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ORIGINAL ARTICLE

# IPI-504, a novel and soluble HSP-90 inhibitor, blocks the unfolded protein response in multiple myeloma cells

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**Abstract**
*Background* Inhibitors of heat shock protein (Hsp) 90 induce apoptosis in multiple myeloma (MM) cells, but the molecular mechanisms underlying this cytotoxic outcome are not clear. Here, we investigate the effect of IPI-504, a novel and highly soluble inhibitor of the Hsp90 ATPase activity, on the unfolded protein response (UPR) in MM cells. The UPR is a stress response pathway triggered by sensors located at the endoplasmic reticulum (ER) membrane whose function is to reduce an excessive accumulation of misfolded protein in the ER. During normal development of B-lymphocytes to antibody-producing plasma cells, a partial UPR has been described, where IRE$\alpha$ and ATF-6 are stimulated, whereas the third sensor, PERK, is not induced.
*Methods* Levels of the activated forms of the three main UPR sensors ATF-6, XBP-1 and PERK/eIF-2 were monitored in two different MM cells lines and one non-MM cell lines under various experimental conditions including incubation with increasing concentration of IPI-504. Also, MM cells were incubated with IPI-504 and several apoptosis markers were monitored.
*Results* We show here that a partial UPR is constitutively activated in plasma cell-derived MM cells and that IPI-504 can potently inhibit this pathway. IPI-504 achieves this by inactivating the transcription factors XBP1 and ATF6. In addition, IPI-504 also blocks the tunicamycin-induced phosphorylation of eIF2 by PERK. Dose-response and time course experiments reveal that IPI-504's inhibitory effect on the UPR parallels its cytotoxic and pro-apoptotic effects on MM cells.
*Conclusion* The results presented here suggest that the IPI-504-induced apoptosis might be, in part, mediated by the inhibition of the partial UPR. Other malignancies that rely on intact and efficient UPR to survive could be considered as new indications for Hsp90 inhibitors.

**Keywords** Unfolded protein response · Hsp90 · Multiple myeloma

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## Introduction

Multiple myeloma is a malignant disease of plasma cells that manifests as lytic bone lesions, monoclonal immunoglobulin (Ig) secretion into the blood or urine and disease in the bone marrow [1]. The high level of Ig secretion requires a highly developed secretory machinery and a steady production of chaperone proteins to allow proper protein translation and folding in the ER. This raises the possibility that these malignant cells possess a constitutively active unfolded protein response (UPR).

The UPR is a threefold signaling pathway triggered by the accumulation of misfolded proteins in the ER (see for review [2–5]). The first step of this response consists of the proteolytic cleavage of an ER-membrane protein [6] and the cytoplasmic release of the ATF6 transcription factor [7, 8]. ATF6 enters the nucleus and binds to a *cis*-acting response element (ER stress element or ERSE [7, 9–12]), triggering the transcription of ER resident chaperones like glucose-regulated proteins BiP/GRP78 and GRP94 [13]. The second phase corresponds to the excision of 26 bases from the transcript encoding the X-box protein, XBP1

[14, 15], allowing the synthesis of a functional, spliced, protein (sXBP1). This unusual cytoplasmic splicing event is carried out by the transmembrane ER-stress sensor IREα [14, 16–19]. sXBP1 translocates into the nucleus and binds to the unfolded protein response element (UPRE) triggering the transcription of numerous genes involved in the ER secretory machinery including the ER-associated degradation (ERAD) system [20]. The third phase consists of the repression of global protein translation via the phosphorylation and inactivation of the elongation factor eIF2, driven by the ER-stress sensor protein kinase R-like ER kinase (PERK) [19, 21].

Whereas the UPR can be triggered by pharmacological agents, it is also activated in a range of normal, physiological and developmental processes [3]. For example, a physiologic UPR is induced during differentiation of B-lymphocytes into Ig-producing plasma cells [20, 22]. Here, the ATF6 and XBP1 pathways are activated to support the high secretion rate [22–24], whereas the PERK/eIF2 pathway is turned off to avoid a detrimental decrease in translation and protein synthesis.

Hsp90 is a chaperone that regulates the stability of many oncoproteins and signaling proteins important in cancer proliferation [25–27]. Recent investigations of the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) have focused on its ability to specifically destabilize proteins that are both client proteins of the Hsp90 chaperone and oncoproteins essential for tumor growth. Examples are oncogenic mutants of the cKIT receptor tyrosine kinase in GIST [28], mutant FLT3 receptors in AML [29], Bcr-Abl fusion proteins in CML [30, 31] or mutant EGFR in non small cell lung cancers [32]. IPI-504 (17-AAG hydroquinone, hydrochloride), currently in phase I clinical trials, is a novel and highly soluble analog of the natural product geldanamycin and its derivative 17-AAG. IPI-504 converts to 17-AAG in vitro and in vivo and maintains all of the potency and selectivity of 17AAG without its solubility and formulation liabilities [33, 34]. In addition, IPI-504 is not only a water-soluble analog, but also an active metabolite of 17-AAG [33–35]. Recently, Hsp90 inhibitors have been shown to efficiently induce apoptosis in multiple myeloma (MM) cells [36]. However, in this case, a key Hsp90 client protein and a clear mechanism of action remain elusive, even though inhibitors of Hsp90 have been shown to block the activation of NFKB [37, 38].

A recent report showed that all three membrane-bound UPR sensors (PERK, IREα, and ATF6) are Hsp90 client proteins, and that 17-AAG can disrupt the UPR in pancreatic tumor cells [39]. This raises the possibility that IPI-504 might block a partial UPR in MM cells, hence facilitating the accumulation of misfolded proteins. These misfolded proteins are not only nonfunctional but also prone to forming aggregates that can interfere with normal cellular functions [40, 41] and a possible concordance between increased protein ubiquitination and cytotoxicity has been suggested [42]. It has been shown that myeloma cells deficient in XBP1 undergo increased apoptosis in response to ER stress [43]. Hence, the cytotoxic effect of IPI-504 could be, in part, associated with its capacity to inhibit the UPR in MM cells.

Here, we show that in resting MM cells ATF6 and XBP1 are present in their active form and eIF2 is not phosphorylated. We also show that IPI-504 treatment inhibits in a dose-dependent manner all arms of the UPR, including UPRE and ERSE-related gene expression as well as tunicamycin-induced eIF2 phosphorylation. Ultimately, IPI-504 treatment leads to cell death via a caspase-dependent pathway. Since the time- and concentration dependence of the observed inhibition of the UPR and the induction of cell death are comparable, our results suggest that the cytotoxic effect of HSP90 inhibitors on MM cells is, at least in part, due to the inhibition of the UPR.

## Materials and methods

### Reagents, cell culture and transient transfection

Hela cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1 ug/ml streptomycin and 1 ug/ml penicillin. U266 and MM.1s were cultured in RPMI 1640 medium containing 15% fetal bovine serum, 1 mM pyruvate, 1 ug/ml streptomycin, and 1 ug/ml penicillin. All the cell lines were maintained at 37°C in a humidified 5% $CO_2$ atmosphere.

### Luciferase reporter gene assay

Recombinant DNA techniques were performed according to standard procedures [44]. Based on published consensus sequences [9, 10] double-stranded oligonucleotides were synthesized, annealed, and inserted into the MluI and XhoI sites of the pGL3-promoter vector (Promega, Fitchburg, WI). Two strands corresponding to 5 tandem repeats of the consensus sequence TGACTG [10] were synthesized. UPRE top strand 5′-ACGCGTACAGGTGCTGACGTGG CATTCACAGGTGCTGACGTGGCATTCACAGGTGCT GACGTGGCATTCACAGGTGCTGACGTGGCATTCAC A GGTGCTGACGTGGCATTC-3′. UPRE bottom strand 5′-CTCGAGAATGCCACGTCAGCACCTGTGAATGCC ACGTCAGCACCTGTGAATGCCACGTCAGCACCTG TG AATGCCACGTCAGCACCTGTGAATGCCACGTC AGCACCTGTA-3′. Also, two strands corresponding to 2 tandem repeats of the ERSE consensus sequence CCAATN9CCACG [9] were synthesized. ERSE top strand 5′-CGCGTCACCAATCGGAGGCCTCCACGACCACC

AATCGGAGGCCTCCACGACC-3′. ERSE bottom strand 5′-CTCGAGGTCGTGGAGGCCTCCGATTGGTGGTCG TGGAGGCCTCCGATTGGTGA-3′. Hela cells were transiently transfected by using Fugene kit reagent (Roche, Palo Alto) according to the manufacturer instructions. MM cells were electroporated using the Amaxa Nucleofactor kit V reagent (Amaxa, Germany). After transfection, cells were incubated for 6 h and then treated with compounds for the following 16 h. Then, both firefly and renilla luciferase activity were simultaneously detected after active lysis using the Berthold Luminometer and the Promega GLO kit (Promega) according to the manufacturer instructions.

### Immunoblotting

For Western analysis, cells were harvested from 6-well plates, washed in PBS, and then dissolved in lysis buffer (50 mM Tris–HCl, pH 7.4, 1% SDS, 40 mM sodium fluoride, 150 mM sodium chloride, 10 mM each of orthovanadate, phenylmethylsulfonyl fluoride, and DTT, and 1 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor). 20–50 μg of protein lysates were loaded onto 4–12% Bis–Tris SDS-PAGE minigels. Proteins were transferred onto PVDF membranes and incubated with primary and secondary antibodies. Proteins were visualized by chemiluminescence using the ECL reagent (Amersham Biosciences, Piscataway, NJ) on BioMax film (Eastman Kodak). Anti-XBP1 antibody was from Biolegend (#619502), ATF6 antibody from Santa Cruz (#H-280), anti-eIF2-p antibody from Cell Signaling (#9721s), anti PERK antibody from Abgent (#AP8054b) and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL read out kit and anti-mouse HRP-linked secondary antibody were from Amersham Biosciences.

### Apoptosis and cell death

Apoptosis was scored by the presence of DNA fragmentation and evaluated using the TUNEL assay kit from Molecular Probes (#A23210). Briefly, after cells were incubated in 96 well plates ± compound, the cells were washed in 50 mM Tris buffer, 0.1% triton (TBST, 2 × 200 μl) and fixed with 1% paraformaldehyde (PFA) for 15 min on ice and then with 100% ethanol at −20°C for 30 min. Fixative was removed by washing twice in TBST, and the fixed cells were blocked and incubated for 1 h at 37°C with the DNA labeling solution (TdT enzyme and BrdUTP). After washing 2 × 200 ul with TBST, each well was then labeled with an HRP linked Anti BrDU antibody (Roche, # 1 585 860) for 1 h at room temperature. Incorporated BrDUTP was monitored using Amersham ECL Detection Reagent.

Viability studies were performed using the vital mitochondrial function stain Alamar Blue (Biosource Int. Camarillo, CA). After cells were incubated in 96-well plates (200 μl) ± compounds, 20 μl of Alamar Blue was added and incubated for 4–6 h at 37°C. The Alamar Blue reduction was monitored using an Envision plate reader (Perkin Elmer, Boston, MA) at $\lambda_{EX} = 544$ nm and $\lambda_{EM} = 590$ nm. The ratios obtained from drug-treated cells versus vehicle treated cells were quantified and plotted against drug concentration to give EC50 values. Caspase-3 and 7 activities were detected using the Caspase Glow kit (Promega) according to the manufacturer instructions.

## Results

### A physiologic, partial, UPR is activated in MM cells

The UPR is a transcriptional response to ER-stress triggered by sensors located in the ER-membrane. In plasma cells, membrane-bound ATF6, one of these sensors, is cleaved upon activation, and a 50 kDa form (p50ATF6) is released into the cytoplasm [20, 22]. To test whether MM cells contain a constitutively active UPR, we compared the levels of p50ATF6 in MM and non-MM cells. Immunoblot analysis (Fig. 1a) indicates that lysates from the MM cell lines MM.1s and—to a lesser extent—U266, display a 50 kDa band whereas Hela cell lysate did not, in good agreement with previous reports showing no p50ATF6 in Hela cells [9, 22]. In plasma cells, the endoribonuclease activity of the second UPR sensor, IREα, splices XBP1 mRNA leading to the production of XBP1 activated form (sXBP1). The presence of sXBP1 was monitored after a 16 h treatment with or without 1 uM tunicamycin (Tm), a protein glycosylation inhibitor known to activate the UPR. Immunoblot of MM cell lysates exhibited a constitutive band corresponding to sXBP1, whereas Hela lysates showed a strong band only in the Tm stimulated sample (Fig. 1b). sXBP1 and p50ATF6 bind to specific DNA response element sequences known as the ERSE and the UPRE. To further investigate the possibility of a constitutive activation of these two sensors, two luciferase reporter gene constructs were designed. One contains the ERSE promoter motif CCAAT-N9-CCACG [9]. This sequence, found in the promoter regions of UPR target genes such as BiP, GRP94 and CHOP [24], is bound and activated by p50ATF6 and, to a lesser extent, by sXBP1 [12, 45]. The second consensus sequence, TGACGTGG, named the UPR element (UPRE) [10], exhibits a much lower affinity for p50ATF6 and is the preferential binding site for sXBP1. Luciferase reporter constructs containing these sequences were electroporated into U266 and MM.1s cells, and luciferase activity monitored after a 16-h treatment with or without

Tm. In U266 and MM.1s cells, levels of ERSE- and UPRE-driven luciferase were equally high in both the non-treated and treated samples (Fig. 1c, d), indicative of a constitutive activity which cannot be further enhanced by tunicamycin treatment. These constitutive activities in MM cells were detected as soon as 2 h after electroporation (data not shown). The same experiment was repeated in Hela cells, after they were transiently transfected with the luciferase reporter constructs described above. Here, only low luciferase activity was detected in non-stimulated cells, whereas treatment with Tm led to a fivefold stimulation. (Fig. 1c, d). A good correlation was found between the p50ATF6 band intensity and the ERSE-driven luciferase production (Fig. 1e). Since electroporation can be considered as a strong and non-specific stress-producing manipulation, its effect on UPR was checked. Luciferase activities were found identical in electroporated and control Hela cells, indicating that the UPR was not stimulated by electroporation itself (data not shown). In addition, electroporated U266 cells did not exhibit phosphorylated form of eIF2 (eIF2-p, data not shown).

In the partial UPR described for plasma cells, the PERK pathway leading to eIF2 phosphorylation and repression of translation is not activated [22]. We checked the level of eIF2-p in MM.1s cells treated with or without Tm for 15 min. As shown in Fig. 1f, eIF2 is weakly phosphorylated in

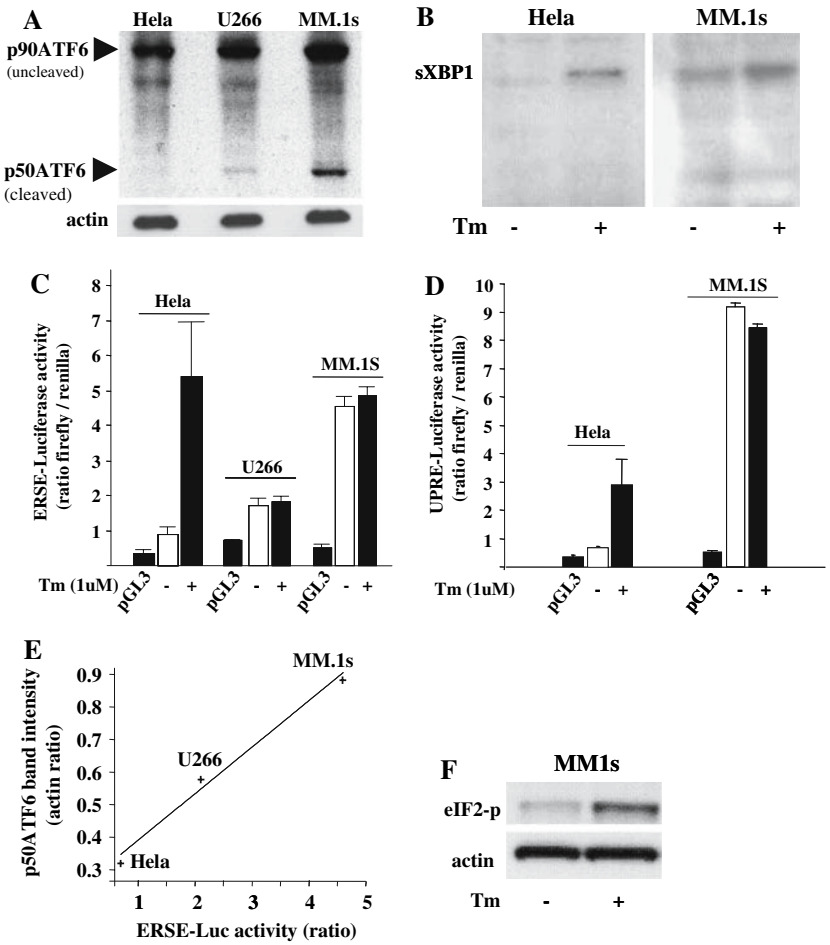


**Fig. 1** A partial, physiologic, UPR is constitutively activated in MM cells. **a** ATF6 cleaved form is only detected in MM cells. An equal amount of cell lysates (30 ug) from untreated MM and non-MM cells (Hela) was loaded in each lane and subjected to immunoblot analysis using anti-ATF6 antibody. **b** sXBP1 is constitutively activated in MM.1s cells but not in Hela cells. An equivalent immunoblot experiment as described above was performed using anti-XBP1 antibody. **c** Activation of ERSE-driven luciferase activity in two MM cell lines, MM.1s and U266, and Hela cells were electroporated with ERSE-Luc together with a tk-renilla construct, ±Tm 2 uM for 16 h, and both renilla and firefly luciferase activities monitored. A ratio of firefly/renilla luciferase activity was calculated to normalize each sample. **d** Activation of UPRE-driven luciferase activity in MM.1s and Hela cells. Cells were electroporated with UPRE-Luc together with a tk-renilla construct and treated as in **c**. **e** Linear correlation between the ERSE-luciferase activity and the ATF6 cleaved band intensities. **f** PERK is not activated in MM cells. MM.1s cells were treated with or without 2 uM Tm for 15 min, directly dissolved into the loading buffer, and loaded onto a polyacrylamide gel. Membranes were then subject to immunoblot analysis using anti-eIF2-p antibody

non-treated MM.1s cells, but phosphorylation is induced after Tm treatment. These results suggest that, similar to plasma cells, the first two arms of the UPR are constitutively active in MM cells whereas the third arm, while functional, is not activated under normal conditions.

## The constitutive UPR in MM is blocked by IPI-504 in a dose dependent manner

IPI-504 is a novel and highly soluble analog of 17AAG, an inhibitor of Hsp90. Since it has been shown that IREα, ATF6 and also PERK are Hsp90 client proteins [39], we studied the effects of IPI-504 on the UPR. Using the luciferase reporter gene assay, we show that IPI-504 can abrogate both the UPRE and ERSE-driven luciferase activity in non-treated U266 and MM.1s cells (Fig. 2a, b) as well as in Tm-treated cells (data not shown). The IC50s for the inhibition of reporter gene activity by IPI-504 were 196 ± 56 nM in U266 and 472 ± 177 nM in MM.1s for UPRE-luc activity and 213 ± 140 nM for the ERSE-driven activity in MM.1s cells. Since the enzymatic activity of a CMV-driven luciferase construct was not affected by 10 uM IPI-504 incubation (data not shown) we conclude that luciferase itself is not an Hsp90 client protein, and that the decrease of luciferase activity is not due to a lack of proper maturation or stability of the reporter-enzyme.

Since the major transcription factors activating the ERSE and UPRE are p50ATF6 and sXBP1 [12], the effects of IPI-504 on the stability of these two proteins were studied. Using immunoblotting and an antibody to ATF6, we show that IPI-504 treatment leads to a dose-dependent decrease of p50ATF6 (Fig. 3a). The EC50, calculated using densitometry to evaluate band intensity (Fig. 3b), was found at 237 nM consistent with the reporter-gene assay. Also, in agreement with the fact that IREα degradation is increased in presence of an Hsp90 inhibitor [39], we show that the level of sXBP1 is decreased in the presence of IPI-504 (Fig. 3c) with an apparent EC50 between 300 nM and 1 uM. Finally, since PERK is also an Hsp90 client protein [39], the effect of IPI-504 on both PERK itself and eIF2-p were examined. MM.1s cells were pre-incubated for 16 h with increasing concentrations of IPI-504 and then stimulated for 15 min using 1 uM Tm. Immunoblots using PERK and eIF2-p specific antibodies (Fig. 3d, e) show a decrease of the specific bands with increasing doses of IPI-504 with apparent EC50s of around 300 nM in both cases. Using the same tools, the time-course of the IPI-504 effects were investigated. The degradation of p50ATF6 took place between 16 and 24 h, whereas sXBP1 protein degradation is detected after 16 h and is nearly completed after 32 h. eIF2-p is not degraded during the first hours of incubation, but is completely eliminated after 16 h (Fig. 4).

## IPI-504 induces apoptosis in MM cells

Since we hypothesized that IPI-504 can induce cell death in MM cells in part because of its ability to block the UPR, the characteristics of IPI-504 growth inhibitory activity were determined. IPI-504 induces cytotoxicity in MM cells in a dose-dependent manner with an EC50 of growth inhibition of 307 ± 51 nM after 72 h incubation (Fig. 5a). This activity is blocked by co-incubation with DEVD-CHO, a caspase 3 inhibitor, indicative of a caspase-dependent apoptotic mechanism for IPI-504 (Fig. 5b). To further confirm the apoptotic nature of the cell death induced by IPI-504, we monitored the induction of DNA fragmentation in MM cells (Fig. 5c). IPI-504 induces DNA fragmentation (measured by the TUNEL assay) with an EC50 of 211 ± 45 nM in MM.1s cells. Lastly, caspase 3/7 activities were shown to be activated in cells incubated with various concentrations of IPI-504, slightly at 16 h and more strongly after 24 h (Fig. 5d).

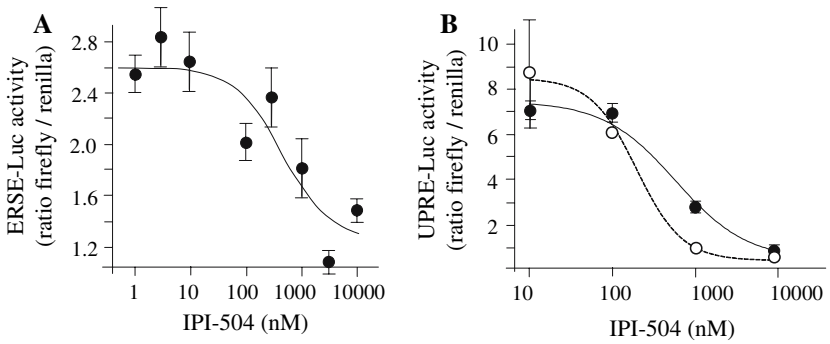


**Fig. 2** ERSE and UPRE-driven gene activation are inhibited by IPI-504 in MM cells in a dose dependent manner. MM.1s (*filled circle*) and U266 (*open circle*) cells were electroporated with ERSE-Luc **a** and UPRE-Luc **b** together with a tk-renilla construct and incubated for 16 h with increasing amounts of IPI-504. Cell lysates were then assayed for both renilla and firefly luciferase activities. A ratio of firefly/renilla luciferase activities was calculated to normalize each sample

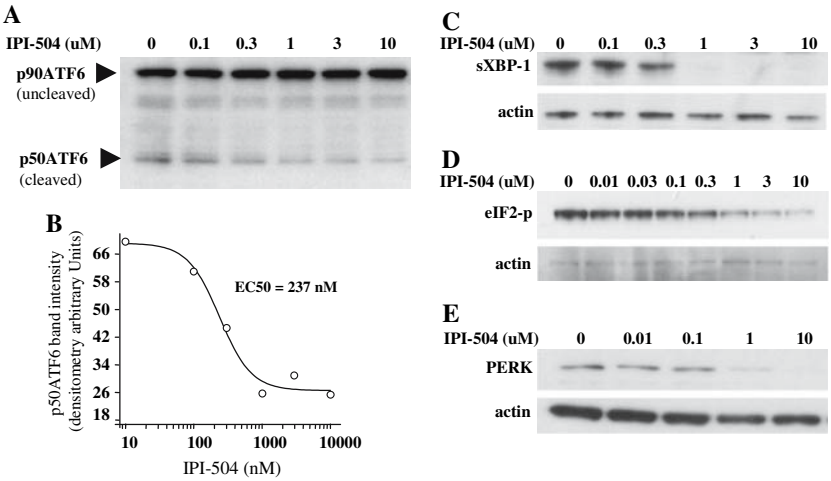


**Fig. 3** IPI-504 inhibits the partial UPR in a dose-dependent manner. MM.1s cells were treated for 16 h with increasing concentrations of IPI-504. An equal amount of cell lysate (30 ug) of treated MM1s cells was loaded in each lane and subjected to immunoblot analysis using the indicated antibodies. **a** Immunoblot analysis was carried out using anti-ATF6 antibody and **b** the p50ATF6 band intensities were evaluated by densitometry and plotted. **c** Immunoblot analysis was performed using antibody against XBP-1, or **d** against the phosphorylated form of eIF2. **e** For the PERK and eIF2-p immunoblot, cells were first treated for 16 h with increasing concentrations of IPI-504 and then stimulated for 15 min with Tm, 2 uM

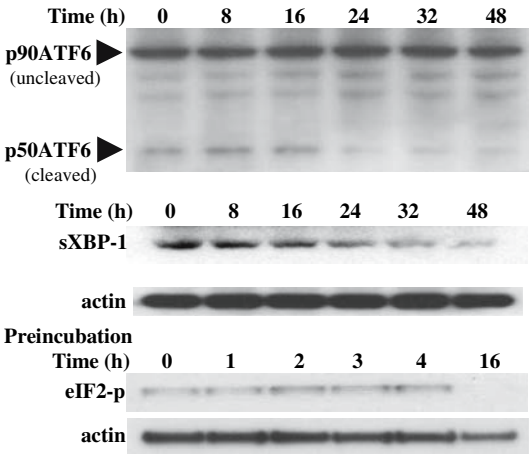


**Fig. 4** IPI-504 inhibits the UPR pathway in a time-dependent manner. MM.1s cells were treated with 1 uM IPI-504 for different times and, in the case of eIF2-p, subjected to 2 uM tunicamycin stimulation for 15 min. An equal amount of cell lysates (30 ug) was loaded in each lane and subject to immunoblot analysis using indicated antibodies

## Discussion

Multiple myeloma is a malignancy derived from plasma cells and it has been shown that during the differentiation of these cells, an unfolded protein response is induced [22]. Nevertheless, it has remained a question in the field of whether the XBP1 arm or other arms of the unfolded protein response are constitutively active in multiple myeloma cells [3]. Should the UPR be constitutively active in MM cells, it becomes an interesting question as to how MM cells react when exposed to an inhibitor of the protein chaperone Hsp90. On the one hand, inhibition of the chaperone should induce more ER stress and lead to a higher UPR response, on the other hand, several of the ER stress sensors have been shown to be Hsp90 clients and therefore an Hsp90 inhibitor could also block the UPR.

We show here that in multiple myeloma cells, in contrast to Hela cells, ERSE and UPRE reporter genes are constitutively on, indicating an active UPR. This activity can be traced back to constitutively active transcription factors sXBP1 and p50ATF6. This ERSE and UPRE reporter gene activity can be efficiently inhibited by the Hsp90 inhibitor IPI-504, indicating an inhibition of the UPR, and we show here that this inhibition is correlated with a degradation of the activated transcription factors.

The UPR is a cellular response to ER stress and there are several published reports suggesting that the UPR might be constitutively activated in MM cells. These cells originate from plasma cells and produce high levels of Ig, which is associated with a certain degree of ER stress. In plasma cells, this stress is alleviated by the activation of the UPR. Indeed, a relationship between Ig production during differentiation of B cells into plasma cells and UPR activation has been reported [22]. It has also been shown that sXBP1, a key UPR effector, is coordinating changes in cellular structures such as the ER, mitochondrial mass and protein synthesis machinery by up-regulating numerous genes [20, 43, 45]. In addition to XBP1, ATF6—but not PERK—are activated in plasma cells, defining a physiologic UPR [20, 22]. This ‘physiologic’ UPR differs from the initially defined pharmacologically-activated UPR.

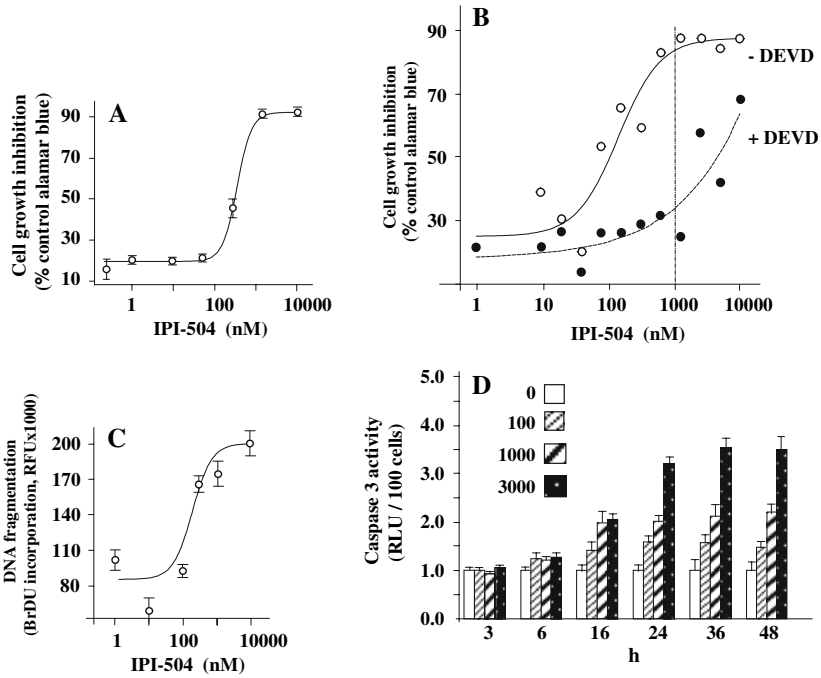


**Fig. 5** IPI-504 induces apoptosis in MM cells. MM.1s cells were incubated for 3 days with increasing concentrations of IPI-504. **a** Alamar blue was added to the wells and the fluorescence was read after a 6-h incubation as described in "Materials and Methods". **b** Cells were either treated with or without 125 nM of DEVD-CHO a caspase 3 inhibitor and treated as in **a**. **c** TUNEL assay was run as described in "Materials and Methods". **d** Cells were incubated with increasing IPI-504 concentrations (see *bar graph legend*, in nM) for 1 h with the caspase 3–7 luminescent substrate, and caspase activities were measured according to manufacturer's instructions. Results are mean values ± SD (*bars*) of three independent experiments

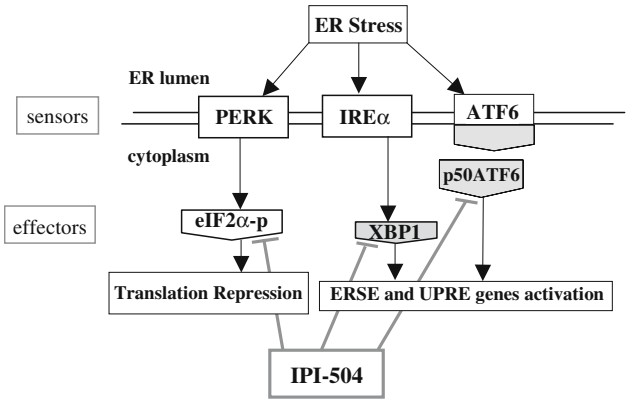


**Fig. 6** A model for the anti-UPR activity for IPI-504. All three arms of UPR are blocked by IPI-504

Here we have provided evidence that in MM cells, but not Hela cells, activated forms of both ATF6 and XBP1 effectors are constitutively expressed, whereas PERK is not active (Fig. 1). This is consistent with a recent gene expression profiling study in MM cell lines, showing that UPR-specific genes, including ATF6 and XBP1, are up-regulated [46, 47]. Also, at the protein level, sXBP1 has been shown to be expressed in MM cells [43]. Here, we report that the two main response elements activated by p50ATF6 and sXBP1, ERSE and UPRE, are constitutively active in MM cells and cannot be further induced by tunicamycin. This suggests a scenario in MM cells where the first two arms of the UPR (p50ATF6 and sXBP1) are constitutively active, whereas the third (PERK) is functional but not constitutive (Fig. 1). While performing experiments to determine sXBP1 levels in MM cells, we noted variability in the abundance of the protein from experiment to experiment. Interestingly, a similar variability has recently been reported in patient samples [48], where in a study of 22 MM patient samples, 10 exhibited a high sXBP1/uXBP1 mRNA ratio, whereas the others had a low ratio. Noteworthy, high levels of XBP1 are correlated with a poor prognosis in that study.

In other cancer cells, the cytotoxicity of Hsp90 inhibitors is generally explained by the degradation of a critical client protein (e.g., Her2, mutant EGFR) that the cancer cell has become dependent on. In MM cells no such client protein has been identified. We show here that, similar to proteasome inhibitors [43], HSP90 inhibitors work to inhibit the UPR [39], which we find constitutively on in MM cells. Concentration and time dependencies indicate that inhibition of the UPR might be a significant contributor in the cell killing activity of geldanamycin derivatives.

IPI-504 inhibits the growth of MM cells with an IC50 of about 300 nM (Fig. 5a), and this cytotoxic activity is associated with caspase activation and DNA fragmentation, two hallmarks of apoptosis (Fig. 5). In addition, incubation of MM cells with the caspase inhibitor DEVD prevents efficient cell killing by IPI-504 (Fig. 5b). To investigate the connection between inhibition of the UPR and induction of cell death by IPI-504, we looked at both concentration and time dependence. The spliced form of XBP1 is most strongly reduced between 0.3 and 1 uM (Fig. 3) and the active form of ATF6 is reduced between 0.1 and 1 uM (Fig. 3). This correlates well with a cell growth inhibition EC50 of about 300 nM (Fig. 5a). By following the time course of sXBP1 and p50ATF6 degradation, sXBP1 disappears

gradually starting at 16 h, while for ATF6 there is a more abrupt change between 16 and 24 h. This again correlates with the time course of induction of caspase 3 activity, which is maximal between 16 and 24 h (Fig. 5d).

These results lead us to suggest that the IPI-504 induced caspase 3 activation—and thereby apoptosis—derives, at least in part, from an inhibition of the UPR. It is not clear how activation or inhibition of the UPR is connected to the induction of apoptosis. For prolonged activation of the UPR, multiple, probably redundant, pathways have been implicated in this process. For example, ER stress activates IRE1 and PERK, which signal through the Jun N-terminal kinase (JNK) to cause apoptosis [49]; the transcription factor CHOP is induced by ER stress and contributes to cell death by up-regulating ER protein synthesis, down-regulating Bcl-2 and by inducing an oxidizing environment in the ER [50, 51]. ER stress has also been shown to activate the murine caspase 12 (caspase 4 in humans) [52]. Lately, the involvement of caspase 12 in this process has been questioned [53, 54] and strong links of the ER to the intrinsic (mitochondrial) apoptosis pathway have emerged. Both the apoptosome component APAF1 has been shown to be involved in ER stress-induced apoptosis and the Bcl-2 related death inducers Noxa and PUMA have been found to be highly up-regulated by ER stress [54, 55]. Since IPI-504 blocks the UPR it might not activate apoptosis by the mechanisms mentioned above [56]. Whatever the exact signaling pathway might be, we have shown here (by TUNEL assay) that IPI-504 leads to cell death by apoptosis in MM cells, and that this cell death is mediated by caspases.

It has been reported that, in CHO and Cos cells, Geldanamycin used at sub-lethal concentrations and analyzed at early time points can induce the transcription of ER chaperones through binding to GRP94 [13]. As we have shown here, in multiple myeloma cells, a "physiological" UPR is already induced without drug treatment, so these GRP94 mediated events might not be detectable in MM cells. In other cells, however, the effect of Hsp90 inhibitors on the UPR might be twofold: at early time points the UPR is induced through inhibition of GRP94, at later time points, as shown here, inhibition of Hsp90 leads to degradation of activated ATF6 and XBP1, thereby shutting down the UPR (Fig. 6).

This is reminiscent of the reported action of the proteasome inhibitor bortezomib on MM cells [43]. Bortezomib [57], as well as Hsp90 inhibitors [39], induce the UPR at early time points but inhibits that response at later time points. This behavior might predict a synergy between HSP90 inhibitors and proteasome inhibitors on MM cells which has in fact been observed [33, 58].

More generally, secretory cells that require an active UPR to ensure proper protein processing may be very susceptible to apoptosis by agents, like IPI-504, that evoke ER stress but disrupt the UPR. Comparison of the UPR status (sXBP1/uXBP1 mRNA ratio) of patient samples and their susceptibility to combination of Hsp90 and proteasome inhibitors might help clarify the importance of this pathway and its clinical relevance.

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