Dual intracellular targeting by ruxolitinib and the Mcl-1 inhibitor S63845 in interleukin-6-dependent myeloma cells blocks *in vivo* tumor growth

Multiple myeloma remains an incurable malignancy with most patients experiencing relapse despite the introduction of novel therapies. While the first monoclonal antibodies have been approved for the treatment of myeloma, small molecule inhibitors of signaling pathways are still investigational. Although the concept of Janus kinase (JAK)/signal transducer and activator of transcription (STAT)3 inhibition in myeloma has shown promising results in preclinical studies, the efficacy of JAK inhibitors as single agents seems to be limited.[1] Ruxolitinib is a potent JAK1/2 inhibitor and approved for the treatment of patients with myeloproliferative disease and for graft-*versus*-host disease.[2] While it has activity as a single agent in multiple myeloma, the combination with the myeloid cell leukemia (Mcl)-1 protein inhibitor S63845 resulted in superior survival in a preclinical *in vivo* model. The results obtained in the INA-6 xenograft model strongly support evaluation of the combination of JAK and Mcl-1 inhibition in humans.

The JAK/STAT3 pathway is activated by cytokines of the gp130 family including interleukin (IL)-6 as the most prominent member with an established pathophysiological role in multiple myeloma.[3,4] Ruxolitinib phosphate

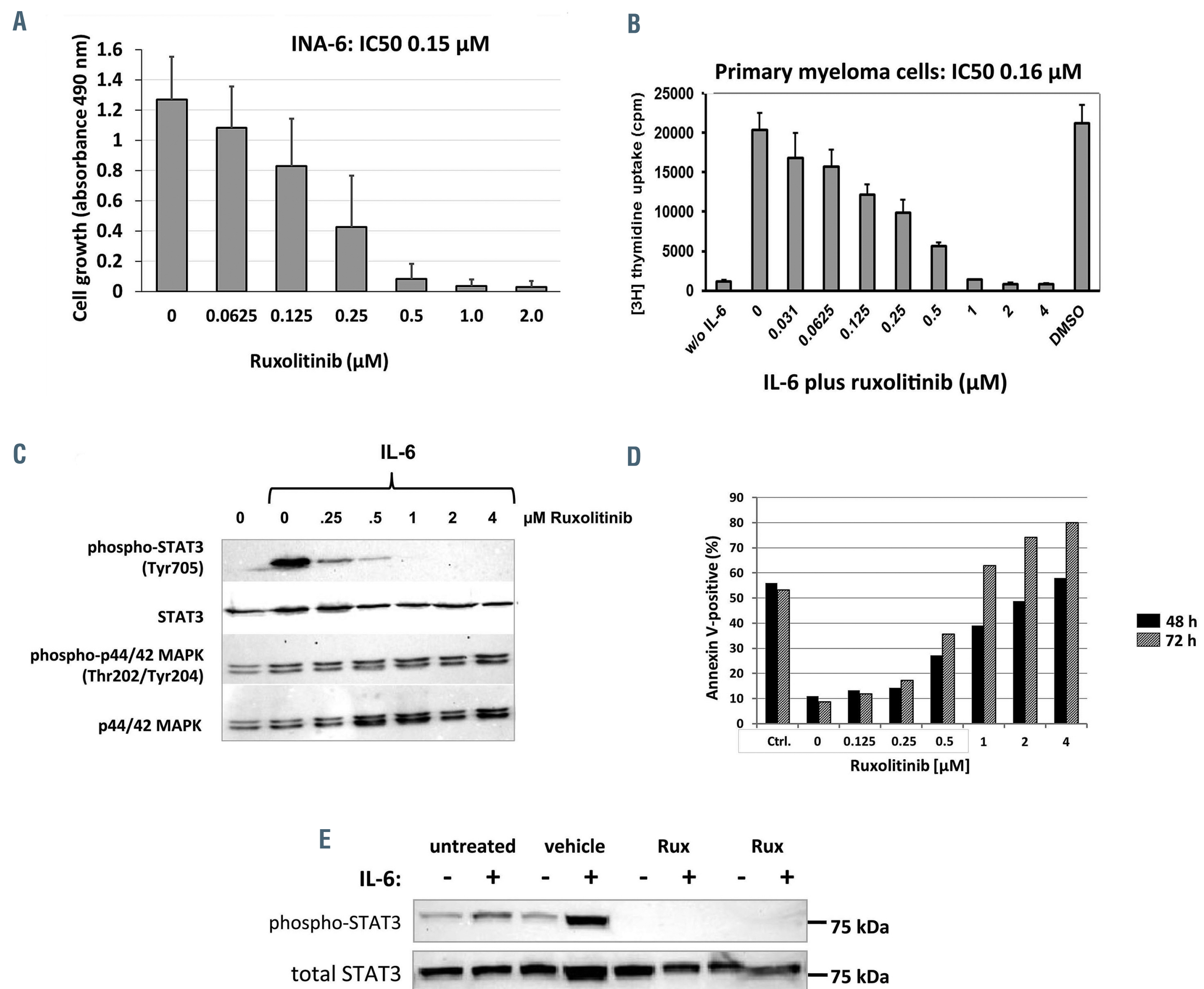

Figure 1. Effects of ruxolitinib on malignant plasma cell growth and STAT3 phosphorylation *in vitro* and *in vivo*. (A) Inhibition of INA-6 growth *in vitro* by ruxolitinib is dose-dependent. Cells were cultured in the presence of 2.5 ng/mL interleukin-6 (IL-6) for 3 days and absorbance was measured in an MTS-based colorimetric assay as described elsewhere.[8] The mean values of ten independent experiments, each performed in triplicate or quadruplicate, are shown. Error bars, standard deviation. The concentration at 50% inhibition was calculated with CalcuSyn software (Biosoft, UK). (B) Inhibitory effect of ruxolitinib on IL-6-stimulated proliferation of primary plasma cells from the peripheral blood of a patient with plasma cell leukemia. 3H-thymidine uptake was measured as described previously.[5] (C) Ruxolitinib dose-dependently inhibits IL-6-induced STAT3 phosphorylation in INA-6 cells, as demonstrated by western blot analysis. INA-6 cells were starved of IL-6 and serum for 4 hours (h), treated with different concentrations of ruxolitinib for 2 h, and then stimulated with 10 ng/mL IL-6 (Gibco®/Life Technologies, Darmstadt, Germany) for 15 min. Control cells did not receive IL-6. Cropped blots are shown. (D) Induction of apoptosis by ruxolitinib as shown by annexin V-FITC/7-AAD staining (Beckman-Coulter) and flow cytometric analysis (FC500). Cells were cultured in IL-6 and different concentrations of ruxolitinib for 48 h and 72 h. Control cells (Ctrl.) did not receive IL-6 or ruxolitinib. (E) Inhibition of STAT3 phosphorylation *in vivo*. A single oral dose of ruxolitinib (60 mg/kg) was given to tumor-bearing mice (at day 27 or day 33 after cell inoculation). One control animal received vehicle (0.5 % w/v methylcellulose, day 33), one engrafted mouse remained untreated. Tumors were explanted 2 h after drug administration. One part of the cells was stimulated *ex vivo* with IL-6 (10 ng/mL) for 10 min (+), the other part remained unstimulated (-). Cell lysates were prepared for sodium dodecylsulfate polyacrylamide gel electrophoresis and western blot analysis. Cropped blots of cell lysates from the two control animals and two ruxolitinib-treated mice are shown.

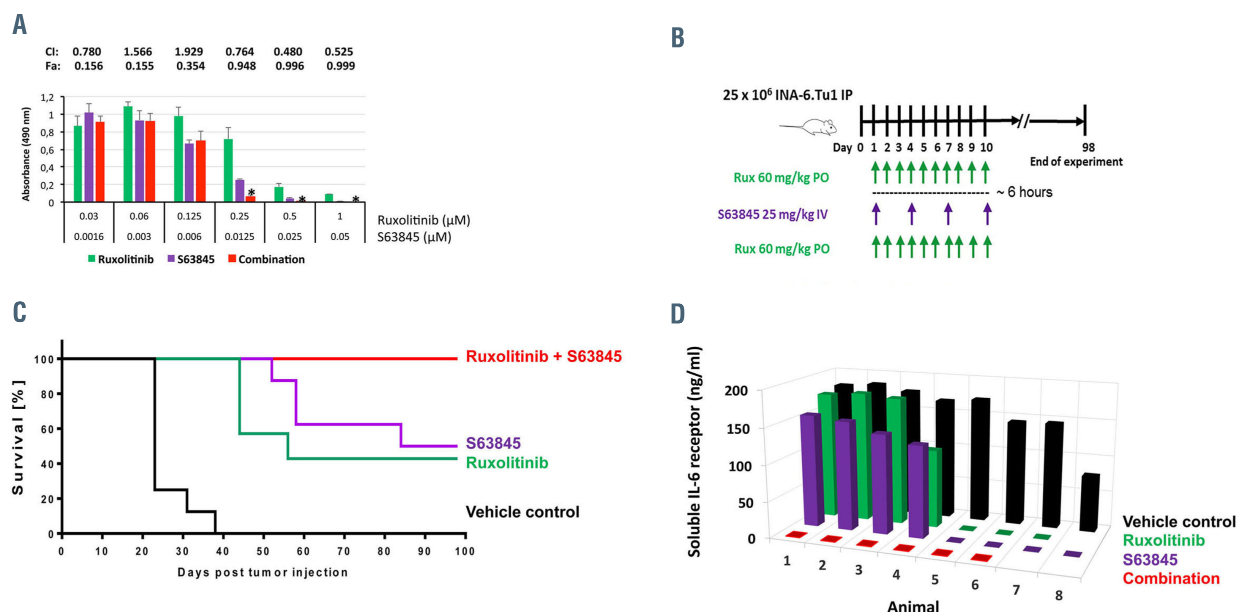

Figure 2. Effects of ruxolitinib, S63845, and their combination on inhibition of plasma cell growth *in vitro* and *in vivo*. (A) Inhibition of INA-6.Tu1 growth *in vitro* by ruxolitinib, S63845, or their combination. Cell growth was measured by an MTS-based colorimetric assay. Drugs were added at the indicated concentrations. The mean values of a representative experiment, performed in quadruplicate, are shown. Error bars, standard deviation. *Significant difference of the effect of the combination from the effect of either single drug ($P<0.05$, unpaired two-tailed *t*-test). The drug combination indices (CI) for experimental values at a constant ratio were calculated with the method of Chou and Talalay with CalcuSyn v2.0 software (Biosoft, UK). CI<1, synergistic effect; CI=1, additive effect; CI>1, antagonistic effect. Fa: affected fraction. (B) Treatment scheme of SCID mice with the combination of ruxolitinib and S63845. Treatment started at day 1 after intraperitoneal (IP) cell inoculation and continued for 10 consecutive days. Ruxolitinib was administered orally (PO) twice daily, with the time between two doses being approximately 6 h. S63845 was injected intravenously (IV) on days 1, 4, 7, and 10. Treatment with ruxolitinib or S63845 as single agents was performed accordingly with vehicle always used as a substitute for the second drug. (C) Survival of SCID mice treated with the combination of ruxolitinib and S63845 (red line) was superior (100% alive) to that of animals treated with ruxolitinib alone (green line; 43% alive; $P=0.0325$) or S63845 as a single agent (purple line; 50% alive; $P=0.0514$). The control group (black line) received vehicle (0% alive; $P\leq0.0001$ against all other groups). There was no significant difference between the ruxolitinib- and the S63845-treated group (P=0.4768). *P*-values were calculated using the log-rank (Mantel-Cox) test: $P<0.05$ is considered significant. (D) Soluble interleukin-6 (IL-6) receptor levels in the serum of mice at the day of sacrifice. Animals with undetectable levels had no visible tumors and survived until the experiment was terminated.

salt (INC424; formerly INCB018424) was supplied by Novartis Pharma (Basel, Switzerland) and Incyte Corp. (Wilmington, DE, USA). Among a number of human myeloma cell lines, the IL-6-dependent INA-6 (established in our laboratory and described in detail elsewhere[5]) was chosen because cytokine pathways after gp130 stimulation are well characterized and the line is sufficiently sensitive to growth inhibition by ruxolitinib (Figure 1A and Table 1). A similar high sensitivity to ruxolitinib in the nanomolar range was observed for growth inhibition of IL-6-stimulated primary plasma cell leukemia cells (Figure 1B). In INA-6, the JAK inhibitor specifically abrogated IL-6-stimulated STAT3 phosphorylation while the MAPK pathway, which is constitutively activated by an N-*RAS* mutation,[5] was not inhibited (Figure 1C). Concomitantly with signaling inhibition, ruxolitinib induced apoptosis in INA-6 cells in a dose-dependent manner (Figure 1D). These findings are consistent with the essential role of STAT3 for the survival of INA-6 cells[6] and other plasma cells.[7] The INA-6 xenograft model also seemed to be particularly suitable for evaluating ruxolitinib given the high *in vivo* activity of gp130 monoclonal antibodies.[8] As pharmacodynamic studies on tumor-bearing mice demonstrate, the constitutive as well as (*ex vivo*) IL-6-stimulated STAT3 activation observed in tumors of untreated or vehicle-treated control mice were inhibited *in vivo* by one single oral dose of ruxolitinib (60 mg/kg) (Figure 1E). Other signaling pathways activated in INA-6 cells *in vitro* and *in vivo*, such as the MAPK pathway constitutively

Table 1. IC_{50} values of ruxolitinib in myeloma cell lines.

Cell line	IC_{50} (μM)
JAK driven	
HEL (*JAK2* V617F)	0.8
IL-6 dependent	
INA-6	0.15
INA-6.Tu1	0.85
B9	0.6
Autonomous growth	
EJM	2.67
JJN3	>8*
JK-6	4.19
L363	>8*
MM1.S	>8*
NCI-H929	>8*
RPMI8226	>8*
U266	>8*

Cell growth was measured by an MTS-based colorimetric assay and half maximal inhibitory concentration (IC_{50}) values were calculated with CalcuSyn v2.0 software (Biosoft, UK). The erythroleukemia line HEL carrying the activating *JAK2* V617F mutation served as a control. With the exception of the murine B9 hybridoma, all cell lines were of human origin. B9 was a kind gift from L. A. Aarden (Central Laboratory Blood Transfusion Service, Amsterdam, the Netherlands); MM1.S was kindly provided by Yu Tzu Tai (Dana Farber Cancer Institute, Boston, MA, USA); INA-6, INA-6.Tu1 and JK-6 were established as described elsewhere.[5,16] All other cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. *Highest concentration evaluated.

activated by an N-*RAS* mutation[5] and the phosphorylation of the S6 ribosomal protein downstream of PI-3 kinase/AKT were not inhibited (*Online Supplementary Figure S1*). For the *in vivo* studies, a subline of INA-6 was used.[5,8] In general, 25x10^{6} INA-6.Tu1 cells were injected intraperitoneally into approximately 8-week-old female SCID/beige (C.B.-17.Cg-Prkdcscid Lystbg/Crl) mice (Charles River, Sulzfeld, Germany). All animal experiments were performed in strict adherence to German laws for animal welfare and were approved by the governmental authorities of Schleswig-Holstein. Animals were kept under specified pathogen-free conditions with free access to food and water in a light-dark cycle of 12 hours.

Blocking one single survival pathway may not be sufficient to eradicate myeloma cells in their tumor environment.[1] The choice of the anti-apoptotic Mcl-1 protein as a second target is based on the knowledge that Mcl-1 is a critical survival factor for myeloma cells and is upregulated by IL-6 produced in the bone marrow microenvironment in a STAT3-dependent manner.[9-11] An additional pathway leading to Mcl-1 upregulation, involving phosphatase of regenerating liver (PRL)-3, has recently been identified.[12] S63845, provided by Novartis, is a potent and selective BH3-mimetic with higher affinity for human than for murine Mcl-1.[13]

Ruxolitinib and S63845 were used in combination and the effects *in vitro* and in animal studies compared with those of the single agents. INA-6.Tu1 cell growth *in vitro* was dose-dependently inhibited by both drugs with a significantly greater effect in combination at higher concentrations (Figure 2A). For the *in vivo* study, INC424 was freshly formulated in 0.5% w/v methylcellulose (Sigma-Aldrich, M0430) in sterile water every 3 to 4 days. S63845 was freshly dissolved in 2% D-α-tocopherol polyethylene glycol 1000 succinate (vitamin E-TPGS) (Sigma-Aldrich) in 0.9% sodium chloride solution shortly before every application. SCID/beige mice were inoculated with INA-6.Tu1 cells as described above and treated for 10 consecutive days starting 1 day after injection of the cells (Figure 2B). Ruxolitinib was administered by oral gavage (60 mg/kg body weight) twice daily with a 6 h interval between the two doses. S63845 was injected intravenously at the dose of 25 mg/kg on days 1, 4, 7 and 10 according to the scheduling described previously.[13] Mice were monitored regularly for signs of tumor growth. The survival time was defined as the time between cell inoculation and the day of sacrifice, which occurred before tumor burden caused paraplegia, cachexia, or any other signs of suffering. Animals without any signs of tumors were sacrificed at the end of the experiment on day 98 (Figure 2B). Treatment was well tolerated in all groups, as indicated by no body weight losses during the first 20 days (*Online Supplementary Figure S2*).

The Kaplan-Meier survival analysis (Figure 2C) shows that all mice of the control group (n=8) developed overt plasmacytomas and had to be sacrificed before day 40. The median survival time in this group was 23 days. A significant delay in tumor growth was observed in four out of seven mice treated with ruxolitinib, while three mice did not show any signs of tumors until the end of the experiment on day 98, resulting in a significantly prolonged median survival time of 56 days ($P<0.0001$ by the log-rank test). Treatment of mice with the Mcl-1 inhibitor (n=8) prevented tumor growth in 50% of the animals and significantly prolonged the median survival time compared to that of the control group ($P<0.0001$). In mice treated with single agents, tumor growth seen in some of the animals was not caused by the development of drug resistance, as the sensitivity to both ruxolitinib and S63845 was retained in explanted tumors (*Online Supplementary Figure S3*). Remarkably, none of the mice treated with the combination (n=6) showed any signs of disease; at day 98 the experiment was terminated and animals were found to be tumor-free. The combination therapy was significantly superior to treatment with ruxolitinib alone, as determined by the log-rank test ($P=0.0325$).

In INA-6-bearing mice, human soluble IL-6 receptors (sIL-6R) accumulate in the blood representing a tool for the detection of minimal residual disease.[5] sIL-6R levels were measured in the serum taken from all mice at the time of sacrifice (Figure 2D). Mice with overt plasmacytomas, i.e., all mice of the control group and four mice each in the ruxolitinib and in the S63845 treatment groups, had measurable sIL-6R levels of up to 180 ng/mL (by enzyme-linked immunosorbent assay; Diaclone, Besançon, France). sIL-6R was not detected in any of the mice with long-term survival. These results strongly indicate that these mice were indeed free of INA-6 tumors.

Ruxolitinib is currently in early clinical evaluation for patients with relapsed/refractory multiple myeloma in combination with steroids, immunomodulatory drugs and proteasome inhibitors. Likewise, clinical trials with the highly selective Mcl-1 inhibitor S64315 (MIK665), a molecule resembling S63845, as well as other Mcl-1 inhibitors are underway.[14] Inhibitors of JAK1/2 and Bcl-2 family proteins are synergistic in myeloid malignancies and are currently being evaluated.[15] In myeloma, simultaneously targeting JAK/STAT3 and Mcl-1 may either disturb one single signaling pathway or, more likely, block more than one pathway to efficiently control myeloma cell growth *in vivo*. The use of ruxolitinib with an Mcl-1 inhibitor in clinical studies is warranted.

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