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論文題目	Discovery of Non-Cysteine-Targeting Covalent Inhibitors by Activity-Based Proteomic Screening with a Cysteine-Reactive Probe		

（論文内容の要旨）

Small molecule pharmaceuticals that form covalent bonds with their targets have historically been avoided due to misconceived concerns including off-target toxicity and potential immune responses. However, after the recent FDA approval of the rationally designed covalent drugs and the appreciation of covalent mechanisms of existing drugs, there has been increasing interest in discovering and designing covalently reacting bioactive molecules. Covalent inhibitors offer potential benefits over classical non-covalent counterparts: prolonged duration of action, less frequent or lower dosing requirements, improved ligand efficiency, drug resistance evasion when the amino acids required for the catalysis of enzymes are targeted, and high selectivity when non-conserved amino acids are targeted. The most popular approach to designing covalent inhibitors has been the incorporation of an electrophilic group into a non-covalent inhibitor. However, discovering brand-new covalent inhibitors, in particular those targeting non-cysteine residues, remains challenging. This thesis describes an intriguing experience during the activity-based proteomic screening of 1,601 electrophilic molecules against cell-lysate proteome, which led to the discovery of non-cysteine-targeting inhibitors with a cysteine-reactive probe.

In the initial screening, proteomic lysates of HEK293 cells were pre-treated with 1,601 electrophilic molecules, followed by treatment with an iodoacetamide-based probe (IA probe). The IA probe used was a conjugate of TAMRA for fluorescent detection with iodoacetamide, which displays broad reactivity with proteins with reactive cysteines. The samples were separated by SDS-PAGE and visualized by in-gel fluorescence scanning. In this context, a compound that reacts with an IA probe-reactive protein will compete with the probe, resulting in the loss or decrease of fluorescence labelling of the protein in the gel profile.

During the screening, an unexpected observation was made. One epoxide molecule, F8, exhibited an increase in the probe labelling of a 40 kDa protein. Mass-spectrometry-based microsequencing analysis showed that the 40 kDa protein was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate (G3P) into 1, 3-bisphosphoglycerate. A combination of mutational and mass spectrometric studies indicate that F8 forms a covalent adduct with an aspartic acid in the active site to displace NAD^+, a cofactor of the enzyme, with concomitant enhancement of the IA probe reaction with the catalytic cysteine.

A number of synthetic or naturally occurring GAPDH inhibitors have been reported; however, all of them have been proposed to react with the catalytic cysteine. The activity-based proteomic screening described in the present thesis led to the discovery of a new class of the epoxide-containing GAPDH inhibitors that covalently react with an aspartate residue. Since the GAPDH inhibitory activities of these molecules remain modest, further structural optimization is needed to generate specific Asp-targeting GAPDH inhibitors. Nevertheless, this case-study on GAPDH exemplifies that activity-based proteomic screening with a cysteine-reactive probe can be used for discovering covalent inhibitors that react with non-cysteine residues.

（論文審査の結果の要旨）

酵素の共有結合阻害剤は医薬品のシーズとしてますます注目されている。しかし、システイン以外のアミノ酸に反応する共有結合阻害剤を発見することは依然として困難である。本論文では、1601 個の反応性低分子化合物のプロテオミクススクリーニングと有機合成展開によって、アスパラギン酸に反応する酵素阻害剤を発見した。

本論文のスクリーニングでは、ライブラリ分子によるシステイン反応性プローブとプロテオーム間の化学反応の競合阻害を網羅的に検出している。予想外なことに、プローブと 40 kDa のタンパク質の反応をライブラリ分子の一つ（F8）が増強することに申請者らは気づいた。質量分析解析により、そのタンパク質はグリセルアルデヒド-3-リン酸デヒドロゲナーゼ（GAPDH）と同定された。

詳細な機構解析により、F8 は活性部位のアスパラギン酸と共有付加体を形成し、酵素の補酵素である NAD+を置換し、触媒システインとプローブの反応を促進することが示唆された。このようなメカニズムと構造活性相関から、より活性の高いアスパラギン酸反応性 GAPDH 阻害剤を同定している。

以上の研究は、システイン反応性プローブによるプロテオミクススクリーニングが、非システイン残基と反応する共有結合阻害剤の発見に利用できることを例証しており、新しい共有結合阻害剤の開発に寄与する。

したがって、本論文は博士（医科学）の学位論文として価値あるものと認める。

なお、本学位授与申請者は、令和 4 年 4 月 20 日実施の論文内容とそれに関連した試問を受け、合格と認められたものである。

要旨公開可能日：　　年　　月　　日　以降