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Letter to the Editor

The rational of the study is to investigate the effect of hypoxia inducible factor (HIF)-1α stable suppression in myeloma cells on the sensitivity to lenalidomide in vivo. We found that the antitumoral in vivo effect of lenalidomide is enhanced by HIF-1α suppression in myeloma cells.

It has been reported that HIF-1α is overexpressed by myeloma cells1-4 and that HIF-1α suppression significantly blocks myeloma-induced angiogenesis and reduces both tumoral burden and bone destruction in vivo in multiple myeloma (MM) mouse models. Actually, the potential effects of HIF-1α modulation on drug sensitivity in MM cells are not known and are under investigation.

The immunomodulatory drugs (IMiDs®) including lenalidomide (LEN) are a class of drugs derived from Thalidomide4 able to exert anti-myeloma effects by the selective cereblon-dependent destruction of IKZF proteins5,6 either through a direct action on MM cell proliferation and survival7, or through indirect immunomodulatory and anti-angiogenic effects.7

Previous data indicated that HIF-1α inhibition not increased the anti-proliferative in vitro effect of Bortezomib on MM cells2,3, accordingly to the strong downregulation of HIF-1α induced by this drug in MM cells.2 On the other hand, we recently reported that HIF-1α knock-down in the human myeloma cell line JJN3 potentiated the in vitro effect of LEN treatment (48-72hrs) on cell proliferation through a significant up-regulation of p27, without changing cell viability.3 Consistently, it has been reported that LEN only slightly down-regulated HIF-1α expression in MM cells.2 These evidences give the rationale to investigate the effect of HIF-1α stable suppression in myeloma cells on LEN sensitivity in vivo.

Thus, in the present study, firstly we inhibited HIF-1α in three human myeloma cell lines (HMCL)s, JJN3, OPM2 and H929, using an anti-HIF1α lentiviral shRNA pool as previously described.3 H929 showed a very high mortality rate after anti-HIF1α lentiviral infection (data not shown) and were not further used for the in vivo experiments.

We assessed the effect of LEN in combination with HIF-1α inhibition in a non-obese diabetic/severe combined immunodeficiency (NOD/SCID) subcutaneous in vivo mouse model.3 Different groups of animals (5 animals for each groups) of two sets of independent experiments, were injected with JJN3-pLKO.1 (empty vector) or JJN3-anti-HIF1α. When tumors became palpable (about 7-10 days after injection) mice were treated with LEN (5mg/kg) (Selleckchem, Houston, TX) or vehicle (DMSO), using
After three weeks, we evaluated tumor volume and weight as previously described\(^3\) and by immunohistochemistry the microvascular density (MVD), checked by CD34 immunostaining (Santa Cruz, Dallas, TX).\(^3\) In addition, in the first set of mice experiment, the expression of p27 (Abcam, Cambridge, UK) was evaluated by immunohistochemistry, and the expression of S-phase kinase-associated protein 2 (SKP2), a p27 inhibitor, and the expression of the HIF-1α target key mediator of glycolysis and tumoral growth, Hexokinase II (HK2), and the levels of pERK 1/2, and total Caspase-3 (Casp-3) were evaluated in the \textit{ex vivo} plasmacytomas lysates by western blot, using the following antibodies: SKP2, Casp-3 (Santa Cruz, Dallas, TX), HK2, pERK 1/2, (Cell Signaling, Danvers, MA). β-actin was used as internal control (Millipore, Darmstadt, Germany). Immunoblots were performed as previously described.\(^8\)

As previously published\(^3\), we found that HIF-1α inhibition decreased the tumoral burden compared to JJN3-pLK0.1 mice. Moreover HIF-1α suppression potentiated LEN treatment with an additive effect, inducing a reduction of the volume of the tumors in mice injected with JJN3 anti-HIF1α as compared to JJN3-pLK0.1 after LEN treatment (Figure 1A) (average variation of tumor volume ± standard deviation: JJN3-pLK0.1 plus LEN versus JJN3-pLK0.1 plus vehicle -62±8%; JJN3 anti-HIF1α versus JJN3-pLK0.1 plus vehicle -60±12%; JJN3 anti-HIF1α plus LEN versus JJN3-pLK0.1 plus vehicle -91±11%). These data were confirmed with OPM2 (Figure 1B) suggesting that the effect of HIF-1α inhibition on LEN treatment were not specific for JJN3.

The p27 nuclear expression was significant increased by LEN treatment in JJN3-anti-HIF1α as compared to JJN3 pLK0.1 mice (mean ± SD: 13.3 ± 2% vs 8 ± 2%; \(P=0.05\)) and compared to JJN3-anti-HIF1α not treated mice (mean ± SD: 13.3 ± 2% vs 6.8 ± 1.6%; \(P=0.006\)). Figure 1C shows p27 expression in one representative tumor grown in mouse for each group of treatment.

By western blot analysis on plasmacytomas, we showed that LEN in combination with HIF-1α inhibition significantly reduced the expression of p-ERK1/2, total Casp-3, HK2 and the p27 inhibitor, SKP2, (Figure 1D). These data suggest that HIF-1α inhibition may increase the \textit{in vivo} anti-tumoral effect of LEN, through the modulation of p27 signaling and consequently cell proliferation and
survival. Based on these in vivo data, we further checked the in vitro effect of long-term LEN treatment on JJN3 pLKO.1 and JJN3-anti-HIF1α viability by MTT assay (Alexis Biochemical, San Diego, CA). We showed that, after 6 days of LEN treatment, HIF-1α inhibition lead to an increased sensitivity to LEN of JJN3 (Figure 1E), that are known to be a human myeloma cell line resistant to this drug.

Because it is well known that LEN exerts its anti-myeloma effect targeting the IKZF proteins, we checked whether the in vivo effect of the combination of HIF-1α suppression and LEN treatment could be mediated by the modulation of these proteins. The expression of IKZF1 (Santa Cruz, Dallas, TX), IKZF3 (Novus Biological, Cambridge, UK) and IRF4 (DAKO, Milan, Italy) were evaluated by western blot in JJN3-anti-HIF1α and JJN3 pLKO.1 treated in vitro with LEN (2-10µM) or vehicle (DMSO) for 72 hours. Interestingly, after LEN treatment at 2 and 10µM, we found that both IKZF1 and IKZF3 were not differentially expressed, whereas IRF4 was down regulated, in JJN3-anti-HIF1α as compared to JJN3 pLKO.1 (Figure 1G-H). This suggests that, outside being a IKZF3 target, IRF4 could be a downstream target of HIF-1α, through a HIF-1a knock-down dependent down-regulation of NF-kB. Moreover, our data indicate that the modulation of IRF4 is involved in the increase of sensitivity to LEN by anti-HIF-1α suppression in LEN resistant MM cells.

Regarding a possible combinatory effect on the in vivo angiogenesis, as expected, we found that both the number of CD34 positive vessels and MVD were reduced in mice colonized by JJN3-HIF-1α as compared to JJN3-pLKO.1, as previously reported. On the other hand, LEN treatment did not further significantly reduce the number of CD34 positive vessels and the MVD (Figure 2A) as shown in one representative ex vivo plasmacytoma for each group of treatment (Figure 2B).

Indeed, to understand the lack of LEN effect on angiogenesis in our in vivo model, we investigated the expression of the main pro-angiogenic molecules in vitro by an angiogenesis PCR array (Roche, Milan, Italy). Accordingly to our in vivo results on the plasmacytoma vascularization, we did not find any significant inhibitory effect of LEN treatment on these molecules in JJN3 anti-HIF1α as compared to JJN3 pLKO.1 even after 72 hours (Figure 2C). Interestingly an up-regulation of CCL2, CCL3, PECAM1 and MMP9 expression was observed in JJN3 pLKO.1 after the treatment with LEN (Figure 2C). This up-regulation was reduced by HIF-1α stable suppression in JJN3. In line with these observations, it was previously reported a paradoxical up-regulation of pro-inflammatory and pro-angiogenic cytokines,
such as TNF-α.\textsuperscript{12} Although it has been demonstrated a direct anti-angiogenic effect of LEN on endothelial cells\textsuperscript{13} as well as \textit{in vivo} in a lymphoma mouse model\textsuperscript{14}, others failed to report a significant reduction of the BM MVD in MM patients treated with new drugs including LEN.\textsuperscript{15}

Overall our data indicate that HIF-1α suppression in MM cells significantly increase the anti-MM \textit{in vivo} effect of LEN, mainly through the inhibition of proliferation signaling pathways rather than to an anti-angiogenic effect. These data suggest that the combination of LEN and HIF-1α inhibition may have a therapeutic rationale in MM.
REFERENCES


FIGURE LEGENDS

Figure 1: The HIF-1α stable inhibition in myeloma cells significantly increased the LEN antitumoral effect in vivo.

Four groups of five NOD/SCID mice each were injected with JJN3-pLKO.1 or OPM2-pLKO.1 (empty vector) or JJN3-anti-HIF1α or OPM2-anti-HIF1α, obtained by anti-HIF1α lentiviral shRNA pool. From 7-10 days after injection mice were treated with LEN (5 mg/kg) or vehicle (DMSO), five days per week, using the intraperitoneal route. After three weeks, we evaluated tumor volume. Box plot represents the median volume of the masses removed from all the mice inoculated with JJN3-pLKO.1 or JJN3-anti-HIF1α. Data were analyzed with Mann-Whitney test (A). Graph bars represent the median volume of the masses removed from all the mice of each experimental groups inoculated with or OPM2-pLKO.1 or OPM2-anti-HIF1α. Data were analyzed with Kruskal-Wallis test ($P=0.022$) (B).

Plasmacytomomas were processed and were analyzed by immunohistochemistry for the expression of p27. Picture shows a representative image of each group (JJN3-pLKO.1, JJN3-pLKO.1 + LEN, JJN3-anti-HIF1α and JJN3-anti-HIF1α + LEN) at 21 day (C).

The protein levels of HK2, SKP2, p-ERK1/2, total Casp-3 and β-actin were evaluated by western blot on ex vivo plasmacytomomas total lysates from mice injected with JJN3-pLKO.1 or JJN3-anti-HIF1α treated with LEN or vehicle (D).

In vitro viability of JJN3-pLKO.1 or JJN3-anti-HIF1α treated with LEN (2 and 10µM) or vehicle (DMSO) for six days was evaluated by MTT assay. Graphs and bars represent the mean±SD. Data were analyzed by a Student’s t-test. (E). The expression of IKZF1, IKZF3, IRF4 and as internal control, β-actin, was evaluated in JJN3-pLKO.1 or JJN3-anti-HIF1α treated in vitro with LEN (2 and 10µM) or vehicle (DMSO) for 72hrs (F). The IRF4 protein bands were quantified by the ImageJ open software and normalized by β-actin (G).

Figure 2: Effect of the combination of HIF-1α stable inhibition and LEN treatment in myeloma cells on in vivo angiogenesis and on the expression of the main pro-angiogenic molecules.

In the ex vivo JJN3 plasmacytomomas, removed from all the mice treated with LEN or vehicle (DMSO) as described above, the number of vessels positive to CD34 and the MVD were evaluated by
immunohistochemistry. Plots and bars represent the single values and the mean± SE of CD34 positive vessels and MVD respectively, of the two independent sets of the in vivo experiments. Data were analyzed by two-tailed t test (A). Pictures show a representative image of CD34 staining of each group of mice at 21 day (JNJ3-pLKO.1, JNJ3-pLKO.1 + LEN, JNJ3-anti-HIF1α and JNJ3-anti-HIF1α + LEN) (B).

The expression of the main pro-angiogenic molecules were analyzed by PCR-array in JNJ3-pLKO.1 or JNJ3-anti-HIF1α treated in vitro with LEN (2 and 10μM) or vehicle (DMSO) for 72hrs. Graph bars represent the mean fold changes ± SD, calculated assuming JNJ3 pLKO.1 DMSO as control condition, of two independent experiments. Data were analyzed by two tailed t-test. (*P<0.05; § P<0.01; # P<0.001) (C).