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学 位 論 文 題 名

Identification of a novel small molecule HIF-1α translation inhibitor

(低酸素誘導因子 HIF-1 α の翻訳を抑制する新規低分子化合物の同定)

学位論文内容の要旨

[Background and Purpose] Solid tumors are hypoxic and this feature renders them more resistant to radiation and chemotherapy. Hypoxia inducible factor-1 α (HIF-1 α) is the central mediator of the cellular response to low oxygen and functions as a transcription factor for a broad range of genes that provide adaptive responses to oxygen deprivation. HIF-1 is over-expressed in cancer and has become an important therapeutic target in solid tumors. Using a HIF-responsive reporter cell-based assay, a 10,000-membered natural product-like chemical compound library was screened to identify novel HIF-1 inhibitors. This led us to discover KC7F2, a lead compound with a central structure of cystamine. In this study, The effects of KC7F2 on HIF-1 transcription, translation and protein degradation processes were analyzed.

[Materials and Methods] Cancer cell lines LN229, LNZ308, U251MG, MCF7, PC3, D54MG, U87MGD, A549 and the human fibroblast cell line HFF-1 were used. In addition, primary cultures of human dermal microvascular endothelial cells (HDMVEC) and primary cultures of mouse neurons were subjected to the experiments. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and 21% O2 (normoxia) or 1% O2 (hypoxia) in a hypoxia workstation (InVivo1,000, Ruskinn). The LN229-HRE-AP reporter cell line for HIF transcriptional activity was used. Cycloheximide (CHX), an inhibitor of protein synthesis, was used to test whether KC7F2 affects the protein degradation rate of HIF-1α. MG-132 (Calbiochem, La Jolla, CA), a proteasome inhibitor, was used to investigate whether KC7F2 affects the protein synthesis of HIF-1α. *Alkaline phosphatase (AP) assay*: LN229-HRE-AP cells were plated onto 96-well plates and incubated with or without chemicals for 24 hrs under hypoxic or normoxic condition. AP enzymatic activity was determined by measuring OD values at 405 nm in a spectrophotometer. S*ulforhodamine B (SRB) assay*: Cells were seeded onto 96-well plates and cultured under normoxia and hypoxia with different concentrations of KC7F2 for 72 hrs or treated for various times with 20 μM KC7F2. For proliferation analysis, cells were fixed with 50% Trichloroacetic acid and stained with 0.4% SRB. Cell proliferation was calculated by measuring OD values at 564 nm using a spectrophotometer. *Clonogenic assay*: D54MG cancer cells or HFF-1 immortalized human fibroblast cells were seeded onto 6-well plates. After 16 hrs, KC7F2 was added to final concentrations of 0, 5, 10 μM and the cells treated for 72 hrs under hypoxia or normoxia. Thereafter, the cells were returned to normoxic conditions. After 11 days, the cells were fixed and stained with crystal violet (0.9 %). Cell colonies were counted and percent of survival was calculated by comparing to colonies obtained in untreated plates. *Western blot analysis*: Cells were washed twice with cold PBS and lysed with conditioned radio-immunoprecipitation assay (RIPA) buffer. The harvested cells were subjected to centrifugation and the collected proteins were electrophoresed on 7.5-12.5% Tris-HCl gels (BioRad, Richmond, CA), then electroblotted to nitrocellulose membranes. The membranes were subjected to immunoblot analysis using primary and corresponding secondary antibodies to detect the target proteins. Northern blot analysis: Cells were harvested by Trizol (Invitrogen, Carlsbad, CA) and total RNA was extracted according to the manufacturer's instructions. Equal amounts of total RNA samples were loaded into 1% agarose-formaldehyde gels, separated by electrophoresis, and

transferred to nylon membranes. HIF-1α and Enolase 1 cDNA probes were labeled with [α-32P] dCTP (Amersham Biosciences, Piscataway, NY) using Prime-It II Random Primer Labeling Kit (Stratagene, Ceder Creek, TX) and hybridized to the membrane. The hybridization was done with ULTRAhyb buffer (Ambion, Austin, TX) at 42°C overnight.

[Results] The inhibitory effects of tested compounds were measured by a reduction of the AP enzymatic activity present in hypoxic cells. Using this bioassay, we screened a 10,000-membered natural product-like combinatorial library, and identified a novel class of compounds, which have a cystamine group as their central structure. Further screening lead us the two most potent compounds, KC7F2 which had an IC50 of 20. KC7F2 markedly inhibited HIF-mediated transcription in cells derived from different tumor types, including glioma, breast and prostate cancers and exhibited enhanced cytotoxicity under hypoxia. Treatment of a variety of cancer cell lines with KC7F2 for 72 hrs demonstrated a clear dose-response cytotoxicity with an IC50 of approximately 15-25 μM. Remarkably, non-tumoral cells (HDMEV and neurons) showed much less susceptibility to KC7F2. The cytotoxicity of KC7F2 was also increased under hypoxia in the SRB experiments whether in dose-response or in long-term time course response. KC7F2 also inhibited colony formation of D54MG cells in a dose-dependent manner and this effect was more significant under hypoxia. In contrast, the immortalized fibroblast cell line HFF-1 was more resistant to KC7F2 treatment in colony formation under both normoxia and hypoxia.

KC7F2 prevented the activation of HIF-target genes such as Carbonic Anhydrase IX, Matrix Metalloproteinase 2 (MMP2), Endothelin 1 and Enolase 1. KC7F2 specifically reduced the protein levels of HIF-1 in a dose-dependent manner under hypoxic conditions, while the levels of β-actin were largely unaffected. These results suggest that KC7F2 inhibits HIF-1 α at the protein level, making it unavailable for HIF-1 mediated transcription. Investigation of the mechanism of action of KC7F2 using CHX and MG-132 and northern blot experiments showed that it does not affect the HIF-1 α protein degradation machineries but its accumulation at the protein translational level without inhibiting HIF-1 α mRNA synthesis. To explore whether the HIF-1 α protein inhibition by KC7F2 was linked to the suppression of the PI3K-Akt-mTOR pathway, LN229 cells were pre-treated for one hour with 40 μM of KC7F2 followed by hypoxia. The levels of phosphorylated 4EBP1 were strongly suppressed by KC7F2 as early as 2 hrs and throughout the 24 hrs incubation under hypoxia. The levels of non-phosphorylated 4EBP1 were not altered until 12 hrs, then at 24 hrs showed a slight drop in expression. In contrast, the levels of phosphorylated Akt, total Akt, phosphorylated mTOR, total mTOR, and total S6K were not affected or showed only modest changes up to 12 hrs in response to KC7F2. At 24 hrs, a ~50% drop in Akt and S6K levels was observed. As phosphorylated S6K was not detectable in LN229, its change in U87MGD glioma cells upon KC7F2 treatment was examined. The level of phosphorylated S6K was affected in a similar fashion to phospho-4EBP1, while total S6K showed a gradual decrease which became more pronounced after 12 hrs. The phosphorylation of 4EBP1 and S6K are important steps for the initiation of protein translation; therefore, these findings provide a mechanistic explanation for the inhibition of KC7F2 on HIF-1 α protein synthesis.

[Conclusion] These results show that KC7F2 is a potent HIF-1 pathway inhibitor and its potential as a cancer therapy agent warrants further study.

学位論文審査の要旨

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(低酸素誘導因子 HIF-1 α の翻訳を抑制する新規低分子化合物の同定)

悪性脳腫瘍や癌組織には低酸素分圧(hypoxia)の状態が観察される。Hypoxia Inducible Factor 1 (HIF-1) はこうしたhypoxiaの腫瘍細胞で広く発現している、α と β のサブユニットからなる転写因子である。このうちHIF-1αはhypoxiaにおいてのみ発現が見られ、細胞増殖、代謝、血管新生などに関係する遺伝子を制御し腫瘍の低酸素適応における中心的役割を担う。それゆえ、HIF-1α の抑制は腫瘍増殖抑制において重要な意味を持つ。本論文ではHIF-1α 抑制作用をもつKC7F2を同定し、その抑制の機序を検証した。

使用した細胞株は脳腫瘍細胞株を含む数種の腫瘍細胞株と正常細胞株、培養は21%O_2 (normoxia)または 1%O_2 (hypoxia)で行った。種々の化合物のスクリーニングにはalkaline phosphatase (AP) assayを施行した。これはHREプロモーター下流にAP遺伝子を持つプラスミドを導入したLN229細胞株を用いたレポーターアッセイである。細胞毒性はSulforohodamine B assayまたはclonogenic assayで評価した。western blot、northern blotは標準的手法で行われた。本研究ではまずAP assayによりKC7F2を選定した後、KC7F2によるAP発現抑制が直接的な細胞毒性やAP酵素活性の低下ではなく、HRE活性抑制に由来すること、並びにKC7F2によりHIF-1α 下流の種々の遺伝子群の発現が抑制されていることが確認された。一方、KC7F2の細胞毒性については正常細胞より腫瘍細胞に、またnormoxiaよりhypoxiaでより強い毒性をあらわすことが示された。更にwestern blotにより、HIF-1αタンパクの発現はKC7F2の濃度依存性に低下することが示され、先のAP assay結果はHIF-1α 抑制に起因すると推察された。また、この抑制はタンパク合成阻害剤のcyclohexiamide、プロテアソーム阻害剤のMG132を使用した実験において、KC7F2がnormoxia及びhypoxiaのいずれの条件でもHIF-1α の合成を抑制したことの結果であることが示された。更にこの合成抑制作用はHIF-α のmRNAレベルではなく、タンパク翻訳レベルにあると考えられたため、HIF-1αタンパク合成の主な経路であるPI3K-Akt-mTOR pathwayの詳細を検討した。その結果、KC7F2はタンパク翻訳開始に重要な4EBP1とS6Kのリン酸化を抑制していることが示され、これによりKC7F2のHIF-1α タンパク合成抑制の機序が説明された。

本論文の考察においては、HIF-1αが高発現している腫瘍細胞に、より細胞毒性が強かったものの、正常細胞にも毒性がみられたことへの説明、及び4E-BP1やS6 Kのリン酸化阻

害の結果を踏まえて、KC7F2 の mTORC1 への作用の検討が今後のさらなる課題とされた。

公開発表における質疑応答では、笠原正典教授より、他の HIF-1α の阻害剤の報告、血管新生に対する阻害効果、KC7F2 が HIF-1α だけを抑制する機序、他の HIF ファミリーへの作用について質問があった。次に畠山鎮次教授からは、KC7F2 の構造と薬効の相関、proline hydroxylation や pVHL complex への直接影響の有無、KC7F2 の細胞毒性の腫瘍細胞に対する選択性、及びこれに関連した正常細胞への癌遺伝子導入実験、KC7F2 と抗生物質の作用機序との相違について質問があった。最後に田仲伸哉教授より HIF-1α 阻害剤の今後の展望について質問があった。いずれの質問に対しても申請者は自ら行った研究やその過程で得られた知見、参考とした文献の引用をもとに、的確に回答した。

癌化学療法、特に脳腫瘍では既存の薬剤の効果が不十分であり、低酸素状態の腫瘍細胞の増殖に重要な役割をもつ HIF-1α の阻害剤は近年注目を集めている。本論文は、この HIF-1α を抑制する新たな化合物 KC7F2 を同定し、その作用メカニズムに迫っている。また、本論文中に示された KC7F2 の腫瘍細胞に対する選択的な毒性は化学療法の上では魅力的な特徴であり、動物実験を含めた今後の詳細な検討により、KC7F2 が新たな癌治療法開発に寄与することも期待される。

審査員一同は、これらの成果を高く評価し、大学院課程における研鑽や取得単位なども併せ申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。