Iron facilitator LS081 reduces hypoxia-inducible factor-1α protein and functions as anticancer agent in hepatocellular carcinoma

Hiroki Tanaka, Zhen Li, Katsuya Ikuta, Lynda Addo, Hiroaki Akutsu, Masao Nakamura, Katsunori Sasaki, Takaaki Ohtake, Mikihiro Fujiya, Yoshihiro Torimoto, Jonathan Glass, Yutaka Kohgo
Iron facilitator LS081 reduces Hypoxia inducible factor-1α protein and functions as anti-cancer agent in hepatocellular carcinoma

Hiroki Tanaka¹*, Zhen Li², Katsuya Ikuta³, Lynda Addo³, Hiroaki Akutsu⁴, Masao Nakamura⁵, Katsunori Sasaki¹, Takaaki Ohtake³, Mikihiro Fujiya³, Yoshihiro Torimoto⁶, Jonathan Glass², Yutaka Kohgo³

¹ Department of Gastrointestinal Immunology and Regenerative Medicine; ³ Division of Gastroenterology & Hematology/Oncology; ⁴ Central Laboratory for Research & Education; ⁵ Department of Chemistry, ⁶ Oncology Center, Asahikawa Medical University, Asahikawa, Hokkaido, Japan. ² Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, Louisiana

Word counts: 3943 words
Number of tables/figures: 6 figures

*To whom correspondence should be addressed:
Hiroki Tanaka, Ph.D.
Department of Gastrointestinal Immunology and Regenerative Medicine, Asahikawa Medical University
2-1-1-1 Midorigaoka-Higashi, Asahikawa, Hokkaido 078-8510, Japan
Phone +81-166-68-2462
Fax +81-166-68-2469
E-mail hiroki-t@asahikawa-med.ac.jp
Summary

Hypoxia inducible factor-1α (HIF-1α) has a central role in cellular oxygen-sensing and its overexpression in many types of cancer is considered important in tumor progression. Thus targeting HIF-1α production/activity has been of great therapeutic interest. In normoxic condition, HIF-1α is hydroxylated by oxygen-dependent prolyl hydroxylases (PHDs), which require a ferrous iron for its activity. The tumor suppressor protein von Hippel Lindau (VHL) binds to the hydroxylated-HIF-1α, which is then ubiquitinated and degraded by proteasomes. We focused on the physiological HIF-1α degradation machinery mediated by PHDs. Previously, we identified a small molecule, LS081 that is capable of stimulating iron uptake into cells. In the present study, we aimed to inhibit the expression of HIF-1α protein and growth of hepatocellular carcinoma by using the iron-facilitating activity of LS081. In the human hepatocellular carcinoma cell lines Hep3B and HepG2, a combination of LS081 and iron (LS081/FeAC) inhibited HIF-1α protein expression but did not inhibit HIF-1α mRNA expression. A mutated HIF-1α protein, whose proline residues were replaced with alanine and transfected into HEK293 cells, was not affected by LS081/FeAC treatment. Furthermore, the iron-facilitating activity of LS081 resulted in Hep3B and HepG2 growth inhibition in vitro and in vivo. These results indicate that the iron-facilitating activity of LS081 inhibits HIF-1α expression through prolyl-hydroxylation of HIF-1α, and might have a therapeutic effect in the treatment of hepatocellular carcinoma.
Introduction

Hypoxia inducible factor-1 (HIF-1) is a transcription factor that enhances the expression of many genes including those involved in angiogenesis, cell proliferation, glucose metabolism, erythropoiesis and cell survival. HIF-1 is composed of α and β subunits, where the β subunit is constitutively expressed, while the α subunit is degraded under normoxic conditions despite the fact that it is continuously synthesized. In the presence of oxygen, HIF-prolyl-hydroxylases (PHD1, 2 and 3) catalyze the iron-dependent hydroxylation of specific prolyl-residues on HIF-1α. Once hydroxylated, HIF-1α binds to von Hippel Lindau tumor suppressor protein (pVHL), is ubiquitinated, and then degraded by proteasomes. Under hypoxic conditions however, HIF-1α is hydroxylated to a lesser extent and imported into the nucleus, where it binds to HIF-1β and other transcription factors and co-activators to transactivate a variety of genes containing the hypoxia response element. In most cancer cells, HIF-1α is overexpressed via either hypoxia dependent or independent mechanisms, resulting in increased HIF transcriptional activity, which helps the cancer cells to survive and grow by enhancing angiogenesis, motility and glycolysis. HIF activities are also involved in resistance to chemotherapy and radiation therapy, therefore, inhibition of HIF activities should be of importance in cancer treatment.

The iron-chelator deferoxamine (DFO), which deprives cells of iron, upregulates the expression of HIF-1α protein, indicating that cellular iron content has an essential role in regulating HIF-1α protein degradation. In fact, FeCl₃ alone or ferri-transferrin reduces HIF-1α expression in hypoxic conditions. Based on these reports, we hypothesized that the facilitation of iron uptake in cancer cells might downregulate the expression of HIF-1α protein by enhancing the activity of PHDs. To date, although HIF-1 inhibitors have been identified, there are no available reports on increasing HIF-1α protein degradation by stimulating iron uptake.

In a previous study, we reported that in mouse hepatocellular carcinoma (HCC) models
HIF-1α was overexpressed and inhibition of HIF-1α mRNA expression resulted in remarkable growth reduction.\(^{11}\) Further, tumor vascularization, that is significantly observed in typical HCC tissues, compared with tumors of other organs proves that the inhibition of HIF-1α could have a great effect in the treatment of HCCs. We also reported that a novel iron facilitator, LS081 inhibited HIF-1α expression in prostate cancer cell lines and inhibited the growth of these cells in cell culture.\(^{15}\) However, neither the mechanism of HIF-1α inhibition nor the effect of LS081 on tumor xenografts were determined. In this study, we present data that LS081 leads to increased activity of PHD with a resulting increased hydroxylation of HIF-1 and consequent decrease of HIF-1 protein expression and that LS081 markedly affects the growth of HCC xenografts.

**Materials & Methods**

**Cell culture.** Human HCC cell lines, Hep3B and HepG2, and the human embryonic kidney cell line, HEK293 were obtained from ATCC. The cells were cultured in DMEM (Wako, Tokyo, Japan), supplemented with 10% FCS, and penicillin-streptomycin (Wako). HEK293 cells were transfected with the expression vectors described below using Lipofectamine LTX (Invitrogen, Carlsbad, CA) and then stable clones were established by treatment with G418 (Sigma-Aldrich, St. Louis, MO). For normoxic cell culture, the cells were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\) and 21% O\(_2\). For hypoxic cell culture, the cells were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\) and 1% O\(_2\) by using a hypoxia workstation (Ruskinn Technology, Pencoed, UK). The cells were treated with the growth medium containing ferric ammonium citrate (FeAC), LS081 (TimTec, Neward, DE) and MG132 (Enzo life sciences, Farmingdale, NY) as indicated for each of the experiments.

**Iron uptake measurements by atomic absorption spectrophotometry.** Hep3B and HepG2 cells were incubated with LS081 at concentrations of 0-30 µM in the presence of 10 µM FeAC for 1hour. The cells were removed from the plates with trypsin, washed extensively with Hepes buffered saline, enumerated and following lysis with 0.1% SDS,
the iron content (fmol/cell) were measured by atomic absorption spectrophotometry using an Hitachi Z8100 Atomic Absorption Spectrophotometer (Hitachi, Tokyo, Japan).

**Western blot analysis.** Cell and tissue samples were lysed in RIPA buffer, separated with polyacrylamide gel and electro-transferred to nitrocellulose membranes. After blocking the membranes with 5% nonfat dry milk in PBST buffer (PBS containing 0.05% Tween-20), the membranes were probed with anti-HIF-1α antibody (Novus biological, Littleton, CO), anti-FLAG antibody (Sigma-Aldrich), anti-Actin antibody (BD biosciences, Franklin Lakes, NJ), and anti-Histone H1 antibody (Santa Cruz, Santa Cruz, CA) followed by incubation with HRP-conjugated anti-mouse IgG secondary antibody (R&D systems, Minneapolis, MN). Antibody binding was then visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA).

**Real time RT-PCR.** Total RNA was isolated from cells with RNA purification system PureLink RNA Mini kit (Invitrogen), and reverse transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real time RT-PCR was then performed using ABI 7300 system (Applied Biosystems) with TaqMan probes for human HIF-1α mRNA (Applied Biosystems). 18S ribosomal RNA was analyzed as internal control, and the ratio of HIF-1α to 18S ribosomal RNA was calculated.

**Immunohistocytometry.** Hep3B cells were cultured under hypoxic conditions for 18 hours, and fixed with 4% paraformaldehyde solution. The cells were then incubated first with the anti-HIF-1α monoclonal antibody (Novus) and then with the Alexa Fluora 488 conjugated anti-mouse IgG antibody (Invitrogen), followed by nuclear staining with DAPI.

**Cell growth assay.** Hep3B and HepG2 cells were cultured both in normoxic and hypoxic conditions with LS081 and/or FeAC for 24 and 48 hours. Cell numbers were enumerated by the MTT assay (Promega, Madison, WI) according to the manufacturer’s protocol. Absorbance was measured at 490 nm using Powerscan HT (DS Pharma, Osaka, Japan), and the ratio against the control at 24 hours was calculated.

**Expression vectors.** To establish an expression vector, which expresses N-terminal
FLAG-tagged wild type HIF-1α (pCI-neo-3×FLAG HIF-1α), cDNA of human HIF-1α was subcloned into pCI-neo-3×FLAG constructed by inserting oligonucleotides encoding the 3×FLAG epitope into pCI-neo mammalian expression vector (Promega). Additionally, the DNA sequences corresponding to the proline residues at amino acids 402 and 564 of HIF-1α were replaced by site-directed mutagenesis, and a vector expressing N-terminal FLAG-tagged HIF-1α whose proline residues were replaced with alanine residues (pCI-neo-3×FLAG HIF-1α P402/564A) was established.

**Xenografts.** Hep3B cells were inoculated into the subcutaneous tissue of the back of Balb/c nude mice (Clea Japan, Tokyo, Japan), and when the tumor volume reached about 400 mm³, daily intraperitoneal injection with LS081 (5 mg/kg/day and 25 mg/kg/day) was started. The controls were treated with vehicle alone (30% polyethyleneglycol in PBS). Tumor diameters were measured with calipers, and the tumor volume was calculated using the formula: volume = d_{short}² × d_{long} / 2. These mice were sacrificed on day 7 of treatment with collection of serum and tissues. All the experimental procedures performed were approved by the institutional committee based on the guidelines for the protection of animals.

**Serum assays for hepatic function, transferrin, and iron content.** Serum analysis for Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvate transaminase (GPT), serum iron and unsaturated iron binding capacity (UIBC) was performed using the automatic serum analyser LABoSPECT008 (Hitachi). Assay reagents used were as follows: L type wako GOT-J2 (Wako) for GOT and L type wako GPT-J2 (Wako) for GPT, Quick auto neo Fe (Sino-test, Tokyo, Japan) for serum iron, Quick auto neo UIBC (Sino-test) for UIBC. Analysis for non-transferrin-bound iron (NTBI) was performed using HPLC with a minor modification as previously reported by our laboratory. Liquid chromatography-mass spectrometry was performed using NanoFrontier eLD (Hitachi). LS081 and/or FeAC were first dissolved in ultrapure water then further diluted in ultrapure methanol. Infusion analysis for these samples was accomplished with a micro syringe at 3 μL/min. flow speed with negative
electrospray ionization. The spectra of samples were compared with the virtual spectra simulated by system software.

Statistics. The Student paired t test was used. $P$ values < 0.05 were considered to be statistically significant.

Results

**LS081 and iron inhibits HIF-1α protein but not mRNA expression in hepatoma cell lines.** The iron facilitating activity of LS081 in Hep3B and HepG2 cells was verified by atomic absorption spectrophotometry. Figure 1A shows that in the presence of 10 µM FeAC, iron uptake is facilitated by LS081 treatment at a dose of 5-30 µM in both cell lines. In order to know whether iron facilitation by LS081 inhibits HIF-1α expression in hepatoma cells, Hep3B and HepG2 cells were cultured both under normoxic and hypoxic conditions with FeAC and/or LS081. The data showed that a combination of LS081 and FeAC significantly inhibited HIF-1α protein expression (Fig. 1B). Nuclear HIF-1α protein also disappeared when FeAC and LS081 was added to the culture medium under hypoxic conditions (Fig. 1C). The presence of HIF-1α protein as detected by immunohistocytometry also showed that the expression of HIF-1α protein both in cytosol and nucleus was decreased when the cells were treated with LS081 and FeAC (Fig. 1D). In contrast, HIF-1α mRNA expression was not significantly affected by FeAC and/or LS081 under normoxic or hypoxic conditions (Fig. 1E). These indicate that LS081-dependent iron facilitation post-transcriptionally inhibits HIF-1α expression.

**Iron facilitation by LS081 enhances HIF-1α prolyl-hydroxylation.** We hypothesized that the reduction in HIF-1α protein expression was caused by an increased activity of HIF-prolyl-hydroxylases with subsequent increased ubiquination and HIF-1α proteosomal degradation. Figure 2A shows that in the presence of the proteasome inhibitor MG132, HIF-1α protein reduction was no longer observed. In order to determine whether the iron-facilitating activity of LS081 decreases HIF-1α protein by enhancing HIF-prolyl-hydroxylation, we established HEK293 cell lines that stably
express FLAG-tagged wild type HIF-1α (293 HIF-1α WT) and FLAG-tagged HIF-1α whose proline residues were replaced with alanine (293 HIF-1α P402/564A) (Fig. 2B). These cell lines were then treated with LS081 and FeAC for 24 hours. As seen in Figure 2C, culture in the presence of LS081/FeAC decreased wild type HIF-1α protein but had no effect on HIF-1α P402/564A expression. Interestingly, the mutated-HIF-1α that lost proline residues was able to evade decrease. These data are consistent with iron facilitation mediated by LS081, thus increasing PHD activity and hence increasing prolyl-hydroxylation of HIF-1α.

**Treatment with LS081 and iron inhibits cell growth in HCCs.** Because HIF-1α transcriptional activity up-regulates many kinds of growth factors and anti-apoptotic factors, inhibition of HIF-1α by LS081 might cause both growth arrest and apoptosis. To investigate the effect of LS081 on cell growth, Hep3B and HepG2 cells were cultured with LS081 and/or FeAC in normoxia and hypoxia for 24 and 48 hours and the cell numbers enumerated (Fig. 3). Growth of Hep3B cells was not affected by LS081, slightly inhibited by FeAC alone, but markedly inhibited by the combination of LS081 and FeAC both under normoxia and hypoxia (Fig. 3A). The combination of LS081 and FeAC inhibited cell growth only under hypoxic conditions in HepG2 cells (Fig. 3B). These observations correspond to the results that HIF-1α protein expression was detectable even under normoxia in Hep3B cells while HIF-1α protein was detectable only in hypoxia in HepG2 cells (Fig. 1A). As the combination of LS081 and FeAC were lower than the controls and they were time-dependently reduced, this treatment may have induced both growth arrest and apoptosis. The data further suggests that LS081 itself does not have a cytotoxic effect on cell growth while a combination of LS081 and iron inhibits cell growth corresponding to HIF-1α expression.

**LS081 treatment inhibits tumor growth of Hep3B xenograft in nude mice.** Hep3B xenografts were initiated and when the tumors reached about 400 mm³, LS081 was given intraperitoneally in the absence of any supplemental iron. Growth curves of the xenografts (Fig. 4A) shows that LS081 significantly inhibited tumor growth.
Western blot analysis after the treatment showed that LS081 reduced HIF-1α expression in the tumor tissue (Fig. 4B). LS081 treatment caused a hemorrhagic necrosis in the tumor tissue, indicating that the treatment induced destruction of intra-tumoral vessels (Fig. 4C). LS081-treated mice showed neither significant body weight change, hepatic damage nor aberrant iron overload in histological analysis (data not shown). Furthermore, the levels of serum hepatic enzymes were not affected by LS081 treatment (Fig. 4D), consistent with a lack of hepatic damage by LS081. These suggest that LS081 might play a role as an anti-cancer agent without hepatic toxicity, despite being active against hepatomas.

**LS081 mainly affects NTBI.** In the animals bearing the xenografts, LS081 treatment did not affect either serum iron levels (i.e. primarily ferri-transferrin) or UIBC (i.e. apo or unsaturated transferrin) (Fig. 5), while LS081 reduced NTBI, which exists as a minor component of body iron (Fig. 5). These results indicate that LS081 may primarily increase serum NTBI into tissues without affecting transferrin bound iron.

**LS081 directly binds to free iron.** Based on our serum analysis, we speculated that LS081 binds to free iron and transports the chelated iron into the cell but that LS081 has a lesser effect on uptake of iron from transferrin. LS081 chelates were analyzed by mass spectrometry of *in vitro* mixtures of LS081 and FeAC (Fig. 6). The spectrum of LS081 (Fig. 6A) shows LS081 to have predominant isotopes with a mass of 363.0725 and 365.0713 daltons. In the mixture of LS081 and FeAC however, the spectrum shifted to a larger mass of about 781 daltons (Fig. 6B). The observed spectra were quite similar to that predicted by computer simulation and are consistent with 2 molecules of LS081 binding 1 molecule of iron.

**Discussion**

Although there has been considerable efforts to identify inhibitors of HIF-1 transcriptional activity, there are no reports that are directly focused on modifying the physiological degradation machinery of HIF-1α as mediated by PHDs nor any reports suggesting that increased intracellular iron could be manipulated to increase PHD activity and hence decrease HIF-1 expression. Our previous report and current studies were
undertaken to determine if compounds that stimulated iron uptake would, in fact, affect HIF expression and alter cancer cell proliferation. In fact, iron facilitation by LS081 significantly decreased HIF-1α protein in a prolyl-hydroxylation dependent manner and, presumably as a consequence, inhibited growth of hepatocellular carcinomas. In some cancer cells, hypoxia independent overexpression of HIF-1α has been observed.\(^{9-11}\)

This phenomenon is thought to be caused by abnormal activation of HIF-1α translation mediated by oncogenic signaling through the PI3/Akt/mTOR pathway. Furthermore, the status of a well-known tumor suppressor p53 may influence HIF-1α down-regulation. In our study, detectable levels of HIF-1α protein were observed in Hep3B cells (p53 null) grown even under normoxic conditions, whereas it was undetectable in HepG2 (p53 wild). These levels were significantly decreased by treatment with LS081/FeAC, suggesting that iron facilitation might have a therapeutic advantage in targeting HIF-1 in some cancers even when normoxic conditions prevail.

Iron content is lower in human hepatomas than in normal tissue.\(^{17}\) In animal models, liver tumors contain reduced amounts of iron and resist iron accumulation.\(^{18-20}\) Furthermore, a key regulator of iron uptake, transferrin receptor 1 (TfR1), which is also known as a transcriptional target of HIF-1, is increased in HCCs.\(^{20}\) In many other cancers, a similar elevation of TfR1 expression and decreased cellular iron content is present, consistent with an iron deficiency phenotype. This decrease could then result in reduced activity of PHDs with HIF-1α protein up-regulation.\(^{21,22}\) Iron depletion observed in HCCs may therefore be a cause of HIF-1α up-regulation. The oxygen and iron dependent PHDs, which are a key factor in HIF-1α degradation, are also known to be transcriptional targets of HIF-1.\(^{23-25}\) Thus HIF-1 dependent up-regulation of PHDs might contribute to a negative feedback control of HIF-1α up-regulation. Even with sufficient oxygen levels in tumor tissues, limited iron content would interfere with the negative feedback control mediated by PHDs. Iron facilitation in this situation may help to reduce HIF-1α expression by enhancing the activity of PHDs. In our present study, the LS081 succeeded in reducing HIF-1α protein levels of HCCs both in cell culture and
animal models. We provided iron facilitation as a novel approach for HIF-1-targeting treatment in hepatocellular carcinomas.

Iron is an essential metal for hemoglobin synthesis in erythrocytes, oxidation–reduction reactions, and cellular proliferation, whereas iron-overload causes organ dysfunction through the production of reactive oxygen species (ROS) production.\(^{(26,27)}\) Most serum-circulating iron binds to transferrin while NTBI exists as a minor component of body iron.\(^{(16)}\) In our study, the treatment with LS081 reduced serum NTBI levels whereas no significant changes were observed in serum iron and UIBC levels. Additionally, mass spectrometry analysis suggested that 2 molecules of LS081 directly bind 1 molecule of free iron. This data suggests that LS081 mainly binds NTBI, and transports it into cells through unknown transporters or receptors without an effect on transferrin bound iron. This selective effect can be explained by the hypothesis that the affinity of transferrin for iron is stronger than LS081 affinity for free iron. Although iron facilitation by LS081 increased ferritin levels in cell culture as previously described,\(^{(15)}\) we did not observe a change in ferritin levels in the Hep3B xenografts in our present study (data not shown). As NTBI levels are extremely low under physiological conditions \textit{in vivo}, LS081 would passively facilitate NTBI uptake into tissues without resulting in iron-overload.

Ponka and colleagues have pioneered the use of hydrazo derivatives to study cellular iron metabolism,\(^{(28,29)}\) many of which are iron chelators that inhibit iron uptake into reticulocytes as well as various cell lines. Our results suggest that LS081, which does chelate iron, facilitates the uptake of iron.\(^{(15)}\) The side-chain composition of LS081 differs from the hydrazone derivatives that inhibit iron uptake.\(^{(15)}\) In the initial screen of the chemical library that identified LS081 as an iron facilitator, other hydrazone derivatives were identified both as facilitators and as iron uptake inhibitors. A detailed structure-activity analysis is being undertaken to determine the side-chain composition that dictates the effects on iron uptake. The observation of hemorrhagic necrosis in the xenografts suggests that the effects of LS081 may not be limited to the cancer cells alone but it remains to be determined if LS081 has a direct effect on endothelium or whether increased iron content in the xenografts leads to the generation of ROS and disruption of the neovascular tissue.

In conclusion, we presented a unique property of a novel iron facilitator LS081 that
enhanced HIF-1α degradation by modulaltion of prolyl-hydroxylation activity. We also succeeded in inhibiting cell growth of HCCs both under normoxic and hypoxic conditions in cell culture and in xenograft models. Furthermore, LS081 itself did not show cytotoxic effects on cell growth in vitro and no hepatic toxicity was observed in the xenografts. Treatment with LS081 might therefore be a novel approach for HIF-1 targeting treatment in cancer.

Acknowledgments


The authors thank Ms. Kotoe Shibusa, Ms. Akemi Kita and Mr. Hiroaki Konishi for their technical assistance in our experiments.

Disclosure

The authors have no conflict of interest.

References

5. Maxwell PH, Wiesener MS, Chang GW et al. The tumour suppressor protein


15. Li Z, Tanaka H, Galiano F, Glass J. Anticancer activity of the iron facilitator


Figure legends

Figure 1. A. Dose response curve of LS081 on iron facilitation in Hep3B and HepG2 cells analyzed by atomic absorption spectrometry. Shown are the means and SD of three independent experiments with each point in triplicate. B. Western blot analysis for HIF-1α and actin (loading control) in whole cell lysates of Hep3B and HepG2 cells treated with LS081 and/or FeAC both under normoxia and hypoxia for 24 hrs. C. Western blot analysis for HIF-1α and Histone H1 (loading control) in nuclear lysates of Hep3B and HepG2 cells treated with LS081 and/or FeAC under hypoxic conditions for 24 hrs. D. Immunohistocytometry detection of HIF-1α in Hep3B cells treated with no addition (Cont.), LS081 and/or FeAC under hypoxic conditions for 24 hrs. Shown is a single optical slice through the level of the nuclei. E. Real time RT-PCR analysis for HIF-1α mRNA in Hep3B (left) and HepG2 (right) cells treated with LS081 and/or FeAC both under normoxic and hypoxic conditions for 24 hrs. The columns represent the means and SD of three independent experiments.

Figure 2. A. Western blot analysis for HIF-1α and actin (loading control) in Hep3B cell treated with 10 μM LS081, 10 μM FeAC and 10 μM MG132 under hypoxic conditions. B. A schematic illustration of the expression vectors used in this study showing that with the loss of the proline residues FLAG-HIF-1α P402/564A could not be hydroxylated by PHDs. C. Left panel: Western blot analysis for FLAG tagged-HIF-1α and actin (loading control) in HEK293 cells treated with LS081/FeAC. Right panel: The ratio of FLAG-HIF-1α to the actin loading control by densitometric analysis. The columns represent the means and SD of three independent experiments. *, P < 0.05, compared with control (HIF-1α WT without treatment).

Figure 3. MTT assay in Hep3B (A) and HepG2 (B) cells cultured with 10 μM LS081 and/or 10 μM FeAC under normoxic and hypoxic conditions for 24 and 48 hours. Left panel: normoxia, Right panel: hypoxia. The columns represent the means and SD of
three independent experiments. *, $P < 0.05$, compared with control.

**Figure 4.** A. The change in tumor volume during the treatments. Each point represents the means and SD of 4 mice. B. Western blot analysis for HIF-1α and Actin (loading control) in the tumor lysates. C. H&E staining of tumor tissue after the treatment (original magnification ×100). D. The levels of serum hepatic enzymes after the treatment. The columns represent the means and SD of 4 mice.

**Figure 5.** Levels of serum iron, UIBC and NTBI in Hep3B xenograft models. The columns represent the means and SD of 4 mice. *, $P < 0.05$, compared with control.

**Figure 6.** A. Left panel: The spectrum of LS081 (overall view). Right panel: Predicted spectrum pattern by computer simulation (upper) and enlarged view of left panel (lower). B. Left panel: The spectrum of the mixture of LS081 and FeAC (overall view). Right panel: Predicted spectrum pattern by computer simulation (upper) and enlarged view of left panel (lower).
Fig. 1

(A) Graph showing the concentration of HIF-1α in Hep3B and HepG2 cells treated with different concentrations of LS081.

(B) Table showing the concentration of HIF-1α in Hep3B and HepG2 cells under 21% and 1% O₂ conditions with 10 μM LS081 and FeAC.

(C) Western blot analysis showing the expression of HIF-1α and Histone H1 in Hep3B and HepG2 cells treated with 10 μM LS081 and FeAC.

(D) Immunofluorescence images showing the expression of HIF-1α and DAPI staining in Hep3B and HepG2 cells treated with Cont., LS081, FeAC, LS081 + FeAC.

(E) Bar graph showing the fold increase in HIF-1α expression in Hep3B and HepG2 cells treated with 10 μM LS081 and FeAC under 21% and 1% O₂ conditions.
Fig. 2

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>HIF-1α WT</th>
<th>HIF-1α P402/564A</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS081/FeAC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MG132</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

FLAG-HIF-1α WT

- FLAG
- P402
- P564
- PHDs

FLAG-HIF-1α P402/564A

- FLAG
- A402
- A564

C

<table>
<thead>
<tr>
<th>Condition</th>
<th>HIF-1α WT</th>
<th>HIF-1α P402/564A</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS081/FeAC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FLAG-HIF-1α</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Graph

LS081/FeAC

- FLAG-HIF-1α WT
- FLAG-HIF-1α P402/564A

<table>
<thead>
<tr>
<th>Condition</th>
<th>FLAG-HIF-1α/Actin ratio (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α WT</td>
<td>+</td>
</tr>
<tr>
<td>HIF-1α P402/564A</td>
<td>-</td>
</tr>
</tbody>
</table>

* indicates a significant difference.
Fig. 4

A

![Graph showing tumor volume (mm$^3$) over time for different treatment groups.]

- Cont.
- 5 mg/kg LS081
- 25 mg/kg LS081

B

![Western blots showing HIF-1α and Actin expression for different treatment groups.]

- Cont.
- 5 mg/kg LS081
- 25 mg/kg LS081

C

![Histological images comparing control (Cont.) and 25 mg/kg LS081 treated samples.]

- Cont.
- 25 mg/kg LS081

D

![Bar graph showing GOT and GPT levels for different treatment groups.]

- Cont.
- 5 mg/kg LS081
- 25 mg/kg LS081
Fig. 6

A

[Chemical structure]

C$_{13}$H$_9$BrN$_4$O$_4$
MW 365.14

Predicted spectrum pattern:

B

[Chemical structure]

781.7516
780.1034
783.7473

Predicted spectrum pattern: