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Dll1/Notch activation contributes to bortezomib resistance by upregulating CYP1A1 in multiple myeloma

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ABSTRACT

One of the greatest challenges in multiple myeloma (MM) treatment is to overcome drug resistance. Many pathways are involved including Notch signaling. Notch receptors are expressed by MM cells and Notch ligand Dll1 is present on bone marrow (BM) stromal cells. In this study, we demonstrate that Dll1 can activate Notch signaling mostly through Notch2 receptor and can contribute to drug resistance to bortezomib, both in murine and human MM cells. Blocking the Notch pathway by DAPT (gamma secretase inhibitor) could reverse this effect and increased sensitivity to bortezomib. We describe the upregulation of CYP1A1, a Cytochrome P450 enzyme involved in drug metabolism, as a possible mechanism of Dll1/Notch induced bortezomib resistance. This was confirmed by inhibition experiments using α-Naphthoflavone or CYP1A1-siRNA that resulted in an increased sensitivity to bortezomib. In addition, in vivo data showed that combination treatment of DAPT with bortezomib was able to increase bortezomib sensitivity and prolonged overall survival in the 5T33MM mouse model. Our data provide a potential strategy to overcome bortezomib resistance by Notch inhibition in MM therapy.

1. Introduction

MM is an incurable B cell disease characterized by the accumulation of malignant plasma cells (PC) in the BM. The BM microenvironment plays a critical role in MM cell growth and survival providing proliferative and anti-apoptotic signals and protecting against currently available anti-myeloma agents [1]. The Notch pathway is a highly conserved pathway regulating cell fate determination, stem cell self-renewal, proliferation and apoptosis [2]. There are four receptors (Notch1–4) and five ligands (Jag1–2, Dll1, Dll3–4) described in this pathway. It is demonstrated that Notch receptors and ligands (such as Notch1–3 and Jag1–2) are expressed in MM patients and MM cell lines [3–6]. Simultaneously, Notch1–3, Jag1 and Dll1 are all detected in MM patients' BM stromal cells [3,7]. The interaction of Notch receptors and ligands between adjacent cells induces proteolytic cleavage and release of the intracellular domain of the Notch receptor, also called NICD. NICD will then enter the nucleus and modify the expression of the downstream target genes [8]. It is reported that Jag1- and Jag2-induced Notch activation promotes MM cell proliferation, while blocking the Notch pathway by gamma secretase inhibitor XII (GSI) alone induces apoptosis and inhibits proliferation of MM cells [5,9–10]. In addition, Notch1 is involved in protection of MM cells from melphalan- and mitoxantrone-induced apoptosis [3]. Previously, we have demonstrated that Notch1–2 receptors are expressed in 5T33MMvt, MMS-1 and LP-1 MM cells while the ligand Dll1 is expressed by human BM stromal cells from normal donors and MM patients and by murine BM stromal cells [11]. We also demonstrated that the Notch pathway can be activated in MM cells by cocultures of MM cells with MS5.Dll1 stromal cells or recombinant Dll1 ligand [11]. In this study, we investigated the role of the Dll1/Notch pathway in MM resistance to bortezomib, currently part of standard anti-myeloma regimens in first-line and advanced disease settings [12,13].

2. Materials and methods

2.1. 5T33MMvv myeloma model and cell lines

The 5T33MMvv murine model of MM was used as described previously [14–16]. Mice were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, Vrije Universiteit Brussel (license No. LA1230281).

Murine 5T33MMvt and human MMS-1, LP-1 and RPMI-8226 MM cells were cultured as described previously [16]. MS5.Dll1 is

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a cell line derived from MS5 stromal cells transduced with human Dll1 and GFP [17]. Stromal cells from MM patients were obtained as described previously [18].

2.2. Cocultures of MM cells with MS5 or MS5.Dll1 stromal cells

MS5 or MS5.Dll1 stromal cells were plated in 6-wells at 10^5 -cells/well. Upon confluence, MM cells (0.5×10^6/well) were added. Cocultured MM cells were harvested for investigation. The potential contamination of stromal cells after harvesting MM cells was checked by FACS analysis, based on GFP expression of stromal cells [17]. Contamination with GFP + cells was less than 3%.

2.3. Soluble Dll1 ligand immobilization and cocultures with MM cells

Recombinant mouse (150 ng/ml) or human (0.5 μg/ml) Dll1 (R&D Systems, USA) were immobilized in 96-wells for 20 h, 4 °C. As control for Dll1, mouse and human IgG (R&D Systems) were used. Plates coated with Dll1 or IgG were washed; MM cells were cultured on Dll1, treated with bortezomib (Millennium Pharmaceuticals, USA) and cell viability was measured by CellTiter-Glo assay (Promega, The Netherlands).

2.4. Flow cytometry

Cells were stained with antibodies and incubated at 4 °C for 30 min. FACS analysis was performed with FACSCanto (Becton Dickinson, USA) using FACS Diva software. Antibodies for mouse Notch1-PE, Notch2-PE, Jag2-PE, Dll4-PE, human Notch1-PE, Dll1-PE and DyLight488 donkey anti-rabbit IgG were from Biolegend (USA). Human Notch2-PE were from eBioscience (USA); mouse Jag1-APC from R&D Systems. Annexin-V-FITC (Becton Dickinson) was used to measure apoptosis.

2.5. Drug resistance and metabolism gene array

Mouse Cancer Drug Resistance and Metabolism RT2 Profiler PCR Array (SABiosciences, USA) was used to compare gene expression of 5T33MMvt cells before and after Dll1 interaction.

2.6. CYP1A1 enzyme activity assay

CYP1A1 enzyme activity was measured by a P450-Glo™ CYP1A1 Assay (Promega).

2.7. Transient transfection of small interfering RNA (siRNA)

LP-1 and MMS-1 cells were transiently transfected with CYP1A1 siRNA (sc-41483) and control siRNA (sc-37007) (Santa Cruz Biotechnology, USA) using the siRNA Reagent System (Santa Cruz Biotechnology). Transfection efficiency with FITC-siRNA was higher than 85%.

2.8. Real-time quantitative PCR

Experiments were performed using SYBR Green Mix (Fermentas, UK) as described previously [19]. Primers for human and mouse genes are listed in Supplementary Table 1 and in our previous study [11]. All primers were synthesized by Thermo (Germany).

2.9. Western blot

Western blot was performed as described previously [19]. Antibodies against NICD1, NICD2 and ActinB were from Cell Signaling (USA). CYP1A1 antibody was from Abcam (UK).

2.10. In vivo survival experiment

C57Bl/KaLwRij mice were injected intravenously on day 0 with 0.5×10^6 5T33MMvv tumor cells. 10 mg/kg DAPT was injected subcutaneously daily from 4 days after tumor inoculation. Bortezomib (0.4 mg/kg twice a week) was injected subcutaneously, starting one week after tumor injection. Treatments were all stopped when terminal disease had developed (hind leg paralysis) in DMSO control mice after approximately 3 weeks of tumor inoculation. Mice were sacrificed upon development of terminal disease.

2.11. Statistical analysis

In vitro experiments were repeated at least 3 times. Data were presented as mean ± SD (standard deviation). Significance between groups from in vitro experiments was determined using Mann–Whitney U test. In vivo experiment was analyzed by Kaplan–Meier survival analysis. Results were considered significant when $P < 0.05$.

3. Results

3.1. Notch is activated by Dll1 interaction mostly through release of NICD2

After ligand/receptor binding, Notch receptor will be cleaved by tumor-necrosis factor-α-converting enzyme/metalloproteinase and the gamma-secretase complex resulting in the release of Notch extracellular domain (NECD) [20–22]. Subsequently, Notch intracellular domain (NICD) will be released in the cytoplasm and transferred into the nucleus to activate the Notch pathway [23]. We analyzed Notch1 and Notch2 surface expression on MM cells after interaction with MS5.Dll1 stromal cells using flow cytometry. Notch1 surface expression was not disturbed (Fig. 1A) while Notch2 was significantly decreased after Dll1 interaction for 2 days (Fig. 1B). Next, we investigated NICD1 and NICD2 expression by Western blot after Dll1/Notch interaction during 12, 24 and 48 h. NICD1 expression did not change after Dll1 interaction in murine 5T33MMvt and human LP-1 and MMS-1 cells (Fig. 1C), while NICD2 is increased after Dll1 interaction (Fig. 1C). Figures only show 48 h time point. These results indicate that Dll1 interaction mostly results in Notch2 activation.

3.2. Dll1/Notch interaction induces bortezomib resistance in MM and upregulates CYP1A1 expression

We investigated whether Dll1/Notch activation could contribute to bortezomib resistance. As demonstrated by viability assays (Fig. 2A), 5T33MMvt cells cocultured with Dll1 ligand were less sensitive to bortezomib compared to control. Furthermore, when we blocked the Notch pathway by DAPT, MM cells became more sensitive to bortezomib, indicating that Dll1/Notch pathway is indeed involved in bortezomib resistance. At that time point, DAPT alone has no effect on the viability of MM cells (results not illustrated). We confirmed murine data with human LP-1, MMS-1 and RPMI-8226 cell lines (Fig. 2B, only MMS-1 is shown). We also investigated apoptosis by annexinV flow cytometry staining. Dll1/Notch activation could reduce MM cell apoptosis induced by bortezomib both in human and murine MM cells (data not shown).

We performed a drug resistance and metabolism gene array to investigate the molecular mechanisms related to Dll1-induced resistance to bortezomib. The most upregulated and downregulated genes after Dll1/Notch interaction in 5T33MMvt cells are shown in Supplementary Table 2. We chose CYP1A1 and AhR (transcription factor of CYP1A1) for further research. CYP1A1 is a

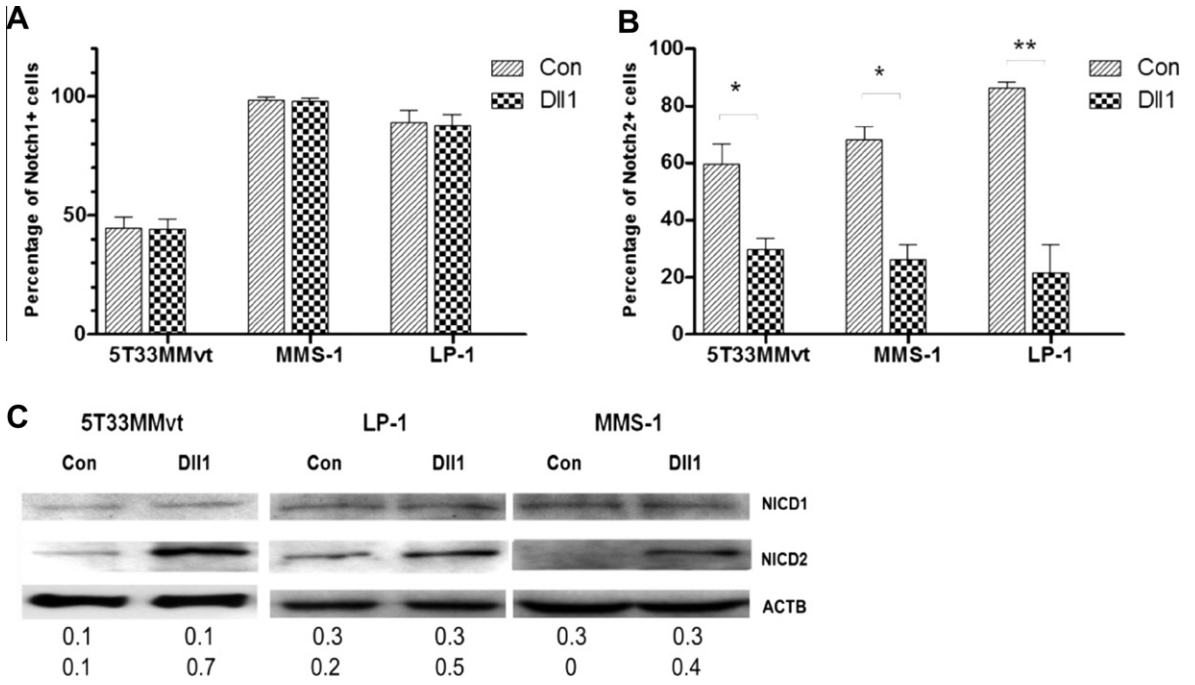

Fig. 1. Dll1/Notch activation mostly mediated by NICD2 release. (A, B) Notch1 and Notch2 expression was investigated by FACS after Dll1/Notch interaction for 2 days. Percentage of Notch1 + and Notch2 + cells was shown as mean ± SD ($n = 3$). Con and Dll1 indicate 48 h cocultures of MM cells with respectively MS5 and MS5.Dll1 stromal cells $^{*}P < 0.05$, $^{**}P < 0.01$ (C) NICD1 and NICD2 were investigated by Western blot analysis after Dll1/Notch interaction for 2 days. The numbers below refer to the relative optical density as measured with NIH ImageJ software. Representative results of 3 independent experiments are shown.

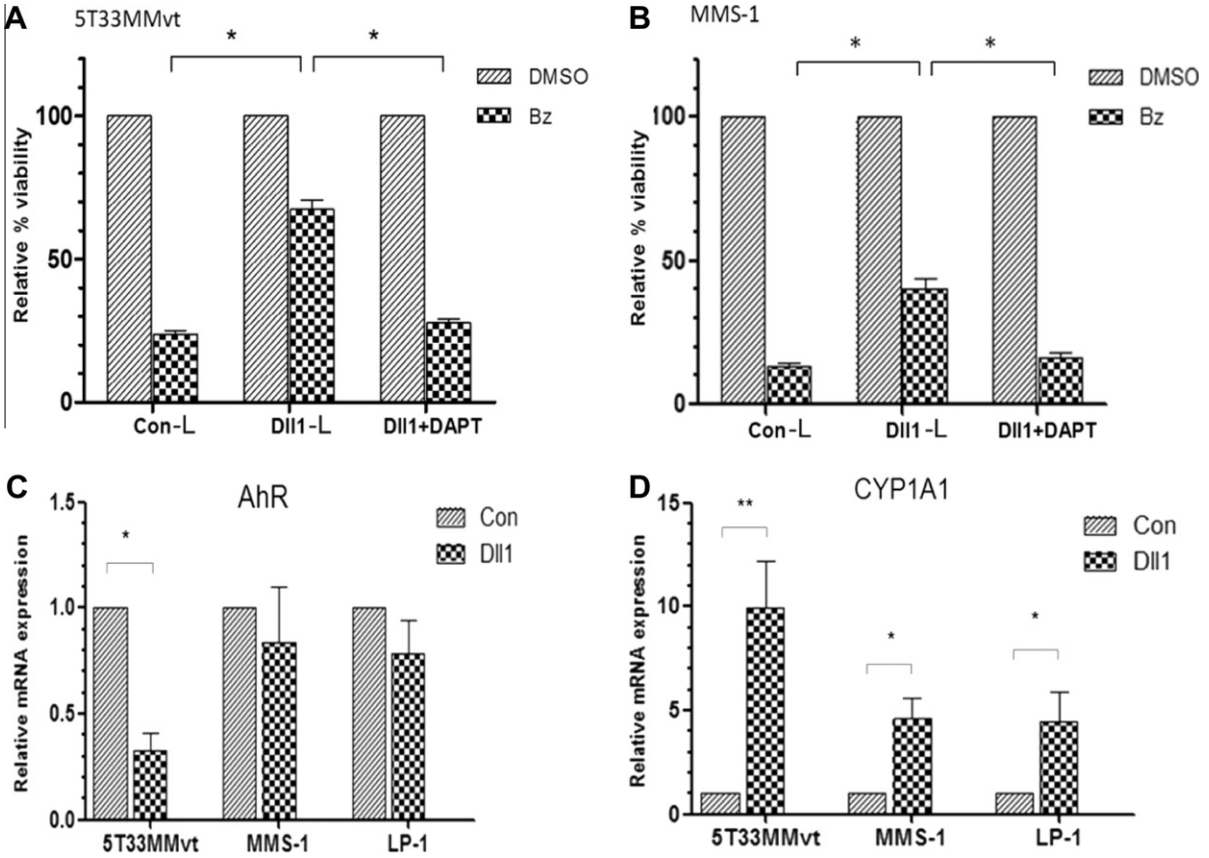

Fig. 2. Dll1/Notch activation induces drug resistance to bortezomib (bz) and upregulates CYP1A1 expression. (A, B) Cell viability assay was performed after bortezomib (5 nM) treatment with or without DAPT (10 μM) for 48 h in 5T33MMvt cells stimulated with mouse Dll1 ligand (A) or in MMS-1 cells stimulated with human Dll1 ligand (B). DAPT alone had little effect and was not shown in the figure. Percentage of alive cells relative to control were shown as mean ± SD ($n = 3$). (C, D) Real-time PCR analysis of AhR and CYP1A1 expression after Dll1 stimulation for 48 h in MM cells ($n = 3$) $^{*}P < 0.05$, $^{**}P < 0.01$.

Cytochrome P450 enzyme that plays a pivotal role in the metabolism of a wide variety of xenobiotic and endogenous compounds [24–25]. Normally, levels of CYP1A1 expression are regulated through transcription factor AhR (Aryl hydrocarbon receptor) in extrahepatic tissues [26]. We investigated the expression of CYP1A1 and AhR after Dll1/Notch interaction. Real-time PCR showed that AhR was down-regulated by Dll1 stimulation in 5T33MMvt cells but not in MMS-1 and LP-1 cells (Fig. 2C). Furthermore, Dll1/Notch activation could increase CYP1A1 expression in 5T33MMvt and human MMS-1 and LP-1 cells both on mRNA level (Fig. 2D) and protein level (data not shown), suggesting that CYP1A1 can be upregulated by Dll1/Notch interaction through an AhR-independent manner.

3.3. Inhibiting CYP1A1 increases sensitivity to bortezomib

To investigate whether CYP1A1 is involved in Dll1-induced drug resistance to bortezomib, we inhibited CYP1A1 activity by NF (α-Naphthoflavone) or CYP1A1-siRNA and analyzed sensitivity to bortezomib. CYP1A1 enzyme activity was partially downregulated after treatment of 5T33MMvt, LP-1 and MMS-1 cells with 20 μM NF (Fig. 3A). Inhibiting CYP1A1 activity by NF could increase sensitivity to bortezomib in 5T33MMvt, LP-1 and MMS-1 cells (Fig. 3B). Subsequently we transfected control siRNA and CYP1A1 siRNA in LP-1 cells; CYP1A1 expression was inhibited both on mRNA level (Fig. 3C) and protein level (data not shown) by CYP1A1 siRNA. As demonstrated by annexinV staining, transfection of CYP1A1 siRNA could increase sensitivity to bortezomib compared to control siRNA (Fig. 3D). Similar results were obtained in MMS-1 cells (data not shown). Next, we investigated whether inhibiting CYP1A1 by NF could reverse bortezomib resistance induced by Dll1 stimulation. Results show that inhibiting CYP1A1 by NF increases bortezomib sensitivity and reverses Dll1-induced drug resistance in MM cells (Fig. 3E). We further cocultured MM cells with patients' derived stromal cells and treated the cultures with DAPT to investigate CYP1A1 expression and bortezomib sensitivity. CYP1A1 expression in MM cells was down-regulated by DAPT and resulted in an increased sensitivity of MM cells to bortezomib (Fig. 3F).

3.4. Combination treatment of bortezomib with DAPT prolongs overall survival in vivo

We have demonstrated that Dll1/Notch interaction could induce drug resistance in MM cells and blocking the Notch pathway with DAPT increases again the sensitivity to bortezomib in vitro. We wondered whether combination treatment of bortezomib with DAPT could increase sensitivity to bortezomib and prolong overall survival in vivo. A survival experiment was performed as illustrated in Fig. 4A. To investigate whether Notch activation could be inhibited by DAPT in vivo, 3 extra DAPT-treated mice were sacrificed when the vehicle group was sick. The expression of Notch downstream genes in BM isolated 5T33MMvv cells was investigated by real-time PCR. Most of Notch targets were inhibited by DAPT in vivo (Fig. 4B). As indicated in Fig. 4C, treatment with this concentration of DAPT alone had no effect on the survival of 5T33MM-diseased mice. Low dose of bortezomib treatment resulted in a longer overall survival. Noticeably, mice treated with the combination of bortezomib with DAPT had a significant prolonged overall survival compared to DAPT alone or bortezomib alone.

4. Discussion

Bortezomib is widely accepted and included in the standard treatment of myeloma patients and has improved clinical outcome. Nevertheless, some patients do not respond to bortezomib or they eventually relapse after response [12–13]. Drugs that overcome this bortezomib resistance are needed. In this study, we investigated the role of Dll1/Notch pathway in MM bortezomib resistance. The role of Notch signaling in MM has been reported by several research groups [5,9–10]. However, to our knowledge, the Dll1/Notch pathway has not been studied yet. Nefedova et al. mentioned that Dll1 is expressed by bone marrow stromal cells [3] and we confirmed that Dll1 is present in human and murine BM [11]. To activate Notch signaling in human and murine MM cells, we used recombinant Dll1 ligand or MS5.Dll1 stromal cells, which overexpress human Dll1 [17]. Comparison of main domains of mouse and human Dll1 on PubMed database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) indicates that they have exact the same domains. It is confirmed in our previous study that using mouse Dll1 ligand with 5T33MMvt cells showed similar Notch activation [11]. Mouse stromal cells on human MM cells can minimize the effect of other adhesive and humoral factors, highlighting the effect of Dll1. We investigated expression of Notch ligands in MS5 and MS5.Dll1 stromal cells by FACS. Jag1, Jag2 and Dll4 are slightly or not expressed on stromal cells, only Dll1 was significantly differential expressed (Supplementary Fig. 1A). Furthermore, there is no significant difference of Notch target gene expression between 5T33MMvt cells and cocultures with control MS5 stromal cells (Supplementary Fig. 1B). In literature, usually one or two Notch downstream genes are reported for Notch activation [5,10,27]; in our studies we investigated 5 downstream genes and the expression is different among different human and murine cell lines. The diverse effects between these downstream genes are not clear and can be an interesting point to study. In this study, expression of target genes was merely investigated to confirm Notch activation after Dll1 stimulation.

Our observations that surface Notch2 expression is decreased and NICD2 expression is increased in MM cells after Dll1 interaction indicate that Dll1 activates Notch mostly through the Notch2 receptor. Our further experiments demonstrate that Dll1/Notch interaction could induce drug resistance to bortezomib in murine and human MM cells while DAPT can reverse the effect. Nefedova et al. reported that BM stromal cells could mediate drug resistance through Notch1 signaling, with a stimulation of Jag1 peptide [3]. To further investigate molecular mechanisms of Dll1-induced drug resistance to bortezomib, we did a drug resistance and metabolism gene array and found that CYP1A1 was upregulated after Dll1/Notch activation. The upregulation of CYP1A1 by Dll1 stimulation seems to be independent of its transcriptional factor AhR. In addition, blocking Notch signaling with DAPT could decrease CYP1A1 expression in MM cells (data not shown). We further analyzed CYP1A1 promoter region and found there are some Notch signaling factor CSL/RBP-Jk binding sequences [28]. These observations indicate that CYP1A1 may be a potential Notch downstream target gene, which is interesting for further investigation. Bortezomib is described as a substrate of CYP1A2 enzyme that could increase the metabolism of bortezomib [29–31]. CYP1A1 is in the same subfamily of Cytochrome P450 enzymes as CYP1A2. Sequences of CYP1A1 and CYP1A2 have a high homology (up to 96%) and they are genetically conserved among human, mouse and rat species (Supplementary Table 3), suggesting that CYP1A1 probably shows similar function than CYP1A2. Our study results suggest that CYP1A1 is involved in Dll1-induced bortezomib resistance in MM cells. Since bortezomib is a proteasome inhibitor and its activity is primarily reliant on the unfolded protein response (UPR) [32], we investigated two primary markers (GRP-78 and CHOP) for UPR [19]. No significant differences of GRP-78 and CHOP mRNA expression after Dll1/Notch activation were observed (data not shown).

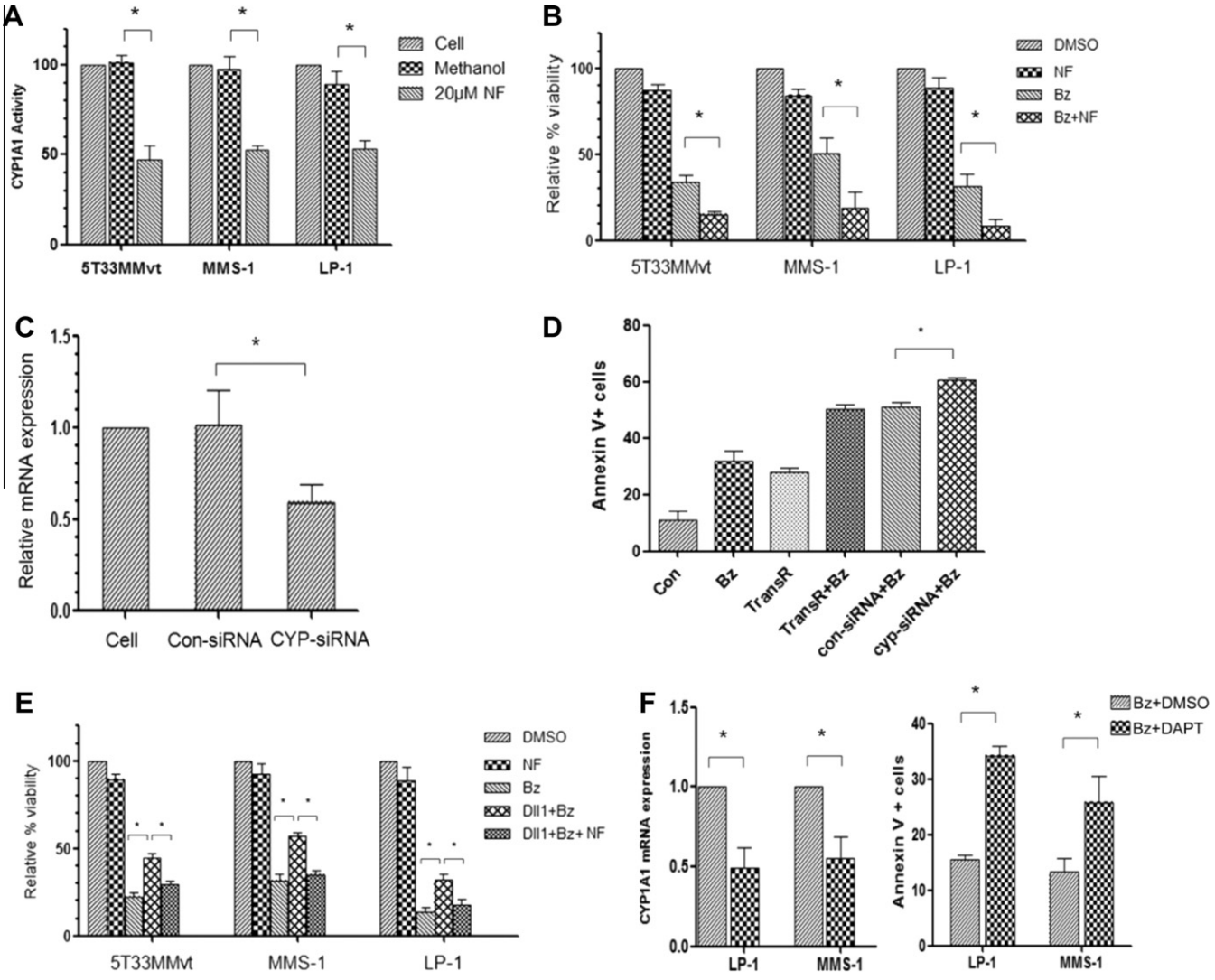

Fig. 3. Inhibiting CYP1A1 increases the sensitivity to bortezomib. (A) CYP1A1 enzyme activity was measured after 20 μM NF (α-Naphthoflavone) treatment for 24 h in 5T33MMvt, MMS-1 and LP-1 cells. Methanol (0.1%) is the control. (B) Bortezomib sensitivity was analyzed by cell viability assays with or without the combination with NF. MM cells were treated for 24 h with 20 μM NF alone, 5 nM Bz alone or both. Percentage of alive cells relative to control were shown as mean ± SD ($n = 3$). (C) CYP1A1 expression was investigated by real-time PCR after CYP1A1 siRNA transfection for 18 h. (D) Bortezomib sensitivity (5 nM) was investigated by apoptosis analysis after CYP1A1 siRNA transfection for 18 h. TransR indicates transfection reagent. Percentage of apoptotic cells (annexin V+) were shown as mean ± SD ($n = 3$). (E) Bortezomib sensitivity (7.5 nM) was analyzed by cell viability assay with or without the combination of Dll1 or NF for 24 h in 5T33MMvt, MMS-1 and LP-1 cells. Percentage of alive cells relative to control were shown as mean ± SD ($n = 3$). (F) CYP1A1 mRNA expression and Bortezomib sensitivity (5 nM) was investigated after cocultures of MM cells with patients' BM stromal cells and treated with DAPT (10 μM) for 48 h. Percentage of apoptotic cells was shown as mean ± SD ($n = 3$) $^*P < 0.05$.

Since we demonstrated in vitro that Notch inhibition with DAPT could increase sensitivity of MM cells to bortezomib, we performed an in vivo survival experiment. 5T33MM-inoculated mice were treated with either DAPT or bortezomib alone or in combination. As side-effects such as gastrointestinal toxicity were reported in mouse models [33–34], we selected a low dose (10 mg/kg) of DAPT in our experiments that could inhibit Notch activation in vivo. The survival showed no difference between vehicle and DAPT group, suggesting that DAPT alone at this concentration has little anti-myeloma effect. This is also confirmed in our previous in vitro study where DAPT could not induce apoptosis in MM cells [11]. No significant weight loss was observed between the DAPT and vehicle group (results not shown). Bortezomib was also administered at a lower dose (0.4 mg/kg) than the optimal dose (0.6 mg/kg) [35] as we wanted to highlight the effect of combination treatment with DAPT. Combination treatment of bortezomib with DAPT increased the sensitivity to bortezomib in vitro and resulted in a better overall survival in vivo.

In conclusion, our results suggest that Dll1 can activate Notch signaling in MM cells through Notch2 receptor and induces drug resistance to bortezomib. We further observed that Dll1-induced bortezomib resistance was due to an upregulation of CYP1A1, a Cytochrome P450 enzyme involved in drug metabolism. In addition, combination treatment of DAPT with bortezomib could increase sensitivity and prolong overall survival in vivo. Previously we have demonstrated that the Notch pathway is involved in clonogenic growth and inhibiting Notch signaling by pretreatment of MM cells with DAPT before injection could significantly delay MM initiation and suppress disease development [11]. It provides a promising therapeutical benefit not only in overcoming drug resistance but also in preventing or delaying MM relapse when combining a Notch pathway inhibitor with traditional chemotherapy.

Conflict of interest

The authors declare no conflict of interest.

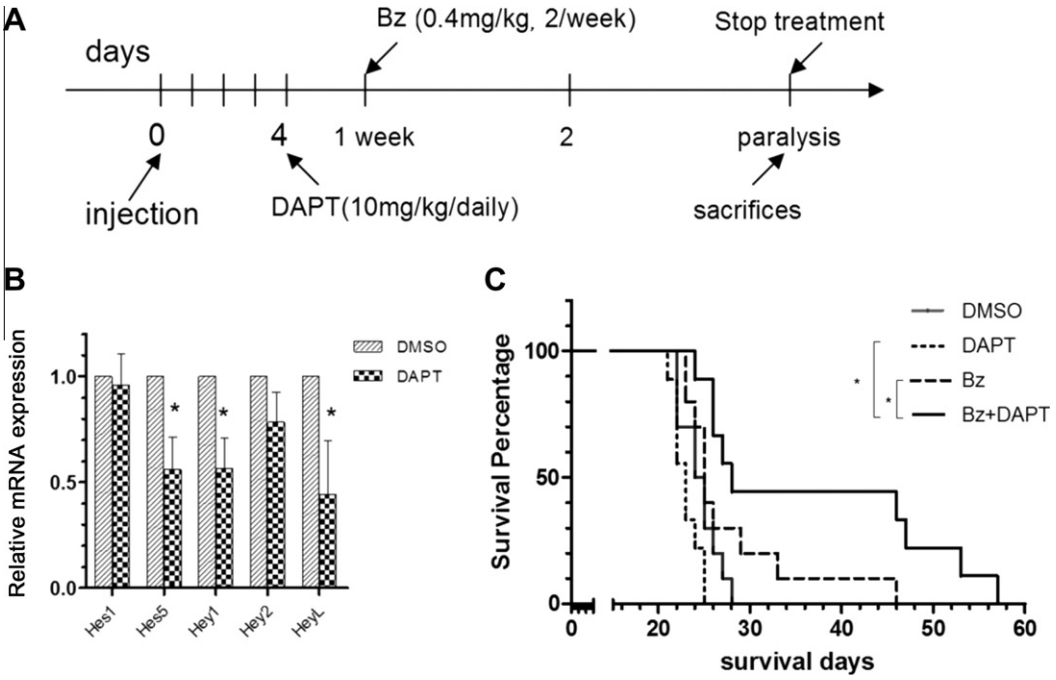

Fig. 4. Combination treatment of bortezomib with DAPT prolongs the overall survival in the 5T33MMvv model. (A) Illustration of the treatment setting in 5T33MMvv inoculated mice. (B) MM cells were collected from the BM of DAPT and DMSO treated mice and Notch downstream genes were analyzed by real-time PCR to investigate Notch pathway inhibition in vivo ($n = 3$) $^{*}P < 0.05$. (C) Kaplan–Meier survival analysis. The *X*-axis represents the survival days from injection to the development of hind leg paralysis and the *Y*-axis represents the survival percentage ($n = 10$/group). Bz vs DMSO, $P < 0.05$; Bz + DAPT vs Bz or DAPT, $P < 0.05$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.071.

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