ABT-737 induces apoptosis in mantle cell lymphoma cells with a Bcl-2^{high}/Mcl-1^{low} profile and synergizes with other anti-neoplastic agents.

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TRANSLATIONAL RELEVANCE:

Prognosis of MCL is considered to be inferior to most of the other NHLs and new therapeutic approaches are warranted. The rational to target Bcl-2 proteins in MCL has been investigated using ABT-737, a BH3 mimetic that targets Bcl-2, Bcl-xl but not Mcl-1. We show that ABT-737 alone induce apoptosis only in Bcl-2<sup>high</sup>/Mcl-1<sup>low</sup> MCL cells. By the use of siMcl-1 transfection, we demonstrate the key role of Mcl-1 in ABT-737 resistance. To overcome Mcl-1 resistance in clinical practice, we investigated two different strategies using three different drugs in combination with ABT-737: (i) to down-regulate Mcl-1 expression using flavopiridol or ara-C and (ii) to neutralize Mcl-1 anti-apoptotic function by increasing Noxa with Bortezomib. Our investigations confirm the synergistic effect of these combinations in ABT-737-resistant cell lines. In conclusion, the present work brings the rationale for the use of ABT-737 alone or in combination according to the apoptotic profile of MCL cells.
ABSTRACT:

Purpose

Mantle cell lymphoma (MCL) is considered to be incurable. ABT-737 is a BH3 mimetic that targets Bcl-2, which is over-expressed in MCL and implicated in drug resistance. The present work investigated the anti-tumor effect of ABT-737.

Experimental Design

Six MCL cell lines and primary MCL cells (n=13) were used. Sensitivity to ABT-737 was assessed and expression levels of Bcl-2 and Mcl-1 were analyzed. Finally, ABT-737 was combined with other cytotoxic agents to promote tailored therapy.

Results

MINO and GRANTA-519 cell lines were highly sensitive to ABT-737 (LD$_{50}$ = 20 and 80 nM, respectively), whereas other cell lines were resistant. In primary MCL cells, 46% of patients’ samples were sensitive to ABT-737. Analysis of protein expression levels revealed that both sensitive cell lines and primary MCL cells are characterized by a Bcl-2$^{high}$/Mcl-1$^{low}$ profile, whereas resistant MCL cells contain high levels of Mcl-1. ABT-737 induced a rapid disruption of both Bcl-2/Bax and Bcl-2/Bik complexes. In addition, silencing of Mcl-1 by siRNA sensitized MCL cell lines to ABT-737. Similarly, flavopiridol which induces Mcl-1 down-regulation resulted in combination with ABT-737 in a synergistic anti-MCL effect in ABT-737 resistant cell lines. This synergy was also observed when ABT-737 was combined with either bortezomib or cytarabine.

Conclusions

The present work demonstrates that ABT-737 induces strong apoptosis in MCL cells expressing a Bcl-2$^{high}$/Mcl-1$^{low}$ profile. In ABT-737-resistant MCL cells, down-regulation of Mcl-1 overcomes Mcl-1-induced resistance and synergizes ABT-737 effects. Our results strongly support the use of ABT-737 according to the Bcl-2/Mcl-1 tumor cell profiles in the treatment of MCL.
INTRODUCTION

Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin’s lymphoma that accounts for approximately 5% of all NHLs (1). Despite recent improvements in therapy, most patients experience relapses and survive an average of approximately 5 years from the time of diagnosis (2). Intensive chemotherapy regimens combined with anti-CD20 antibodies with or without autologous stem cell transplantation are widely used but not well-tolerated in elderly or unfit patients. Indeed, there is an urgent need for new targeted molecular approaches using novel compounds. New insights into the pathogenesis of MCL tumor cells have recently opened windows of opportunity for innovative targeted therapies (3, 4). One of these new therapeutic approaches is to induce apoptosis via the intrinsic pathway and/or to overcome MCL cell resistance by antagonizing the anti-apoptotic Bcl-2 family proteins. This approach is prompted by several investigations demonstrating that the anti-apoptotic protein Bcl-2 is constitutively over-expressed in MCL and plays a major role in drug resistance (5, 6). These findings highlight the potential of Bcl-2-targeted therapy in MCL.

Members of the Bcl-2 family are critical regulators of apoptosis and the interactions between pro-survival and pro-apoptotic members are major determinant of cell fate (7). Structural and functional characteristics divide them into three subgroups: multidomain anti-apoptotic members (Mcl-1, Bcl-2, Bcl-xL, Bcl-W and A1), multidomain pro-apoptotic members (Bax and Bak) and BH3-only members (Bim, Bid, Bad, Bik, Puma and Noxa). In response to a wide range of derangement signals, BH3-only members are activated (8). Certain BH3-only proteins, including Bim and Bid, are called activators because of their ability to activate Bax and Bak (9). In contrast, the anti-apoptotic molecules antagonize cell death by sequestering either BH3-only proteins or multidomain pro-apoptotic members. The presence of Bax or Bak is required to mediate mitochondrial damage. First, these multi-domain pro-apoptotic family members have to be activated to oligomerize; subsequently, oligomers form pores in the mitochondrial outer membrane. The apoptotic cascade is frequently, if not always, altered in tumor cells, including MCL cells (3).

ABT-737 is a new compound that selectively binds with high affinity to Bcl-2 and Bcl-xL (10). ABT-737 displaces pro-apoptotic BH3-only from Bcl-2 or Bcl-xL, leading to activation of Bax and Bak and downstream caspases (9). Due to the low affinity of ABT-737 for Mcl-1, high basal levels of Mcl-1 have been associated with ABT-737 resistance (11-14). Previous
studies have also shown that ABT-737 is effective as a single agent against some leukemia/lymphoma cell lines both in vitro and in vivo (11, 15-17).

This present study was focused on the anti-tumoral effect of ABT-737 in MCL. We also aimed to identify prognostic biomarkers that may predict MCL tumor cell responses to ABT-737. Using this approach, we have developed a rationale for ABT-737-based tailored therapeutic strategies in MCL.
DESIGN AND METHODS:
MCL cell lines and primary MCL cells.

JEKO-1, MINO, REC-1, GRANTA-519 and JVM-2 MCL cell lines were purchased from DSMZ (Braunschweig, Germany). The UPN-1 MCL cell line was kindly provided by Dr. V. Ribrag (IRG, Villejuif, France). JEKO-1, MINO, REC-1, GRANTA-519 and JVM-2 were maintained in RPMI-1640 medium supplemented with 10 % FCS and 2 mM glutamine. UPN-1 was maintained in MEM α medium supplemented with 10 % FCS and 2 mM glutamine. Primary MCL cells were obtained from patients treated in our institution who had provided their written informed consent and been diagnosed with de novo or relapsed MCL; diagnosis was confirmed according to the WHO classification (18). MCL cells from blood or ascites were obtained following gradient density centrifugation using Ficoll-hypaque. For Western blotting analysis, MCL cells were purified with CD19-immuno-magnetic beads.

Antibodies (mAbs) and reagents.

Antibodies used in this study were: Anti-Bcl-2 (clone 124, Dako), Anti-Mcl-1 (S19, Santa Cruz Biotechnology) anti-caspase 3 (E-8, Santa Cruz Biotechnology), anti-Bik (N19, Santa Cruz Biotechnology), anti-Bcl-xL (BD Biosciences), anti-Actin (Chemicon), anti-Noxa (Alexis Coger), anti-Bax (clone 4F11, Immunotech), anti-Bim (Millipore), anti-Puma (Ab-1, Calbiochem), ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL, USA). Flavopiridol and cytarabine-arabinoside were obtained from Sigma Aldrich. Bortezomib was kindly provided by Millenium Laboratory (Raleigh, NC, USA).

Flow cytometric analysis of apoptosis and Bax/Bak activation.

Cell death in MCL cell lines was assessed by Apo-2.7 staining. Cell death in primary MCL cells was assessed by both Apo-2.7 and CD19 staining combined with an analysis of altered cellular morphology (lower FSC). Flow cytometry analysis was performed on a FACSCalibur using Cell Quest software (Becton Dickinson, San Jose, CA, USA). For Bax and Bak immunofluorescence staining, 5x10^5 cells were fixed using the Intra Prep Permeabilisation Reagent Kit (Immunotech) following the manufacturer’s recommendations. The cells were incubated with anti-Bax (clone 6A7) mAb, anti-Bak (BD Biosciences, Pharmigen) or IgG1 isotype control for 20 min. The cells were then incubated with anti-mouse- or anti-rabbit-FITC antibodies (Immunotech) for 20 min, washed once in PBS and resuspended in PBS-1% formaldehyde. The flow cytometry analysis was performed as above.
Immunoprecipitation and Immunoblotting.

Cells (10x10^6) were lysed in 1% CHAPS containing lysis buffer. Whole cell lysates were obtained, pre-cleared with Protein A-sepharose and incubated overnight with 10 μg of the specific antibody. Immunocomplexes were captured with either protein A-sepharose. The beads were pelleted, washed three times and boiled in SDS sample buffer. The presence of immunocomplexes was determined by western blot analysis. Western blotting analysis were performed as previously described (19).

RNA isolation and quantitative real-time PCR.

RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). Two micrograms of total RNA was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers (Amersham Biosciences). Quantitative PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) in a MX4000 instrument (Stratagene). BCL2 (Hs00608023_m1), MCL1 (Hs00172036_m1) and RPL37a (Hs01102345_m1) TaqMan gene expression assays were purchased from Applied Biosystems. The thermal cycling parameters used consisted of 50°C for 2 min for optimal AmpErase UNG activity, then 40 cycles of 95°C for 30 s and 60°C for 1 min. To control specificity of the amplified product, a melting curve analysis was performed. No amplification of nonspecific product was observed. Amplification of the housekeeping gene RPL37a was conducted for each sample as an endogenous control.

siRNA

Mcl-1 and control siRNAs duplexes used were ON-TARGET plus siRNA pools of four oligos purchased from Dharmacon. One hundred pmol of siRNA were mixed with 10^6 cells and electropfected using the Amaxa Nucleofector™ apparatus (Amaxa, Cologne Germany). Two days after transfection, the cells were incubated for 24h with ABT-737. The gene-silencing effect was evaluated by Western blot analysis.
RESULTS:

Sensitivity of MCL cell lines to ABT-737

The sensitivity of MCL cell lines to ABT-737 was investigated in normal culture conditions. After a 24-hour treatment, cell death was assessed using APO 2.7 staining (Fig 1A). MINO and GRANTA-519 were highly sensitive to ABT-737 with a lethal dose (LD)\textsubscript{50} of 25 and 80 nM, respectively. In contrast, the four other MCL cell lines (JEKO-1, REC-1, UPN-1 and JVM-2) were resistant to ABT-737 (LD\textsubscript{50}> 8 µM). To determine the pathway by which ABT-737 induces apoptosis, the activation of caspases 9 and 3 was investigated. Cleavage of caspases 3 and 9 was detectable within 2 hours following treatment and was coupled to Bax and Bak activation demonstrating that apoptosis occurs via the intrinsic apoptotic pathway (Fig 1B and 1C). Because ABT-737 selectively binds to anti-apoptotic Bcl-2 and Bcl-x\textsubscript{L} but not Mcl-1, the respective expression level of these proteins was investigated by Western blotting analysis (Fig 1 D). MINO and GRANTA-519 both showed high expression of Bcl-2 but low levels of Mcl-1 (a so-called Bcl-2\textsuperscript{high}/Mcl-1\textsuperscript{low} profile). Interestingly, resistant MCL cell lines (JEKO-1, REC-1, UPN-1 and JVM-2) all expressed high Mcl-1 levels. In contrast, the level of Bcl-x\textsubscript{L} did not appear to be related to ABT-737 sensitivity. To confirm the correlation between sensitivity to ABT-737 and the Bcl-2/Mcl-1 profile, the mRNA levels of Bcl-2 and Mcl-1 for the six MCL cell lines using quantitative RT-PCR were determined (Fig 1E). As observed at the protein level, sensitive MCL cell lines had high Bcl-2/Mcl-1 mRNA ratios (3.4 and 10.2 for MINO and GRANTA-519, respectively), whereas resistant MCL cell lines had low Bcl-2/Mcl-1 mRNA ratios (≤1).

Sensitivity of primary MCL cells to ABT-737

ABT-737 induced apoptosis was also evaluated in primary MCL tumor cells from 13 patients. Characteristics of patients are summarized in Supplemental Table. After 24 hours of ABT-737 treatment, cell death was quantified in the MCL cell compartment (CD19 positive cells) and a combined analysis of altered cellular morphology (lower FSC) was also performed (Fig 2A). ABT-737 induced apoptosis in MCL cells in 6 samples (with LD\textsubscript{50}s ranging from 15 to 150 nM), whereas 7 samples were classified as resistant to ABT-737 (median LD\textsubscript{50} not reached). In 8 samples, the number of MCL cells was sufficient to evaluate Bcl-2 and Mcl-1 expression by Western blotting (Fig 2B). As observed above in MCL cell lines, only Bcl-2\textsuperscript{high}/Mcl-1\textsuperscript{low}
primary tumor cells were sensitive to ABT-737 and a high expression of Mcl-1 was consistently associated with resistance to ABT-737.

**ABT-737 triggered a rapid disruption of Bcl-2/ Bax and Bcl-2/Bik complexes**

To determine the mechanism of action of ABT-737, the constitution of Bcl-2 complexes and their dynamic upon short time ABT-737 treatment were examined. For this purpose, Bcl-2 immunoprecipitations were realized after 2 hours ABT-737 treatment in the two sensitive cell lines. In untreated GRANTA-519 cells, Bcl-2 was associated with both the effector protein Bax and the BH3-only proteins Bim, Puma and Bik (Fig 3). In contrast to GRANTA-519, MINO does not express Bim. In MINO, endogenous Bcl-2 was mainly associated with Bax, Puma and Bik. Of note, in both cells no interaction between Bcl-2 and Bak was found (result not shown). In GRANTA-519 cells, an examination of Bcl-2 heterodimers after a two-hours ABT-737 treatment showed a strong decrease of Bcl-2/Bax complexes and a very weaker decrease of both Bcl-2/Bik and Bcl-2/Puma complexes. Consistent with the strong decrease of Bcl-2/Bax complexes, there was an increase of Bax in the immunoprecipitate supernatant (Fig 3). In MINO treated by ABT-737, an important decrease of both Bcl-2/Bax and Bcl-2/Bik complexes were observed. Similarly to GRANTA-519, a weak decrease of Bcl-2/Puma complex was found. Finally, while no disruption of the Bcl-2/Bim complex was observed after short time ABT-737 treatment in both cell lines, a longer treatment of eight hours led to a partial disruption of Bcl-2/Bim complexes (data not shown). This last result is in agreement with the fact that Bim displayed the highest affinity for Bcl-2.

**Mcl-1 protects MCL cells against ABT-737-induced apoptosis**

In MCL cells, a high level of anti-apoptotic protein Mcl-1 was observed in resistant cells, suggesting that Mcl-1 expression level might be associated with ABT-737 resistance. To investigate the specific role of Mcl-1 in ABT-737 resistance, Mcl-1 was silencing using RNAi technology. After 48 hours of transfection with siMcl-1, a marked reduction of Mcl-1 level was observed (Fig 4A). The knockdown of Mcl-1 sensitized both JEKO and JVM2 cells to ABT-737 demonstrating that Mcl-1 level plays a major role in resistance to this drug (Fig 4B). Thus, this result underscores the interest of drug combinations that downregulate Mcl-1 expression.
Combination of ABT-737 and flavopiridol overcomes the Mcl-1 associated resistance of MCL cells to ABT-737

Flavopiridol, a semisynthetic flavonoid, is known to inhibit mRNA transcription by inhibiting kinases that activate RNA polymerase II (20). Inhibition of Mcl-1 transcription by flavopiridol has been reported in chronic lymphoid leukemia (CLL) and multiple myeloma (MM) cells (21, 22). ABT-737 resistant MCL cell lines were cultured with flavopiridol (250 nM) for 24 hours. A drastic diminution of Mcl-1 protein level was observed, whereas neither Bcl-2 nor Bcl-xL levels were affected by flavopiridol exposure (Fig 4C). Interestingly, the decrease of Mcl-1 level occurred rapidly, after 2 hours, and became undetectable after 4 hours of treatment with flavopiridol. This decrease was observed prior to caspase 3 cleavage, which was detected only after 6 hours of treatment (Fig 4D). Following 2 hours of flavopiridol exposure, qRT-PCR analysis confirmed a decrease in Mcl-1 mRNA levels for up to 90%. (Fig 4E). Taken together, these results demonstrate that Mcl-1 protein down-regulation in flavopiridol treated cells is not due to a caspase-dependant cleavage, but occurs at the mRNA level. Because flavopiridol induces a rapid and strong caspase-independent down-regulation of Mcl-1, we hypothesized that flavopiridol might overcome the putative Mcl-1-associated resistance to ABT-737. The four ABT-737-resistant MCL cell lines were then treated with suboptimal doses of ABT-737 or flavopiridol or a combination of both drugs. Cell viability was assessed after a 24-hour exposure (Fig 4F). The measurement of induced apoptosis by the combination of ABT-737 plus flavopiridol shows a major synergistic effect with suboptimal doses of each drug, as demonstrated by a combination index (CI) <1 according to the Chou-Talalay method (23).

ABT-737 synergizes with cytarabine arabinoside (Ara-C) or bortezomib in ABT-737 resistant MCL cell lines.

Ara-C and, more recently, bortezomib have been shown to be two important compounds in the treatment of MCL patients (24-26). Ara-C exposure can induce a rapid decrease of Mcl-1 that occurs independently of caspase-3 (Fig 5A). In contrast, bortezomib exposure does not modified Mcl-1 but induce a strong increase of the BH3-only Noxa protein (Fig 5B). Interestingly, Noxa binds selectively Mcl-1 and thus counteracts its anti-apoptotic function (27). The same results were observed in JVM-2 cell line (data not shown). As observed with flavopiridol, a strong synergistic effect was measured (CI < 1 according to the Chou-Talalay method (23).
method) when both compounds were combined with ABT-737 in resistant JEKO-1 and JVM-2 (Fig 5C).
DISCUSSION

The present study evaluated the antineoplastic activity of ABT-737 in MCL cells. It shows that MCL cells are quite heterogeneous regarding their sensitivity to ABT-737 (with LD_{50} values ranging over 1000 fold). Indeed, the less sensitive MCL cell lines show only limited induced apoptosis when exposed to high doses of ABT-737 (in the micromolar range), whereas the most sensitive lines undertake massive apoptosis after exposure to extremely low concentrations of ABT-737 (in the nanomolar range). Investigations performed with fresh tumor cells from MCL patients have confirmed this finding and revealed that about half of the patient samples are highly sensitive to ABT-737 alone. Interestingly, the level of sensitivity in these samples is comparable to the one observed in sensitive MCL cell lines. Indeed, our results support the use of ABT-737 in MCL but also highlight the need for biological markers that could predict ABT-737 responsiveness.

Analysis of proteins expression level in MCL cells reveals that the subset of highly sensitive MCL cells is consistently characterized by a Bcl-2^{high}/Mcl-1^{low} profile. This is in accordance not only with the molecular mechanism of action of ABT-737 but also with previous studies conducted in other hematological malignancies. Indeed, CLL cells have been reported to be extremely sensitive to ABT-737, and like ABT-737-sensitive MCLs, express high levels of Bcl-2 but always low levels of Mcl-1 (11, 28). It is also interesting to notice that ABT-737 induces apoptosis at a higher drug concentration (5-15 µM) in MM cell lines, which are known to express high levels of Mcl-1 (29). Therefore, Mcl-1 expression level appears as one valuable biomarker regardless of the level of Bcl-2 (12-14) and regardless the nature of the tumor cell. Indeed, several studies and ours have identified a uniform profile (Bcl-2^{high}/Mcl-1^{low}) for ABT-737-sensitive cells. Therefore, both biomarker profiling (Bcl-2^{high}/Mcl-1^{low} and Mcl-1 levels) could be used to predict responses to ABT-737 exposure. These biomarkers are of major interest for future targeted therapies using ABT-737 alone in MCL.

Because half of the MCL samples did not undergo apoptosis in the presence of ABT-737 alone and because our findings demonstrate that the silencing of Mcl-1 overcomes ABT-737 resistance the second part of the present work was devoted to investigate strategies that may overcome MCL resistance to ABT-737 by down-regulating Mcl-1 expression. We observe that the level of Mcl-1 protein is highly variable from one patient to another and from one MCL cell line to another. As reported by Khoury and co-workers, high Mcl-1 expression level
has been associated with a more aggressive biological behavior of the MCL cells (30). More recently, Perez-Galan and colleagues reported that Mcl-1 is also involved in bortezomib resistance in MCL cells (27). Flavopiridol is known to effectively inhibit Mcl-1 transcription in CLL and MM (21, 22). In MCL, flavopiridol was reported to induce apoptosis (31). Therefore, flavopiridol is currently under investigation for treatment of several hematological malignancies, including both CLL and MCL (32-34). Our investigation confirms that flavopiridol down-regulates Mcl-1 at both the mRNA and protein levels in MCL cells and demonstrates a highly synergistic apoptotic effect when combined with ABT-737 in ABT-737-resistant cell lines. The effectiveness of combining ABT-737 with compounds that inactivate Mcl-1 firmly establishes the validity of such a therapeutic approach in the treatment of MCL (12, 35-37). A similar synergistic effect is measured when ABT-737 is combined with Ara-C, which is a heavily-prescribed drug in the treatment of MCL (24, 38, 39). As reported with flavopiridol, Ara-C induce Mcl-1 down-regulation at the mRNA level (40). Of note the regulation of Noxa protein expression level was not significantly modified under both combinations (data not shown). Thus, both flavopiridol and Ara-C, which decrease Mcl-1 expression, appear to be promising supplements in ABT-737 therapy of patients presenting high Mcl-1 levels. A second investigated strategy to overcome Mcl-1 is to modify Mcl-1 complexes and its protein network. Bortezomib is among the most promising compounds in the treatment of MCL (25). Interestingly, the mixture of ABT-737 with bortezomib shows a synergistic effect even though no decrease of Mcl-1 protein expression is observed in the presence of bortezomib for up to 24 hours. Among the various pro-apoptotic effects of bortezomib, one involves the drastic increased of Noxa, a BH-3-only protein that is known to specifically interact with Mcl-1 (41). The up-regulation of Noxa leads to an increase of Noxa/Mcl-1 heterodimers. Thus, it counteracts the anti-apoptotic role of Mcl-1 and then Mcl-1 fails to protect cells from apoptosis (27). Taken together, our results show that several strategies might be employed to overcome ABT-737 resistance. One would be to include compounds that decrease Mcl-1 expression (such as ABT-737 treatment in combination with flavopiridol or Ara-C) and another approach would be to inhibit Mcl-1 anti-apoptotic action by increasing Noxa expression using bortezomib. Recent publications suggested that ABT-737 exposure may enhance Mcl-1 up-regulation, leading to ABT-737 resistance (36, 42). This putative role played by ABT-737 as a Mcl-1 up-regulator further enhances the potential of Mcl-1 targeted therapy used in combination with ABT-737.
In MCL sensitive cells, our findings demonstrate that short exposure to ABT 737 induces a rapid disruption of both Bcl-2/Bax and Bcl-2/Bik complexes. Bax is a multi domain pro-apoptotic protein which permeabilizes the outer mitochondria membrane leading to cytochrome C release while Bik is a sensitizer BH-3 only protein. The major impact of the dissociation between Bcl-2 and Bax in ABT-737 induced apoptosis in MCL is in agreement with the work from Van Delft and colleagues (14). In contrast, Bcl-2/Bim and Bcl-2/Puma complexes are weakly affected by ABT-737 that could be explained by their high affinity to Bcl-2 (9). In contrast, we did not observed a correlation between Bcl-xL expression level and response to ABT-737 in MCL.

The present work strongly supports the use of ABT-737 in MCL and opens the window for selected ABT-737 tailored therapies that use biomarkers able to predict patient responses. Indeed, MCL patients presenting a Bcl-2<sup>high</sup>/Mcl-1<sup>low</sup> profile may be good candidates for ABT-737 monotherapy, whereas ABT-737 combined with a Mcl-1-targeted therapy (such as flavopiridol, Ara-C or bortezomib) would be more appropriate for MCL patients with high Mcl-1 levels. These biomarkers are easily assessed prior treatment induction. In our experiments, Bcl-2 and Mcl-1 protein level assessment is a powerful tool to determine Bcl-2/Mcl-1 patient profiles, but other methods such as flow cytometry or quantitative PCR analysis could also evaluate these markers effectively. The unique mechanism of action of ABT-737 offers the opportunity to treat patients by using a targeted therapy based on their Bcl-2/Mcl-1 profile in tumor cells. The present investigation provides the biological rationale for these future clinical trials to evaluate ABT-737 alone or in combination with Mcl-1 reducing agents in MCL patients. Indeed, ABT-263 (navitoclax, Abbott) which is an orally bioavailable BH3-mimetic compound of the same class as ABT-737 and currently under investigation in hematological and solid malignancies could also be used as ABT-737 (13, 43-46).
REFERENCES


**FIGURE LEGENDS:**

**Figure 1. ABT-737 induces apoptosis in MCL cell lines and correlates with Bcl-2/Mcl-1 profile.**

**A:** Sensitivity of MCL cell lines to ABT-737. Cells were cultured for 24 hours with increasing concentrations of ABT-737 (from 20 nM to 20 µM). Cell death was quantified by APO 2.7 staining. The lethal dose 50 (LD50) is indicated for each cell line. Data represent means +- Standard deviation from three independent experiments.

**B:** ABT-737 induces rapid apoptosis via the intrinsic pathway in sensitive MCL cell line MINO. MCL cell line MINO was cultured with 100nM ABT-737 as indicated. Immunoblot analysis of MINO lysate (70 µg of protein) reveals the cleavage of caspase 9 and 3. Actin was used as a loading control.

**C:** ABT-737 induces apoptosis via Bax and Bak activation. MINO cells were cultured for 24 hours with 100nM ABT-737. Bax and Bak activation were detected by flow cytometry.

**D:** Bcl-2/Mcl-1 protein profile correlates with ABT-737 sensitivity. Cell lysates were separated by SDS-PAGE and then electrotransferred onto PVDF membranes. The membranes were probed with the respective antibodies. Actin was included as a protein loading control.

**E:** Bcl-2/Mcl1 mRNA ratio correlates with ABT-737 sensitivity. Bcl-2 and Mcl-1 mRNA levels were measured by qRT-PCR at baseline and determined normalized according to JEKO-1 mRNA levels. Data represent means +- Standard deviation from three independent experiments.

**Figure 2. Sensitivity of MCL patient samples to ABT-737.**
A: Sensitivity of MCL patient samples to ABT-737. MCL cells from blood samples of 13 patients were cultured for 24 hours with different concentrations of ABT-737 (20, 80, 300, 1250 nM). Cell death was quantified by FACS analysis of APO 2.7 and CD19 stained cells.

B: Western-blotting analysis of Bcl-2 and Mcl-1 protein expression in MCL patient samples. CD19+ purified cell lysates were analyzed by immunoblotting with the respective antibodies. Actin was included as a protein loading control. Sensitive MCL samples (in grey) present high levels of Bcl-2 and low levels of Mcl-1, contrary to resistant MCL samples (in black) that express high levels of Mcl-1.

Figure 3. ABT-737 mainly disrupts Bcl-2/Bax and Bcl-2/Bik complexes.

Cells were treated with ABT-737 (100nM) for 2 hours and cell extracts were subjected to immunoprecipitation with an anti-Bcl-2 antibody followed by immunoblotting analysis for the indicated antibodies. Non-treated cells were used as a control.

Figure 4. Flavopiridol induces a rapid down–regulation of Mcl-1 at both the protein and mRNA level and synergizes with ABT-737.

A and B: Mcl-1 silencing sensitizes JEKO and JVM2 to ABT-737 induced-apoptosis. MCL cells were transfected with either siControl or siMcl-1. At 48 hours after transfection, cells were used to prepare cell lysates and Mcl-1 level was determined by immunoblotting (A). Transfected cells were treated with ABT-737 for 24 hours before to be stained by Apo2.7 and analyzed by flow cytometry (B). Results are means ± SD from at least three independent siRNA transfections.

C: MCL cell lines resistant to ABT-737 were cultured for 24 hours with or without 250 nM flavopiridol. Western blot analysis of Mcl-1, Bcl-2 and Bcl-xL protein expression is shown.

D: MCL cell line JEKO-1 was cultured for 2, 4, 6 and 24 hours with 500nM flavopiridol. Western-blot analysis of Mcl-1 and caspase 3 proteins is shown.

E: Mcl-1 mRNA level was measured by quantitative RT-PCR at baseline and after 2, 4 and 6 hours of treatment with flavopiridol (500 nM) in JEKO-1.

F: Effect of combining ABT-737 and flavopiridol in ABT-737 resistant cell lines. ABT-737 resistant MCL cell lines were cultured for 24 hours with the indicated drugs. In all
experiments, drugs were added simultaneously. Cell death was quantified using APO 2.7 staining. Sub-optimal concentrations of ABT-737 (150 nM) and flavopiridol (250 nM for JEKO-1, REC-1 and JVM-2, 150 nM for UPN-1) were used. Synergy was confirmed by combination index (CI) <1 according to the Chou-Talalay method. Data represent means ± SD from three independent experiments.

**Figure 5. ABT-737 synergizes with bortezomib and cytarabine in ABT-737 resistant cell lines.**

A: Effect of Ara-C on McI-1 expression. JEKO-1 was cultured for 2, 6 and 24 hours with 20 ng/mL Ara-C. Western blot analysis of McI-1 and caspase 3 expression is shown.

B: Effect of bortezomib on Noxa expression. JEKO-1 was cultured for 2, 6 and 24 hours with 10 nM bortezomib. Western-blot analysis of McI-1 and Noxa protein expression is shown.

C: ABT-737 resistant MCL cell lines JEKO-1 and JVM-2 were cultured for 24 hours with the indicated drugs. Sub-optimal concentrations of ABT-737 (150 nM), bortezomib (10 nM) and Ara-C (20 ng/mL for JEKO-1, 80 ng/mL for JVM-2) were used. Death was quantified using APO 2.7 staining. Synergy was shown by a combination index (CI) <1 according to the Chou-Talalay method. Data represent means ± SD from three independent experiments.

**Supplemental Table. Characteristics of patients' samples.**

MNC: mononuclear cells
Figure 1.

A

% APO2.7 positive cells

ABT-737 (nM)

MINO (LD₅₀ = 25 nM)

GRANTA-519 (LD₅₀ = 80 nM)

JEKO-1 (LD₅₀ = 8000 nM)

REC-1 (LD₅₀ = 10 000 nM)

JVM-2 (LD₅₀ = 9000 nM)

UPN-1 (LD₅₀ = 9000 nM)

B

Caspase 3

cleaved

Caspase 9

cleaved

Actin

ABT-737 (hrs)

C

Relative cell number

Fluorescence Intensity

Bak

Ct

ABT-737

Bax

D

Mcl-1

Bcl-2

Bcl-xL

Actin

Bcl-2/Mcl-1 ratio

5.5 4 0.4 0.5 0.6 0.1

E

Bcl-2 / Mcl-1 mRNA ratio

MINO

GRANTA-519

JVM-2

JEKO-1

REC-1

UPN-1

3.4

10.2

0.8

1

0.9

0.01
Figure 2.

A

Bcl-2 Mcl-1 Actin

Bcl-2/Mcl-1 ratio

B
Figure 3.

**GRANTA-519**

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**MINO**

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Figure 4.

A

Si Mcl-1
- + - +
Mcl-1
- + - +
Actin
- + - +

B

JEKO-1
P=0.016
P=0.031
P=0.003
P=0.010

JVM-2
P=0.003
P=0.010

% APO 2.7 positive cells
ABT-737 (μM)

C

JEKO-1 REC-1 UPN-1 JVM-2
Flavo
- + - + - + - +
Mcl-1
- + - + - + - +
Bcl-2
- + - + - + - +
Bcl-XL
- + - + - + - +
Actin
- + - + - + - +

D

Flavo
0 2 4 6 24 hours
Mcl-1
Caspase 3
Actin
full length
cleaved

E

MCL1 mRNA relative expression
0 2h 4h 6h

F

% APO 2.7 positive cells
ABT-737
Flavopiridol
ABT-737 + Flavopiridol
JEKO-1 REC-1 UPN-1 JVM-2
CI = 0.2 CI = 0.4 CI = 0.3 CI = 0.2
Figure 5.

A

[Images showing protein expression levels over time with labels such as Mcl-1, Caspase 3, Actin, Ara-C (hours), and Bortezomib (hours).]

B

[Images showing protein expression levels with labels like Mcl-1, Noxa, Actin, and Jeko-1.]

C

[Bar charts showing % APO2.7 positive cells with CI values 0.4 and 0.9 for Jeko-1, and CI values 0.7 and 0.3 for JVM-2.]

Legend:
- ABT-737
- Ara-C
- ABT-737 + AraC
- Bortezomib
- ABT-737 + Bortezomib
Clinical Cancer Research

ABT-737 induces apoptosis in mantle cell lymphoma cells with a Bcl-2\textsuperscript{high}/Mcl-1\textsuperscript{low} profile and synergizes with other anti-neoplastic agents.

Cyrille Touzeau, Christelle Dousset, Bodet Linda, et al.

*Clin Cancer Res* Published OnlineFirst August 5, 2011.

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