Targeting Mcl-1 for multiple myeloma (MM) therapy: Drug-induced generation of Mcl-1 fragment Mcl-1\(^{128-350}\) triggers MM cell death via c-Jun upregulation

Fengjuan Fan\(^a\), Giovanni Tonon\(^b\), Muhammad Hasan Bashari\(^a\), Sonia Vallet\(^a\), Elena Antonini\(^b\), Hartmut Goldschmidt\(^d\), Henning Schulze-Bergkamen\(^a\), Joseph T. Opferman\(^e\), Martin Sattler\(^c\), Kenneth C. Anderson\(^c\), Dirk Jäger\(^a\), Klaus Podar\(^a,\ast\)

\(^a\) Medical Oncology, National Center for Tumor Diseases (NCT), University of Heidelberg and German Cancer Research Center (DKFZ), Heidelberg, Germany
\(^b\) Functional Genomics of Cancer Unit, Division of Molecular Oncology, San Raffaele Scientific Institute, Milan, Italy
\(^c\) Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA
\(^d\) Section Multiple Myeloma, Department of Internal Medicine V, National Center for Tumor Diseases (NCT), University of Heidelberg, Heidelberg, Germany
\(^e\) St. Jude Children’s Research Hospital, Memphis, TN, USA

**A R T I C L E  I N F O**

Article history:
Received 27 July 2013
Accepted 27 September 2013

Keywords:
Mcl-1
c-Jun
Multiple myeloma
Apoptosis
MEFs
Glioblastoma

**A B S T R A C T**

Myeloid cell leukemia-1 (Mcl-1, HGNC: 6943), a pro-survival member of the Bcl-2 family, plays a crucial role in Multiple Myeloma (MM) pathogenesis and drug resistance, thus representing a promising therapeutic target in MM. A novel strategy to inhibit Mcl-1 activity is the induction of ubiquitin-independent Mcl-1 degradation. Our own and other previous studies have demonstrated caspase-dependent generation of a 28 kDa Mcl-1 fragment, Mcl-1\(^{128-350}\), which inhibits MM cell proliferation and survival. Here, we show that similar to bortezomib, the novel proteasome inhibitors carfilzomib and ixazomib, as well as staurosporine and adaphostin, induce the generation of Mcl-1\(^{128-350}\) in MM cells. Next, the molecular sequelae downstream of Mcl-1\(^{128-350}\), which mediate its pro-apoptotic activity, were delineated. Surprisingly, we observed nuclear accumulation of drug-induced or exogenously overexpressed Mcl-1\(^{128-350}\), followed by elevated mRNA and protein levels of c-Jun, as well as enhanced AP-1 reporter activity. Moreover, drug-induced AP-1 activity was blocked after introducing a point mutation into the highly conserved Mcl-1 caspase-cleavage site Asp127, but not Asp157. Consequently, drug-triggered cell death was significantly decreased in MM cells transfected with Mcl-1 D127A, but not Mcl-1 D157A. Consistent with these data, treatment with bortezomib triggered c-Jun upregulation followed by apoptosis in Mcl-1\(^{wt}\)/wt but not Mcl-1\(^{A\ast\ast}\) murine embryonic fibroblasts (MEFs). Transfection of a plasmid carrying Mcl-1\(^{wt}\) into Mcl-1\(^{A\ast\ast}\)Murine MEFs restored bortezomib-induced Mcl-1 fragmentation, c-Jun upregulation and AP-1 reporter activity. Finally, our data indicate that drug-induced generation of a pro-apoptotic Mcl-1 fragment followed by c-Jun upregulation may also be a novel therapeutic approach in other tumor entities.

© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Multiple myeloma (MM), the second most common blood cancer in adults, is characterized by bone marrow (BM) plasmacytosis, monoclonal protein in blood and/or urine, bone lesions, renal compromise, and immunodeficiency. Despite unprecedented advances in our understanding of disease pathogenesis and treatment during the last decade, median overall survival of MM patients remains at 7–8 years. Therefore, the identification and validation of targets for novel therapeutic approaches is urgently needed.

In tumor cells, the finely tuned homeostatic balance between cell growth and cell death is disturbed. Deregulated expression, degradation and function of anti-apoptotic and pro-apoptotic members of the B-cell lymphoma-2 (Bcl-2) family have been
implicated in the development of virtually all hematologic and solid malignancies [1]. Myeloid cell leukemia-1 (MCL-1) gene, located at chromosome 1q21, encodes a strong anti-apoptotic protein of the Bcl-2 family. Functionally, Mcl-1 is involved in cell homeostasis and differentiation; in the development and maintenance of B and T cells; in the survival of hematopoietic stem cells [2–4]; and in the maintenance of long-lived plasma cells in their BM niche [5]. Structurally, Mcl-1 is unique among the Bcl-2 family. As other anti-apoptotic Bcl-2 family proteins, Mcl-1 contains Bcl-2 homology regions ( BH 1–4 domains) and a transmembrane domain, which confer the ability to heterodimerize with other family members (e.g. pro-apoptotic proteins Bak and Bax) or to bind to membranes (e.g. cell membrane, mitochondrial membrane, nuclear envelope). Mcl-1 differs from its pro-survival family members in its larger size of 350 residues due to the N-terminal presence of two weak and two strong polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST domain). The PEST domain is rich in regulatory residues and motifs including sites for cleavage, ubiquitination and phosphorylation. Dependent on cellular conditions such as the presence of growth factors and cytokines or the interaction with other cells, these residues and motifs are responsible for Mcl-1 stability, localization, dimerization, and thereby for its function [6].

Caspase-induced Mcl-1 cleavage occurs at two distinct, highly conserved sites, Asp127 and Asp157. Mcl-1 cleavage at Asp127 results in the generation of two fragments, 28 kDa Mcl-1128–350 and 17 kDa Mcl-111–127; whereas cleavage at Asp157 results in 23 kDa Mcl-1158–350 and 21 kDa Mcl-1151–157 cleaved products [7–9]. The two C-terminal Mcl-1-cleaved products, Mcl-1128–350 and Mcl-1158–350, have lost the N-terminal BH4 domain and are structurally similar to Bax. Besides impairing the ability of Mcl-1 to sequester pro-apoptotic proteins (e.g. Bax, Bak and Bim) and thereby to promote survival, caspase-induced cleavage converts Mcl-1 to a pro-apoptotic protein [9–11]. Taken together, Mcl-1 cleavage is an important process by which cell viability may be rapidly regulated. Induction of Mcl-1 cleavage is therefore a promising novel strategy for MM therapy [12].

In MM, Mcl-1 plays a key role in tumor cell survival and drug resistance [13–17]. Indeed, Mcl-1 is among the significantly up-regulated molecules in MM versus normal plasma cells in an identical genotypic background [18]. Increasing data emerge on regulation and mechanistic sequelae of Mcl-1 in MM [19]. Based on these findings, Mcl-1 becomes a promising target for MM therapy. Preclinical approaches to deplete Mcl-1 in MM cells include targeting the ubiquitin-dependent pathway (e.g. deubiquitinase USP9X inhibitors) [20]; BH-3 mimetics (e.g. obatoclax, CX015-070) [21]; the galec-tin-3 antagonist GCS-100 [22]; and overexpression of microRNA-29b [23,24]. Several ongoing clinical trials evaluate the activity of obatoclax in a variety of solid and hematologic malignancies including MM (http://www.clinicaltrials.gov/).

Our own and previous studies have shown that bortezomib-induced generation of Mcl-1 fragment Mcl-1128–350, but not Mcl-111–157, triggers Bax-dependent apoptosis in MM cells [11,25,26]. However, exact molecular mechanisms by which pro-apoptotic Mcl-1128–350 triggers MM cell death are unknown. Here, we show that a number of preclinical and clinical compounds induce generation of Mcl-1128–350 in MM cells. Functionally, the particular novelty of our data is the demonstration of a new molecular mechanism by which pro-apoptotic Mcl-1128–350 triggers upregulation of c-Jun mRNA and protein levels, followed by increased AP-1 activity in MM cells. Importantly, our data suggest that this mechanism is operable not only in MM but also in other hematologic and solid malignancies, which express high levels of Mcl-1, gliblastoma in particular.

2. Materials and methods

2.1. Materials

Bortezomib, ixazomib (MLN9708), carfilzomib, and adaphostin (NSC 680410), the adamatyl ester analogue of the tyrophaoin AGC957 were purchased from Selleck Chemicals; staurosporine was from Sigma; MG-132 was from Merck. Antibodies against human Mcl-1 (S-19), c-Jun (H-79), extracellular signal-regulated kinase 2 (ERK2) were from Santa Cruz Biotechnology; antibodies against histone deacetylase 1 (HDAC1), and PARP were from Cell Signaling Technology; anti-γ-tubulin was from Sigma; anti-murine Mcl-1 was from Rockland Immunochemicals; Alexa Fluor 488 goat anti-rabbit IgG was from Molecular Probes. The anti-1 (S-19) antibody, raised against a peptide mapping amino acid residues 121–138 of human Mcl-1, is able to detect full length Mcl-1, as well as the fragments Mcl-1128–350 and Mcl-11128–350 [7,10].

2.2. Cell culture

All human MM cell lines as well as K-562, HT-29 and ACHN cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories), 1% penicillin/streptomycin and 2 mM L-glutamine (all from Gibco). T98G, U-87 MG, Hela and 786-0 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 2 mM L-glutamine. MEM cell lines Mcl-11127 and Mcl-11128 were generated by SV40 large T transfection followed by Tat-Cre-mediated deletion. Single cell clones were selected and then grown in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 2-mercaptoethanol (Sigma) and Minimum Essential Medium (MEM) non-essential amino acids (Gibco) from early passages. Both Mcl-11127 and Mcl-11128 were extensively characterized as being hypersensitive to various death stimuli with restorable resistance upon re-expression of Mcl-1 [4].

2.3. Plasmids and cell transfection

Human and murine Mcl-1 expression plasmids pcDNA3.1-hMcl-1 and pcDNA3-HA-mMcl-1 [27, 35-AP-1 reporter [28] were obtained from Addgene. Mcl-1128–350 was a kind gift from Dr. Graham Packman (University of Southampton, UK).

For the generation of Mcl-1 DI27A and Mcl-1 DI57A mutants, Asp127 and Asp157 were individually mutated to alanines using GeneArt Site-Directed Mutagenesis System (Invitrogen) with pcDNA3.1-hMcl-1 as the template following the manufacturer’s instructions. The primers used for Mcl-1 DI27A were 5′-GAGACGGCACCTCCTCACCTCG-3′ and 5′-TGTTTAAGCTGTGCCACCTGTT-3′. The primers for human c-Jun were 5′-ACTCGGACCTCCTCACCTCG-3′ and 5′-ATCTCTCGGTACCTTCGGGAGC-3′. The mutated Mcl-1 constructs were verified by sequencing.

MM1S cells were transiently transfected with indicated plasmids using the Amaxa Cell Line Nucleofector Kit V (Lonza) according to the manufacturer’s instructions. Mcl-11127 and Mcl-11128 MEFs were transiently transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen).

2.4. Cell lysis, fractionation and western blot analysis

Whole cell lysates were prepared in Fracleton’s lysis buffer (10 mM Tris-Cl, 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, pH 7.05) supplied with Halt Protease and Phosphatase Inhibitor Cocktail (Pierce), Extracts of cytoplasmic and nuclear fractions were prepared using Nuclear Extract Kit (Active Motif) according to the manufacturer’s instructions. Western blot analysis was performed as previously described [11].

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was purified with RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions, then reverse transcribed and synthesized to complementary DNA (cDNA) using Omniscript reverse transcriptase (Qiagen). PCR amplification was performed using Taq DNA polymerase (Qiagen). The primers used to amplify human Mcl-1 were 5′-ATCTCTCGGTACCTCCTCACCTCG-3′ and 5′-CTTGTAGCTGGAGGGCGAC-3′. The primers for human c-Jun were 5′-ATCCTGGACCCTCCTCACCTCG-3′ and 5′-TGTTTAAGCTGTGCCACCTGTT-3′. The primers for the human 18S RNA were 5′-GACCCAACCGAGATGGAGCA-3′ and 5′-TAGTACGACGCGGGCTGTG-3′. cDNA samples were also analyzed by quantitative real-time PCR using QuantFast SYBR Green PCR kit (Qiagen) with primers: 5′-GACCATCACCCGGACCTT-3′ and 5′-TGGTATGCTACGGTTCG-3′ for human Mcl-1, 5′-CTCAATGCGCCGAAAAGAGACGAC-3′ and 5′-CACTGTTCCCTTGAGACG-3′ for human c-Jun, 5′-TGCTGTCATCTGGTTGATGTATCT-3′ and 5′-TCTCTGCTGCCACCTCATTAGT-3′ for human p-2-microglobulin.
2.6. Luciferase assay

Cells were transiently transfected with 3×AP-1 reporter or pGL2-basic vector, together with pRL-CMV Renilla luciferase reporter as an internal control. In some experiments, cells were co-transfected with plasmids expressing c-Jun, Mcl-1128–350, Mcl-1 wild-type or its mutants. After the indicated treatment, cells lysates were prepared and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) with a single tube luminometer (Berthold Detection System) according to the manufacturer’s instructions. The firefly luciferase relative light units (RLU) were normalized to Renilla luciferase RLU.

2.7. Immunofluorescence

MM.1S cells treated with or without bortezomib were collected and 3×10⁴ cells in 100 μl medium were attached on glass slides by centrifugation at 300 rpm for 5 min using a Shandon Cytospin Cytocentrifuge (Thermo Scientific). Then cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with primary and secondary antibodies, sequentially. Slides were counterstained with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories). Immunofluorescence was observed using a Zeiss Axioplan fluorescence microscope with a 40×/0.75 numerical aperture objective. Images were captured by a CCD camera with Axiovision software (Carl Zeiss Microscopy). After data acquisition, images were processed using ImageJ software (NIH).

2.8. Apoptosis assay

Cells were treated as indicated, then washed with PBS and co-stained with FITC-labeled Annexin V and propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) following the manufacturer’s instructions. Apoptosis was analyzed on a FACSCanto II flow cytometer (BD Biosciences).

2.9. Statistics

Statistical analysis was done using the Student’s t-test. P-values are indicated by asterisks (∗∗ P < 0.01, ∗ P < 0.05).

Fig. 1. Drug-induced generation of Mcl-1 fragment Mcl-1128–350 correlates with c-Jun upregulation. (a) mRNA levels of Mcl-1 in MM cells. Total RNA was prepared from indicated human MM cell lines and used in reverse transcription-polymerase chain reaction (RT-PCR) to determine the relative expression level of Mcl-1 with 18S rRNA as a control. (b) Protein levels of Mcl-1 in MM cells. Cell lysates were prepared from the indicated MM cells. Aliquots of cell extracts (40 μg) were used in western blot with antibodies against Mcl-1. Tubulin served as a loading control. (c) MM.1S cells were treated with 5 μM melphalan (Mel), 100 nM doxorubicin (Dox), 100 μM thalidomide (Thal), 5 μM adaphostin (Ada), 500 nM staurosporine (STS), 10 nM bortezomib (Bort), or left untreated (control, Ctrl). After 24 h, cell lysate was immunoblotted with indicated antibodies. Arrow indicates the position of the 28 kDa Mcl-1 fragment. (d) Bortezomib-induced generation of Mcl-1128–350 is associated with c-Jun upregulation in different MM cell lines. Indicated MM cell lines were treated with or without bortezomib for 24 h, followed by immunoblot analysis of the lysates for the expression of Mcl-1 and c-Jun. Tubulin was used as a loading control. (e) Induction of c-Jun mRNA expression by drug treatment. Total RNA was purified from MM.1S cells treated as described in (c). The relative expression levels of Mcl-1 and c-Jun were analyzed by RT-PCR with 18S rRNA as a control. For Mcl-1, the 444 bp product corresponds to the full-length transcript, while the smaller product (196 bp) represents the short alternative splice variant [48]. (f) Quantitative PCR (qPCR) analysis of Mcl-1 and c-Jun mRNA levels in samples from (e). Beta-2-microglobulin served as an endogenous control. The symbols of * and ** indicate P < 0.05 and P < 0.01, respectively. (g) Other proteasome inhibitors that trigger Mcl-1128–350 generation and c-Jun upregulation. MM.1S cells were treated with carfilzomib (Carf), ixazomib (Ixaz), MG-132 (MG), or left untreated. After 24 h, total cell lysate was immunoblotted with Mcl-1, c-Jun and Tubulin.
3. Results

3.1. Drug-induced generation of Mcl-1\(^{128–350}\) correlates with c-Jun upregulation

Numerous preclinical studies have indicated the requirement of anti-apoptotic protein Mcl-1 for MM cell survival. Here, we examined the expression of Mcl-1 in six human MM cell lines (RPMI 8226, DOX40, U266, OPM2, MM.1S, MM.1R) and confirmed high levels of Mcl-1 mRNA (Fig. 1a) and protein (Fig. 1b) in MM cells. Several anti-MM drugs have been reported to downregulate expression levels of full length Mcl-1 via a ubiquitin-dependent pathway, thereby inducing apoptosis. Our own and other previous data have demonstrated the ability of bortezomib to induce caspase-dependent generation of pro-apoptotic Mcl-1\(^{128–350}\) fragment [11,25]. To identify additional compounds which are able to trigger generation of Mcl-1\(^{128–350}\) cleaved protein in MM cells, we tested a number of preclinical and clinical compounds including melphalan, doxorubicin, thalidomide, adaphostin, bortezomib, and staurosporine. All drugs investigated induced MM cell apoptosis (data not shown). However, generation of Mcl-1\(^{128–350}\) was only observed upon treatment with bortezomib, staurosporine and adaphostin (Fig. 1c).

We next sought to determine the downstream molecular sequelae which trigger anti-MM effects of Mcl-1\(^{128–350}\). Based on our previous studies which have shown that upregulation of c-Jun in MM cells inhibits proliferation and induces apoptosis, we investigated whether generation of Mcl-1\(^{128–350}\) is associated with the pro-apoptotic function of c-Jun [29]. Indeed, drug-induced generation of Mcl-1\(^{128–350}\) correlated with c-Jun protein upregulation in MM cell lines MM.1S (Fig. 1c), RPMI 8226, DOX40, U266, OPM2, and MM.1R (Fig. 1d). Consistent with these data, RT-PCR analysis showed increased levels of c-Jun mRNA in MM cells treated with adaphostin, staurosporine and bortezomib, but not melphalan, doxorubicin and thalidomide (Fig. 1e). These results were further confirmed by quantitative real-time PCR (Fig. 1f). Interestingly, similar to bortezomib, the next generation proteasome-inhibitors carfilzomib and ixazomib, as well as the investigational proteasome inhibitor MG-132 also triggered generation of Mcl-1\(^{128–350}\) and upregulation of c-Jun protein (Fig. 1g).

![Fig. 2. Mcl-1\(^{128–350}\) triggers c-Jun upregulation and AP-1 transcriptional activity. (a) Induction of c-Jun mRNA by Mcl-1\(^{128–350}\) expression. MM.1S cells were transfected with plasmids encoding empty vector (pcDNA3), wild-type Mcl-1 (Mcl-1\(^{wt}\)), the Mcl-1 fragment (Mcl-1\(^{128–350}\)) or c-Jun. After 24 h, total RNA was purified and reverse transcribed to cDNA. Mcl-1 and c-Jun were detected by PCR, with 18S rRNA as a control. Note that the primer pair for Mcl-1 was able to amplify the cDNA region encoding the Mcl-1\(^{128–350}\). (b) Mcl-1\(^{128–350}\) triggers upregulation of c-Jun protein levels. MM.1S cells were transfected with pcDNA3, Mcl-1\(^{wt}\) or Mcl-1\(^{128–350}\). Cell lysates were immunoblotted with antibodies against Mcl-1 and c-Jun. ERK2 was used as a loading control (upper panel). Lower panel: densitometric analysis of Mcl-1 full length relative to ERK2 from three independent experiments. (c) MM.1S cells were transfected with empty vector or c-Jun. Cell lysates were immunoblotted with antibodies against Mcl-1 and c-Jun. ERK2 was used as a loading control. (d) Accumulation of Mcl-1 fragment in the nucleus is associated with nuclear c-Jun upregulation. MM.1S cells were transfected with pcDNA3 or Mcl-1\(^{128–350}\). Cytoplasmic and nuclear fractions were prepared and immunoblotted with antibodies as indicated. HDAC1 and Tubulin were used as purity controls of nuclear (HDAC1) and cytoplasmic fractions (Tubulin). (e) Mcl-1\(^{128–350}\), but not full length Mcl-1, induces AP-1 activity. MM.1S cells were transiently transfected with indicated constructs. Transfection mixes also contained a plasmid encoding Renilla luciferase for normalization. After 18 h, the whole cell lysate was analyzed by dual-luciferase reporter assay. Relative firefly luciferase reporter activity was determined as the percentage of the Renilla luciferase activity. The results were presented as mean ± standard deviation (SD) of triplicate samples. Insert panel: positive control for the AP-1 reporter luciferase assay using c-Jun expression plasmid.](image-url)
3.2. Mcl-1128–350 triggers c-jun upregulation and AP-1 transcriptional activity

In order to investigate the potential functional interrelation between Mcl-1128–350 and c-jun upregulation, we transfected MM.1S cells with plasmids carrying Mcl-1wt, Mcl-1128–350 or control vector and measured c-jun expression. The results showed increased levels of c-jun mRNA (Fig. 2a) as well as c-jun protein (Fig. 2b) in Mcl-1128–350-expressing MM cells. In contrast, overexpression of c-jun did not induce the generation of Mcl-1 fragment Mcl-1128–350 (Fig. 2c). Interestingly, we found that overexpressed Mcl-1128–350 but not full-length Mcl-1, accumulated in the nuclear fraction (Fig. 2d). These data suggest a role for Mcl-1128–350 in the regulation of c-jun transcription. Consequently, overexpression of Mcl-1128–350, but not Mcl-1wt, enhanced the transcriptional activity of c-jun in MM cells (Fig. 2e). AP-1 activity triggered by transfection of MM cells with c-jun expression plasmid served as a positive control (Fig. 2e, insert). Taken together, these data demonstrate for the first time a regulatory role of Mcl-1128–350 in AP-1 transcription and activity.

3.3. Drug-induced generation of Mcl-1128–350 triggers pro-apoptotic c-jun activity

Similar to exogenous Mcl-1128–350, drug-induced Mcl-1 fragment Mcl-1128–350 predominantly localized within the nucleus (Fig. 3a and b) and triggered transcriptional activity of c-jun (Fig. 3c).

To confirm a specific role of the Mcl-1 fragment Mcl-1128–350 in the induction of c-jun transcriptional activity, we generated constructs carrying Mcl-1 mutants D127A and D157A. Specifically, the highly conserved Mcl-1 caspase-cleavage sites Asp127 and Asp157 were individually mutated to alanines by site-directed mutagenesis with the primers indicated (Fig. 4a). Consistent with our hypothesis, bortezomib-triggered induction of c-jun activity was significantly inhibited in MM cells transfected with Mcl-1 D127A, but not with Mcl-1 D157A (Fig. 4b). Consequently, bortezomib-induced MM cell apoptosis was significantly decreased in Mcl-1 D127A-overexpressing MM cells (Fig. 4c). Taken together, these results demonstrate that drug-induced generation of Mcl-1128–350 enhances pro-apoptotic c-jun expression and activity.

3.4. Drug-induced generation of a pro-apoptotic Mcl-1 fragment might also be a novel therapeutic approach in other tumor entities

To further verify our findings, we next utilized wild-type (Mcl-1wt/wt) and Mcl-1-null (Mcl-1△null) murine embryonic fibroblasts (MEFs) [4]. Indeed, bortezomib triggered c-jun upregulation in Mcl-1wt/wt MEFs, but not in Mcl-1△null MEFs (Fig. 5a). Importantly, re-expression of wild-type murine Mcl-1 (mMcl-1wt) in Mcl-1△null MEFs restored the effect of bortezomib on c-jun expression (Fig. 5b). Similar to MM cells, bortezomib-induced Mcl-1128–350 was predominantly localized within the nuclear fraction (data not shown). Consistently, treatment with bortezomib significantly elevated AP-1 transcriptional activity in Mcl-1wt/wt, but not Mcl-1△null, MEFs (Fig. 5c). Moreover, when Mcl-1△null MEFs were transfected with a plasmid carrying mMcl-1wt, bortezomib-induced upregulation of AP-1 activity was rescued (Fig. 5d). The presence of the Mcl-1 fragment correlated with induction of MEF apoptosis (data not shown).

Mcl-1 overexpression has been extensively reported in hematologic cancers including MM, where it is also associated with poor prognosis (Suppl. Fig. 1). However, until recently its relevance in
other tumor types, we identified a pattern, consistent across several tissues when compared with their normal counterparts. Among them, an extensive survey of published datasets to uncover tumor types demonstrating a particularly strong overexpression of Mcl-1 in cancer types including epithelial cancers, suggesting that Mcl-1 deregulation exerts a major role not only in hematologic cancers, but also in solid cancers. Indeed, in neuroblastoma, the role of Mcl-1 as an anti-apoptotic cancer gene has been challenged [30]. Notwithstanding, a recent survey of copy number aberrations in a panel of 3,131 cancer specimens belonging to 26 histological malignancies [36–39], suggesting that tumor cells develop effective strategies to sustain the increased expression of Mcl-1. Indeed, a somatic mutation affecting USP9X, with the substitution of a lysine with an asparagine, has been recently reported in MM, although its functional impact remains to be ascertained [40]. Compounds targeting the proteasome-dependent pathway of Mcl-1 degradation may not be effective. Therefore, the induction of non-proteasomal degradation of Mcl-1 may represent an alternative to be explored for cancer therapy in general, and MM in particular. Indeed, sequencing results of Mcl-1 in 23 MM cell lines did not show the existence of point mutations in Mcl-1 caspase-cleavage sites, excluding a potential mechanism of drug resistance in MM [Suppl. Fig. 3].

Figure 4. Mutation of the Mcl-1 residue Asp127, but not Asp157, significantly decreases bortezomib-induced c-Jun upregulation and MM cell apoptosis. (a) Schematic representation of wild-type (wt) Mcl-1 and the mutants D127A, D157A. Numbers indicate amino-acid residues. The highly conserved Mcl-1 caspase-cleavage sites Asp127 and Asp157 were individually mutated to alanine by site-directed mutagenesis with the primers indicated. Mutations were verified by sequencing. (b) Drug-induced upregulation of AP-1 activity is significantly decreased after introduction of a point mutation into Mcl-1 caspase-cleavage site Asp127. MM.15 cells were transfected with 4 μg Mcl-1 wt, D127A or D157A, 4 μg AP-1 reporter and 0.8 μg Renilla luciferase vector, and then treated with bortezomib or left untreated. Whole cell lysates were analyzed by dual-luciferase reporter assay. The relative fold change of luciferase activity is shown as mean ± SD from three independent experiments. The luciferase activity in Mcl-1 wt group without drug treatment was set to 1. * Indicates P < 0.05 by Student’s t-test. (c) Drug-triggered MM cell apoptosis is significantly reduced after introduction of a point mutation into Mcl-1 caspase-cleavage site Asp127. MM.15 cells were transfected with Mcl-1 wt, D127A or D157A, and then treated with or without bortezomib. Cell apoptosis was analyzed by Annexin V/PI staining. The percentage of cells staining positive for Annexin V is shown as mean ± SD of three independent experiments. ** Indicates P < 0.01.

4. Discussion

Mcl-1 is required for long-term survival of plasma cells and intimately involved in MM pathogenesis and drug resistance. Based on these findings, Mcl-1 represents a promising therapeutic target in MM [12,34,35].

E3-ubiquitin ligases Mule, β-TrCP, and SCFβTrCP, as well as the deubiquitinase USP9X, are key factors, which regulate Mcl-1 ubiquitination and proteasome-dependent Mcl-1 degradation. Induction of proteasome-dependent Mcl-1 degradation has been investigated as a potential therapeutic strategy in cancer treatment including MM. However, mutational inactivation of enzymes associated with proteasomal Mcl-1 degradation occurs in a variety of malignancies [38–39], suggesting that tumor cells develop effective strategies to sustain the increased expression of Mcl-1. Indeed, a somatic mutation affecting USP9X, with the substitution of a lysine with an asparagine, has been recently reported in MM, although its functional impact remains to be ascertained [40]. Compounds targeting the proteasome-dependent pathway of Mcl-1 degradation may not be effective. Therefore, the induction of non-proteasomal degradation of Mcl-1 may represent an alternative to be explored for cancer therapy in general, and MM in particular. Indeed, sequencing results of Mcl-1 in 23 MM cell lines did not show the existence of point mutations in Mcl-1 caspase-cleavage sites, excluding a potential mechanism of drug resistance in MM [Suppl. Fig. 3].

Using a drug screen to identify Mcl-1128–350-generating agents in MM cells, our results show that, similar to bortezomib [11,25], staurosporine and adaphostin, as well as next-generation proteasome inhibitors carfilzomib and ixazomib, induce Mcl-1128–350 generation. One mechanism underlying the observation that proteasome inhibitors induce Mcl-1 fragmentation might be enhanced stabilization and accumulation of full-length anti-apoptotic Mcl-1 leading to generation of sufficient levels of pro-apoptotic Mcl-1128–350. Based on these data, we hypothesize that the anti-MM activity of proteasome inhibitors, at least in part, is mediated via generation of Mcl-1128–350. In ongoing studies, we are utilizing a large-scale drug screen to identify additional compounds which are able to induce Mcl-1128–350 generation.

Given that drug-induced generation of Mcl-1128–350 is a promising novel therapeutic approach in MM therapy, we next sought to decipher molecular sequela downstream of Mcl-1128–350 which mediate its pro-apoptotic effect. The pivotal functional role of subcellular Mcl-1 localization has been emphasized in a number of recent studies [1,41]. Surprisingly, we observed nuclear accumulation of drug-induced or exogenously transfected Mcl-1128–350 in MM cells. These results suggested a potential regulatory role of Mcl-1128–350 in gene transcription and cell cycle control in MM cells. Sequence analyses using both the PredictProtein server (https://www.predictprotein.org/) [42] and YLoc (http://abi.inf.
uni-tuebingen.de/Services/YLoc/webloc.cgi) [43] showed that full length Mcl-1 is located in the cytoplasm. Furthermore, the PredictProtein server failed to identify a classical nuclear localization signal (NLS) in Mcl-1 protein sequence, and the NetNES software (http://www.cbs.dtu.dk/services/NetNES/) [44] failed to find a nuclear export signal (NES) in the protein (data not shown). Therefore, our ongoing studies aim to identify proteins which interact with Mcl-1\textsubscript{128–350} to provide the necessary NLS function and facilitate its nuclear import in MM cells.

Functionally, our present data demonstrate that the generation of Mcl-1\textsubscript{128–350} induces marked elevation of c-Jun mRNA and protein levels, as well as enhanced AP-1 reporter activity. Consistent with these findings, drug-induced upregulation of pro-apoptotic c-Jun was blocked after introduction of a point mutation into the highly conserved Mcl-1 caspase-cleavage site Asp127. In contrast, introduction of a point mutation into the Mcl-1 cleavage site Asp157 did not decrease bortezomib-induced c-Jun upregulation, further emphasizing a key role of Mcl-1\textsubscript{128–350} in c-Jun transcription. However, the DNAbind web server (http://www.enzim.hu/~szia/dnabind.html) [45] predicted Mcl-1 as a non DNA-binding protein, based on its sequence. Thus, future studies will focus to further explore the functions of Mcl-1 fragment in the nucleus.

Although most studies identified c-Jun as an enhancer of proliferation, a tumor promoter and oncogene, recent studies show that c-Jun also mediates cell death either by acting as a transcriptional regulator of survival/death genes (e.g. Fas ligand, DP5, Bim, p14\textsubscript{ARF}/p19\textsubscript{ARF}, Dmp1) and as an inducer of DNA repair responses, or by triggering caspase-mediated cleavage of numerous molecules (e.g. fodrin, poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, and protein kinase C) [46]. More recently, the apoptosis-antagonizing transcription factor (AATF) has been identified as a nucleolar-confined c-Jun cofactor whose expression levels and spatial distribution determines the levels of c-Jun-mediated apoptosis [47]. Potential explanations for its opposing effects include cell type specificity, the availability of external and internal survival factors, and specific spectra of binding partners [46]. Exact molecular mechanisms conferring c-Jun-induced cell death in MM cells are still elusive and under active investigation.

To further support our findings, we examined effects of Mcl-1 fragment-generating drugs in Mcl-1\textsuperscript{wt/wt} MEFs and Mcl-1\textsuperscript{Δnull} MEFs [4]. Our results show that bortezomib significantly increased c-Jun protein levels as well as c-Jun transcriptional activity in Mcl-1\textsuperscript{wt/wt} but not Mcl-1\textsuperscript{Δnull} MEFs. These data suggest a potential therapeutic role for drug-induced generation of the Mcl-1

Fig. 5. Drug-induced generation of a pro-apoptotic Mcl-1 fragment might also be a novel therapeutic approach in other tumor entities. (a) Bortezomib triggers c-Jun upregulation in Mcl-1\textsuperscript{Δnull} MEFs, but not in Mcl-1\textsuperscript{wt/wt} MEFs. Mcl-1\textsuperscript{wt/wt} and Mcl-1\textsuperscript{Δnull} MEFs were cultured in the presence or absence of 10 nM bortezomib for 48 h. (b) Expression of mMcl-1\textsuperscript{wt} in Mcl-1\textsuperscript{Δnull} MEFs restores bortezomib-triggered c-Jun upregulation. Mcl-1\textsuperscript{Δnull} MEFs were transiently transfected with pcDNA3 or pcDNA3-HA-mMcl-1. After 12 h, cells were treated with or without 10 nM bortezomib for 48 h. Cell lysates were examined by immunoblot analysis with indicated antibodies. Tubulin served as a loading control (a and b). (c) Bortezomib induces AP-1 activity in Mcl-1\textsuperscript{wt/wt} MEFs, but not in Mcl-1\textsuperscript{Δnull} MEFs. Mcl-1\textsuperscript{wt/wt} and Mcl-1\textsuperscript{Δnull} MEFs were transfected with AP-1 reporter and Renilla luciferase vector. (d) Expression of mMcl-1\textsuperscript{wt} in Mcl-1\textsuperscript{Δnull} MEFs restores bortezomib-induced AP-1 activity. Mcl-1\textsuperscript{Δnull} MEFs were transfected with AP-1 reporter, Renilla luciferase vector, as well as pcDNA3 or pcDNA3-HA-mMcl-1. Transfections were performed in duplicate in a 12-well plate format. MEFs were treated with or without 10 nM bortezomib for 48 h. Whole cell lysates were used in a dual-luciferase assay, and the fold change of luciferase activity is shown as the mean ± SD. The relative luciferase activities without bortezomib treatment were set to 1, respectively. * Indicates P < 0.01 (c and d). (e) Upregulation of Mcl-1 in glioblastoma (GBM). Oncomine analysis of samples isolated from glioblastoma patients shows a significant upregulation of Mcl-1 expression when compared with expression values of samples isolated from healthy individuals (based on TCGA publicly available data: 515 patient samples versus 10 healthy controls, Affymetrix U133A array, probe set 200798_s_at). (f) Bortezomib-induced generation of Mcl-1\textsubscript{128–350} is associated with c-Jun upregulation in glioblastoma cell lines. T98G and U-87 MG cells were treated with or without bortezomib for 24 h, followed by immunoblot analysis of cell lysates with antibodies against Mcl-1 and c-Jun. Tubulin was used as a loading control.
fragment also in settings other than MM. Indeed, recent evidence from the literature points to a much wider role for Mcl-1 in carcinogenesis, including solid cancers as well as hematologic malignancies. Our own survey on published datasets identified several tumor cell lines including glioblastoma, chronic myelogenous leukemia, renal adenocarcinoma, colorectal adenocarcinoma, and renal adenocarcinoma.

In summary, the present study confirms Mcl-1 as a promising target in MM therapy. Specifically, our data propose a new therapeutic approach by which specific compounds induce proteasome-independent generation of the pro-apoptotic Mcl-1 fragment Mcl-1128–350, followed by c-Jun upregulation, increased AP-1 activity, and the induction of MM cell death (Fig. 6). Finally, our results suggest that this mechanism may be operative not only in MM, but also in other malignancies.

Conflict of Interest

All authors declare no conflicts of interest.

Acknowledgements

We thank Dr. G. Packham (University of Southampton School of Medicine, Southampton General Hospital, Southampton, UK) for kindly providing Mcl-1128–350 plasmid; and Drs. M. Trier and D. Bohmann for kindly providing c-Jun plasmid. K. Podar is a recipient of a B. Braun Stiftungs-Grant.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.09.042.

References

Q. Zhong, W. Gao, F. Du, X. Wang, Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis, Cell 121 (2005) 1085–1089.


