cell suspensions of 5 hyperplastic tonsils and 14 MCL cases, as well as 5 tonsils and 37 MCL paraffin-embedded tissue samples were obtained from the department of Pathology. All protocols for obtaining and studying human tissues and cells were approved by the institution’s review board for human subject research.
MCL cell lines Jeko-1, Granta-519 (DSMZ, Braunschweig, Germany), UPN-1 and HBL-2 (Dr. W. Klapper, Kiel) and CLL cell lines JVM-3 (prolymphocytic leukaemia), MEC1 and MEC2 (DSMZ), MO1043 (Dr. Ricardo Dalla-Favera (Columbia University, New York, NY, USA) were used for functional studies[14-17].

Cell purification and treatment

For T cell depletion, cells were incubated with anti-CD3 (OKT3, LCG, Middlesex, UK) and depleted with Dynal magnetic beads (Invitrogen, Breda, The Netherlands).

BCR ligation was performed with anti-human IgM F(ab')2 (Southern Biotechnology Associates, Birmingham, AL). Stimulation through CD40L and IL-4 was performed with CD40L (Alexis Biochemicals, Lausen, Switzerland) and IL-4 (R&D Systems, Minneapolis, MN, USA). For Western blotting cells were lysed in 1x SDS Sample Buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). For MTT assays MCL cell lines were cultured during 72 hours in the presence of CD40L and IL-4 and different concentrations of SP600125, an inhibitor of Jun N-terminal kinase [18] (Biaffin Gmbh & Co KG, Kassel, Germany).

For cell cycle analysis MCL cell lines were cultured during 72 hours in the presence of 5µM or 10µM YC 137, a selective BCL-2 inhibitor [19] (Merck, Darmstadt, Germany), SP600125, ERK1/2 inhibitor [20] and SB203580[21], a selective p38MAPK inhibitor (Biaffin). Optimal concentrations of inhibitors were checked by western blot.

MAPK array and antibody

Purified B cells were used for MAPK array detection by a commercially available kit (R&D). An ELISA for phosphorylated (p)-JNK ELISA was used according to the manufacturer’s instructions (Cell Signaling Technology, Boston, MA, USA). Antibodies against p-SAPK/JNK (Thr183/Tyr185) (81E11), p-p38 MAPK (Thr180/Tyr182) (12F8), p-GSK-3α/β (Ser21/9) (37F11), p-p44/42 MAPK (Thr202/Tyr204) (20G11), p-c-Jun (Ser73), p-ATF-2 (Thr69/71), EGR1 (15F7), p21 (DCS60), survivin (71G4B7E), Cyclin D1 (92G2) were purchased from Cell Signaling Technology. P27 (Y236), CDC2 (E53), and BCL-2 (E17) antibodies were purchased from Epitomics (Burlingame, CA, USA). HRP-labeled rabbit anti mouse antibody, HRP-labeled goat anti rabbit antibody were purchased from DAKO (Glostrup, Denmark).

Immunohistochemistry (IHC)

Routinely obtained histological slides were used according to standard protocols. Antigen retrieval was performed according to the protocols of the manufacturers. Immunostaining was amplified by incubation with the appropriate HRP-conjugates and the reaction was visualized by dianminobenzidin. Appropriate positive and negative controls were used.
Figure 1. p-MAPK array analysis shows a high JNK activity in mantle cell lymphoma. 
A: p-MAPK array data (in duplicate) for purified B cells from 2 hyperplastic tonsils and 3 MCL samples. Each bar represents mean ± SD pixel density. *p < 0.05 and #p < 0.01 are significantly different from tonsil. B. Quantification of p-JNK expression levels in B cells of 5 hyperplastic tonsils and 14 MCL samples. Cell lysates are used and p-JNK expression levels are detected by p-SAPK/JNK sandwich ELISA. Each point represents the OD492 reading number of the samples. C. Immunohistochemistry shows high expression of p-JNK in mantle cell lymphoma. A, B: p-JNK (Thr183/Tyr185) is weakly expressed in tonsil, especially in germinal center cells (A), but is strongly expressed in MCL cells showing nuclear staining (B). C, D: p-p38 alpha (Thr180/Tyr182) is highly expressed in both tonsil (C) and MCL cases with nuclear staining (D). E, F: p-GSK-3α/β (Ser21/9) is widely expressed in tonsil with intermediate staining (E). A higher expression level was observed in the MCL samples (F). GC: germinal center; MZ: mantle zone. Magnifications 400X.
Western Blot

Cell lysates were separated on polyacrylamide gels and electroblotted onto nitrocellulose membranes. Blots were blocked in blocking buffer (TBS with 0.05% Tween 20, pH 7.6 with 5% skimmed milk), washed and incubated with primary antibodies at 4 °C overnight. Immunostaining was amplified by incubation with HRP-conjugated antibodies and chemiluminescence was detected with ECL (Pierce, Rockford, USA).

MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to cells and incubated for 4 hours at 37°C. The cells were centrifuged and the supernatant was removed. DMSO (Sigma) was added and absorption was measured.

Figure 2: p-JNK is involved in CD40 signaling pathway in MCL cell lines. A. Western blot analysis of the total cell lysate the MCL cell lines for p-SAPK/JNK (Thr183/Tyr185), p-ERK (Thr202/Tyr204), p-p38 (Thr180/Tyr182), p-c-Jun (Ser73), and p-ATF2 (Thr69/71) before and after CD40L (330ng/ml) and IL-4 (100ng/ml) or after IgM (10μg/ml) stimulation. p-SAPK/JNK (Thr183/Tyr185) is obviously upregulated after CD40L and IL-4 stimulation and slightly upregulated after IgM stimulation. After CD40L and IL-4 stimulation, there is a slight downregulation of p-ERK and no obvious change of p-p38 is observed in MCL cell lines. p-ATF2, a downstream target for JNK, is also obviously upregulated after the CD40L and IL-4 stimulation, whereas there is no obvious change of phospho-c-Jun. Similar results are found in all four MCL cell lines. JEKO-1 is shown as a representative example. p: Phosphorylated B. Quantification of the p-JNK expression level before and after stimulation by Phospho-SAPK/JNK sandwich ELISA. CD40L and IL-4 treated cells show higher expression levels than control (untreated cells) and IgM treated cells in all cell lines. Each bar represents mean value for the 4 cell lines ± SD of OD492 readings. *p < 0.05 is significantly different from control (untreated cells).
at 540nm in an ELISA reader.

**Alamar blue assay**

MCL cell lines cells incubated with vehicle (DMSO) alone or with 20µM SP600125 were washed after 2 days and cultured in fresh medium. Alamar blue (Invitrogen) was added and fluorescence was measured at several later time points with an ELISA reader at 570/600 nm.

**Cell cycle analysis**

Cells were washed in PBS with 0.1% BSA. Hypotonic DNA staining buffer (0.1% Sodium citrate; 0.3% Triton–x 100; 0.01% Propidium iodide, 0.002% Ribonuclease A) was added to the pellet and mixed well. Acquisition was performed on the flowcytometer (Calibur, Becton Dickinson, San Jose, CA). The percentage of G2-M cell cycle cells was analyzed by ModFit LT3 by using the one cell cycle analysis model; the percentage of polyploid cells using the two cell cycle analysis model.

**Statistical analysis**

All data were derived from at least three independent experiments. Quantity one software was used to quantify the MAPK array spots and Western blot bands. Significant differences between the groups were determined using unpaired Student’s t-test.

**Results:**

**JNK is constitutively active in mantle cell lymphoma**

Five out of 21 MAPK kinases of the MAPK array were predominantly active in both the 2 samples of purified tonsillar B cells and all (3 out of 3) MCL samples (Figure 1A). From these 5 kinases, p38 alpha had the highest activity, and p38 gamma and GSK3A/B an intermediate activity. JNK2 and pan JNK showed a low activity in both tonsils but a significantly higher activity in the MCL cases (p<0.05). Activity of other kinases such as ERK1/2 and JNK1 was inconsistent in the 3 MCL cases (supplementary data).

We validated p-JNK expression in purified B cells from 5 tonsils and 14 MCL samples by p-SAPK/JNK ELISA. The p-JNK level in MCL samples was about 2-fold higher than in tonsil samples (p<0.001; Figure 1B).

We also investigated the expression of p-SAPK/JNK, p-p38 alpha and p-GSK3A/B in 5 reactive tonsils and 37 MCL cases by immunohistochemistry (Figure 1C). In the tonsils, p-JNK was only detected in few germinal center cells. In contrast, 30 of 37 of the MCL cases showed a strong nuclear p-JNK staining. p-p38 alpha was highly expressed in all reactive tonsils and MCL cases. For p-GSK3A/B, a high expression level was observed in the MCL samples and an intermediate level in normal tonsils.
Figure 3: Effect of JNK inhibitor SP600125 on MCL cell lines. A: Four MCL cell lines were treated with the indicated concentrations of SP600125 and tested for cell growth by the MTT assay at 48 h after treatment. Results were expressed as percentage of cell growth in cells not treated with any inhibitor. Each bar represents the mean value for the 4 cell lines ± SD. *p < 0.05 is significantly different from vehicle. B: MCL cell lines were incubated with different doses of SP600125 (10µM or 20µM) in the presence or absence of CD40L (330ng/ml) and IL-4 (100ng/ml) for 2 days, and % cell growth compared with controls was measured by MTT. Each bar represents the mean value for the 4 cell lines ± SD. *p < 0.05 is significantly different. C: MCL cell line cells were treated with SP600125 for periods up to 3 days and analyzed by flow cytometry after propidium iodide staining. Relative DNA content is represented on the x-axis (arbitrarily set at “2” of the first peak for cells in G0/G1 phase of the cell cycle for that respective cell line) and the number of cells counted is represented on the y-axis. There was an increase in the percentage of cells in the G2-M phase at day 1 and an obvious increase in cells with endoreduplication at day 2 and day 3. HBL-2 is shown as a representative example. D: Cell morphology of the MCL cell lines after treatment with vehicle or 20µM SP600125 for 2 days. UPN1 with usually 43 chromosomes has relatively typical mantle cell lymphoma morphology with intermediately sized cells with irregular nuclei (treated with vehicle) (A). After treatment with 20µM SP600125, they have increased in cell size and show multilobated nuclei; only very few multinucleated cells were seen. Karyotyping showed that 50% of the cells were tetraploid (B). HBL-2 with usually 46 chromosomes has a more pleomorphic morphology with already large lobulated cells (treated with vehicle) (C). After treatment these features were much more prominent; 85% of the cells were tetraploid and few octaploid by karyotype analysis (D). UPN1 and HBL-2 are shown as representative examples. All microphotographs were taken at a 400x magnification.
p-JNK is involved in CD40 signaling in MCL

Using western blot analysis of the 4 MCL cell lines, we observed increased p-SAPK/JNK (Thr183/Tyr185) after 10 min stimulation by CD40L and IL-4 stimulation and to a lesser extent after IgM stimulation (Figure 2A). In particular JNK-2 was activated after CD40L and IL-4 stimulation. There was some downregulation of p-ERK but no obvious change of p-p38. p-ATF2, a downstream target of JNK, was significantly upregulated, whereas there was no obvious change of p-c-Jun at this interval.

We additionally used an ELISA to detect endogenous levels of p-SAPK/JNK protein in the 4 MCL cell lines after stimulation by IgM or CD40L/IL-4. The highest induction of p-SAPK/JNK was observed when the cells were cultured with CD40L/IL-4 (P<0.05; Figure 2B).

JNK inhibition induces growth arrest and cell cycle deregulation in MCL cell lines

To determine the efficacy of JNK inhibition in MCL cell lines, cells were treated with increasing concentrations of the JNK inhibitor SP600125 for periods up to 3 days and analyzed for cell viability with the MTT assay. As shown in Figure 3A, concentrations of SP600125 greater than 10 μM significantly decreased cell viability in all 4 MCL cell lines.

To test whether the effects of JNK inhibition by SP600125 could be reversed by CD40 signaling, we treated lymphoma cells with a combination of anti-CD40 and IL-4. This significantly reduced the growth inhibition caused by 20 μM SP600125, but not up to the level of untreated cells (p<0.01; Figure 3B).

To determine whether SP600125 altered the cell cycle distribution, cells were treated with SP600125 for up to 3 days and analyzed by DNA flow cytometry. Two main changes were observed. First, there was a three- to ten-fold increase of cells in the G2-M phase at day 1 (Table 1). Secondly, there was an obvious increase in cells with endoreduplication on day 2 and day 3 (Figure 3C). There was no obvious increase of the percentage of cells in the sub-G1 phase up to day 3. We also did not find a significant increase in apoptotic cells as measured by change in mitochondrial membrane potential (data not shown).

Endoreduplication leading to a high content of tetraploid cells (and thus octaploidy at G2/M phase) was confirmed by karyotype analysis in all 4 cell lines: for example untreated HBL-2 contained approximately 96% diploid and 4% tetraploid cells, whereas the SP600125 treated cell line contained approximately 15% diploid and 85% tetraploid cells; untreated Jeko-1 showed only cells with approximately 75 chromosomes, but the treated cell line almost only cells with 150 chromosomes (data not shown). This was accompanied by major changes in cytomorphology, nuclei
Figure 4: JNK inhibitor SP600125 specifically causes G2/M arrest at day 1  

A: Different concentrations of SP600125, ERK1/2 inhibitor or SB203580 are represented on the x-axis and the percentage of cells in various phases of the cell cycle as determined by flowcytometry is represented on the y-axis. The UPN1 cell line as a representative cell line is shown. JNK inhibitor SP600125 effects in G2/M arrest, while the ERK1/2 inhibitor and the p38 inhibitor SB203580 have no effect. 

B: Total cell lysates (Cell line UPN1 is shown as a representative cell line) were prepared in the presence or absence of SP600125 20µM for 24 hours followed by Western blot analysis with cell cycle signaling specific antibodies. Actin is used as a loading control. SP: SP600125.

Table 1: The percentage cells in the G2-M phase after 1 day incubation with different concentrations of SP600125.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>5µM</th>
<th>10µM</th>
<th>20µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeko-1</td>
<td>8.97±1.98</td>
<td>14.39±2.26</td>
<td>22.62±2.60</td>
<td>29.38±4.34</td>
</tr>
<tr>
<td>HBL-2</td>
<td>6.06±1.99</td>
<td>9.86±1.33</td>
<td>56.12±4.15</td>
<td>60.70±7.02</td>
</tr>
<tr>
<td>Grant-519</td>
<td>7.75±1.16</td>
<td>17.85±3.15</td>
<td>42.53±6.18</td>
<td>50.44±9.08</td>
</tr>
<tr>
<td>UPN-1</td>
<td>9.71±1.54</td>
<td>18.95±1.47</td>
<td>31.49±6.47</td>
<td>44.30±2.44</td>
</tr>
</tbody>
</table>

Average percentages in the G2-M phase cells of 3 experiments with standard deviation.
JNK in MCL

becoming much bigger and multilobated, reminiscent of the pleomorphic blastoid variant of MCL (Figure 3D).

**JNK-inhibition leads to G2/M cell cycle arrest**

In order to rule out the possibility that other members of the MAPK family convey the same cell cycle responses, we tested an ERK1/2 inhibitor and the p38 inhibitor SB203580. Neither inhibitor led to significant changes in the cell cycle distribution (Figure 4A).

To investigate proteins that could be involved in the G2/M cell cycle arrest, we analyzed cell cycle regulators 24 hrs after start of SP600125 treatment. There was a reduction in EGR1 in cells treated with 10 µM or 20 µM SP600125 but no changes in p21, p27, CDC2, survivin, Cyclin D1 and BCL-2 (Figure 4B). Thus, JNK may act by upregulation of EGR1 expression to promote cell cycle progression.

**MCL cell lines cells are sensitive to SP600125-induced polyploidization**

Many blastoid MCL cases are tetraploid, a clearly distinguishing feature from other types of B-cell lymphomas[9]. Recent studies on the human leukemia U937 cells have demonstrated that SP600125 significantly increases mitotic arrest and cell polyploidy upon overexpression of BCL-2 [22]. Like native MCL all four cell lines showed high expression of BCL-2. In comparison, we also analyzed the endoreduplication status in 3 CLL cell lines (MEC-1, MEC-2, MO1043), and one PLL cell line (JVM-3), which all highly express BCL-2. After 2 and 3 days of 20µM SP600125 treatment the 4 MCL cell lines contained approximately 70% and the CLL cell lines approximately 30% polyploid cells (Figure 5A). Thus the MCL cell lines cells seemed more sensitive to SP600125-induced endoreduplication, independent of the BCL-2 status.

**Table 2: Percentage of polyploid^1 cells after different treatments for 2 days.**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>YC137 10µM</th>
<th>SP600125 20µM</th>
<th>20µM+YC137 10µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeko-1</td>
<td>0.22±0.04</td>
<td>23.12±4.41</td>
<td>81.38±7.45</td>
<td>2.90±0.14</td>
</tr>
<tr>
<td>HBL-2</td>
<td>1.35±0.49</td>
<td>20.75±1.05</td>
<td>86.45±7.98</td>
<td>30.36±4.15</td>
</tr>
<tr>
<td>Granta-519</td>
<td>3.78±0.60</td>
<td>8.97±0.46</td>
<td>69.01±5.41</td>
<td>8.56±1.06</td>
</tr>
<tr>
<td>UPN-1</td>
<td>4.10±0.55</td>
<td>21.17±0.23</td>
<td>52.60±2.94</td>
<td>15.48±1.41</td>
</tr>
</tbody>
</table>

Average percentages of polyploidy cells of 3 experiments with standard deviation. ^1: The percentage of cells with a octaploid DNA index relative to the base line status of each individual cell line was used.

Using Alamar Blue assay, we compared the growth rate between the untreated and treated (mainly polyploid cells, 20µM SP600125 treatment 2 days) up to 28 hours after washing the inhibitor away and culturing in fresh medium. SP600125 treated cells showed a slower growth rate than untreated cells (Figure 5B).
Figure 5: JNK inhibition induces polyploidization in MCL cell lines on day 2 and 3. A: MCL and CLL cell lines were incubated with 20µM SP600125 or vehicle alone for 2 days. Cell cycle distribution was analyzed by flow cytometry. The percentage of polyploid cells was analyzed by ModFit LT3.1 software. B: MCL cell lines cells incubated with vehicle (untreated) or 20µM SP600125 (treated) for 2 days, and then cells were washed and cultured in fresh medium. Cell growth is shown as measured with Alamar Blue for up to 28 hours. Results for HBL-2 are shown. All experiments in triplicate. C: Untreated and SP600125 treated cells were washed by PBS and cultured in fresh medium in the presence or absence of the BCL-2 inhibitor YC 137 (10 µM) for 24 hours. Cell viability was tested by MTT assay at 24 h after treatment. Results are expressed as percentage of cell growth when compared with the cells that were incubated with vehicle. The average for all 4 cell lines is shown. *p < 0.05 is significantly different between the two groups. D: MCL cell lines were incubated with 20µM SP600125 in the presence (IV) or absence of 10 µM YC 137 (II) or vehicle control (DMSO) alone for 2 days. Cells were incubated with both agents simultaneously. Cell cycle distribution was analyzed by flowcytometry. DNA content is represented on the x-axis (arbitrarely set at “4” for the second peak of that respective cell line) and the number of cells counted is represented on the y-axis. Experiments were done 2 to 3 times with similar results; results for Granta-519 are shown.
Recent reports showed a selective resistance of tetraploid cancer cells against DNA damage-induced apoptosis[23], which might be enhanced upon BCL-2 overexpression. We therefore compared the effect of the BCL-2 inhibitor YC137 on the SP600125 treated cells versus untreated cells. To that end, cells were first treated with SP600125 for 2 days, washed and subsequently incubated with or without 10 μM YC137 for 24 hours. Viability was assessed by the MTT assay. These results showed that the polyploid cells were more resistant to the BCL-2 inhibitor (Figure 5C).

We also investigated the role of BCL-2 in SP600125-mediated endoreduplication by using the BCL-2 inhibitor YC137. In all MCL cell lines simultaneous incubation with 10 μM YC137 and 20 μM SP600125 for 48 h prevented the formation of SP600125-mediated polyploidy (Table 2; Figure 5D).

Discussion:

The c-Jun N-terminal kinases (JNKs) are recognized as key regulators of many cellular events, including apoptosis and proliferation[24, 25]. Ten JNK isoforms are created by alternative splicing of messenger RNA transcripts derived from three genes: JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously distributed. By contrast, JNK3 is found mainly in neuronal tissue and the testis[26-28]. In B cells, JNKs can be activated by the ligation of CD40[29]. JNKs can promote survival and migration of B lymphocytes [30]. The pro-survival role for JNK in B-lymphoma has been demonstrated by in vivo studies in a mouse model of B cell lymphoma[8]. Recently, JNKs have become a new therapeutic target and SP-600125, an anthrapyrazolone inhibitor of JNK, has been used for the treatment of autoimmune and neurodegenerative diseases and ovarian cancer[31-33].

Our data showed that CD40L/IL-4 can significantly enhance the JNK phosphorylation status. Inhibition of JNK activation with SP600125 resulted in growth arrest in all 4 MCL cell lines. Furthermore, the growth inhibitory effect of SP600125 could be reversed by simultaneous stimulation through anti-CD40/IL-4. Several in vitro studies have shown that CD40 signaling plays an important role in enhancing cell viability and cell cycle progression of MCL cells[1, 3, 34]. In addition, constitutive activation of the CD40 pathway in mammary epithelial cells promoted cell transformation and neoplastic growth[35]. By using specific inhibitors, we found that JNK, but not ERK or p38MAPK signaling, plays an important role in G2/M arrest in MCL cell lines. Extensive studies on many other cell lines have shown that inhibition of JNK by SP600125 leads to G2/M cell cycle arrest[36-39]. Our results are in agreement with these findings.

In breast cancer cells the effect of JNK inhibition was independent of p53[37]. Of these cell lines Jeko-1 is carrying a non-sense mutation for p53, UPN-1 was originally described with a missense mutation, while Granta 519 has wild type p53 with a deletion
of INK4a/ARF. Inactivation of p53 might play a role in G2/M cell cycle arrest in these cell lines. In addition, we found that EGR1 was downregulated after SP600125 treatment in cell lines, which suggests that G2/M cell cycle arrest is due to the change in EGR1 expression. EGR1 is an important downstream target of JNK and plays an important role in cell cycle regulation, cell proliferation, metastasis and apoptosis[40, 41]. EGR1 was first identified as a putative G0/G1 switch regulatory gene in lymphocyte cultures and named G0S30[42]. There are constitutive and inducible levels of EGR1 expression in self-renewing B-1 lymphocytes[43]. EGR1 is regulated by the Interleukin-1-JNK-MKK7-c-Jun pathway and impedes interleukin-1-inducible tumor growth arrest[44]. Thus, EGR1 might play an important role in MCL cell cycle regulation as a JNK signaling pathway downstream target.

Our results show that there is a higher percentage of polyploid cells after treatment with SP600125 in MCL cell lines than in CLL cell lines. MCL cells can be divided into two major subtypes based on morphology: a common and a blastoid or pleomorphic variant. Blastoid MCL subtypes have a tendency to harbor chromosome numbers in the polyploid range, a feature clearly separating MCL from other types of B-cell lymphoma[9]. Many studies have demonstrated that basal JNK activity is very important to maintain a diploid state[37, 45-47]. Our results are in agreement with these findings. Our results also showed that polyploidization is irreversible after depletion of SP600125 treatment and that these cells have a slower growth rate.

Although polyploid MCL cells (treated with SP600125 2 days) had a slower growth rate than the original cells, they were more resistant to BCL-2 inhibitor treatment. A recent publication showed resistance of polyploid cancer cells against DNA-damaging agents such as cisplatin, gamma- and UVC-irradiation[23]. Polyploidization may be due to prolongation of mitotic progression. This might be caused by inefficient JNK mediated phosphorylation and in consequence sustained activity of BCL-2 during this phase of the cell cycle[48]. A recent report[49] showed that BCL-2 can suppress general DSB repair and V(D)J recombination by inhibiting the nonhomologous end-joining pathway and lead to an accumulation of DNA damage and genetic instability. Our results demonstrated that the small molecule BCL-2 inhibitor YC137[19] led to complete inhibition of SP600125-induced endoreduplication in 4 MCL cell lines. These results are in line with recent results showing that SP600125 treatment of human leukemia cell line U937 and in particular U937/BCL-2 cells (cells with ectopic BCL-2 expression) led to G2/M phase arrest and endoreduplication, which could be reverted by simultaneous treatment with the BCL-2 inhibitor HA14-1[22].

In summary, our data in MCL cell lines suggest that JNK expression is mediated via CD40-CD40L interaction and that JNK plays an essential role in stabilizing G2/M phase progression. Together with high BCL-2 expression JNK inhibition can induce G2/M
phase arrest as well as high levels of endoreduplication, mimicking the tetraploid state of a subset of MCL cases.

References:


Chapter 4


Hideshima T, Hayashi T, Chauhan D, Akiyama M, Richardson P, Anderson K. Biologic sequelae of c-Jun NH2-terminal kinase (JNK) activation in multiple myeloma cell lines. Oncogene 0 AD;22(54):8797-801.

Mingo-Sion AM, Marietta PM, Koller E, Wolf DM, Van Den Berg CL. Inhibition of JNK reduces G2/M transit independent of p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells. Oncogene 0 AD;23(2):596-604.


Supplementary data

Pixel density of the MAPK array for tonsil and MCL cases

<table>
<thead>
<tr>
<th></th>
<th>ERK1</th>
<th>ERK2</th>
<th>JNK1</th>
<th>JNK2</th>
<th>JNK pan</th>
<th>p38 alpha</th>
<th>p38 delta</th>
<th>p38 gamma</th>
<th>RSK1</th>
<th>RSK beta</th>
<th>GSK3A/B</th>
<th>GSK3B</th>
<th>AKT1</th>
<th>AKT2</th>
<th>AKT3</th>
<th>Akt pan</th>
<th>HSP27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonsil 1</td>
<td>145.47</td>
<td>223.50</td>
<td>331.78</td>
<td>938.05</td>
<td>NO</td>
<td>486.02</td>
<td>9740.67</td>
<td>1316.82</td>
<td>2577.09</td>
<td>NO</td>
<td>1171.65</td>
<td>2193.29</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Tonsil 2</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>1030.00</td>
<td>NO</td>
<td>300.00</td>
<td>11919.75</td>
<td>3171.99</td>
<td>3057.06</td>
<td>NO</td>
<td>2907.65</td>
<td>3579.68</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>MCL1</td>
<td>NO</td>
<td>NO</td>
<td>2075.10</td>
<td>3291.15</td>
<td>NO</td>
<td>5890.64</td>
<td>12545.50</td>
<td>2867.13</td>
<td>4579.54</td>
<td>3000.26</td>
<td>7123.68</td>
<td>8292.26</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>2877.11</td>
</tr>
<tr>
<td>MCL2</td>
<td>185.85</td>
<td>NO</td>
<td>NO</td>
<td>4893.64</td>
<td>NO</td>
<td>4337.74</td>
<td>9158.15</td>
<td>1328.99</td>
<td>2304.87</td>
<td>6254.90</td>
<td>7699.96</td>
<td>1048.10</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>1395.91</td>
</tr>
<tr>
<td>MCL3</td>
<td>14079.47</td>
<td>13774.91</td>
<td>4465.52</td>
<td>5185.33</td>
<td>4495.16</td>
<td>4513.00</td>
<td>13816.65</td>
<td>4807.58</td>
<td>10858.49</td>
<td>4744.67</td>
<td>4757.38</td>
<td>4762.48</td>
<td>NO</td>
<td>5443.31</td>
<td>8051.85</td>
<td>NO</td>
<td>6594.70</td>
</tr>
</tbody>
</table>

1. NO: no detectable signal after the film exposure
2. The number means the average pixel density of phospho-MAPK array data (in duplicate) for purified B cells from 2 hyperplastic tonsils and 3 MCL samples.