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# ユビキチンシステムの多機能性を活用した脳神経系加齢性病態の克服に関する研究

（H17－ゲノム－一般－009）

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総 括 研 究 報 告 書

# ユビキチンシステムの多機能性を活用した脳神経系加齢性病態の克服

(H17−ゲノム−一般−009)

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本研究では、ユビキチンシステムが不用蛋白質の分解系として機能するだけでなく、多数の蛋白質の活性制御に関与し様々な生命現象に深く係わるという多機能性を活用し、脳神経系の老化ならびに老化がもたらす病態について、ユビキチンシステム、特に脱ユビキチン化酵素から見た克服法を開発する。なかでもUCH-L1は酵素として作用する以外にそれぞれユビキチンキャリア蛋白として機能しアポトーシス関連因子、細胞生存因子を統合的に制御することで神経細胞の生存と死に密接に関わっていることが研究代表者の和田らにより明らかにされてきた(Nat Genet, 1999; Hum Mol Genet, 2003; Am J Pathol 2004 など)。このように研究代表者は脱ユビキチン化酵素の生物学的研究で先駆的成果を収めてきたが、本研究ではこれらの成果をさらに発展させ、脱ユビキチン化酵素の機能変化がもたらす神経細胞老化の初期変化を解明するとともに、脱ユビキチン化酵素による老化関連蛋白質制御の分子的実体を明らかにする。研究開始後これまでに UCH-L1 がユビキチンシステムとリソソームシステムの機能連関に基づき細胞傷害性である酸化ストレスを検出し老化関連蛋白質の分解制御を行うこと、シナプス可塑性や記憶学習行動に関わることを明らかにした。中でも今年度はUCH-L1は酸化ストレスによりそれ自身が酸化された際にtubulinを始めとする細胞内蛋白質との相互作用が更新することを見出した。またUCH-L1に類縁のUCH-L3が欠損したマウスでは網膜においてカスパーゼ非依存的神経細胞死が見出されるものの寿命の延長が果たされることを見出した。このように UCH-L1, UCH-L3 はともに神経細胞の機能と生存に関して重要な因子であることが明らかになったことで脱ユビキチン化酵素を標的にした治療法開発をめざしたin silico drug screeningを新たに開始し、新規のUCH-L3阻害剤3種を同定した。

## A. 研究目的

本研究では、現時点では有効な予防診断法の乏しい脳の加齢変化に対してより信頼性と確度の高い生物学的検出法を提供し、さらには神経細胞老化がもたらす認知症等の病態の修復法を開拓し、その実施を通して健全な社会を実現することに貢献することを目標とする。その達成にむけて今回はこれまで研究代表者が精力的に研究を続けてきたユビキチンシステムに焦点を当て、脱ユビキチン化酵素、UCH-L1とUCH-L3を機軸にした神経細胞老化の分子メカニズムの解明と脱ユビキチン化酵素の機能モニタリングによる神経系老化の評価系の構築をめざす。

研究最終年度にあたる本年度は、UCH-L1、UCH-L3を題材に、UCH-L1については、UCH-L1自体が酸化修飾を受けるだけでなく、酸化型UCH-L1は非修飾型UCH-L1と比較してtubulinを始め細胞の機能と生存に関わる細胞内蛋白質との相互作用が亢進することを見出した。また、UCH-L1と相同性の高い UCH-L3 の欠損マウスでは寿命が延長することを見出した。さらに、UCH-L3 に作用する薬剤の in silico drug screening を試み、5万個の

pilot screening から酵素活性阻害脳を有する3個の化合物を同定した。

B. 研究方法

(1) 酸化修飾がもたらす UCH-L1 の機能変化の解析

生化学的手法を用いて、UCH-L1 の酸化修飾体を作成し、他の神経系機能蛋白質との相互作用の有無、その程度を非修飾型 UCH-L1 と比較した。さらに、点突然変異体を使用し、酸化修飾を受けるアミノ酸残基を解析した。

(2)寿命における UCH-L3 の役割解明

昨年度に引き続き UCH-L3 欠損マウスと野生型対照マウスの寿命を観察し、生存曲線を作成し統計処理を行った。

(3)UCH-L3作用薬の開発

UCH-L3の 3 次元構造のデータを公的サイトから入手し in silico drug screening の系を構築した。

(4)UCH-L3 作用薬の機能解析

Ubiquit5in-AMC を基質にした UCH-L3 の脱ユビキチン化酵素活性に及ぼす各作用薬の効果を酵素化学的に解析した。

(倫理面への配慮)

動物を使用する研究計画はすべて国立精神・神経センター神経研究所動物実験倫理問題検討委員会で審議され承認を受けた。実際の動物使用に当たっては国の法律・指針並びに米国 NIH の基準を守り動物が受ける苦痛を最小限に留めた。ヒト標本を用いた研究は実施しなかった。

C. 研究結果

(1)酸化修飾がもたらす UCH-L1 の機能変化の解析

複数のカルボニル修飾剤により酸化型 UCH-L1 を作成した。いずれの酸化体も、免疫沈降法などにより、非修飾型と比較して多数の蛋白質との相互作用が亢進していた。相互作用する蛋白質を質量分析計を用いた解析により同定したところ tubulin が含まれることが見出された。この tubulin と酸化型 UCH-L1 の相互作用は直接的であることが免疫共沈法でも確認された。さらに、tubulin の重合に及ぼす影響を検討したところ、酸化型 UCH-L1 は tubulin との結合性が野生型に比して亢進することから UCH-L1 の酸化修飾は tubulin の重合化を促進するという結果を得た。また UCH-L1 と tubulin との結合には UCH-L1 の Arg-63, His-185 のアミノ酸残基が重要である結果を得た。他方、酸化修飾には Cys-90、Cys-152 が関わることが示された。ついで酸化修飾がどのような構造変換を遂げるかを円二色偏光法で解析したところ、ベータシートの組成率が高くなることが判明し、その結果不溶性が亢進する結果を得た。

(2)寿命における UCH-L3 の役割解明

UCH-L3 欠損メスマウスの寿命は有意差を持って野生型対照マウスの寿命に比べ延長していること判明した。

(3)UCH-L3作用薬の開発

既に報告されている結晶化 UCH-L3 の X 線散乱法による解析結果を利用し分子モデルを構築した。また、血液脳関門を透過すると考えられる化合物 5 万個のデジタルデータを入手し、UCH-L3 と各化合物の docking simulation を市販コンピュータ3台で行った。その結果、高い結合性を予測する 10 個の化合物を同定した。

(4)UCH-L3 作用薬の機能解析

前項で同定された 10 種の化合物を実際に入手し、Ubiquit5in-AMC を基質にした UCH-L3 の脱ユビキチン化酵素活性に及ぼす影響を酵素化学的に解析したところ 3 種の化合物が阻害活性を示すことを見出した。

D. 考察

研究代表者は以前神経軸索ジストロフィーを主病変に持つ gracile axonal dystrophy (gad)マウスの原因遺伝子が UCH-L1 であることをみいだした。神経

軸索ジストロフィーは脊椎動物神経系で認められるもっとも普遍的な加齢所見を考慮すれば、脱ユビキチン化酵素が老化と密接に関わっていることを示す貴重な発見であった。UCH-L1 を始めとする脱ユビキチン化酵素の生物学的意義に着目した研究を展開したところ、UCH-L1 が多機能蛋白質として機能し、神経細胞体においては神経細胞死との関連性において抗アポトーシス蛋白質や prosurvival 蛋白質と機能的リンクを形成し神経細胞の生存に密接に関わることが明らかになった。研究開始初年度は UCH-L1 が神経発生・新生制御にも関わっていることを見いだし、また UCH-L3 が神経細胞死の重要な規定因子であることを示すなど脱ユビキチン化酵素の神経細胞における生物学的意義の一端を明らかにした。さらに 2 年目の昨年度は UCH-L1 についてはリソソームシステムと機能連関を形成することで老化関連蛋白質の分解制御を行うこと、細胞酸化傷害時には UCH-L1 自身が酸化修飾を受け前記リソソームシステムとの連関に変動を来し老化関連蛋白質の分解制御が変動することを見出した。また、UCH-L1 には遺伝子多型(S18Y)が存在しS型に比べY型が酵素活性が高くまた神経系の老化に伴う病態に防護的であることから、UCH-L1 標的にした活性化剤の in silico drug screening の系を構築した。さらに UCH-L1 と相同性の高い UCH-L3 の欠損マウスではミトコンドリアの変化を伴うカスパーゼ非依存的神経細胞死が増加すること(Am J Pathol, 2006)、さらには UCH-L3 が寿命の重要なキー蛋白質である可能性の高いことを見いだした。最終年度の今年度はこれらの成果を発展させ、UCH-L1 の酸化修飾は UCH-L1 蛋白のベータシートの組成を高めるなど構造変化を来たすことをみいだした。その結果他の蛋白質との結合性が高まることが予想されたが実際生化学的解析から複数の蛋白質との結合性が高まることを見出した。これらの蛋白質の中には、tubulin という神経細胞の生存と機能発現に無くてはならない蛋白質も含まれていた。酸化修飾の結果、UCH-L1 は tubulin の重合を促進するという結果を得たことから、UCH-L1 の酸化修飾は微小管形成などに影響を与える可能性が考えられる。また、酸化修飾、tubulin との結合に重要なアミノ酸残基をそれぞれ見出すことに成功したので、今後 UCH-L1 を標的にした治療法・治療薬開発に寄与すると考えられる。また、UCH-L3 についても今年度 in silico drug screening を実施し、5 万化合物の中から酵素活性阻害剤 3 種を新たに同定することに成功した。今後これらの薬剤の薬理効果を動物で検証する予定であるが、治療薬開発の新たな方法を提示できたことは価値が高いと考える。

今年度の成果は脱ユビキチン化酵素、UCH-L1 とUCH-L3を機軸にした神経細胞老化の分子メカニズムの解明と脱ユビキチン化酵素の機能モニタリングによる神経系老化の評価系の構築をめざすうえで十分基盤形成を果たしたと考える。今後両分子の機能変化が醸し出す細胞老化の分子機序をゲノム、プロテオームの面から明らかにし老化プロセスの解明に新たなメスを入れるとともに、治療戦略上必要不可欠な遺伝子・蛋白質素子を抽出することを行うが、今年度の成果は目標達成に向けて研究が着実かつ独創性高く展開されていることを示すものである。高齢者社会を迎えた我が国においては老化がもたらす様々な病態の克服は医療行政だけでなく、健全な国家財政の形成のためにも必要不可欠な社会的急務である。本研究の継続発展はこれら社会的要請に対して革新的治療法の提供という回答を出すだけでなく、老化の初期過程の検出という予防診断法を提供すると期待される。

E. 結論

UCH-L1、UCH-L3 はそれぞれ神経系老化、寿命の重要な調節因子であると結論される。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的所有権の出願・登録状況(予定を含む)

1. 特許取得

(出願中)

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2. 実用新案登録

なし

3. その他

なし

別紙５

研究成果の刊行に関する一覧表レイアウト

書籍

| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書　籍　名 | 出版社名 | 出　版　地 | 出 版 年 | ページ |
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Review

# The functions of UCH-L1 and its relation to neurodegenerative diseases

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**Abstract**

Parkinson's disease (PD) and Alzheimer's disease (AD), the most common neurodegenerative diseases, are caused by both genetic and environmental factors. Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is a deubiquitinating enzyme that is involved in the pathogenesis of both of these neurodegenerative diseases. Several functions of UCH-L1, other than as an ubiquitin hydrolase, have been proposed; these include acting as an ubiquitin ligase and stabilizing mono-ubiquitin. This review focuses on recent findings on the functions and the regulation of UCH-L1, in particular those that relate to PD and AD.


*Keywords:* UCH-L1; Ubiquitin; Parkinson's disease; Alzheimer's disease; Gad mouse; Oxidative stress; Mono-ubiquitination

**Contents**



## 1. Introduction

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), also known as PGP9.5, is a protein of 223 amino acids (Wilkinson et al., 1989). Although it was originally characterized as a deubiquitinating enzyme (Wilkinson et al., 1989), recent studies indicate that it also functions as a ubiquitin (Ub) ligase (Liu et al., 2002) and a mono-Ub stabilizer (Osaka et al., 2003). It is one of the most abundant proteins in the brain (1–2% of the total soluble protein) and immunohistochemical experiments demonstrate that it is exclusively localized in neurons (Wilson et al., 1988). Thus, its role in neuronal cell function/dysfunction was predicted. Indeed, the lack of UCH-L1 expression in mice results in gracile axonal dystrophy (*gad*) phenotype (Saigoh et al., 1999). Down-regulation and extensive oxidative modification of UCH-L1 have been observed in the brains of Alzheimer's disease (AD) patients as well as Parkinson's disease (PD) patients (Castegna et al., 2002; Choi et al., 2004; Butterfield et al., 2006). Moreover, administration of UCH-L1 was shown to alleviate the β-amyloid-induced synaptic dysfunction and memory loss associated with a mouse model of AD (Gong et al., 2006). In addition, an isoleucine 93 to

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methionine amino acid mutation (I93M) of UCH-L1 was identified as a cause of autosomal dominant PD (Leroy et al., 1998). Our recent analysis of transgenic (Tg) mice expressing UCH-L1$^{I93M}$, showed an age-dependent loss of dopaminergic neurons, which is one of the pathological hallmarks of PD (Setsuie et al., 2007). On the contrary, a polymorphism that results in the amino acid substitution of serine 18 to tyrosine in UCH-L1 (UCH-L1$^{S18Y}$) was linked to decreased susceptibility to PD in some populations (Maraganore et al., 1999; Wintermeyer et al., 2000; Wang et al., 2002; Elbaz et al., 2003; Toda et al., 2003; Maraganore et al., 2004; Facheris et al., 2005; Tan et al., 2006; Carmine Belin et al., 2007). Together, all of these aspects indicate that the precise regulation of UCH-L1 is essential for neurons to survive and to maintain their proper function. In this review, we would like to summarize recent findings on UCH-L1, mostly those that relate to PD and AD.

## 2. The molecular functions of UCH-L1

UCH-L1 was first discovered as a member of the ubiquitin carboxy-terminal hydrolase family of deubiquitinating enzymes (Wilkinson et al., 1989; Nijman et al., 2005). *In vitro* analysis indicated that UCH-L1 can hydrolyze bonds between Ub and small adducts or unfolded polypeptides (Fig. 1). It can also cleave Ub gene products, either tandemly conjugated Ub monomers (UbB, UbC) or Ub fused to small ribosomal protein (S27a), very slowly, to yield free Ub, *in vitro* (Fig. 1) (Larsen et al., 1998). However, all of the activities detected *in vitro* are significantly lower than those of any other known Ub hydrolases, and its *in vivo* substrate has not yet been identified. Indeed, X-ray crystallography analysis of UCH-L1 indicates that it might exist in an inactive form on its own, and binding partners that regulate its activity may be warranted (Das et al., 2006).

In 2002, a group identified another enzymatic activity in UCH-L1, Ub ligase activity, *in vitro* (Liu et al., 2002). UCH-L1 was shown to exhibit dimerization-dependent Ub ligase activity (Fig. 1). Thus, from their observations, it is assumed that UCH-L1 might function as a hydrolase in a monomeric form and as a ligase in a dimeric form. Neither dimerization nor ligase activity were observed in the isozyme UCH-L3. In contrast to the well-recognized ubiquitination pathway (using E1, E2 and E3 ligases), which requires ATP to activate free Ub in order to conjugate Ub to the substrate, UCH-L1 does not require ATP, a notable characteristic of this ligase.

In addition, our group reported another function of UCH-L1, a mono-Ub stabilizing effect *in vivo*, which is independent of enzymatic activity (Osaka et al., 2003). We found that a large amount of mono-Ub is tightly associated with UCH-L1, inhibiting the degradation of mono-Ub in the brain. When UCH-L1 was over-expressed in SH-SY5Y cells, the half-life of mono-Ub was extended. Moreover, the expression level of UCH-L1 affected the level of mono-Ub in the mouse brain; *gad* mice showed a decreased level and UCH-L1$^{WT}$ Tg mice showed an increased level of mono-Ub compared with wild-type mice. Thus, these results indicated that UCH-L1 functions as an Ub-stabilizing factor, regulating the pool size of mono-Ub *in vivo*

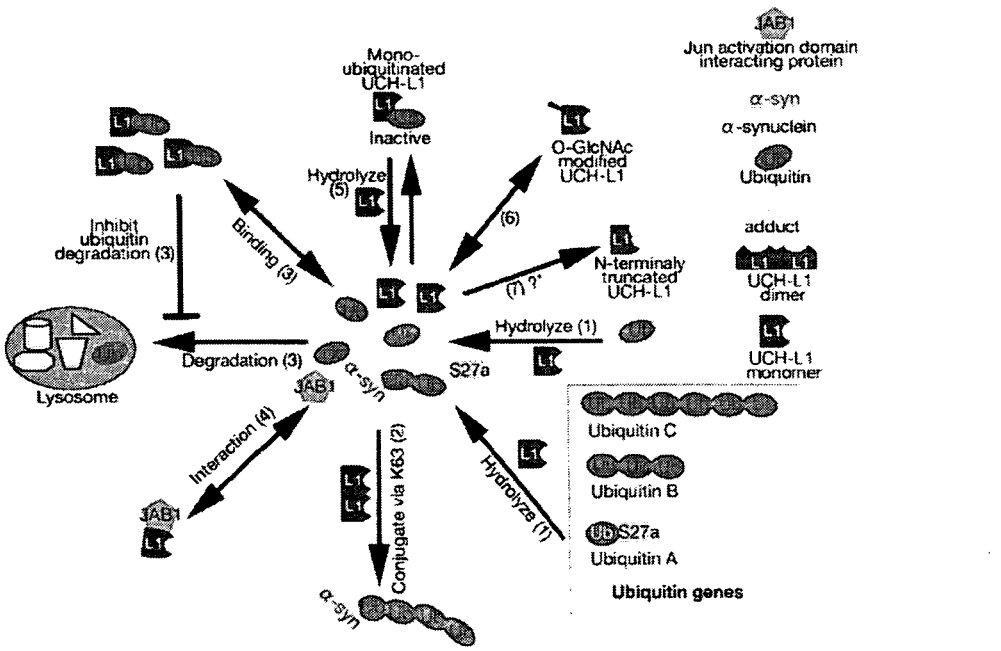


Fig. 1. Proposed functions and regulations of UCH-L1. (1) Under monomeric form, UCH-L1 can hydrolyze bond between Ub and small adduct or unfolded polypeptide *in vitro*. It can also cleave Ub gene products *in vitro*. (2) Under dimeric form, UCH-L1 ligase activity can produce K63 linked Ub chains to its substrate *in vitro*. One of its presumed substrate is di-ubiquitinated α-synuclein. (3) UCH-L1 is bound to mono-Ub *in vivo*. This interaction inhibits the degradation of mono-Ub. (4) UCH-L1 is shown to interact with Jun activation domain binding protein (JAB1). (5) Mono-ubiquitination and inactivation of UCH-L1 can occur reversibly. (6) *O*-GlcNAc-modified UCH-L1 is found in the synaptosome fraction. (7) N-terminally truncated forms of UCH-L1 are also found. *The truncated forms of UCH-L1 might occur as a result of either N-terminal processing of full length UCH-L1 or the translation from the different methionine. (8) The oxidatively modified UCH-L1 is also found in the diseased brains but is not shown in this figure. Please see the text for details.

(Fig. 1). Importantly, this mono-Ub stabilizing effect of UCH-L1 was independent of its enzymatic activity, as the C90S mutant, which lacks enzymatic activity but retains its Ub-interacting ability, still showed a mono-Ub stabilizing effect in cells.

As mentioned earlier, UCH-L1 is a highly expressed protein. Thus, the elucidation of the mechanisms involved in the regulation of UCH-L1 should be an important issue. Recently, a post-translational modification of UCH-L1 that controls the function of UCH-L1 was identified (Meray and Lansbury, 2007). The type of modification is mono-ubiquitination, which may occur reversibly to a lysine residue near the active site (probably K157) of UCH-L1 (Fig. 1). Mono-ubiquitinated UCH-L1, as mimicked by an Ub-UCH-L1 fusion protein, failed to bind mono-Ub and to increase mono-Ub levels in the cell. The enzymatic activity of UCH-L1 may also be inhibited by this modification because it prevents binding to the ubiquitinated targets. In addition, mono-ubiquitinated UCH-L1 was hydrolyzed in intra-molecular manner (Fig. 1). Thus, UCH-L1 might regulate its functional capability by auto-deubiquitination.

In addition to ubiquitination, the existence of a beta-*N*-acetylglucosamine (*O*-GlcNAc)-modified UCH-L1 in the synaptosome fraction of rat brain was reported (Fig. 1) (Cole and Hart, 2001). Moreover, amino-terminally truncated forms of UCH-L1 were found in human brains (Fig. 1), and the levels of this truncated form were shown to be decreased in AD brains but not in PD brains (Choi et al., 2004). Further effort to elucidate the physiological significance of these modifications and their relationship to the pathogenesis of AD and PD should be made.

## 3. *Gad* mice and the physiological function of UCH-L1 in the brain

*Gad* mice exhibit an autosomal recessively inherited disorder caused by an in-frame deletion that includes exons 7 and 8 of *Uchl1*, leading to a lack of UCH-L1 expression (Saigoh et al., 1999). These mice show sensory ataxia at an early stage, followed by motor ataxia at a later stage. Pathologically, the mutant is characterized by 'dying-back'-type axonal degeneration and formation of spheroid bodies in nerve terminals. In addition, *gad* mice show abnormal accumulation of APP, β-amyloid (Ichihara et al., 1995), Ub, and proteasome subunit-positive deposits (Saigoh et al., 1999) in the degenerating neuronal axons. These results clearly indicate that UCH-L1 is essential for the functional maintenance of some subsets of neuronal axons.

On the contrary, most neurons show no signs of degeneration in the brains of *gad* mice. By analyzing these neurons in *gad* mice, we found that a lack of UCH-L1 protects cells from acute stress-induced apoptosis (Harada et al., 2004). In wild-type mouse retina, light stimuli and ischemic retinal injury induced strong Ub expression in the inner retina with an expression pattern similar to that of UCH-L1. On the other hand, *gad* mice showed reduced Ub induction after light stimuli and ischemia, whereas the expression levels of anti-apoptotic and pro-survival proteins were significantly higher. Consistently, ischemia-induced caspase activity and neural cell apoptosis were suppressed to ~70% in *gad* mice. The heat-induced apoptosis of testicular cells was also suppressed in *gad* mice (Kwon et al., 2004). These reports demonstrate that UCH-L1 is involved in the regulation of stress-induced apoptosis, presumably through Ub induction.

## 4. Oxidative modification of UCH-L1 and neurodegeneration

Recently, an increased amount of oxidatively modified UCH-L1 in the brains of AD and PD patients, compared to normal brains, was reported (Castegna et al., 2002; Choi et al., 2004; Butterfield et al., 2006). The oxidative stress may cause such modifications to the protein. At present, several methionine residues and one cysteine residue of UCH-L1 have been reported as possible targets of oxidation; these form methionine sulfoxide and cysteinic acid (Cys–$SO_3H$), respectively, in PD and AD brains. Furthermore, the level of carbonyl-modified UCH-L1, which is also induced by oxidative stress, was found to be increased in PD and AD brains (Choi et al., 2004).

Consistent with the above data, addition of 4-hydroxy-2-nonenal (HNE; one of the carbonyls) induced the HNE modification of recombinant UCH-L1 *in vitro* (Nishikawa et al., 2003). HNE is an endogenous neurotoxin and a candidate mediator of oxidative stress caused by lipid hyperoxidation, known to trigger the cell death of neurons. In addition, proteins modified by HNE at lysine, histidine and/or cysteine residues are accumulated in the nigral neurons and the Lewy bodies of PD patients (Yoritaka et al., 1996; Castellani et al., 2002) and in the neurofibrillary tangles of AD patients (Montine et al., 1997). Cysteine (C90) and histidine (H161) form the active center of UCH-L1 along with asparagine (N176). Thus, the alteration of UCH-L1 activity was presumed to occur as a result of HNE modification. In agreement with this hypothesis, the hydrolase activities of HNE-modified UCH-L1 were reduced to about 40–80% of non-modified UCH-L1, and were inversely correlated with the degree of modification (Nishikawa et al., 2003). Oxidative stress is now recognized as an important factor, which is implicated in the pathogenesis of a number of age-related neurodegenerative diseases including PD and AD (Halliwell, 2006; Lin and Beal, 2006). Thus, the oxidative modification and subsequent decrease in the enzymatic activity of UCH-L1 may affect the function and survival of neurons, leading to the pathogenesis of AD and PD.

## 5. Decreased level of UCH-L1 and AD

As mentioned above, UCH-L1 is often present in the Ub-positive inclusions known as neurofibrillary tangles found in AD (Lowe et al., 1990). A recent report indicated that brains from patients with sporadic AD contain decreased levels of soluble UCH-L1, which is inversely proportional to tangle number (Choi et al., 2004). In addition, *gad* mice show an accumulation of amyloid precursor protein (APP) and β-amyloid, typical proteins accumulated in the inclusions of AD brains (Ichihara et al., 1995). Thus, a reduction in the levels of functional UCH-L1 was speculated to contribute to the

pathogenesis of AD. Recently, a group showed that the introduction of UCH-L1 rescued the synaptic and cognitive function of AD model mice (Gong et al., 2006). They used double Tg mice, over-expressing APP together with presenilin 1 (PS1), as an AD mouse model. At a young age following β-amyloid elevation, these mice showed cognitive defects such as inhibition of long-term potentiation (LTP), a type of synaptic plasticity related to memory. The protein level of UCH-L1 was significantly decreased in the hippocampi of these APP/PS1 Tg mice. Remarkably, synaptic function was restored to normal level when UCH-L1 protein fused to the transduction domain of HIV-transactivator protein (TAT) was transduced to hippocampal slices from APP/PS1 Tg mice. In fact, introduction of TAT-UCH-L1 to APP/PS1 mice, over time, improved their contextual learning. This therapeutic effect may be dependent on the enzymatic activity of UCH-L1 because the C90S mutant did not show any significant effect. These findings clearly demonstrate a link between decreased UCH-L1 function and the pathogenesis of AD. Further analysis may prove UCH-L1 to be a useful therapeutic target for treating AD.

## 6. I93M mutation with gain of toxic function of UCH-L1 and PD

In 1998, a cytosine to guanine (C277G) mutation in the *UCHL1* gene was reported in a German family affected with PD (Leroy et al., 1998). This missense mutation leads to an I93M amino acid substitution in the UCH-L1 protein. In this German family, four out of seven family members were affected with the autosomal dominant form of PD. All of the patients clinically resembled those with sporadic PD. However, there was an unaffected presumed carrier of this mutation in the family. Moreover, gene linkage analysis of *UCHL1* in other PD families failed to discover new families carrying this mutation. Therefore, the link between the I93M mutation in UCH-L1 and the development of PD has been questioned, with the assumption that the C277G alteration in the *UCHL1* gene is a rare polymorphism. To clarify the link between *UCHL1* mutation and PD, a series of experiments, including the *in vitro* biochemical analysis of mutant UCH-L1 and an analysis of Tg mice expressing UCH-L1$^{I93M}$, were performed.

The analysis of recombinant UCH-L1$^{I93M}$ showed a decrease in its deubiquitinating activity to about 55% of the UCH-L1$^{WT}$ activity level, using the model substrate Ub-amino methyl cumarine (AMC) (Table 1) (Leroy et al., 1998; Nishikawa et al., 2003). However, *gad* mice, which bear no activity of UCH-L1, show no signs of dopaminergic cell loss, the typical pathological hallmarks of PD. In addition, heterozygous mice, which are presumed to show half of the activity level seen in wild-type mice, are asymptomatic (Saigoh et al., 1999). Despite the species difference between mice and humans, these results indicate that the molecular mechanism involved in PD cannot simply be explained by decreased enzymatic activity (Saigoh et al., 1999).

We next compared the secondary structures of UCH-L1$^{WT}$ and UCH-L1$^{I93M}$ using recombinant proteins. Circular dichroism analysis showed that the UCH-L1$^{I93M}$ contains a decreased level of α-helix compared with UCH-L1$^{WT}$ (Table 1) (Nishikawa et al., 2003). It is reported that the SH-SY5Y cells expressing UCH-L1$^{I93M}$ form an increased number of UCH-L1-positive aggregates compared with cells expressing UCH-L1$^{WT}$ or UCH-L1$^{C90S}$, an enzymatic activity-defective mutant (Ardley et al., 2004). Thus, the I93M mutation may change the conformation of UCH-L1, leading to altered biochemical properties.

To ascertain if the I93M mutation gives rise to a gain of toxic function *in vivo*, we made a transgenic (Tg) mouse expressing UCH-L1$^{I93M}$ (I93M Tg mouse) and analyzed this mouse to determine if UCH-L1$^{I93M}$ could induce dopaminergic neuron loss. The I93M Tg mice showed several pathological changes related to PD (Setsuie et al., 2007). They showed an age-dependent decline in the number of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the substantia nigra. The striatal dopamine content also decreased in parallel with the decrease in the number of dopaminergic neurons. Although we did not find any signs of Lewy body formation, we found silver staining-positive argyrophilic grains and abnormal electron dense core vesicles, which are also found in the autopsied brains of PD patients. In addition, we found aggregates containing both UCH-L1 and Ub in the perinuclei of dopaminergic neurons in the substantia nigra of I93M Tg mice. Therefore, the gain of toxic function caused by the I93M mutation in UCH-L1 might be the main factor contributing to the pathogenesis of PD.

Table 1
Association between UCH-L1 mutants and PD

| | WT | I93M | S18Y | References |
|---|---|---|---|---|
| Incidence of PD | | ↑ | ↓ | [a] |
| Functional comparison | | | | |
| Hydrolase activity | (100%) | ↓↓ | ↑ | Leroy et al. (1998), Nishikawa et al. (2003) |
| Ligase activity | (100%) | ↓ | ↓↓ | Liu et al. (2002) |
| Mono-Ub binding affinity | + | ND | ND | Osaka et al. (2003) |
| Structural comparison | | | | |
| α-Helix content | (Normal) | ↓ | ± | Nishikawa et al. (2003), Naito et al. (2006) |
| Globularity[b] | + | +++ | ± | Naito et al. (2006) |

[a] For references please see the text.
[b] The spherical shape is indicated as ± and the ellipsoidal degree is indicated by +.

## 7. S18Y polymorphism in UCH-L1 and PD

A polymorphism in UCH-L1 resulting in the amino acid substitution of serine 18 to tyrosine was first reported in 1999 with the possible protective effect against PD (Maraganore et al., 1999). This polymorphism is relatively common in Japanese (allele frequency is 39–54%) and Chinese (~50%) populations, but is rare in European (14–20%) populations (Liu et al., 2002). Further analysis indicated that this inverse association between this polymorphism and PD exists in some populations, such as in Japanese and Chinese but not in others (Maraganore et al., 1999; Mellick and Silburn, 2000; Wintermeyer et al., 2000; Levecque et al., 2001; Wang et al., 2002; Elbaz et al., 2003; Toda et al., 2003; Maraganore et al., 2004; Facheris et al., 2005; Healy et al., 2006; Tan et al., 2006; Carmine Belin et al., 2007). This association was most apparent for younger cases of PD compared with younger controls. In addition, the protection was dependent on the S18Y allele dosage.

A group showed that the Ub ligase activity of UCH-L1, as mentioned above, is responsible for this reduced risk for PD associated with the S18Y polymorphism (Liu et al., 2002). Ub ligase activity of UCH-L1 was shown towards α-synuclein (probably di-ubiquitinated α-synuclein) as a substrate, leading to Ub chain formation (elongation) through lysine 63 of the Ub molecules (Fig. 1). When substrates are poly-ubiquitinated via lysine 63 of Ub, they escape from Ub-proteasomal system (UPS)-dependent protein degradation leading to the stabilization of the substrate. UCH-L1$^{WT}$ tended to form dimers in contrast to UCH-L1$^{S18Y}$, leading to increased ligase activity in UCH-L1$^{WT}$ (Table 1). Thus, the stability of α-synuclein may be enhanced in the presence of UCH-L1$^{WT}$ compared to UCH-L1$^{S18Y}$. This difference may reduce the protein level of α-synuclein and reduce the risk of PD in subjects with the S18Y polymorphism. From these experiments, the authors proposed a mechanism in which the ligase activity of UCH-L1 might affect the morbidity of PD in the brain.

Using small angle neutron scattering (SANS), we compared the structural differences that exist between UCH-L1 variants, wild type, I93M and S18Y in aqueous solution (Naito et al., 2006). SANS is an effective method to analyze detailed protein configuration in solution. Using this method, all of the recombinant UCH-L1 variants formed dimers in water. I93M was more ellipsoidal compared with wild-type protein, and S18Y promoted globularity compared with wild-type protein (Table 1). Thus, the shape of the mutant UCH-L1 in water correlated with the risk of PD. Although further analysis should be performed to determine the significance of UCH-L1 dimerization and the S18Y polymorphism for neurodegeneration, the experiments performed in these two laboratories have provided some clues.

## 8. Concluding remarks and future prospects

UCH-L1 is indicated as a multi-functional protein (Fig. 1) with abundant expression in neurons. In addition, it has become apparent that UCH-L1 may contribute to the pathogenesis of PD and AD. Thus, it is a probable diagnostic and medicinal target of these diseases. However, the mechanism of neurodegeneration induced by I93M mutation and the mechanisms underlying the decreased expression, amino-terminal truncation and increased oxidative modification of UCH-L1 in neurodegenerative diseases have yet to be revealed. In addition, there are several unresolved issues regarding the molecular functions and regulation of UCH-L1. The *in vivo* substrates need to be defined. The ways in which the function and localization of UCH-L1 are regulated are largely unknown. The identification and the analysis of the interacting partners might give us some clues, one of which is Jun activation domain binding protein (JAB1) in H1299 cell, a lung cancer cell line (Fig. 1) (Caballero et al., 2002), though their interaction in the brain is unknown. Recently, a physiological function of an isozyme UCH-L3 was identified in the oxidative stress-induced apoptosis of photoreceptor cells, neurons that reside in the retina (Semenova et al., 2003; Sano et al., 2006). In addition, a reciprocal function of UCH-L1 and UCH-L3 has been proposed in the heat stress-induced apoptosis of testis in mice (Kwon et al., 2004). Thus, the functional diversity between UCH-L1 and UCH-L3 should also be defined. In addition to neurodegeneration, UCH-L1 is thought to be involved in the regulation of ATP receptors in neurons (Manago et al., 2005), in the morphology of neuronal precursors (Sakurai et al., 2006), in the normal function of the testis and the ovary (Kwon et al., 2005; Sekiguchi et al., 2006) and in various human diseases such as cancer (Hibi et al., 1999). Thus, UCH-L1 might contribute to more diverse phenomena than were previously thought.

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# Identification of novel chemical inhibitors for ubiquitin C-terminal hydrolase-L3 by virtual screening

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**Abstract**—UCH-L3 (ubiquitin C-terminal hydrolase-L3) is a de-ubiquitinating enzyme that is a component of the ubiquitin–proteasome system and known to be involved in programmed cell death. A previous study of high-throughput drug screening identified an isatin derivative as a UCH-L3 inhibitor. In this study, we attempted to identify a novel inhibitor with a different structural basis. We performed in silico structure-based drug design (SBDD) using human UCH-L3 crystal structure data (PDB code; 1XD3) and the virtual compound library (ChemBridge CNS-Set), which includes 32,799 chemicals. By a two-step virtual screening method using DOCK software (first screening) and GOLD software (second screening), we identified 10 compounds with GOLD scores of over 60. To address whether these compounds exhibit an inhibitory effect on the de-ubiquitinating activity of UCH-L3, we performed an enzymatic assay using ubiquitin-7-amido-4-methylcoumarin (Ub-AMC) as the substrate. As a result, we identified three compounds with similar basic dihydro-pyrrole skeletons as UCH-L3 inhibitors. These novel compounds may be useful for the research of UCH-L3 function, and in drug development for UCH-L3-associated diseases.


## 1. Introduction

The ubiquitin–proteasome system is responsible for the regulation of cellular proteolysis. In this system, ubiquitination serves as a targeting signal for proteolysis.[1] Ubiquitin C-terminal hydrolase-L3 (UCH-L3) is one of the components of the ubiquitin–proteasome system and hydrolyzes ubiquitin C-terminal adducts for the recycling of cellular ubiquitin.[2] Ubiquitin with C-terminal adducts is a substrate for UCH-L3, and ubiquitin with a free C-terminus is recycled within the ubiquitin–proteasome system. There is some evidence that UCH-L3 plays an important role in programmed cell death. Programmed cell death is implicated in a number of human diseases, including neurodegenerative disease,[3] autoimmune disease,[4] cancers[5,6], etc. Loss of UCH-L3 leads to programmed cell death by apoptosis of certain type of cells in vivo, germ line cells and photoreceptor cells.[7,8] High-level expression of UCH-L3 genes and proteins, and acceleration of UCH-L3 enzymatic activity is reported in multiple types of cancer cells,[5,6] suggesting that UCH-L3 activity may be required for cancer cell survival. Therefore, UCH-L3 is a potential target for drug development to control programmed cell death in specific types of cells including cancer cells.

Structure-based drug design (SBDD) is a method used to discover novel leads for drug development as it enables more rapid hit identification than the classical screening methods of in vitro or in vivo biological assays. The computer-based approach for drug screening, using molecular docking, is a shortcut method when the crystal structure of a target protein is available. Key methodologies for docking small molecules to protein were developed during the early 1980s,[9] and various types of docking simulation software are now available, for example, DOCK,[10] GOLD, and FlexX.[11] BCR-ABL tyrosine kinase inhibitors ($IC_{50}$ values ranging from 10 to 200 μM) were successfully

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identified by virtual screening of 200,000 compounds against crystal structures using DOCK,[12] implemented by the anchor-and-grow algorithm with respect to ligand flexibility.[10] Human thymidine phosphorylase inhibitor ($IC_{50}$ = 77 μM) was also identified by virtual screening of 250,521 compounds using DOCK.[13] Furthermore, metallo-β-lactamase inhibitors ($IC_{50}$ values less than 15 μM) were identified through virtual screening by GOLD,[14] using the genetic algorithm for ligand flexibility.

The advantage of chaining different docking programs was evaluated and the results suggested that virtual ligand screening is performed faster with reasonable accuracy by using chained screening, than by using a single program with default parameters.[15] In this study, the results of chained docking against UCH-L3 crystal structure were examined by UCH-L3 hydrolysis activity assay to validate the efficacy of the DOCK–GOLD SBDD method. We identified three inhibitors ($IC_{50}$ = 100–150 μM) of UCH-L3 by the DOCK–GOLD virtual screening of 32,799 compounds.

## 2. Results and discussion

### 2.1. Protein preparation and chemical database

In the 3D structure of the UCH-L3-ubiquitin complex, ubiquitin C-terminus is buried in the active site cleft among four active site residues of UCH-L3: Gln89, Cys95, His169, and Asp184.[16,17] During the virtual screening process by DOCK and GOLD, the protein–ligand interacting site was restricted to the binding site of the three ubiquitin C-terminal amino residues (as described in Section 4), in order that the outcome could be verified by a ubiquitin C-terminal hydrolase enzymatic assay. The first DOCK screening was performed against 32,799 compounds of CNS-Set, which was prefiltered by RPBS under the most modest filtering condition.[18]

### 2.2. DOCK and GOLD screenings

To screen for compounds that bind to the active site, the first screening was performed by DOCK, and the protein–ligand interaction area was restricted to the ubiquitin binding site of UCH-L3 (see Section 4). The top-scoring 1780 compounds (5.4% of the initial 32,799 compounds) with energy scores of less than −30 kcal/mol were selected for further screening. These compounds were then re-screened by GOLD twice, with different genetic algorithm (GA) settings. To predict binding ability to the active site cleft accurately, the protein–ligand interacting area was defined in approximately the same way as in the first DOCK screening step (see Section 4). Screening by GOLD consisted of two rounds. Using the GOLD score, we initially extracted the top scoring 100 compounds from 1780 compounds, using the 7–8 times speed-up GA parameter settings. These 100 compounds were then re-scored using the default GA settings (see Section 4) to more accurately predict binding ability. Ten compounds with GOLD scores of over 60 were predicted to bind to the UCH-L3 active site; that is, 0.03% of the total number of chemical compounds was screened.

### 2.3. $IC_{50}$ determination

A previous study demonstrated that compounds with GOLD scores of about 60 may inhibit enzyme activity with $IC_{50}$ values of 10–100 μM.[19] An enzyme assay was performed among the top 10 chemicals to address whether they actually bind to the UCH-L3 active site with the predicted affinities (Table 1 and Fig. 1).

Ubiquitin-7-amido-4-methylcoumarin (Ub-AMC; AMC attaches to the carboxyl terminus of ubiquitin) is a fluorogenic substrate of UCH-L3 and other UCH isozymes. UCH-L3 is known to hydrolyze Ub-AMC into free ubiquitin and AMC,[20,21] and the hydrolyzed AMC group is excited at light wavelength of 355 nm and emits fluorescence at 460 nm. Hydrolysis activity of UCH-L3 is inhibited if a compound binds to its active site and thus blocks interaction between the active site of UCH-L3 and the ubiquitin C-terminus. Inhibition of hydrolysis of Ub-AMC leads to a lower concentration of free AMC and hence a lower level of fluorescence intensity.

We experimentally determined the affinity constant ($K_m$) of Ub-AMC hydrolysis by human UCH-L3 as 83.3 ± 1.5 nM (mean ± SEM, from three independent experiments). The candidate compounds identified by

**Table 1.** GOLD scores of the top 10 ranked chemicals after GOLD calculation[a]

| Docking rank/Compound No. | Compound name | GOLD scores |
|---|---|---|
| 1 | 1-Benzyl-3-hydroxy-4-(5-methyl-2-furoyl)-5-(3-pyridinyl)-1,5-dihydro-2*H*-pyrrol-2-one | 66.01 |
| 2 | 3-[4-Methyl-5-({[3-(2-thienyl)-1,2,4-oxadiazol-5-yl]methyl}thio)-4*H*-1,2,4-triazol-3-yl]-1*H*-indole | 65.62 |
| 3 | *N*-{4-[1-(2-Furoyl)-5-(2-furyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl}methanesulfonamide | 64.85 |
| 4 | $N^1$-Cyclopropyl-$N^2$-(4-methoxyphenyl)-$N^2$-[(4-methylphenyl)sulfonyl]glycinamide | 64.76 |
| 5 | *N*-{3-[1-Acetyl-5-(2-thienyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl}ethanesulfonamide | 64.23 |
| 6 | 3-Hydroxy-5-(4-methoxyphenyl)-1-(1,3,4-thiadiazol-2-yl)-4-(2-thienylcarbonyl)-1,5-dihydro-2*H*-pyrrol-2-one | 62.96 |
| 7 | 5-(4-Fluorophenyl)-3-hydroxy-4-(5-methyl-2-furoyl)-1-(3-pyridinylmethyl)-1,5-dihydro-2*H*-pyrrol-2-one | 62.73 |
| 8 | $N^1$-Cyclopropyl-$N^2$-[(4-methoxyphenyl)sulfonyl]-$N^2$-(4-methylphenyl)glycinamide | 62.52 |
| 9 | $N^1$-Cyclopentyl-$N^2$-(3-methoxyphenyl)-$N^2$-(phenylsulfonyl)glycinamide | 62.39 |
| 10 | 4-({[5-(2-Furyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl]thio}methyl)-1,3-thiazol-2-amine | 62.35 |

[a] Ten compounds are listed according to the top 10 rank of GOLD scores and assigned the number corresponding to GOLD score ranks.

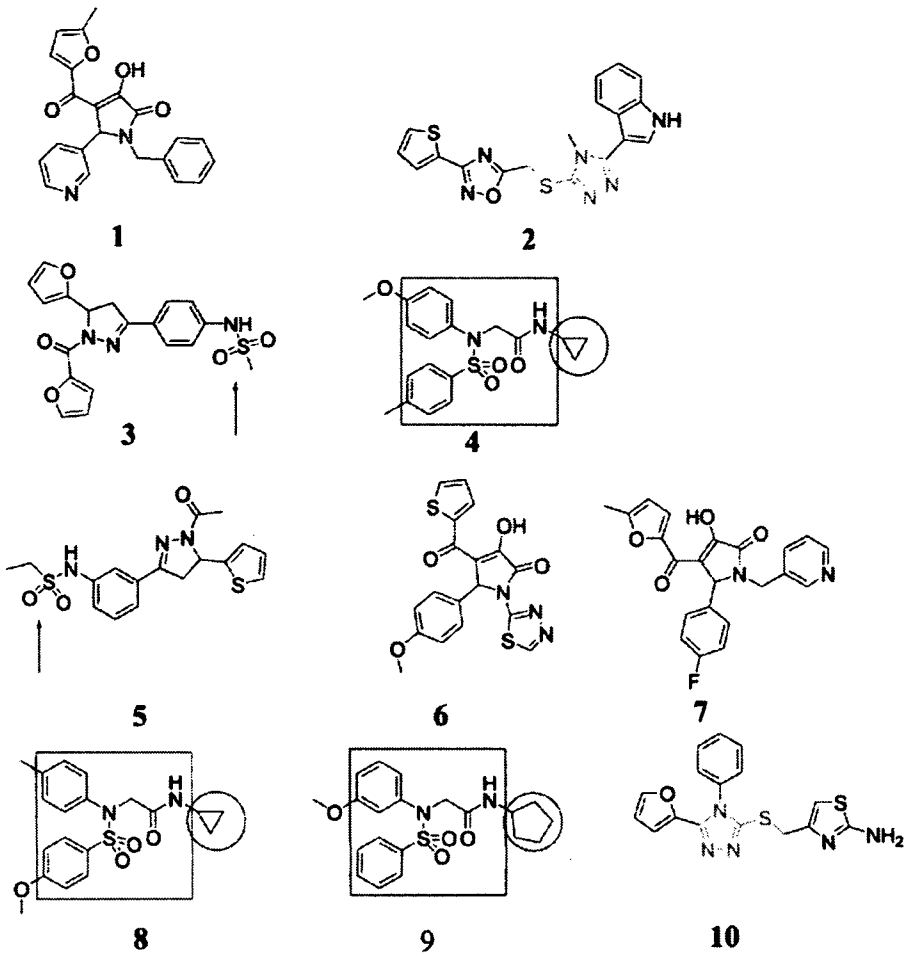


**Figure 1.** Top 10 ranked compounds identified by DOCK and GOLD screening. Note that there are several shared basic skeletons and functional groups: 1,5-dihydro-2*H*-pyrrol-2-one (drawn in red, compounds **1**, **6**, and **7**), glycinamide (boxed in red, compounds **4**, **8**, and **9**), cycloalkane group (circled in red, compounds **4** and **8**; cyclopropyl, compound **9**; cyclopentyl), 4,5-dihydro-1*H*-pyrazol-3-yl phenyl (drawn in blue, compounds **3** and **5**), sulfonamide (pointed, compounds **3** and **5**), and 4*H*-1,2,4-triazol-3-yl (drawn in green, compounds **2** and **10**).

DOCK–GOLD chained docking screening were tested for their ability to inhibit the hydrolysis activity of UCH-L3, at the Ub-AMC concentration equivalent to the $K_m$ value. Four compounds among these candidates inhibited enzyme activity (Fig. 2a). We did not test the inhibitory effects of compound **3**, as it is a fluorogenic chemical with an emission wavelength of 460 nm. Compounds **1**, **6**, and **7** significantly inhibited the hydrolysis activity of UCH-L3 (initial velocity of Ub-AMC hydrolysis; nM/s [Fig. 2b]). Compounds **1** (401 μM), **6** (375 μM), and **7** (350 μM) inhibited the hydrolysis activity by 83.2 ± 1.5%, 76.5 ± 0.6%, and 76.8 ± 1.0%, respectively, as compared with control DMSO ($p < 0.01$, vs control; Dunnett's test). The $IC_{50}$ value of compound **2** should hypothetically be several hundred μM. Although compound **2** (380 μM) inhibited hydrolysis activity by 16.2 ± 2.1% as compared with control DMSO, the difference was not found to be significant by Dunnett's test. Five other compounds were unable to inhibit the UCH-L3 hydrolysis activity: compound **4** (334 μM; final concentration), compound **5** (331 μM), compound **8** (401 μM), compound **9** (386 μM), and compound **10** (387 μM) (Fig. 2b). Experimentally determined $IC_{50}$ values of compounds **1**, **6**, and **7** (Fig. 3) were as follows: compound **1** (103 μM), compound **6** (154 μM), and compound **7** (123 μM).

### 2.4. Competitive inhibitor

To show that the identified compounds bind to the active site of the UCH-L3, various concentrations of compound **1** and iodoacetamide (108 mM) were added to UCH-L3/Ub-AMC reaction buffer. Iodoacetamide is a non-competitive inhibitor of UCH-L3 (Fig. 4a). It is a thiol alkylating agent of the UCH-L family and derivatizes and inactivates the active site leading to loss of UCH-L3 enzymatic activity.[22] In the presence of compound **1** and iodoacetamide, the percentage of active UCH-L3 reduced by iodoacetamide treatment was recovered in comparison with the control, and the recovery was dependent on the concentration of compound **1** (Fig. 4b). Our results showed that compound **1** is a competitive inhibitor of UCH-L3. This suggests that compound **1** bound to the UCH-L3 active site to prevent iodoacetamide from inactivating it.

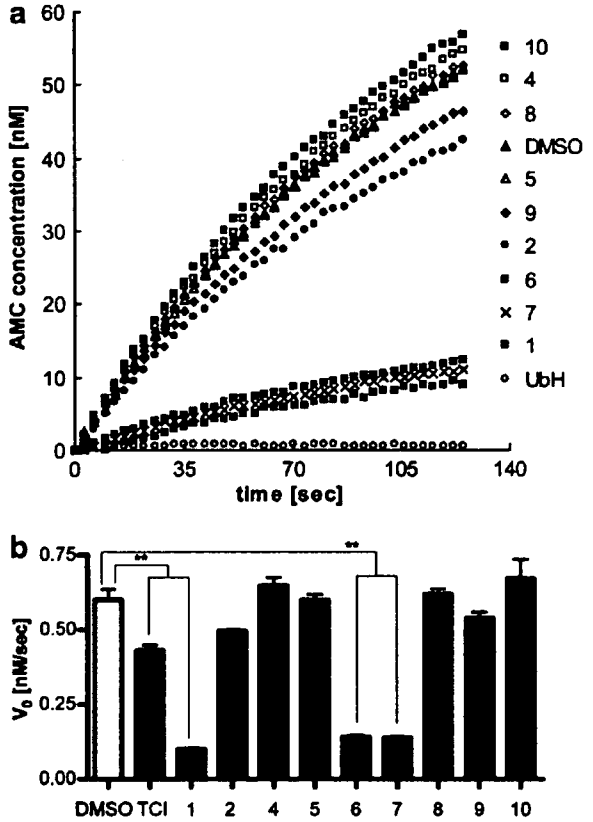


**Figure 2.** Analysis of UCH-L3 inhibitory effects of compounds **1**–**10**. (a) Kinetics of UCH-L3-catalyzed hydrolysis of Ub-AMC with the compounds. Fluorescence intensity was converted to AMC concentration by subtracting the intensity of fully hydrolyzed substrate from that of solution without substrate. Concentrations of compounds are as follows: compound **1** (401 μM); compound **2** (380 μM); compound **4** (334 μM); compound **5** (331 μM); compound **6** (375 μM); compound **7** (350 μM); compound **8** (401 μM); compound **9** (386 μM); and compound **10** (387 μM). As a known inhibitor, ubiquitin-aldehyde (Ub-H, 120 nM) was used. Each value represents the mean of three independent experiments. (b) Inhibitory effects of compounds on initial velocity of hydrolysis ($V_0$) are shown. Fluorescence intensity was converted by the same method described in (a). 4,5,6,7-Tetrachloroindan-1,3-dione (TCI, 20 μM) was used as a UCH-L3 selective inhibitor with $IC_{50}$ of 600 nM.[22] Each value represents the mean ± SEM of three independent experiments. Dunnett's multiple comparison test was performed using GraphPad Prism software (**: $p < 0.01$, DMSO as control).[29]

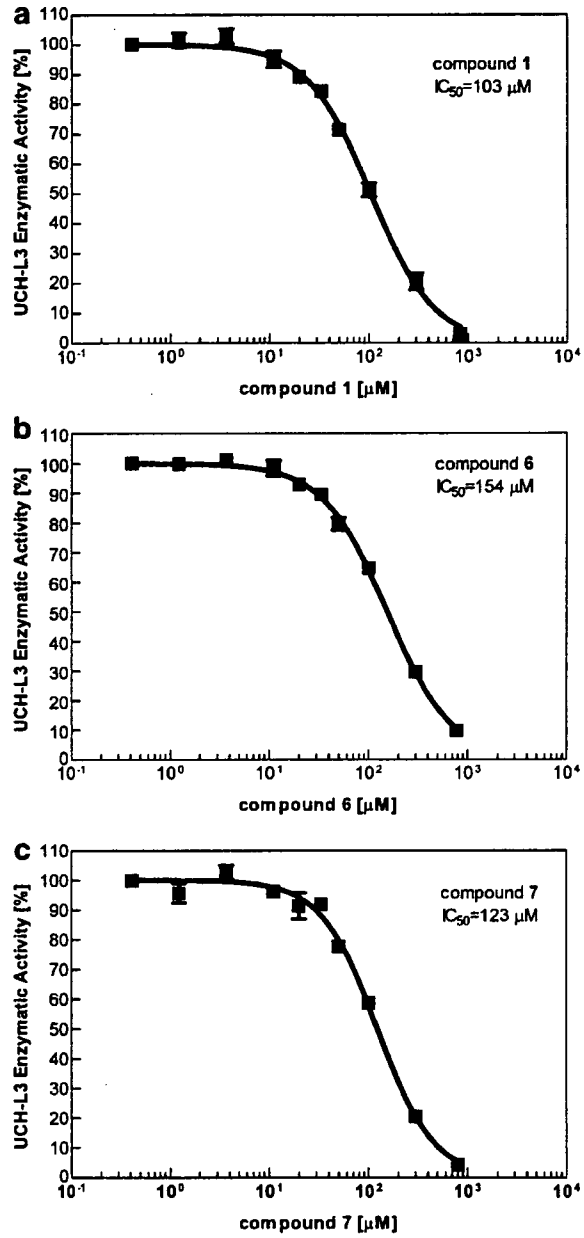


**Figure 3.** $IC_{50}$ curves of compounds for UCH-L3 enzymatic activity. (a) Compound **1**, (b) compound **6**, and (c) compound **7**. The horizontal axis shows the concentration of each compound. The vertical axis shows the relative UCH-L3 enzymatic activity [%] in comparison with maximal initial velocity. $IC_{50}$ values are shown in graphs. Each plotted value represents the mean ± SEM of three independent experiments.

In order to show that the compounds **1**, **6**, and **7** bind to UCH-L3, Biacore 100 analysis was conducted. Biacore 100 analysis detects interaction between a small molecule and protein and enables quantification of the interaction.[23] The results showed that binding of each compound to UCH-L3 increased and was dependent on the concentration of the compound **6** (data not shown).

### 2.5. Predicted binding mode

Figure 5 shows the predicted binding modes of compounds **1**, **6**, and **7** to UCH-L3. Since chemical formulae of the three compounds are similar to each other, the predicted docked structures of these and UCH-L3 have similar binding modes. Two hydrogen bonds were observed between the docked ligand and two amino acid residues in the predicted compound **1**/UCH-L3 complex structure; the carbonyl group of compound **1** appears to form a hydrogen bond to the NH group of Ala11, and the pyrrole C=O appears to form a hydrogen bond to the hydroxyl group of Thr157. Three hydrogen bonds were predicted between the docked ligand and two amino acid residues in the compound **6**/UCH-L3 complex structure; the thiadiazole group of compound **6** appears to form a hydrogen bond to the NH group of Leu9, and