Preclinical Activity of the Novel Orally Bioavailable HSP90 Inhibitor NVP-HSP990 against Multiple Myeloma Cells

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Abstract. *Background: HSP90 inhibitors effectively reduce expression and activity levels of oncogenic survival proteins. However, their clinical anti-multiple myeloma (MM) activity has been found to be rather weak, spurring the exploration of combination therapies and development of compounds with improved physicochemical properties. Materials and Methods: Preclinical effects of the novel orally bioavailable HSP90 inhibitor NVP-HSP990 on the viability, apoptosis and client protein levels of MM cells (established cell lines and clinical specimens) were tested alone and in combination with other drugs. Results: NVP-HSP990 exerted profound activity against MM cells, with a molecular mode of action conforming well with its role as HSP90 inhibitor. Enhanced activity was most obvious in combination with melphalan. Combination with a phosphatidylinositol-3-kinase (PI3-kinase)/mammalian target of rapamycin (mTOR) inhibitor, rendered the HSP90 blockade-mediated stress response ineffective and considerably increased the anti-MM toxicity. Conclusion: Given the current interest in both HSP90 and PI3-kinase/mTOR as potential clinical targets, these observations could broaden the therapeutic utility of either class of inhibitor in MM.*

The therapy of multiple myeloma (MM), a fatal plasma cell neoplasm, which accounts for about 13% of haematologic types of cancer, has been substantially improved over the last decade (1, 2). Altered chemotherapeutic regimens, transplantations and successful novel drugs such as bortezomib, thalidomide and lenalidomide, are having a significant impact on the extent of remissions and on overall survival (3). However, the large majority of patients will eventually be confronted with refractory disease and certain genetically defined subproups of patients, as well as those with extramedullary manifestations, face particularly dire prognoses (4, 5). In order to explore additional and complementary therapeutic approaches, inhibitors of the heat shock protein of 90 kDa (HSP90) are being tested in a number of preclinical and clinical settings in MM (6-9). HSP90 is an ATP-dependent molecular chaperone that promotes correct folding and conformational stability of a number of proteins (termed 'clients'), many of which are implicated in sustaining the malignant phenotype (10, 11). Additionally, overexpression of HSP90 in malignant cells (12, 13), and a high retention time of HSP90 inhibitors (14), as well as their better affinity for HSP90 in tumor tissue (15), are characteristics assumed to contribute to the strong therapeutic index observed in preclinical evaluations. Nevertheless, clinical effectivity of HSP90 inhibitors in single-agent trials for myeloma has, at best, been very modest, although these studies were performed in heavily pretreated patient collectives (16). A better result was obtained with a combination of the HSP90 inhibitor 17-AAG (Tanespimycin) and the proteasome inhibitor bortezomib, suggesting that the combination yields better responses than bortezomib alone in at least some patients with relapsed MM (9). Although ansamycin-based compounds have been the foremost in preclinical and clinical testing (17), ongoing efforts are being directed at diversifying the molecular scaffolds of HSP90 inhibitors in order to enhance their solubility (to achieve orally administrable drugs), to achieve improvements regarding pharmacokinetics and toxicity and to provide safeguards against potential structure-related resistance mechanisms, such as down-regulation of the enzyme NQ-diaphorase in the case of

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ansamycins (11, 18). In this study, we characterized the preclinical anti-myeloma efficacy of NVP-HSP990, a highly orally bioavailable HSP90 inhibitor based on a novel chemical core structure, alone and in combination with other clinical and experimental compounds.

Materials and Methods

HSP90 inhibitor NVP-HSP990 and other small molecule compounds. The synthesis and properties of the novel HSP90 inhibitor NVP-HSP990 ((R)-2-amino-7-((R)-4-fluoro-2-(6-methoxypyridin-2-yl)phenyl)-4-methyl-7,8-dihydropyrido[4,3-d]pyrimidin-5(6H)-one) will be described elsewhere. Stock solutions (20 mM) in H_2O-free dimethylsulphoxide (DMSO) were stored at –20˚C and all working dilutions were always freshly prepared from these stocks. Drugs tested in combination experiments with NVP-HSP990 were bortezomib (LC Laboratories, Woburn, MA, USA), melphalan (Sigma, Deisenhofen, Germany), doxorubicin (Merck Chemicals, Darmstadt, Germany), and PI103 hydrochloride (Axon Medchem, Groningen, the Netherlands).

Cell culture. The culture of human MM cell lines (AMO-1, INA-6, JJN-3, KMS-11, KMS-12-BM, L363, MM.1s, OPM-2, RPMI-8226, U266), as well as acquisition, purity assessment and culture of primary MM cells and of bone marrow stroma cells (BMSC) has been described in detail in Stühmer *et al*. (19). All primary materials were obtained from routine diagnostic samples after obtaining informed consent of patients and permission by the Ethik-Kommission der Medizinischen Fakultät der Universität Würzburg (reference number 73/05). Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density centrifugation from buffy coats obtained from healthy donors. MM cell lines were either purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), or from LGC Standards (Wesel, Germany). Each purchased cell line was immediately expanded to create a stock bank (consisting of 40-50 frozen aliquots) and from one of these aliquots a working bank (again consisting of 40-50 frozen aliquots) was generated. In order to maintain cell culture authenticity and consistency, a fresh working bank aliquot was thawed and used for experiments every 3-4 months (dead-end cell culture).

Drug combination analyses. To determine combination effects of NVP-HSP990 and other drugs according to the method of Chou and Talalay (22), single drug and drug combination dose effect curves were generated using the Alamar blue assay in 96-well formats (25000 cells per well for AMO-1, 40000 for L363 and MM.1s; 2 wells per concentration). The molar ratio for combination of NVP-HSP990 with other drugs was determined by the respective single-drug (EC_{50}) values. In addition to the combination at EC_{50}s, at least four lower and three higher concentrations were also chosen (single ray – constant ratio design (22)). Single-drug and combination experiments were simultaneously performed (drug exposure for 3 days) and the results were analyzed with CalcuSyn software (version 2.1; Biosoft, Cambridge, UK). Only drug concentrations that resulted in effects exceeding 2% but not exceeding 98% (*i.e.* values that essentially determine the shape of the respective dose-effect curve) were included in the analysis and at least four values were required to represent this range. An experiment was not evaluated if the correlation coefficient for any of the three dose-effect curves involved was lower than 0.95. A combination index (CI) of 1 represents additivity while lower values indicate synergy and higher values antagonistic effects.

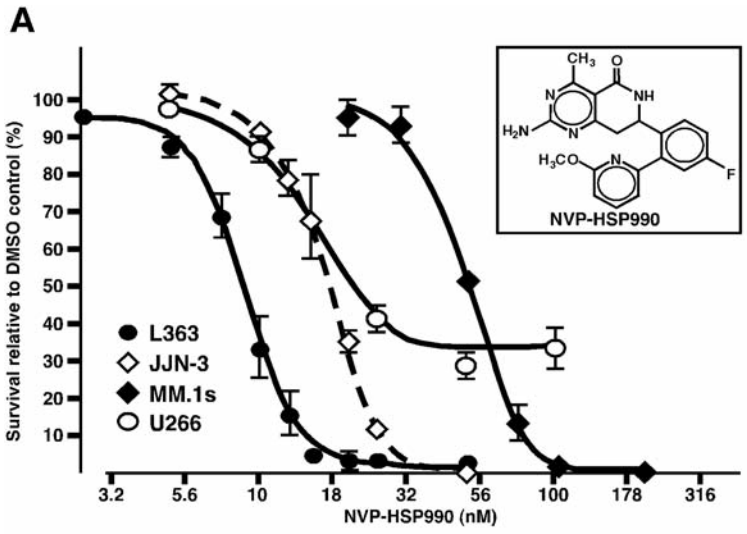
Apoptosis assay. Cell death was assessed through annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining as described elsewhere (20). Dose-response curves were calculated from at least three independent experiments by non-linear regression analysis (variable slope dose-response curve) using GraphPad Prism 3.0 (GraphPad Software, La Jolla, CA, USA). For apoptosis inhibition experiments the caspase inhibitor zVAD-FMK (PeptaNova GmbH, Sandhausen, Germany) was added at a concentration of 50 μM at the same time as NVP-HSP990. For longer incubations zVAD-FMK was replenished after 24 h.

Viability assay. The Alamar blue colorimetric assay was used to appraise drug effects on the viability (metabolism/proliferation/cell death) of MM cells. Between 25000 and 50000 cells were seeded per well (96-well plates) and each test concentration was prepared in triplicate. The colour reaction measurement in a microplate reader and calculation were performed as described in the manufacturer's manual (MorphoSys, Oxford, UK). Effects were quantified in relation to DMSO-treated controls (100% values).

Western blot analysis. Cell lysis was carried out for 15 min on ice in 30 mM Tris-HCl, 120 mM NaCl, 10% glycerol, 1% Triton X-100 (pH 7), with addition of complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktails I and II (Sigma). After clearing (centrifugation at 16,000 x*g*, 15 min), all procedures were performed as described elsewhere (21). The primary antibodies used were: anti-β-actin (Sigma), anti-pan-AKT (Cell Signaling Technologies (CST), Frankfurt am Main, Germany), anti-phospho-AKT (Thr308) (CST), anti-HSP27 (Stressgen Bioreagents, Ann Arbor, MI, USA), anti-HSP72 (Stressgen Bioreagents), anti-HSP90β (Millipore, Schwalbach, Germany), anti-inhibitor of nuclear factor kappa B kinase alpha (IKKα) (BD Biosciences, Heidelberg, Germany), anti-IKKβ (CST), anti-IKKγ (Santa Cruz, Heidelberg, Germany), anti-poly (ADP ribose) polymerase 1 (PARP-1) (Santa Cruz), anti-v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) (Santa Cruz), anti-receptor interacting protein (RIP) (BD Biosciences). The secondary antibodies used were directed against either rabbit (Jackson ImmunoResearch Laboratories, Newmarket, UK) or mouse (Jackson ImmunoResearch Laboratories).

Results

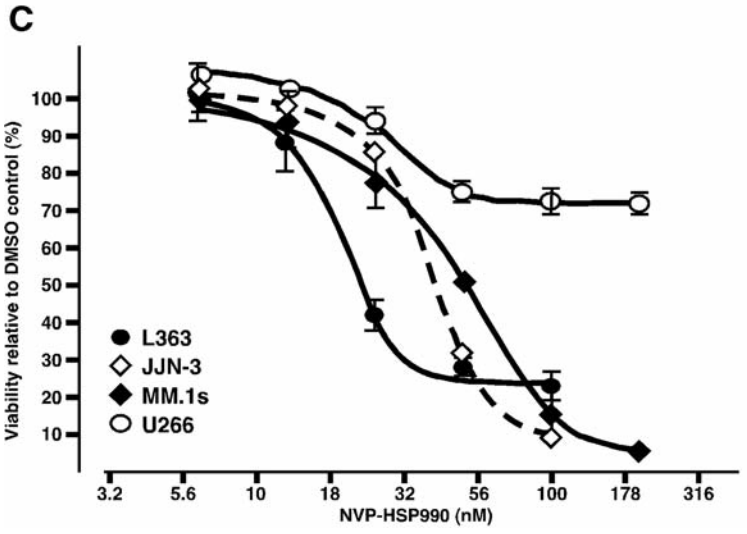
Toxicity of NVP-HSP990 in MM cells versus nonmalignant cells. We started our analysis of the effects of NVP-HSP990 in MM cells by establishing kill curves (annexin V-FITC/propidiumiodide apoptotic assay) and viability curves (Alamar blue metabolic assay) for a range of MM cell lines (Figure 1). Out of the nine MM cell lines tested, six (AMO-1, INA-6, JJN-3, L363, OPM-2, RPMI-8226) could be described as being very sensitive to NVP-HSP990, given the fact that they displayed steep and full kill curves, with EC_{50}s ranging from 8-21 nM, and EC_{90}s from 14-41 nM) (Figure 1 A,B).



B

	NVP-HSP990 (nM)	
MM line	EC 50	EC 90
AMO-1	18	24
INA-6	18	24
JJN-3	17	26
KMS-11	20	60
KMS-12-BM	31	n.r.
L363	8	14
MM.1s	54	75
OPM-2	21	41
RPMI-8226	15	28
U266	18	n.r.

n.r. = not reached at 1 μM



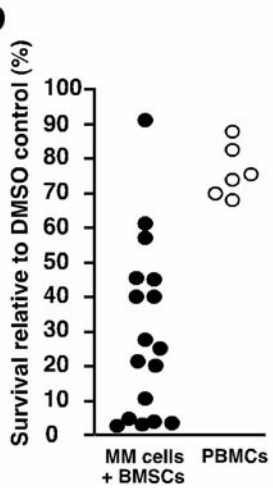


Figure 1. *Cytotoxicity of heat-shock protein 90 inhibitor NVP-HSP990 in multiple myeloma (MM) cells. A: Exemplary kill curves determined by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) measurements after 3 days' exposure to rising concentrations of NVP-HSP990. Inset: chemical structure of NVP-HSP990. B: Overview of the 50% and 90% effective concentrations (EC_{50}/EC_{90}) for NVP-HSP990-induced cell death for 10 MM cell lines. C: Viability curves as determined by the Alamar blue assay after 3 days' exposure of selected MM cell lines to increasing concentrations of NVP-HSP990. Similarity of the effect curves to those in (A) suggests that this assay is also primarily a reflection of cell death. D: Survival (annexin V-FITC/PI assay) relative to dimethyl sulphoxide (DMSO)-treated controls of purified primary MM cells co-cultured with bone marrow stromal cells (BMSCs) (n=17), or of peripheral blood mononuclear cells (PBMCs) obtained from the blood of healthy donors (n=6) after 3 days' treatment with 100 nM NVP-HSP990.*

Cell lines KMS-11 and MM.1s were slightly less sensitive, but still showed full dose-response relationships (EC_{50}s: 20 and 54 nM, EC_{90}s: 60 and 75 nM, respectively), whereas MM cell lines KMS-12-BM and U266 displayed shallow kill curves, with imcomplete effects, even at higher doses (EC_{90}s not reached at up to 500 nM NVP-HSP990). This pattern, as well as the effective concentrations, were roughly mirrored by the viability assay (Figure 1C), showing that this assay also most likely reflects the effects of cell death rather than arrested proliferation or metabolic restriction. However, treatment of the rather sensitive MM cell lines L363 and JJN-3 with 50 nM of NVP-HSP990, or of the less sensitive lines MM.1s and U266 with 150 nM, either with or without concomitant blockade of caspases with zVAD-FMK, showed that a unifying mechanism of cell death governing the sensitivity of MM cells to the novel HSP90 inhibitor did not appear to be existant. Thus, caspase inactivation substantially improved survival of L363 and U266 cells, arguing for a principally

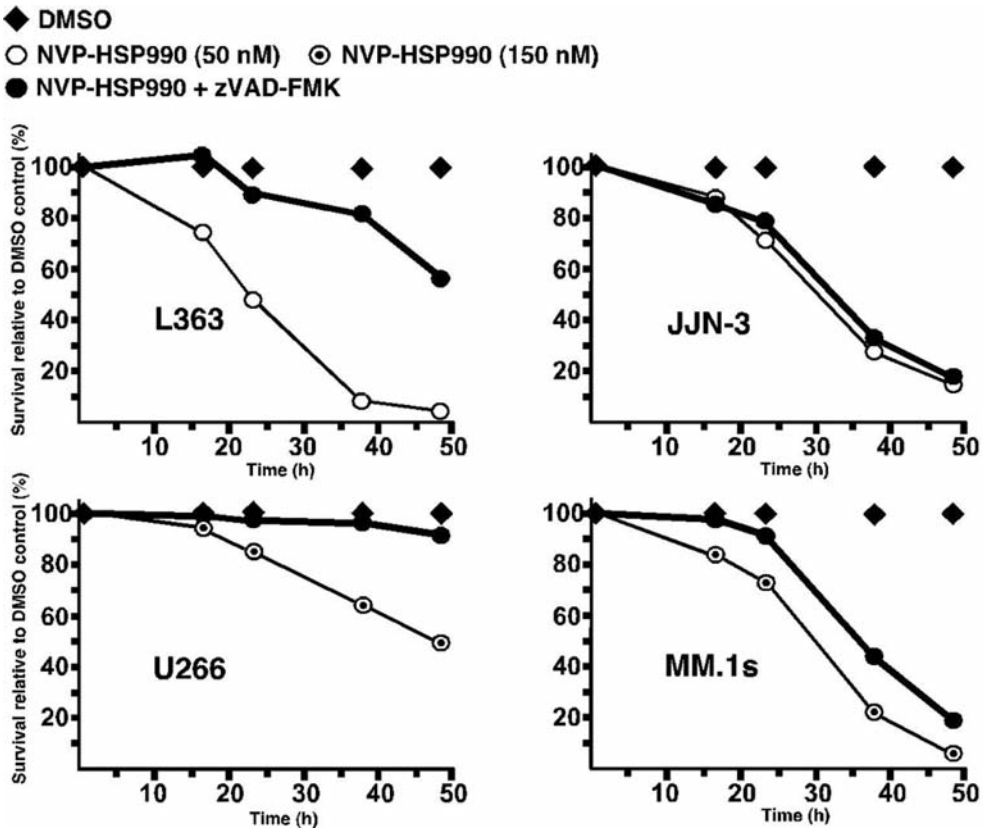


Figure 2. *Mechanism of cell death induced by HSP90 inhibitor NVP-HSP990 in multiple myeloma (MM) cells. Four MM cell lines were treated either with or without NVP-HSP990, and with HSP90 inhibitor in combination with caspase inhibitor zVAD-FMK. Cell death was determined by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and FACS analysis at the time points indicated. The caspase inhibitor was replenished after 24 h. The concentrations of NVP-HSP990 (either 50 or 150 nM) were chosen to represent values that just about suffice to induce the maximal achievable kill effect in the respective MM cell lines.*

apoptotic mechanism of the drug, whereas little (MM.1s) or no (JJN-3) effects were observed for the other two MM cell lines, suggesting that mainly non-apoptotic mechanisms are at work (Figure 2). Primary MM cell samples (n=17), which, for these experiments were always co-cultured with primary BMSCs, also showed a clear measure of heterogeneity in their sensitivity to NVP-HSP990 (Figure 1D; only the effect of treatment with 100 nM NVP-HSP990 for 3 days is shown). About 60% of samples were strongly sensitive (defined as <30% survival with respect to DMSO-treated controls), and the remainder showed intermediate effects (35%-65% survival), except for one resistant sample. Toxicity of 100 nM NVP-HSP990 against nonmalignant PBMCs was in the range of 10-30% compared to DMSO-treated controls (n=6). This effect remained unchanged even at the highest concentrations of the HSP90 inhibitor tested (1 μM) and was mainly due to loss of the CD14-positive fraction, which we have also found to be most sensitive against a number of other drugs (data not shown).

NVP-HSP990 acts as a bona fide HSP90 inhibitor in MM cells. NVP-HSP990 was developed as a structurally novel ATP-competitive inhibitor of HSP90. To test its efficacy in down-regulating the level of HSP90 client proteins in MM cells, we treated cells from four different MM cell lines for 14 h with 50 nM (L363, JJN-3) or 100 nM (MM.1s, U266) of NVP-HSP990 and analyzed the effects on protein levels by western blotting (Figure 3). All MM cell lines responded by strong up-regulation of HSP72, a well-established stress response of cells challenged with HSP90 inhibition, whereas levels of HSP90 itself remained unchanged (Figure 3 exemplarily shows HSP90β). Conversely, protein levels of well-established HSP90 clients, such as RAF-1, AKT, RIP and components of the IKK complex, were often found to be strongly reduced (Figure 3). Strikingly, even though the HSP72 up-regulation in JJN-3 cells was as pronounced as in the other MM cell lines and the cell line as such is very sensitive to the HSP90 inhibitor, most HSP90 clients displayed little, if any, down-regulation under the conditions

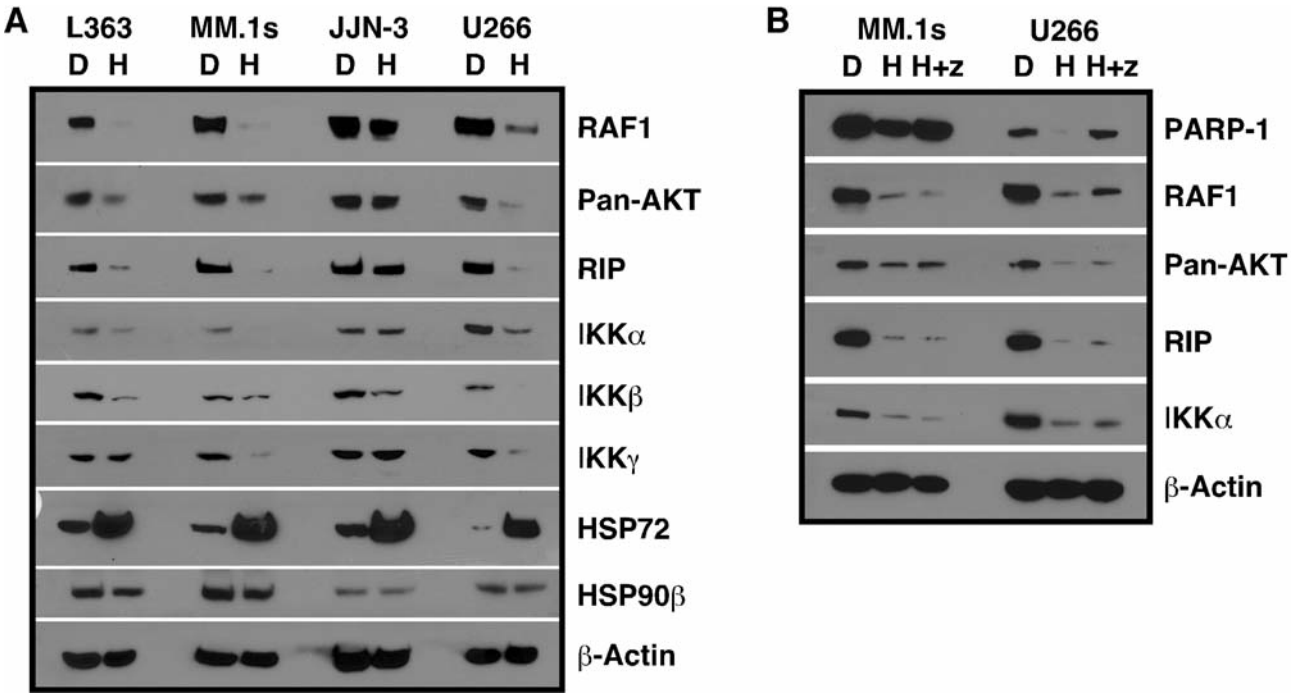


Figure 3. *Heat-shock protein of 90 kDa (HSP90) client protein depletion in multiple myeloma (MM) cells after treatment with the HSP90 inhibitor NVP-HSP990. A: MM cells were incubated overnight with either 50 nM (L363, JJN-3) or 100 nM (MM.1s, U266) NVP-HSP990 (H) or with control amounts of solvent dimethyl sulphoxide (DMSO) (D) and western blot analysis performed for established HSP90 client proteins (RAF1, AKT, IKKα, β and γ; RIP) and non-clients (HSP90, β-actin) as controls. HSP72 staining shows strong up-regulation in all drug-treated samples, indicative of HSP90 inhibition. B: MM cells were treated as in (A) and with concomitant caspase inhibition with zVAD-FMK (H+z) to distinguish caspase from non-caspase-mediated cleavage. In contrast to the caspase target PARP-1 no contrast in the levels of HSP90 client protein depletion is observed between caspase-active and caspase-blocked samples, indicating that within this time frame, the effect on client protein depletion is entirely mediated via HSP90 inactivation by NVP-HSP990.*

tested. Because the NVP-HSP990 kill curve for JJN-3 cells remained essentially unchanged after caspase inactivation (Figure 2), we mechanistically verified the action of NVP-HSP990 by probing protein levels in MM.1s and U266 cells after treatment with 100 nM NVP-HSP990 for 14 h with or without caspase inactivation by zVAD-FMK (AKT depletion, for example, might be either the result of instability due to HSP90 blockade or due to cleavage through caspases, since it is also a caspase substrate). Whereas caspase inactivation (H+z; Figure 3B) completely voided the full or partial cleavage visible for the caspase substrate PARP-1, the treatment had no effect on the depletion of HSP90 clients, underpinning the functionality of NVP-HSP990 as a *bona fide* HSP90 inhibitor in MM cells.

Inhibition of HSP72 up-regulation after blockade of HSP90 with NVP-HSP990 and of PI3-kinase with PI103. The up-regulation of HSP72, interpreted as a cellular response to relieve the stress imparted by application of the HSP90 inhibitors, is suspected to play a role in the ability of cancer cells to attenuate the apoptotic effects of such drugs (23, 24). Because we have recently observed that inhibition of PI3-kinase can preclude HSP90 blockade-mediated up-regulation of HSP72, we tested this effect in a number of MM cell lines. Pretreatment for 1.5 h with 2 μM of the PI3-kinase/mTOR inhibitor PI103, followed by addition of NVP-HSP990 (100 nM for another 5 h), nearly completely suppressed HSP72 up-regulation in L363 and OPM-2 cells, strongly inhibited it in MM.1s and to a somewhat lesser extent in JJN-3 cells, but had little effect in KMS-11 cells (Figure 4A), even though the applied concentration of PI103 in all cases completely suppressed AKT phosphorylation at position Thr308 (the PDK1 phosphorylation site, and thus directly downstream of PI3-kinase), indicating full blockade of PI3 kinase. The less pronounced up-regulation of HSP27, which is also part of the HSP90 inhibition-mediated stress response, was also curbed in the PI103/NVP-HSP990 combination when background HSP27 levels were low (MM.1s, OPM-2, JJN-3; Figure 4A). In cell lines with very high constitutive levels of HSP27 (L363, KMS-11) no differences were observed, although small changes might have been obscured by the sheer intensity of the signal. Cell death measurements after treatment of MM

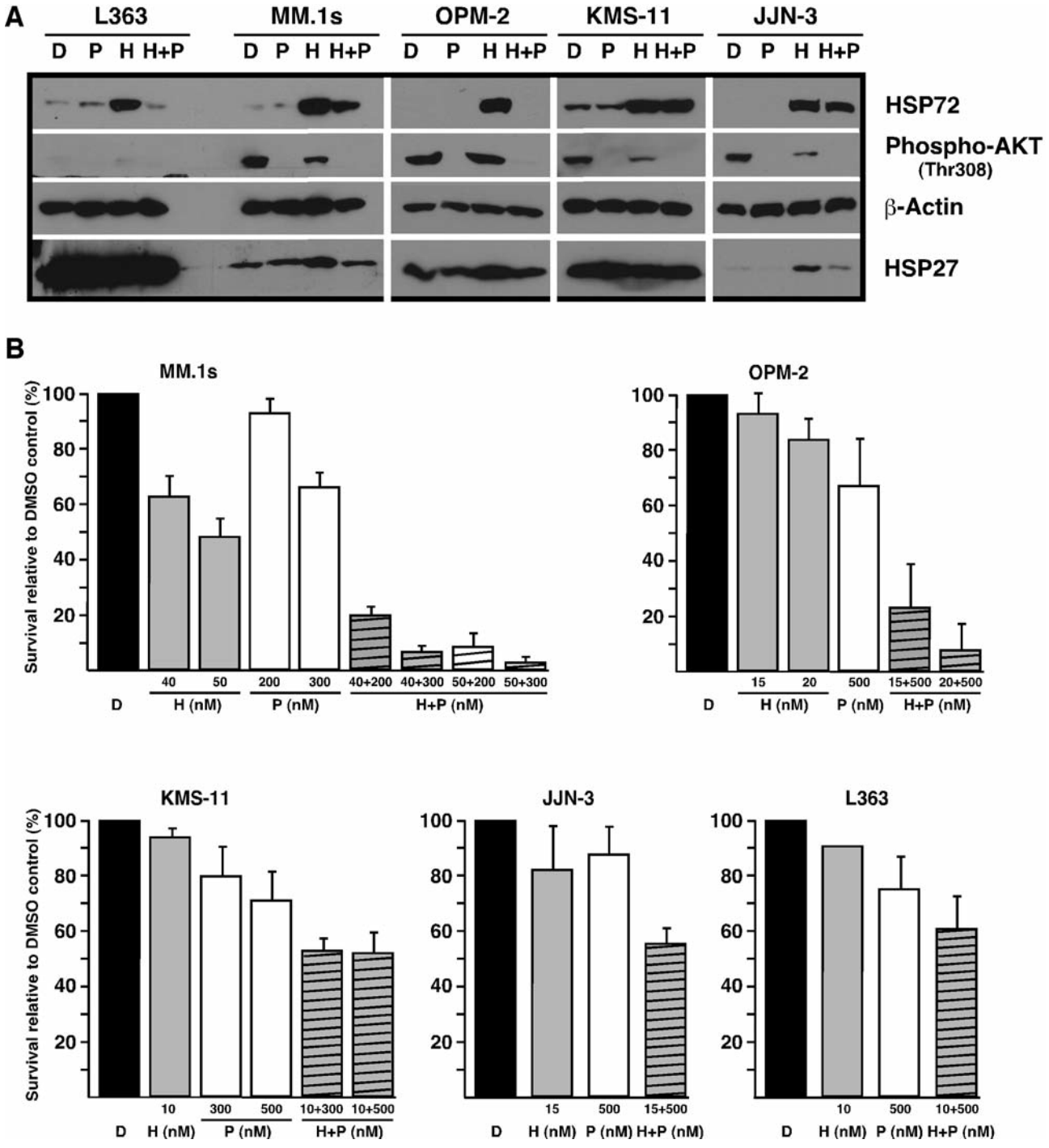


Figure 4. *Concomitant inhibition of PI3-kinase and of heat-shock protein of 90 kDa (HSP90) with the HSP90 inhibitor NVP-HSP990 can prevent the up-regulation of HSP72 in multiple myeloma (MM) cells. A: Western blot analysis of MM cells after preincubation with 2 μM PI3-kinase inhibitor PI103 (P) followed by additional treatment for 5 h with 100 nM NVP-HSP990 (H). The pronounced drug-induced up-regulation of HSP72 was entirely prevented by the combination in L363 and OPM-2 cells, strongly reduced in MM.1s cells, but little affected or virtually unaffected in JJN-3 and KMS-11 cells, respectively. B: Cell death induction as measured by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining after 3 days' treatment with low effective concentrations of NVP-HSP990 and with and without PI103.*

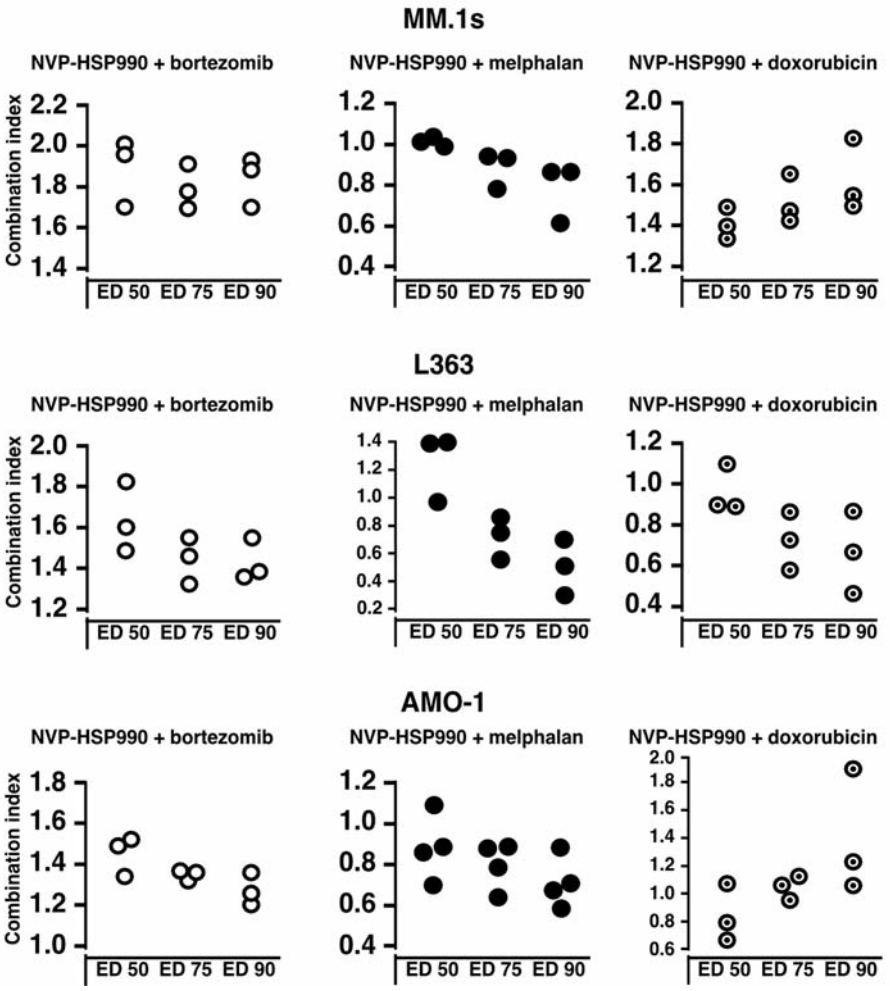


Figure 5. *Combination of HSP90 inhibitor NVP-HSP990 with drugs relevant to the treatment of multiple myeloma (MM). MM cell lines MM.1s, L363 and AMO-1 were treated with NVP-HSP990, bortezomib, melphalan or doxorubicin individually and each of the latter three drugs also in combination with the HSP90 inhibitor (see Materials and Methods section for specific experimental design). Combination indices (CIs) are given for three effect levels (ED 50, ED 75 and ED 90), with each point at a specific effect level derived from a separate experiment. A CI of 1 represents additivity while lower values indicate synergy and higher ones antagonistic effects.*

cells for 3 days with subeffective concentrations (*i.e.* EC_{10} – EC_{40} in the single-drug kill curves) of NVP-HSP990 and PI103 led to strong supra-additive effects in MM.1s and OPM-2 cells. The combination effects were closer to additivity in JJN-3, KMS-11 and L363 (Figure 4B).

Combination of NVP-HSP990 and clinically relevant drugs for the treatment of MM. Because the further development of HSP90 inhibitors for the potential treatment of MM patients is likely to take place in the context of combination therapies, we tested the effects of NVP-HSP990 on the survival of AMO-1, L363 and MM.1s cells, when combined with either bortezomib, melphalan or doxorubicin (Figure 5). Alamar blue assays were used to determine the viability of samples over a range of single and combination drug concentrations, with combinations pipetted to cover the effective range at a constant ratio determined by the EC_{50} concentrations of the single-drug viability curves (constant ratio design (22)). Analyses of combination indices (CI) with the program CalcuSyn of three separate experiments for each drug combination for each cell line showed a tendency from additivity to synergy at higher effect levels for all MM cell lines for the combination of NVP-HSP990 with melphalan, and for NVP-HSP990 plus doxorubicin in L363. The latter combination became increasingly antagonistic at higher effect levels in AMO-1 and MM.1s cell lines. The combination with bortezomib was slightly antagonistic at all effect levels in these three MM lines (Figure 5).

Discussion

The concept of HSP90 inhibition as anticancer therapy is still relatively new, and a number of HSP90 inhibitors are currently in different phases of clinical testing (www.clinicaltrials.gov). However, application of the furthest developed (17-AAG, Tanespimycin) in a heavily pretreated MM patient collective yielded little clinical benefit (16). The combination of 17-AAG and bortezomib has led to better results, with an objective response rate of 27% reported for a group of 67 evaluable patients (9). It is therefore commonly assumed that if HSP90 inhibitors are to be effectively applied in MM treatment, this will require combination therapies. Another important issue with clinical development of HSP90 inhibitors is toxicity, which has generally been reported to be manageable in the clinical trials thus far. However, dose-limiting toxicities are currently still being probed and evaluated, and it remains unclear whether sufficient target inhibition is attainable at permissible dosages. It will also be important to distinguish between toxicities due to on-target effects (*i.e.* blockade of HSP90) and off-target effects because the latter may substantially differ between drugs or drug classes and can also be related to the specific formulations, especially if compounds are not water soluble.

NVP-HSP990 represents one of the most potent oral HSP90 inhibitors reported thus far, and addresses all major issues [*i.e.* efflux by P-glycoprotein, metabolism by cytochrome P450 3A4 or NAD(P)H quinone oxidoreductase 1 (NQO1), hepatotoxicity, difficulty in synthesis and formulation, low oral bioavailability] that have hindered the clinical development of 17-AAG. Our preclinical assessment of the molecular and biological effects of the drug on multiple myeloma cells confirmed strong and selective depletion of HSP90 client proteins, which, as befits a *bona fide* HSP90 inhibitor, is independent of the activation status of caspases. NVP-HSP990 also strongly induces the heat-shock stress response (*e.g.* up-regulation of HSP72) commonly observed after HSP90 blockade. Cell death by apoptotic and non-apoptotic mechanisms was observed at low nanomolar concentrations for MM cell lines. Additionally, the majority of primary MM cells in co-culture with BMSCs were susceptible to treatment with NVP-HSP990, whereas PBMCs remained largely unaffected even at 10-fold higher concentrations of the drug. Collectively, these data show that NVP-HSP990 successfully blocks its target and that this translates into efficient MM cell death in our *in vitro* settings.

The reasons for the low single-agent efficacy of HSP90 inhibitors in clinical trials are not actually clear. The heat shock factor protein 1 (HSF1)-mediated stress response has been touted as a possible pro-survival escape route for cancer cells because it is fast and strongly activated on HSP90 blockade and leads to up-regulation of a number of potentially anti-apoptotic heat-shock proteins, most notably of HSP72 (24). HSP72 knockdown has been shown to enhance the apoptotic effect of HSP90 inhibition in MM (24) and solid tumour-derived cell lines (23), but HSP70 has so far remained an elusive target for direct pharmacological inhibition (25, 26). It had previously been found that certain treatments that curtail the HSP90-blockade-induced stress response can attenuate HSP72 up-regulation, for example treatment with transcriptional incapacitators of HSF1 (25). Here we show that the combination of NVP-HSP990 with PI103, an inhibitor of PI3-kinase (p110α) and mTOR (as well as DNA-dependent protein kinase) can be very effective at preventing the HSP90 blockade-induced up-regulation of HSP72 in MM cell lines, and that the combination can lead to strongly enhanced cell death in some MM cell lines. However, as the example of KMS-11 and JJN-3 cell lines shows, HSP72 up-regulation may only be partially prevented or hardly affected at all, and a range of susceptibilities regarding the extent and speed of this effect may therefore be expected if still larger cohorts of cell lines or primary MM samples are analysed. Up-regulation of HSP27, another member of the HSP90 blockade-induced stress response can also be observed, but constitutive HSP27 levels are extremely variable across MM samples and its role in apoptosis prevention is therefore difficult to judge. Of note, MM.1s and OPM-2 cells, which showed the most impressive induction of cell death up on use of combination of the two inhibitors, represent the two cell lines with, by far, the strongest constitutive AKT activation ((27) and data not shown). Since the activity of AKT is affected by HSP90 blockade (as an HSP90 client), as well as through blockade of PI3-kinase/mTOR, this might provide a relatively straightforward mechanistic point of convergence for the synergistic cell death activity of the drug combination. In that respect, the combination of the ansamycin class HSP90 inhibitor 17-demethoxy-17-[[(2-dimethylamino) ethyl]amino]geldanamycin (17-DMAG) and perifosine, an alkylphospholipid that interferes with AKT activation, led to synergistic cytotoxicity in OPM-2 and MM.1s cells, also corroborating the potential utility of HSP90 and PI3-kinase pathway blockade (28). The clinical testing of NVP-HSP990 with PI3-kinase (with/without mTOR) inhibition may be particularly attractive since the existence of useful therapeutic windows for both components is expected, and the latter inhibitors comprise a large spectrum of compounds currently in preclinical and clinical development.

The reduction in viability of MM cell lines through combination of NVP-HSP990 with either bortezomib, melphalan or doxorubicin followed a pattern previously observed with other HSP90 inhibitors in EC_{50}-based constant ratio combination analyses (29, 30). In essence, for all three cell lines tested the combination effects were largely additive, with a slight antagonistic bias for bortezomib (*i.e.* antagonistic in the sense that although the combination was more effective

than any single drug treatment alone, this was less pronounced than expected from the sum of both single-drug effects). Conversely, the synergy between NVP-HSP990 and melphalan reflects a slightly higher than expected decrease in viability (but quite pronounced at high effect levels in the case of cell line L363) in the combination treatments. Collectively, these data show that while NVP-HSP990 enhances the anti-myeloma effect of conventional therapeutic drugs, this is generally within the range expected for a combination of compounds that do not mechanistically interfere with each other to substantially affect cell death in either a negative (antagonistic) or positive (synergistic) way.

Lastly, it is conceivable that the combination of two structurally different HSP90 inhibitors might provide a road to better clinical efficacy. It is hard to gauge to what extent HSP90 is eventually inhibited in cancer cells of patients under treatment, and whether sufficient blockade for apoptosis induction is actually achieved at tolerable doses (11). The most widely employed surrogate marker for drug efficacy, up-regulation of HSP72, does not necessarily correlate with clinical activity (31) and even in *in vitro* settings, HSP72 is up-regulated at HSP90 inhibitor concentrations that do not yet induce apoptosis (M.C. personal communication). HSP90 inhibitors are therefore being tested for clinical efficacy up to the point of their dose-limiting toxicity, which may, however, not be a consequence of HSP90 blockade but of unrelated side-effects or, as in the case of HSP90 inhibitors with low aqueous solubility, due to solvent-related issues. As more clinical information on the efficiency, optimal dosing regimens and side-effect profiles of different and improved HSP90 inhibitors becomes available, it should become possible to judge if their combination might lead to enhanced target blockade against a backdrop of reduced toxicity. The favourable physicochemical properties of NVP-HSP990 would make it an immediate candidate drug for such an approach.

Taken together, these data show that clinical evaluation of NVP-HSP990 should proceed. In addition to the potential benefits that the improved properties of the drug could offer, the successful future application of NVP-HSP990 (or other HSP90 inhibitors) in myeloma may be determined by factors that are currently still topics of preclinical research. This includes determination of optimal drug combination partners, but also the vastly expanding possibilities of MM genome sequencing which could help to identify patient subgroups, for example those that harbour rare *BRAF* mutations (32), which might show particular promise for therapeutic responses due to HSP90 blockade-mediated client protein depletion.

Conflict of Interest Statement

Zhenhai Gao and Michael R. Jensen are employees and stockholders of Novartis whose potential product is featured in this paper.

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