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ORIGINAL ARTICLE

The anti-myeloma effects of the selective JAK1 inhibitor (INCB052793) alone and in combination in vitro and in vivo

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Abstract

The Janus kinase (JAK) pathway has been shown to play key roles in the growth and resistance to drugs that develop in multiple myeloma (MM) patients. The anti-MM effects of the selective JAK1 inhibitor INCB052793 (INCB) alone and in combination with anti-MM agents were evaluated in vitro and in vivo. Significant inhibition of cell viability of primary MM cells obtained fresh from MM patients, and the MM cell lines RPMI8226 and U266, was observed with single agent INCB and was enhanced in combination with other anti-MM agents including proteasome inhibitors and glucocorticosteroids. Single-agent INCB resulted in decrease in tumor growth of the MM xenograft LAGκ-1A growing in severe combined immunodeficient mice. Mice dosed with INCB (30 mg/kg) showed significant reductions in tumor volume on days 28, 35, 42, 49, 56, and 63. Similarly, INCB at 10 mg/kg showed anti-tumor effects on days 56 and 63. Tumor-bearing mice receiving combinations of INCB with carfilzomib, bortezomib, dexamethasone, or lenalidomide showed significantly smaller tumors when compared to vehicle control and mice treated with single agents. These results provide further support for the clinical evaluation of INCB052793 alone and in combination treatment for MM patients.

Keywords Multiple myeloma · Janus kinase inhibitor · Tumor · In vivo · Xenograft

Introduction

Multiple myeloma (MM) is a bone marrow (BM)-based plasma cell malignancy, which is currently incurable [1–3]. It is well established that pristane, a liquid plastic or hydrocarbon, induces murine plasmacytomas (PCTs) in BALB/cAnPt mice [4]. Initial studies identified a cytokine produced by a murine macrophage cell line as a PCT-growth factor due to its ability to promote the establishment of PCTs in vitro [5]. The effects of this cytokine were shown to be very similar to a protein called B cell stimulatory factor 2, and it was produced by macrophages. It was eventually renamed interleukin-6 (IL-6) [6–8]. Mechanistic studies showed that binding of IL-6 to its receptor (R), IL-6R, promoted its dimerization, which further triggered a cascade of events leading to phosphorylation of the cytoplasmic protein gp130 and phosphorylation of Janus kinases (JAKs) [9–11]. Phosphorylation of JAKs and gp130 induces signal transducers and activators of transcription (STAT) 3 binding, its phosphorylation resulting in its activation via STAT3 dimerization. This allows STAT3 dimers to translocate to the nucleus and bind to the regulatory regions of target genes, causing the transcriptional upregulation of genes involved in cell proliferation and survival, including of MM cells [9–11].

IL-6 plays a critical role in promoting the growth proliferation and survival of MM cells [11–15]. Specifically, IL-6 is required by normal murine plasma cells to transform into malignant PCTs and by some human MM cell lines for their proliferation and survival. However, in some cases, subsequent generations of these cell lines are able to bypass the need for IL-6 and thus acquire independence from this cytokine [16]. Previous studies have demonstrated an ambiguous relationship between IL-6 dependence and the activation status of STAT3

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and JAK. For example, STAT3 was shown to be constitutively active in the IL-6-dependent U266 human MM cell line [9] and in IL-6-independent murine plasmacytomas and hybridomas together with JAK1 action, whereas constitutive activation of STAT3 was not observed in IL-6-dependent murine plasmacytomas and hybridomas [16]. Results from several independent laboratories have shown that inhibition of the JAK pathway decreases MM cell survival and proliferation [1, 17, 18] and that dual JAK1/2 inhibitors, such as INCB16562, can significantly inhibit the viability of IL-6-dependent human MM cell lines (INA-6 and U266) [19]. Collectively, these findings provide evidence that the IL-6/JAK/STAT pathway contributes to the pathogenesis of MM and its targeting with inhibitors has shown anti-MM efficacy in vitro and in vivo.

Thus, we performed studies to assess the anti-MM effects of the selective JAK1 inhibitor INCB052793 alone and in combination with conventional anti-MM agents. Herein, in vitro experiments were conducted to determine if enhanced inhibition of cell viability was observed when using suboptimal concentrations of INCB052793 in combination with the proteasome inhibitors (PIs) carfilzomib (CAR) or bortezomib (BOR) or the glucocorticosteroid dexamethasone (DEX). Specifically, inhibition of cell viability was assessed with the MTS assay, using MM cell lines and primary MM cells obtained fresh from MM patients in our clinic. Additionally, we examined the in vivo anti-tumor effects of INCB as a single agent and in combination with CAR, BOR, DEX, or lenalidomide (LEN), using our human MM xenograft model LAGκ-1A.

Materials and methods

Anti-MM agents and in vivo drug administration

INCB052793 was provided by Incyte Corporation (Wilmington, DE) and dissolved in 100% dimethylacetamide (DMAC) with 160 mg INCB052793 + 1 ml DMAC to generate an initial stock solution, which was further diluted in 0.5% methylcellulose (19 ml) to generate a stock final solution of 8 mg/ml. The final stock solution (8 mg/ml) was further diluted in 0.5% methylcellulose to generate the three doses of INCB052793 (3, 10, and 30 mg/kg) used in the in vivo studies for the single agent studies and 10 mg/kg for all combination studies. The vehicle was made in the same manner as that described above for making INCB052793, but the only difference was that it did not contain any of the JAK1 inhibitor. CAR was obtained from the clinic as a stock solution (2 mg/ml) and diluted in 5% dextrose to a dose of 3 mg/kg. BOR was obtained from the clinic at a stock solution of 1 mg/ml and diluted in 0.9% sodium chloride (NaCl) to generate a dose of 0.25 mg/kg. DEX was obtained as a stock solution (10 mg/ml from the clinic and diluted in 0.9% NaCl to a dose of 0.5 mg/kg. LEN was obtained from the clinic in the form of pills, diluted in 0.5% methylcellulose to generate a stock solution of 5 mg/ml and further diluted in 0.5% methylcellulose to generate a dose of 15 mg/kg. Formulations were prepared weekly. The dosing volume for the vehicle control, INCB052793, and LEN groups was 200 μl/injection, and the dosing volume of all remaining drugs was 100 μl/injection. INCB052793 and vehicle control were administered twice daily via oral gavage injection (p.o.), throughout study duration. BOR was administered twice weekly, on Mondays and Wednesdays via intravenous (i.v.) injection, and CAR was administered twice weekly on Mondays and Tuesdays via i.v. injection. DEX was administered once daily via intraperitoneal injection (i.p.). LEN was administered once daily, five times per week, via oral gavage. The following two-drug combinations were evaluated: INCB052793 + CAR, INCB052793 + BOR, INCB052793 + DEX, INCB052793 + LEN, and LEN + DEX.

MM xenograft models

All animal studies were conducted according to the protocols approved by the Institutional Animal Care and Use Committee (approval no. 13.006). For all MM xenograft experiments conducted, 4-week old male CB17 severe combined immunodeficient (SCID) *Mus musculus* (strain 236) were obtained from Charles River Laboratories (Wilmington, MA) and housed under sterile conditions. They were maintained in a specific pathogen-free area in our animal resources facility for 1 week, prior to initiation of experiments. LAGκ-1A tumors were used for the in vivo studies. To establish the LAGκ-1A tumor, a BM biopsy was obtained from a female MM patient who had progressed on LEN treatment but subsequently responded to melphalan and BOR combination therapy. The biopsy tissue was surgically implanted in the hind limb of an anesthetized SCID mouse and passaged through succeeding generations [20]. This human MM tumor has been maintained in vivo for more than a decade and has been used to evaluate new drugs and novel combinations. In the current study, LAGκ-1A tumors were excised from anesthetized (with isoflurane) donor mice, sectioned into 20 to 40 mm^3 pieces, and surgically implanted into the left superficial gluteal muscle of anesthetized naïve/recipient SCID mice. Tumors were allowed to grow for 1 week before mice were randomized into treatment groups, based on human IgG or human B cell maturation antigen (BCMA) levels in their plasma (detailed below) based on our prior studies showing the latter plasma biomarker accurately assesses tumor burden in SCID mice-bearing human MM [21].

Determination of plasma hIgG or BCMA levels

Enzyme-linked immunosorbent assays (ELISAs) were used to determine the levels of human (h) IgG (Bethyl Laboratories,

Montgomery, TX) or hBCMA (R&D Systems, Minneapolis, MN) in the plasma of mice bearing the human MM tumor LAGκ-1A. Mice-bearing MM tumors were anesthetized and bled retro-orbitally using heparinized capillary tubes on a weekly basis. Samples were spun at 13,000 rpm for 5 min, plasma collected, and hIgG or hBCMA ELISAs used according to the manufacturer's specifications. Absorbance at 450 nm with a reference wavelength of 550 nm was determined on a μQuant microplate spectrophotometer (Multiskan EX) with Ascent software.

Statistical analysis

Tumor growth curves and cell viability assay graphs were analyzed in terms of treatment group means and standard error of the mean (SEM). Statistical significance of differences observed in drug-treated groups versus the vehicle group was determined using a Student's *t* test. The minimal level of significance was $P < 0.05$. Data graphed is the mean ± SEM, with $n = 7–8$ mice/group and quadruplicate cultures for the in vivo and in vitro experiments, respectively.

MM cell lines and primary MM cells

All studies involving human tissue were approved by the Western Institutional Review Board (WIRB BIO 001), and informed consent was obtained in accordance with the Declaration of Helsinki. Patients were diagnosed with MM and their clinical status determined according to the International Myeloma Working Group criteria [22, 23]. The human MM cell lines RPMI8226 and U266 were obtained from American Type Culture Collection (ATCC, Rockville, MD). BM aspirates were collected in heparinized tubes, and mononuclear cells (MCs) were isolated using density-gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Bone marrow mononuclar cells (BMMCs) were cultured in RPMI1640 (Omega Scientific, Tarzana, CA) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, 2 M glutamine, 1 M sodium pyruvate, 25 M HEPES, 200 units/ml penicillin, and streptomycin at 37 °C in an atmosphere of 5% carbon dioxide (CO_2).

Cell viability assay

MM cell lines RPMI8226 and U266 and fresh BMMCs from 3 MM patients were seeded at 1×10^5 cells/100 μL/well in 96-well plates in RPMI1640 media with FBS for 24 h prior to treatment. Cells were then treated with either control, INCB alone, or in combination with anti-MM agents at various concentrations for 48 h. After each incubation period, cell viability was quantified using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Each well was treated with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) for 1 to 4 h, after which absorbance was recorded at 490 nm. The quantity of formazan product as measured by absorbance at 490 nm is directly proportional to the number of living cells in culture. Absorbance at 450 nm with a reference wavelength of 550 nm was determined on a μQuant microplate spectrophotometer (Multiskan EX) with Ascent software and data graphed using GraphPad Prism 4 software (La Jolla, CA).

Apoptotic assay using annexin V and propidium iodide staining

To quantify apoptosis in response to drug treatment, cells from fresh BMMCs from MM patients or MM cell lines (5×10^5 cells per well) were incubated with vehicle, INCB052793, anti-MM agents (CAR, BOR and DEX), or the doublet combination of INCB052793 plus each of these anti-MM agents, at 37 °C and 5% CO_2 for 48 h. Cells were then washed twice with phosphate-buffered saline, re-suspended in binding buffer (100 mM HEPES/NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM calcium dichloride), and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and with the fluorescent dye propidium iodide (PrI), according to the manufacturer's protocol (BioVision, Mountain View, CA). For each drug treatment, 1×10^5 gated events were recorded. Cells negative for both PrI and annexin V staining were considered live; annexin V-positive, PrI-negative cells were considered early apoptotic; and annexin V-positive, PrI-positive cells were considered late apoptotic. Flow cytometric analyses were performed using a Beckman Coulter FC500 cytometer with Cytomics cxp software (Beckman Coulter, Fullerton, CA).

Results

Anti-proliferative effects of INCB052793 and PIs on primary MM cells and MM cell lines

The anti-MM effects on cell viability resulting from INCB052793 alone and in combination with the PIs CAR or BOR, were evaluated using the MTS assay. For all cell viability experiments (MTS assay), *P* values denoting statistical significance are listed within the figures. Primary MM cells from two MM patients (2276 and 2560) and two MM cell lines (RPMI8226 and U266) were cultured in the presence of CAR or BOR for 48 h. Suboptimal concentrations of single agents were used to determine whether INCB052793-containing combination treatments would inhibit cell viability more than either agent alone. The combination treatment consisting of both INCB052793 and CAR induced a concentration-dependent inhibition of cell viability in the primary MM cells (MMBM 2276 and 2560; Fig. 1a, b) and both

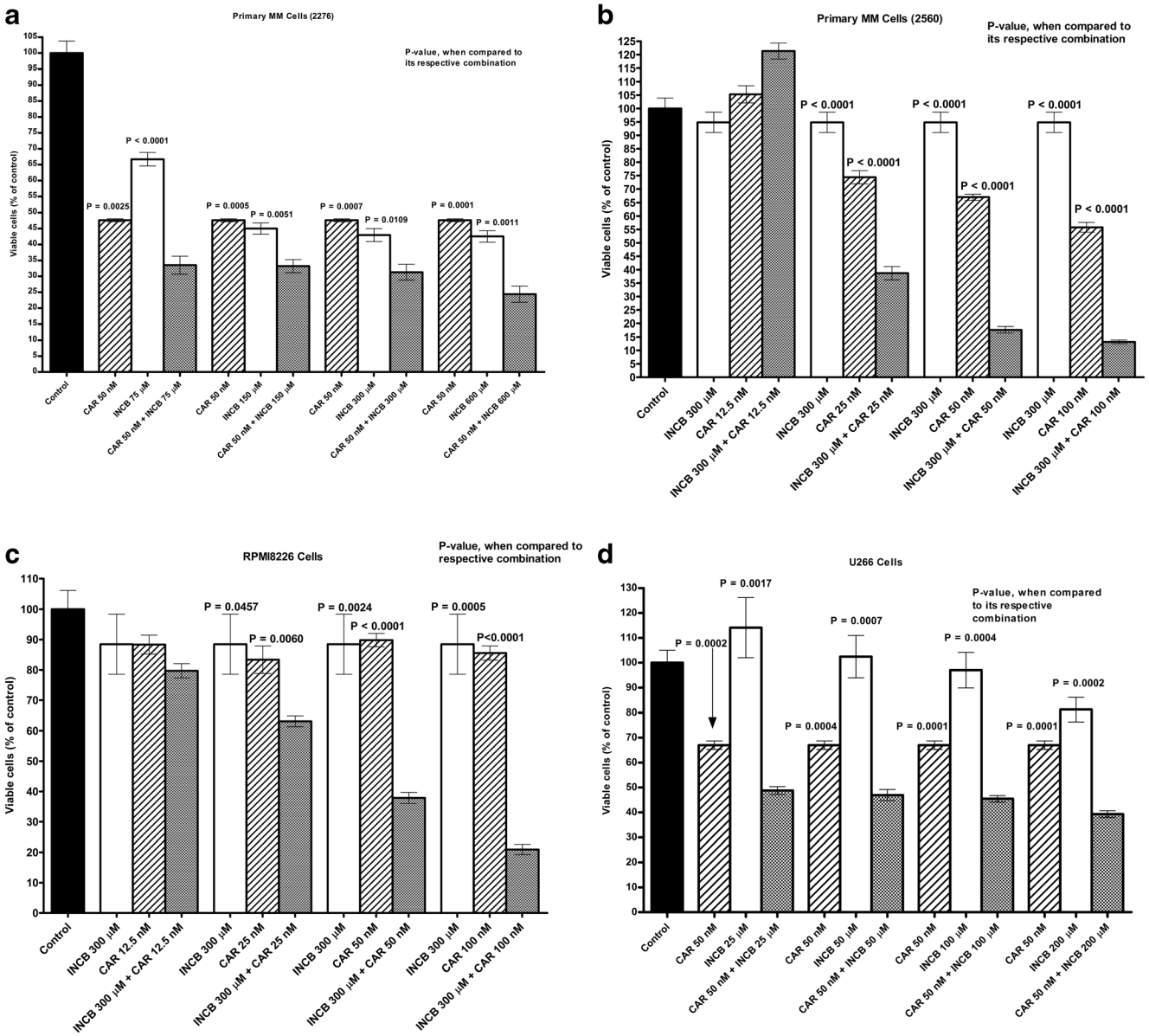
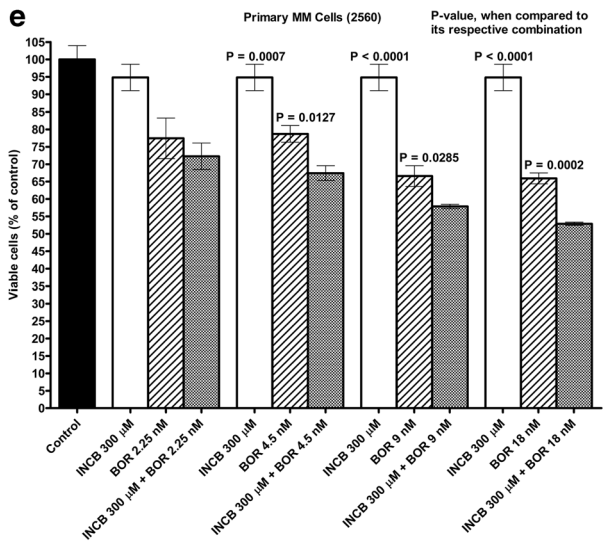
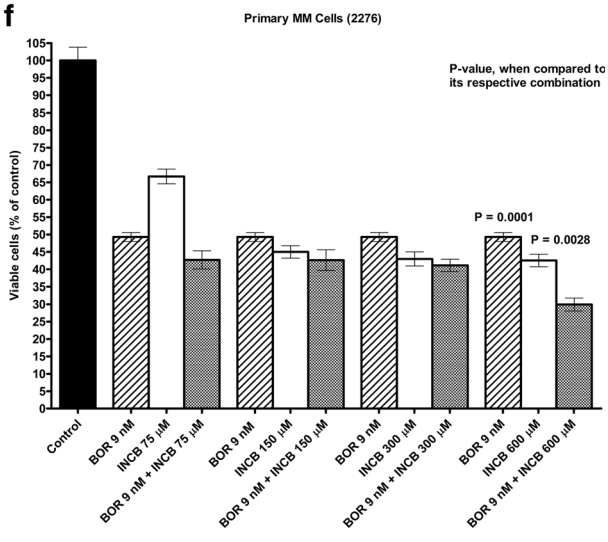
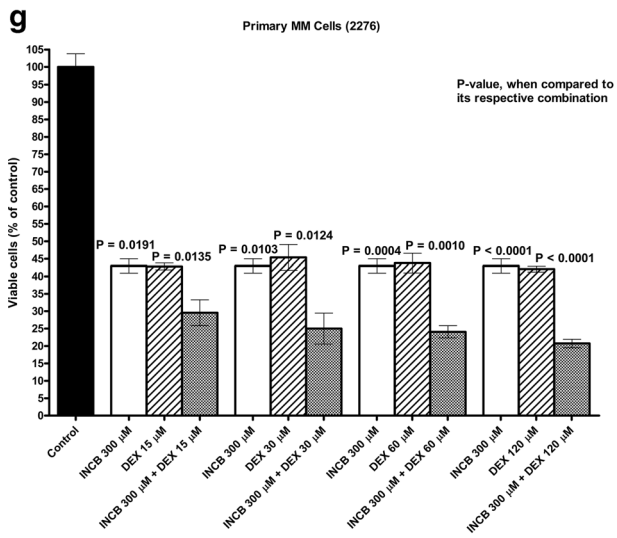


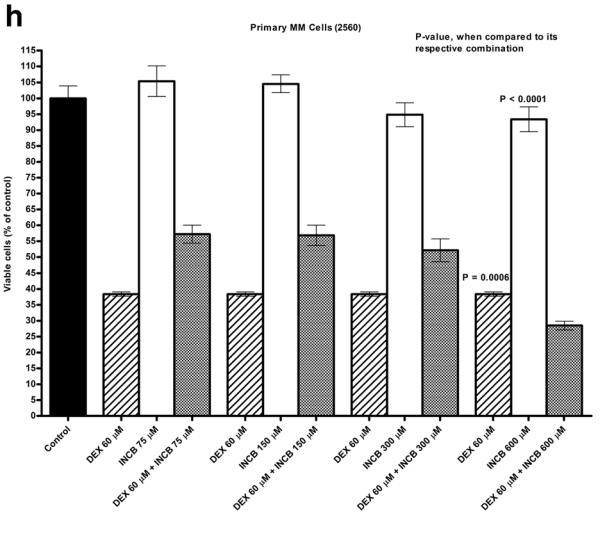
Fig. 1 The INCB052793 in combination treatment with anti-MM agents demonstrates greater inhibition of cell viability than either agent alone. **a** Primary BMMCs from MM patient 2276 were cultured in the presence of vehicle (black bar), INCB052793 (white bars), CAR (striped bars), or both agents (hatched bars) at the concentrations indicated for 48 h, and cell viability quantified with the MTS assay. **b** The MTS assay was used to quantify cell viability of primary cells from MM patient 2560 cultured in the presence of vehicle (black bar), INCB052793 at a fixed concentration (300 μM; white bars), escalating concentrations of CAR (concentrations indicated; striped bars), or both agents (hatched bars) at the concentrations indicated for 48 h. **c** Inhibition of RPMI8226 cell viability: INCB052793 (300 μM; white bars), CAR (concentrations indicated; striped bars), or both agents (hatched bars) at the concentrations indicated. **d** Inhibition of U266 cell viability: INCB052793 (concentrations indicated; white bars), CAR (50 nM; striped bars), or both agents (hatched bars). **e** Primary MM cell (2560) inhibition of viability: INCB052793 (300 μM; white bars), BOR (concentrations indicated; striped bars), or both agents (hatched bars). **f** Inhibition of viability of primary MM cells from patient 2276: BOR (9 nM; striped bars), INCB052793 (escalating concentrations; white bars), or both agents (hatched bars). **g** Significant inhibition of primary MM cell (2276) viability: INCB052793 at a fixed concentration (300 μM; white bars), escalating concentrations of DEX (concentrations indicated; striped bars), or both agents (hatched bars) at the concentrations indicated for 48 h. **h** Inhibition of primary MM cell (2560) viability: INCB052793 (concentrations indicated; white bars), DEX (60 μM; striped bars), or both agents (hatched bars). **i** Inhibition of RPMI8226 cell viability: INCB052793 (300 μM; white bars), DEX (concentrations indicated; striped bars), or both agents (hatched bars). Using GraphPad Prism 4 software, data graphed are the mean ± standard error of the mean using four replicates.

of the MM cell lines RPMI8226 and U266 (Fig. 1c, d). Using the same experimental conditions as those in Fig. 1a–d, a different PI, BOR, was used in combination with INCB052793 and also showed a concentration-dependent inhibition on cell viability from primary MM cells obtained from the same two MM patients (Fig. 1e, f).









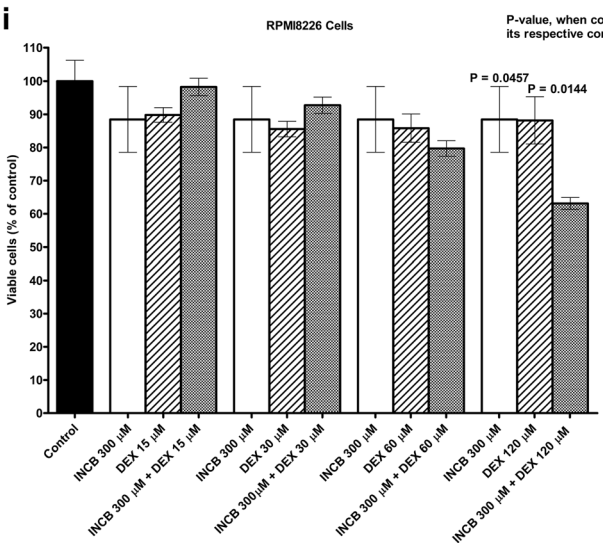


Fig. 1 (continued)

INCB052793 in combination with DEX inhibits primary MM cell and MM cell line viability

We also determined the anti-proliferative effects of INCB052793 alone and in combination with the glucocorticosteroid DEX. The combination of INCB052793 and DEX induced a concentration-dependent inhibition of cell viability significantly more pronounced than the single agents, in all MM cells tested: both primary MM cells (MM 2276 and 2560; Fig. 1g, h) and the MM cell line RPMI8226 (Fig. 1i).

INCB052793 in combination with anti-MM agents induces apoptosis of primary MM cells and the RPMI8226 MM cell line

To determine if the reduction in cell viability after treatment of primary MM cells with INCB052793 was due to apoptosis, primary BMMCs from MM patient 2276 were cultured in the presence of INCB052793 (300 μM) plus BOR (9 nM), CAR (50 nM), or DEX (60 μM) for 48 h. The fractions of cells which stained with the viability dye (PrI) and the apoptosis marker (annexin V) were measured. The percentage of cells in early (PrI−/Annexin V+) and late apoptosis (PrI+/Annexin V+) and necrosis (PrI+/Annexin V−) was greater among cells cultured with the combination of INCB plus BOR (54.8%) than with either INCB052793 alone (8.7%) or BOR (15.6%) alone (Fig. 2a–d). At this time point, the proportion of cells in late apoptosis (PrI+/Annexin V+) was higher in the INCB052793 + BOR treatment group at 29.2%, whereas the proportion of cells in late apoptosis was only 0.5% in the single agent BOR or INCB052793 groups. Similarly, using the same primary MM cells, the combination of INCB052793 and CAR resulted in a higher proportion of total cell apoptosis and necrosis of 42.7%, when compared to the total cell apoptosis and necrosis of 17.6% or 8.7% with single agent CAR or INCB052793, respectively (data not shown). Furthermore, using the same primary MM cells from this patient, the doublet of INCB052793 and DEX also resulted in a higher proporiton of total cell apoptosis and necrosis of 31.8% than with either CAR alone (17.6%) or INCB052793 alone (8.7%; data not shown). Thus, when INCB052793 was combined with BOR, CAR, or DEX for 48 h, the proportion of primary tumor cells from MM patient 2276 in late apoptosis was higher than the proportion of cells in early apoptosis in all three of these combinations.

Using the same experimental model and the same INCB-containing doublets evaluated as shown in Fig. 2, freshly isolated primary tumor cells from a second MM patient, 2655, were also tested. The results are shown in Table 1. Primary MM cells from this patient incubated with any of the INCB052793-containing doublets consistently had a higher proportion of dead cells. Specifically, the proportion of total cell death (early and late apoptosis plus necrosis) in the doublets of INCB052793 plus CAR, DEX, or BOR was 44.6%, 36.4% and 35.6%, respectively. In contrast, total cell death in cells exposed to the single agents INCB052793, CAR, DEX, or BOR was only 9.9%, 7.4%, 9.2%, and 16.2%, respectively. Additionally, the combination of these same INCB052793 doublet combinations, when incubated with the human MM cell line RPMI8226, resulted in a higher proportion of total cell apoptosis (early and late) and necrosis (Table 2).

The proportion of total cell death resulting from the doublet combinations of INCB052793 plus CAR, BOR or DEX was 49.1%, 38.8%, and 31.7%, respectively. The proportion of total apoptosis induced by these INCB052793-containing doublets was higher than among cells exposed to the respective single agents (INCB052793, CAR, BOR, and DEX were 10.9%, 14.2%, 21.3%, and 7.8%, respectively). With the exception of the INCB052793 plus BOR combination, the proportion of RPMI8226 cells in early apoptosis (PrI−/Annexin V+) was greater following treatment with the combination of INCB052793 plus CAR or DEX (Table 2).

Single agent INCB052793 at three dose levels reduced growth of the human MM tumor LAGκ-1A in vivo

The anti-MM effects of INCB052793 were evaluated using the human MM xenograft model, LAGκ-1A. The left superficial gluteal muscles of SCID mice were surgically implanted with 20–40 mm^3 LAGκ-1A tumors. They were allowed to grow for 1 week before mice were randomized into treatment groups, based on human IgG levels in their plasma. On days 28, 35, 42, 49, 56, and 63, significant anti-MM effects were observed in mice treated with single agent INCB052793 at 30 mg/kg compared with vehicle alone ($P = 0.0006$, $P = 0.0002$, $P = 0.004$, $P = 0.0008$, $P = 0.0003$, $P = 0.0014$, respectively; Fig. 3a).

At the next lower dose, 10 mg/kg, on days 56 and 63, significant anti-MM effects were also observed in mice treated with single agent INCB052793 ($P = 0.0125$ and $P = 0.0162$, respectively). The drug was well tolerated with all mice alive in the vehicle control and the three INCB052793 treatments groups alive on day 63. Human IgG levels were also measured in these same mice. Animals administered INCB052793 at 30 mg/kg showed lower IgG levels on days 49 and 63 ($P = 0.0282$, $P = 0.0274$, respectively), but not on day 56 (Fig. 3b).

INCB052793 in combination with the PIs CAR or BOR inhibited growth of human MM in vivo

Given the reduction in cell viability and increase in apoptosis observed in primary MM cells and MM cell lines when cultured in the presence of INCB052793 and PIs, we also evaluated the in vivo anti-MM effects of INCB052793 plus CAR or BOR in LAGκ-1A-bearing SCID mice. On day 7 post-tumor

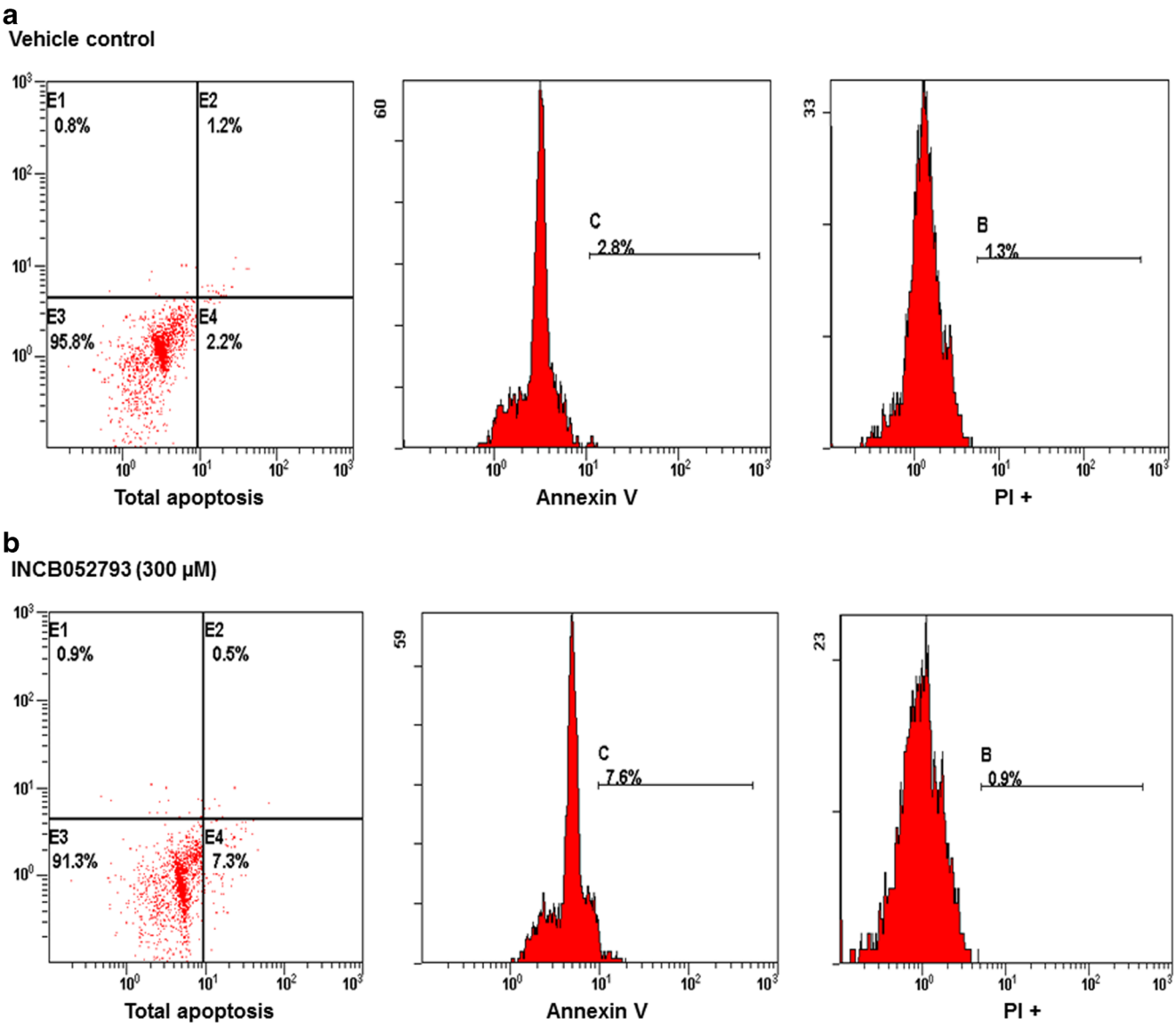


Fig. 2 INCB052793 plus BOR induces apoptosis of primary MM cells. BMMCs from MM patient 2276 were incubated with **a** vehicle control, **b** INCB052793 (300 μM), **c** BOR (9 nM), or **d** INCB052793 and BOR (300 μM and 9 μM, respectively) for 48 h, and the percentage of positive staining for early apoptosis (PrI−/Annexin V+) and late apoptosis (PrI+/Annexin V+) was quantified using flow cytometric analysis. One representative set of data from three experiments is shown

implantation, treatment was initiated with single agent INCB052793 or CAR or the combination of INCB052793 with CAR. On day 56 post-tumor implantation, significant anti-MM effects were observed in mice treated with INCB052793 when compared to vehicle control and single agent CAR ($P = 0.0023$ and $P = 0.0475$, respectively) (Fig. 4a). In contrast, single agent CAR did not have significant anti-MM effects when compared to vehicle control (Fig. 4a). Mice treated with the combination of INCB052793 with CAR showed anti-MM effects when compared to vehicle alone ($P < 0.0001$), CAR alone ($P = 0.0024$) and single agent INCB052793 ($P = 0.0429$; Fig. 4a). Overall, mice survived the INCB052793 treatment regimens with 100% survival in all treatment groups at the time of study termination.

The combination of another PI, BOR, with INCB052793 was also evaluated using this same human MM xenograft model. On day 7 post-tumor implantation, treatment was initiated with single agent INCB052793 or BOR or the combination of INCB052793 and BOR. On day 56 post-tumor implantation, anti-MM effects were observed in mice treated with INCB052793 when compared to vehicle control ($P = 0.0023$) and statistical significance was almost reached when compared to single agent BOR ($P = 0.064$; Fig. 4b). In contrast, single agent BOR did not have significant anti-MM effects when compared to vehicle control. The combination of INCB052793 and BOR resulted in significant anti-MM effects when compared to vehicle control ($P < 0.0001$), BOR ($P = 0.0014$), and single agent INCB052793 ($P = 0.0469$; Fig. 4b).

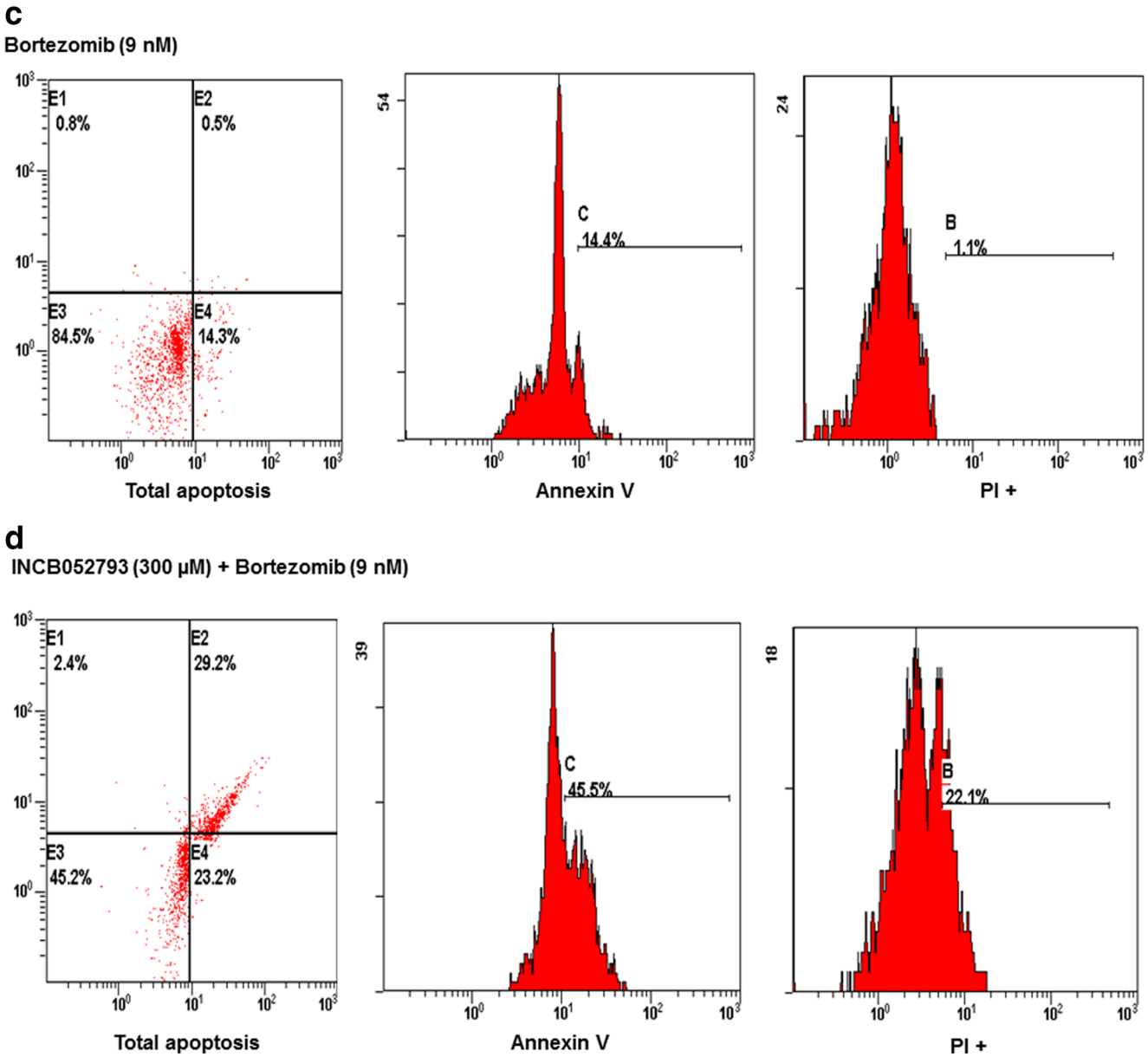


Fig. 2 (continued)

Administration INCB052793 alone and in combination with the immunomodulatory agent LEN or glucocorticosteroid DEX inhibits the growth of the human MM tumor LAGκ-1A in SCID mice

We evaluated the anti-MM effects of INCB052793 alone and in combination with DEX or LEN in LAGκ-1A-bearing mice. Treatment was initiated at day 7. On day 35 post-tumor implantation, tumor-bearing mice dosed with single agent INCB052793 had smaller xenografts ($P = 0.0348$) when compared to the vehicle control group, whereas mice treated with single agent DEX or LEN did not show anti-MM effects. In contrast, mice dosed with single agent LEN did not have smaller tumors when compared to the vehicle control group at any time point. Beginning on day 42, and weekly thereafter, on days 49, 56 and 63, mice dosed with single agent INCB052793 had significantly smaller tumors, with $P = 0.0078$, $P = 0.0142$, $P = 0.0162$, and $P = 0.0186$, respectively when compared to the vehicle control group. In contrast, mice dosed with single agent DEX did not have significantly smaller tumors when compared with the vehicle control group on day 42. Tumors in single agent DEX dosed mice were eventually significantly smaller, but 1 week later than single agent mice dosed with INCB052793, on days 49 ($P = 0.0372$), 56 ($P = 0.0337$), and 63 ($P = 0.0399$), when compared to the vehicle control group. Mice receiving the doublets of INCB052793 with DEX or LEN had smaller tumors ($P = 0.0002$ or $P < 0.0001$, respectively), as did mice treated with

Table 1 Summary of flow cytometric apoptosis results: INCBO52793 + anti-MM agents on primary MM cells from patient 2655

	Total apoptotic cells (%)	Annexin V+ (%)
Control	3.6	2
INCB052793 (300 μM)	9.9	7
Carfilzomib (50 nM)	7.4	4.5
Carfilzomib (50 nM) + INCB052793 (300 μM)	44.6	31.7
Dexamethasone (60 μM)	9.2	5.8
Dexamethasone (60 μM) + INCB052793 (300 μM)	36.4	22.9
Bortezomib (9 nM)	16.2	9.9
Bortezomib (9 nM) + INCB052793 (300 μM)	35.6	19.8

DEX plus LEN ($P = 0.0081$; Fig. 5), when compared to the vehicle control group as early as day 35. The significant inhibition of tumor growth at day 35 post-tumor implantation with these doublet combinations was observed throughout the remainder of the study, when compared to the vehicle control group. Specifically, INCB052793 + DEX showed smaller tumors on days 42, 49, 56, and 63 with $P = 0.0010$, $P = 0.0008$, $P = 0.0005$, and $P = 0.0013$, respectively, as did INCB052793 + LEN on days 42, 49, 56, and 63 with $P = 0.0003$, $P = 0.0004$, $P = 0.0003$, and $P = 0.0007$, respectively, when compared to vehicle treated mice. Similarly, LEN + DEX-treated mice showed smaller tumors on days 42, 49, 56, and 63 with $P = 0.0181$, $P = 0.0143$, $P = 0.0175$, and $P = 0.0156$, respectively, when compared to vehicle alone-treated mice. Despite LEN + DEX combination treatment resulting in slightly smaller tumors in mice than among mice receiving treatment with DEX alone or mice receiving treatment with INCB052793 alone, at no time throughout the study did the administration of this doublet combination (LEN + DEX) result in mice showing significantly smaller tumors than mice dosed with either of these agents alone. Additionally, tumors in mice receiving INCB + DEX were smaller on days 56 and 70 ($P = 0.0101$ and $P = 0.0089$, respectively, but not on day 63) than mice receiving LEN + DEX. Mice dosed with the doublet combination of INCB052793 + LEN had significantly smaller tumors than mice dosed with LEN + DEX on days 35, 42, 49, 56, 63, and 70 with $P = 0.0352$, $P = 0.0306$, $P = 0.0084$, $P = 0.0017$, $P = 0.0110$, and $P = 0.0037$, respectively (Fig. 5).

Table 2 Summary of flow cytometric apoptosis results: INCBO52793 + anti-MM agents on the MM cell line RPM18226

	Total apoptotic cells (%)	Annexin V+ (%)
Control	4	2.3
INCB052793 (300 μM)	10.9	10.3
Carfilzomib (50 nM)	14.2	13.9
Carfilzomib (50 nM) + INCB052793 (300 μM)	49.1	31
Bortezomib (9 nM)	21.3	15.1
Bortezomib (9 nM) + INCB052793 (300 μM)	38.8	26.9
Dexamethasone (60 μM)	7.8	6.8
Dexamethasone (60 μM) + INCB052793 (300 μM)	31.7	22.2

Discussion

Since MM patients will inevitably develop resistance to their therapies [24], new treatment regimens are needed to prolong their survival. Thus, the anti-MM effects of new and novel classes of drugs are needed including inhibitors of the JAK pathway, as this pathway has been shown to play key roles in growth and resistance of MM to currently available treatments [9, 11, 12, 14, 15]. In this study, we evaluated the anti-MM effects of the selective JAK1 inhibitor INCB052793 that is currently in clinical development.

These preclinical studies demonstrate that this JAK1 inhibitor shows anti-MM activity alone and enhances the efficacy of conventional anti-MM agents, including PIs (CAR or BOR), immunomodulatory agents (LEN), and glucocorticosteroids (DEX). In the MM cell line RPMI8226, the proportion of apoptotic and necrotic myeloma tumor cells among cells treated with INCB052793 in combination with CAR was markedly higher than cells exposed to single agents. Similarly, using fresh tumor cells from MM patients 2276 and 2655, this same doublet combination produced a higher percentage of total cell death (early and late apoptosis and necrosis) than exposure of these tumor cells to single agents. When evaluating INCB052793 with a different PI, BOR, the proportion of apoptotic and necrotic myeloma tumor cells among cells treated with INCB052793 in combination with this agent was markedly increased in the RPMI8226 MM cell line, when compared to cells treated with either single agent alone. This same doublet combination was also studied in primary MM cells from patients 2276 (Fig. 2) and 2655 (Table 1), with the combination having a higher percentage of total cell death, whereas treatment with single agents resulted in a much lower proportion of cells showing cell death. Additionally, INCB052793 in combination with the glucocorticosteroid DEX was also highly effective at reducing cell viability in tumor cells from three MM patients and the RPMI8226 cell line in vitro. These same drug combinations significantly inhibited tumor growth in SCID mice bearing the human MM tumor LAGκ-1A in vivo. Furthermore, the combination of INCB052793 with LEN was highly effective at inhibiting

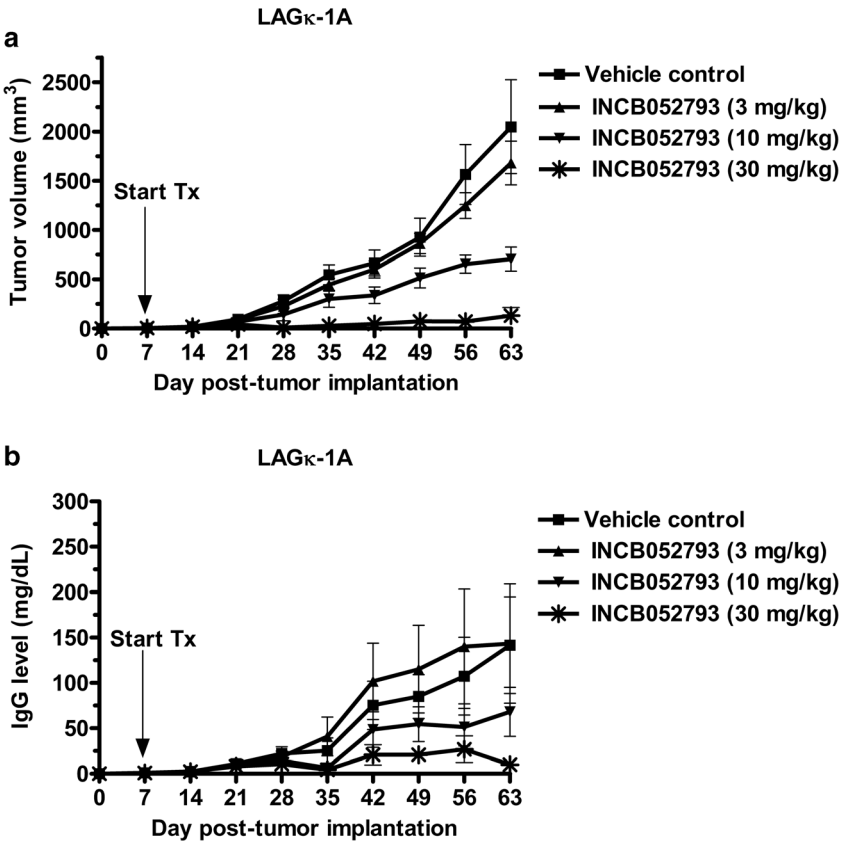


Fig. 3 Single agent INCB052793 significantly inhibited the growth of the human MM xenograft LAGκ-1A in SCID mice. **a** LAGκ-1A-bearing mice were dosed with vehicle control and increasing doses of INCB052793 (3 mg/kg, 10 mg/kg and 30 mg/kg). On days 56 and 63 post-tumor implantation, mice dosed with INCB052793 at 10 mg/kg had smaller tumors when compared to animals receiving only the vehicle control. Tumor volumes were also reduced on days 28, 35, 42, 49, and 56 in mice dosed with INCB052793 at 30 mg/kg. **b** Mice-bearing LAGκ-1A tumors showed a marked reduction in plasma hIgG levels on days 49 and 63 post-tumor implantation. Data are presented as means ± standard error of the mean and graphed using GraphPad Prism 4 software. There were no drug-related deaths in any mice in any group throughout the study

tumor growth among mice bearing LAGκ-1A, when compared to either agent alone. Taken together, our in vitro and in vivo studies demonstrate that the JAK1 inhibitor INCB052793 enhances the anti-MM efficacy of PIs,

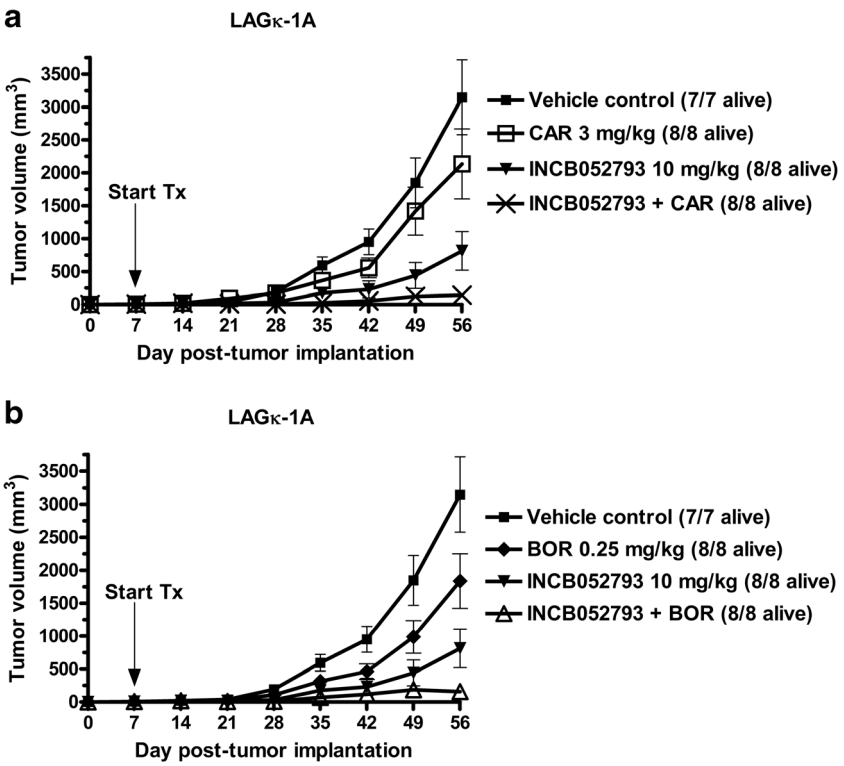


Fig. 4 INCB052793 combined with proteasome inhibitors significantly inhibited the growth of MM tumors. **a** The combination of INCB052793 and CAR markedly reduced LAGκ-1A tumor volumes in mice on day 56 post-tumor implantation. **b** At this same time point, mice dosed with the combination of INCB052793 plus BOR had significantly smaller tumors when compared with the single agent BOR, single agent INCB052793, and vehicle control groups. There were no drug-related deaths in any mice in any group throughout the study. Data are presented as the mean ± standard error of the mean and graphed using GraphPad Prism 4 software

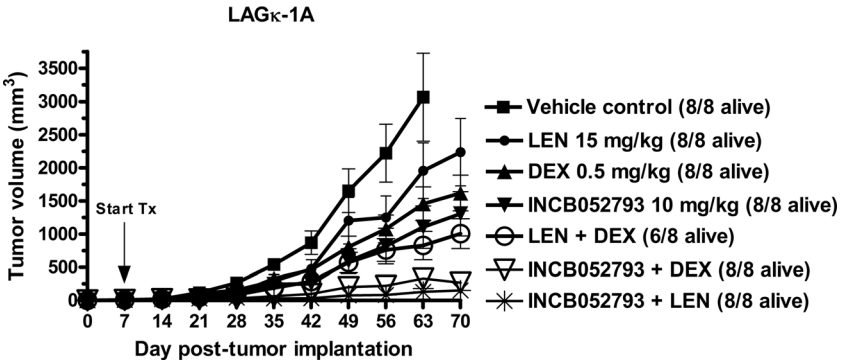


Fig. 5 INCB052793 combined with DEX or LEN significantly inhibited the growth of LAGκ-1A tumors. The combination of INCB052793 and LEN significantly inhibited tumor volumes in mice beginning on day 35 post-tumor implantation and weekly thereafter. Similarly, beginning on day 35 post-tumor implantation, significantly smaller tumors were observed in mice dosed with the doublet combination of INCB052793 plus DEX with this trend continuing weekly thereafter until study termination. Data, graphed using GraphPad Prism 4 software, is the mean ± SEM, with eight mice per group

immunomodulatory agents, and glucocorticosteroids and provides continued support for the ongoing clinical evaluation of these drug combinations for the treatment of MM patients.

Given the significance of the IL-6/JAK/STAT pathway in the pathogenesis of MM, the targeting of the components of this pathway would appear to be a promising therapeutic target for anti-MM drug development; however, no specific inhibitors of the IL-6/JAK/STAT pathway have been approved by the Food & Drug Administration for the treatment of MM. Previous JAK inhibitors tested in MM were shown to have undesired effects, such as weakening the efficacy of traditional anti-MM agents, or were effective only in a small percentage of MM patients. In one preclinical study, retreatment of the human MM cell line U266 with a JAK inhibitor antagonized the anti-MM effects of DNA damaging chemotherapeutic drugs [17]. An anti-IL-6 antibody was only effective in a small proportion of MM patients who showed less than 18 μg/day of IL-6 in their blood [25].

Furthermore, an array of ambiguous results pertaining to the function of various IL-6/JAK/STAT pathway inhibitors in IL-6-dependent versus IL-6 independent MM cell lines has further prevented the development of these types of agents for use in MM patients. For example, serum starved U266 and RPMI8226 (IL-6-dependent and IL-6 independent, respectively) MM cells cultured in the presence of IL-6 with and without the dual JAK1/2 inhibitor, INCB16562, demonstrated significantly elevated and markedly reduced p-STAT3 expression levels in non-drug exposed cells and drug-treated cells, respectively [19]. However, when these MM cell lines were cultured in the presence of serum and INCB16562, inhibition of U266 but not RPMI8226 cell viability was observed [19]. Additionally, when cultured under the same experimental conditions as the U266 and RPMI8226 cell viability experiments described above, the IL-6-dependent INA-6 MM cell line also demonstrated significantly reduced p-STAT3 expression levels and inhibition of cell viability but no reductions in the expression levels of the anti-apoptotic proteins Bcl-xL and Bcl-2 were observed [19]. Furthermore, our in vitro experiments clearly demonstrate that the selective JAK1 inhibitor INCB052793 plus conventional anti-MM agents significantly inhibited cell viability and enhanced apoptosis of both IL-6-dependent U266 and IL-6-independent RPMI8226 MM cells. Despite the fact that we did not evaluate for IL-6 dependence and STAT3 activation in the primary MM cells and the LAGκ-1A MM xenograft model evaluated in our studies, we demonstrated that INCB052793 alone and when combined with conventional agents (CAR, BOR, LEN, and DEX) shows significant anti-MM activity when compared to their respective single agent treatment groups.

The collective results from previous studies evaluating both traditional IL-6/JAK/STAT pathway inhibitors, the novel and selective JAK1 inhibitor INCB052793, and conventional anti-MM therapies, suggest that when exposed to anti-MM agents, MM cells acquire resistance and are able to adapt and utilize alternative pathways for their survival. One of the known mechanisms through which MM cells become resistant to LEN is upregulation of MUC1 expression [26]. Recently, it has been demonstrated that MUC1 expression is markedly downregulated through inhibition of JAK [26] providing support for the combination of INCB052793 and LEN to treat MM, which is supported by our in vitro and in vivo experiments. Recently, we have also demonstrated that inhibition of the JAK pathway markedly reduces expression of Trib1 [27], a protein that enhances tumor stimulatory M2 macrophage polarization [28]. Furthermore, we have shown that inhibition of JAK also reduces M2 polarization in monocytes [29] providing support for also indirect bone marrow microenvironmental effects of JAK inhibition on reducing MM growth.

Despite attempts to selectively eliminate only MM cells, anti-MM agents also affect cellular mechanisms in normal cells in ways that are not currently understood. The lack of the desirable clinical efficacy of traditional JAK inhibitors is likely due to the promiscuity of agents' targeting and lack of understanding of the alternative survival pathways used by

MM cells to overcome drug resistance. Therefore, learning the mechanisms which contribute to the pathogenesis of MM and the resistance of these tumor cells to treatments, along with evaluating novel and more selective JAK1 and/or JAK2 inhibitors, such as INCB052793 and ruxolitinib, alone and in combination with currently available drugs is needed to elucidate these different mechanisms and help support the further development of these agents to treat MM patients. A phase 1 trial is currently evaluating the JAK1/2 inhibitor ruxolitinib in combination with LEN and steroids for relapsed or refractory MM patients (NCT03110822) with promising clinical activity [30]. In addition, a clinical trial is evaluating INCB052793 for patients with advanced malignancies including hematologic (NCT02265510). The current preclinical results from this study evaluating the JAK1 inhibitor INCB052793 alone and in combination with PIs, immunomodulatory drugs, and glucocorticosteroids for treating MM both in vitro and in vivo provide support for clinical trials evaluating this drug alone as well as with these other active MM agents for treatment of MM patients.

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Compliance with ethical standards

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). All institutional and national guidelines for the care and use of laboratory animals were followed. Informed consent was obtained from all patients for being included in the study. All animal studies were conducted according to the protocols approved by the Institutional Animal Care and Use Committee (approval no. 13.006).

Conflict of interest This research was supported by a grant from Incyte Corporation. J. Berenson is a consultant/advisory board member for Incyte Corporation.

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