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論文題目	**Chemical biology research on the UCHL1–HIF axis toward development of molecular targeted anticancer drugs** **（分子標的抗がん剤開発を指向した UCHL1-HIF 経路に関するケミカルバイオロジー研究）**		

Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is a deubiquitinating enzyme that aggravates tumor malignancy. Recently, UCHL1 has been reported to stabilize Hypoxia-inducible factor 1, alpha subunit (HIF-1α), which transactivates a variety of oncogenic genes to promote metastasis and lead to poor prognosis in multiple cancers. Therefore, the development of novel UCHL1 inhibitors is of great significance in the treatment of malignant tumors. In this research, a novel UCHL1–HIF axis inhibitor was successfully developed through drug repositioning and bioassays. I firstly manifested in spheroid models the feasibility of UCHL1 inhibition against HIF-1-related tumor malignancy **(Chapter 1)**. Next, a highly potent UCHL1 inhibitor was discovered through *in vitro* high-throughput screening and *in silico* instructed structural modification. Finally, this novel inhibitor was found to show improved specificity and high potency in blocking the UCHL1–HIF-1 axis using cell models, leading to inhibited HIF activity and cell migration, which consequently demonstrated its efficacy **(Chapter 2)**. Overall, the strategy of blocking the UCHL1–HIF axis and the identification of the novel UCHL1 inhibitor is expected to contribute to further treatments of UCHL1-related tumors.

Chapter 1: UCHL1 promotes HIF-1-dependent tumor cell malignancy in spheroid models

First, I investigated the impact of pharmacological inhibition of the deubiquitinating activity of UCHL1 on HIF-1-dependent tumor malignancy. In 2D monolayer culture, UCHL1 inhibition by siRNA or LDN57444, a well-known UCHL1 inhibitor, drastically lowered HIF-1α protein levels in UCHL1-expressing cells. In UCHL1 non-expressing cells, ectopic expression of UCHL1 significantly increased HIF-1α protein expression levels, which was canceled by the treatment of LDN57444. When further analyzed whether the UCHL1 inhibitor could affect the HIF-dependent transactivation, LDN57444 dose-dependently inhibited the HIF activity and decreased the transcription of HIF downstream genes. Finally, LDN57444 significantly blocked cell migration in UCHL1 expressing cells, suggesting a crucial role of the enzyme in tumor migration.

In 3D spheroid culture models, which has been more widely applied in the preclinical studies to provide more physiologically relevant information, ectopic expression of UCHL1 significantly upregulated malignancy-related factors such as solidity, volume, as well as viable cell number in a HIF-1α dependent manner. Conversely, inhibition of the UCHL1–HIF-1 pathway downregulated these malignancy-related factors and abolished the UCHL1–HIF axis mediated cell proliferation and invasiveness. Finally, inhibition of UCHL1 promoted HIF-1α degradation and lowered the expression of HIF-1 target genes in the 3D model, as also observed in 2D monolayer culture models.

Chapter 2: Development of a novel UCHL1 inhibitor by computational drug repositioning and bioassays

As known UCHL1 inhibitors have all been reported with severe side-effects such as the induction of ER stress and abnormal synapse protein expressions, the development of more effective and selective UCHL1 inhibitors remains a major challenge for the treatment of malignant tumors. Therefore, I then focused on the development of novel UCHL1 inhibitors through drug repositioning. A high-throughput screening of 4,500 known drugs and reagents was performed and seven compounds were demonstrated to be effective UCHL1 inhibitors. By further examined with their inhibition against the UCHL1–HIF axis, only two out of seven compounds were qualified to dose-dependently inhibit HIF activity as well as the UCHL1-inducued HIF-1α stabilization. In further confirmation of phenotypic migration inhibition, only one compound, Compound A, showed relatively strong selectivity towards UCHL1 harboring cells at low concentrations. Since Compound A was originally developed as a protein kinase X inhibitor that blocks protein synthesis and therefore potently

inhibits cell viability, thirty Compound A analogues were designed by adding amide or bis-amide groups into the long acyl chains. Molecular docking simulations was then applied to find an analogue that has predictably the highest inhibition against UCHL1 as well as the lowest inhibition against protein kinase X. Top three ranking analogues were synthesized and only Compound A_5, was shown to exhibit a much-increased inhibitory effect against UCHL1 as well as a decreased inhibitory effect against protein kinase X.

In the analysis of the predicted interaction between UCHL1 and Compound A_5, the amino group was determined to form new hydrogen bonds with UCHL1 and helps the formation of additional interaction between UCHL1 and Compound A_5. Furthermore, ASP155 and ASP156 of UCHL1 was confirmed to be essential in the binding with Compound A_5 by newly forming charge interactions and hydrogen bonds. Therefore, UCHL1 recombinants mutated in their ASP 155 or ASP 156 or both was created to apply *in vitro* kinetics analysis and found Km to be increased significantly in UCHL1 D155A or D156A mutants. Moreover, Ki of Compound A_5 was also much lower compared with Compound A against UCHL1 WT, D155A, D156A and D155, 156A mutants. These results revealed the significance of ASP155 and ASP156 of UCHL1 in the binding with Compound A_5. Finally, in an *in vitro* kinase assay that determines the kinase activity of protein kinase X, the inhibitory effect of Compound A_5 turned out to be almost eliminated when compared with Compound A. Taken together, Compound A_5 is a promising UCHL1 specific inhibitor.

In the determination of cytotoxicity, which is one of the strongest impacts of protein kinase X inhibition, cytotoxicity of Compound A_5 in DLD-1, MDA-MB-231, MDA-MB-436 cells was 4.5, 4.9 and 12.6 times lower respectively, indicating a lower cytotoxicity. Then it was tested whether Compound A_5 could specifically inhibit UCHL1–HIF axis to prevent metastasis in cellular models, Compound A_5 showed lower IC_{50} of HIF activity inhibition in UCHL1 overexpressing cells than in UCHL1 non-expressing cells. In wound healing assays, inhibitory effects on cell migration was confirmed in only MDA-MB-436 cells at a concentration of 1 or 2.5 μM of Compound A_5. One micro mol/mL of Compound A led to a decrease of HIF-1α accumulation in only UCHL1 expressing cells and eliminated the UCHL1-overexpression led HIF-1α accumulation. Finally, Compound A_5 showed no inhibitory effect against the phosphorylation of protein kinase X target protein while Compound A abundantly lowered the phosphorylation. These results above demonstrated the higher specificity and potency of our novel UCHL1 inhibitor.

In conclusion, this thesis represents a systematic work on the demonstration of the effectiveness of UCHL1 inhibitors in HIF-1 dependent tumor malignancy as well as the development of novel UCHL1 inhibitors through computational drug repositioning and bioassays. Compound A_5 is an ideal lead compound for the development of more potent and specific analogues. At the same time, the combination of in vitro HTS assay and computational with bioassays renders a more efficient developing process for UCHL1–HIF-1 axis. I am confident that my results will contribute to the discovery of effective UCHL1–HIF-1 axis inhibitors in future therapies of metastasis in UCHL1-related tumors, such as breast and pulmonary cancers.

（続紙 ２ ）

（論文審査の結果の要旨）

著者は、新しいがん化学療法の開発の基盤研究として、低酸素シグナル応答に着目した分子標的抗がん剤の開発研究を行った。特に、脱ユビキチン化酵素 UCHL1 が活性化する低酸素誘導因子 HIF-1 経路に注目して、UCHL1 の機能理解及び分子標的抗がん剤リード化合物の取得を目的として本研究を行った。

はじめに、乳がん細胞株 MDA-MB-436 (UCHL1 発現株) 及び MDA-MB-231(UCHL1 非発現株)を用いたスフェロイド培養系を確立して、UCHL1-HIF 経路が、がん細胞の転移促進に加えて、増殖・悪性化に強く影響することを生化学的手法及びケミカルバイオロジー的手法を用いて明らかにした。続いて、UCHL1-HIF 経路を標的としたハイスループットスクリーニング系を構築後、既存薬剤のリプロファイリング研究を行った結果、UCHL1 阻害剤として有望な化合物 A を見出した。また、データベース上、入手可能な UCHL1 の立体構造の活用、化合物 A の標的酵素の立体構造のモデリングなどを in silico 解析で行い、より選択的に UCHL1 を阻害する化合物群の設計を行った。その過程で、UCHL1 の複数のアミノ酸変異体などを作製・精製後、活性及び阻害剤感受性試験を行うことで、化合物 A の UCHL1 活性阻害に必要な UCHL1 内のアミノ酸残基の推定・実証を行った。さらに、in vitro 及び培養細胞レベルで化合物 A より優れた選択性を有する化合物 B の取得に成功した。これらの知見は、UCHL1-HIF 経路を標的とした分子標的抗がん剤開発に貢献することが期待される。

よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、令和2年2月13日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。

なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、当該論文の全文に代えてその内容を要約したものとすることを認める。

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