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COMMUNICATION

GGCT fluorogenic probe: Design, synthesis and application to cancer-related cells

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Cancer-related γ-glutamyl cyclotransferase (GGCT) specifically converts γ-glutamyl amino acids (γ-Glu-Xaa) into pyroglutamate and the corresponding amino acids (Xaa). Here we report a novel GGCT fluorogenic probe "LISA-101" containing a masked *O*-acylated fluorophore "resorufin" on the side chain of the P_1' amino acid (Xaa). Upon GGCT treatment, the P_1' amino acid was liberated and spontaneously released the intact fluorophore. Thus, the fluorescence was regained. LISA-101 will expand the strategies for cancer studies.

Introduction

We have identified human chromosome 7 ORF 24 (C7orf24) as a tumor-related protein.[1] C7orf24 is involved in regulation of the glutathione homeostasis cycle as a γ-glutamyl cyclotransferase (GGCT).[2] GGCT specifically converts γ-glutamyl amino acids (γ-Glu-Xaa) into pyroglutamate (pyroGlu) and the corresponding amino acids (Xaa) as shown in Scheme 1A. By contrast, GGCT does not recognize γ-glutamyl peptides. Although its definite role remains unclear, GGCT has been found to be over-expressed in a range of cancers[3] and silencing of the gene by small interfering RNA (siRNA) showed an antiproliferative effect on cancer cell lines.[1,4] Thus, it is believed that GGCT accelerates cancer progression and its inhibitor should function as an anti-cancer drug. Moreover, because the glutathione cycle is a critical system for life phenomena, GGCT has recently attracted the interests of researchers in areas of not only cancer[5] but also neurogenesis[6] and botany.[7] Especially, it is interesting that systemic administration of siRNA of GGCT could retard the tumor growth and induce necrosis of tumor tissue while showing no obvious toxicity to normal tissues. Thus, GGCT is a promising target of cancer treatments.

To gain further understanding of GGCT, we designed the first-in-class fluorogenic probe "LISA-4" to realize the concept of the "CHELA (CHemical and Enzymatic Ligand Activation) system" in 2013 (Scheme 1B).[8] Prior to this, the singular substrate preference of the enzyme had long hampered its chemical probe development. Namely, the presence of an α-carboxyl group at the P_1' site is essential for the enzyme-substrate interaction. Thus, none of the conventional chromogenic/fluorogenic probes including γ-Glu-pNA could be processed by GGCT. Our strategy to develop a fluorogenic probe is as follows: a) a canonical substrate-mimicking non-fluorescence dipeptide "γ-Glu-Xaa", where Xaa possesses a masked *O*-acylated fluorophore on its side chain, is used; b) GGCT-mediated release of Xaa from the dipeptide probe causes its intramolecular chemical cyclization reaction to liberate the inherent fluorophore upon breaking the *O*-acyl structure linking with the fluorophore; c) consequently, the fluorescence can be regained (Scheme 1C). For example, the fluorogenic probe "LISA-4" [γ-Glu-Ser(CO-methylumbelliferone)] was effectively processed by GGCT, and the subsequent chemical cyclization reaction immediately liberated the intact fluorophore "methylumbelliferone". As a result, LISA-4 enabled visualization of GGCT activity for the first time. The carbonate bond in this molecule, however, was not sufficiently stable under neutral conditions, and this instability hampered its application to cell-based assays. Here we report the design and synthesis of a stable GGCT fluorogenic probe, LISA-101, containing an *N*-ethyl urethane bond. This allows the probe to be successfully applied to cell-based assays.

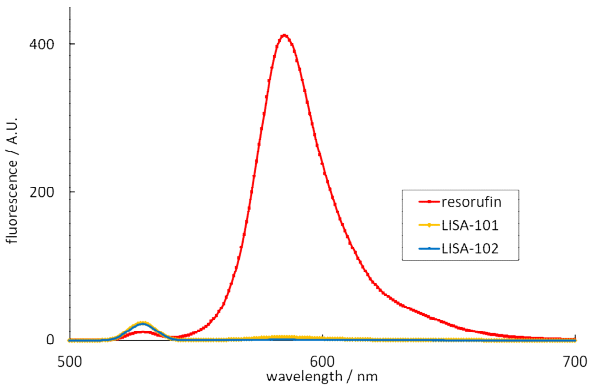


Fig. 1 Fluorescence spectra of resorufin, LISA-101 and LISA-102 (excitation at 530 nm).

Results and Discussion

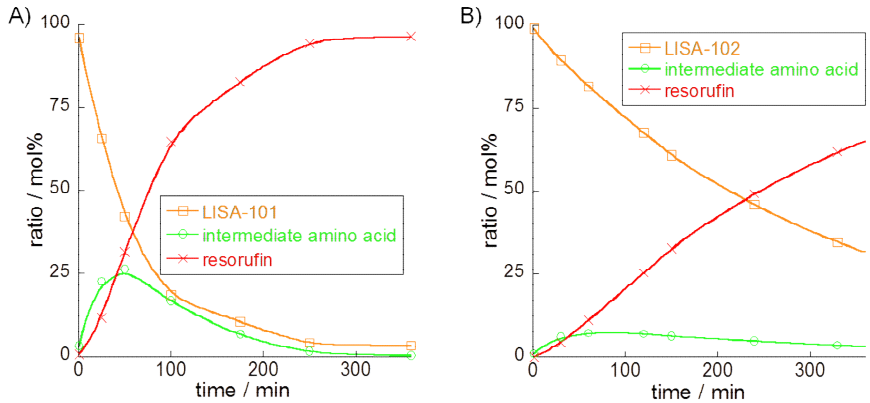
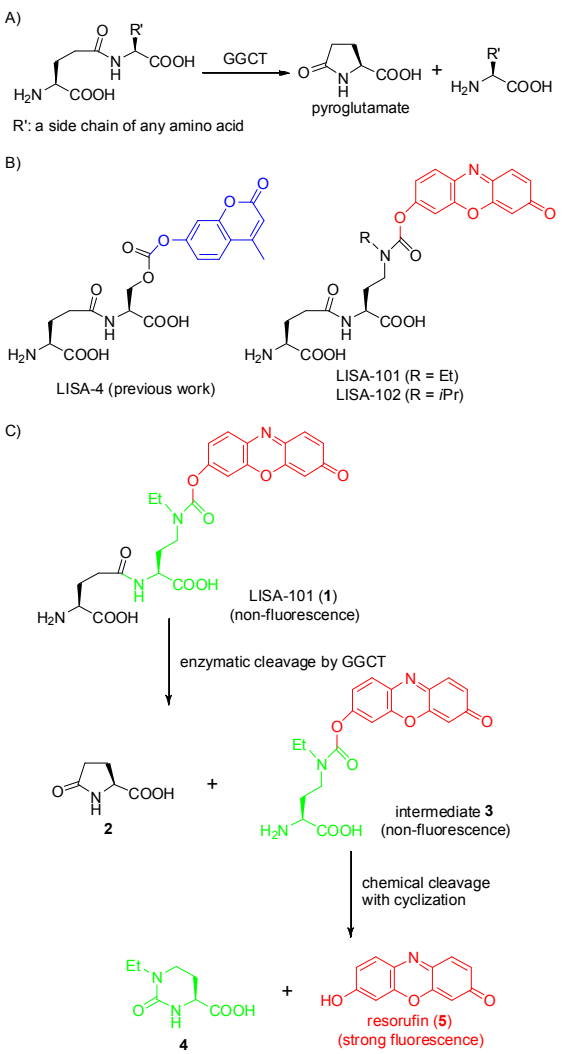
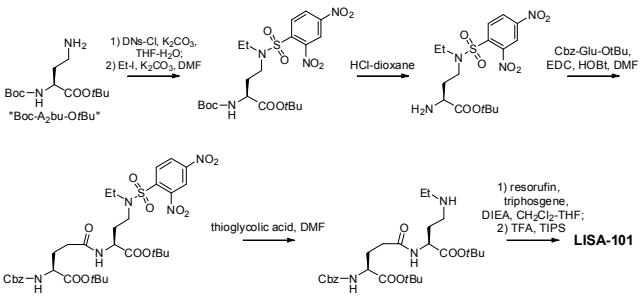


Fig. 2 LISA compounds were reacted with GGCT in pH 8 tris buffer at room temperature, and the reaction was monitored by HPLC. [A) LISA-101 and B) LISA-102]. Resorufin was not released in the absence of GGCT (Closed circles in A).



Scheme 1 A) Model scheme of GGCT reaction. B) Structures of LISA compounds. C) Mechanism of fluorogenic GGCT probes, illustrated using LISA-101.

We designed a stable GGCT probe by modifying the structure of LISA-4. The connection of the fluorophore with the side chain of Xaa was replaced by an *N*-alkyl urethane structure, which is known to be more stable than the carbonate bond used in LISA-4.



Scheme 2 Synthesis of LISA-101. LISA-102 was synthesized in a similar manner.

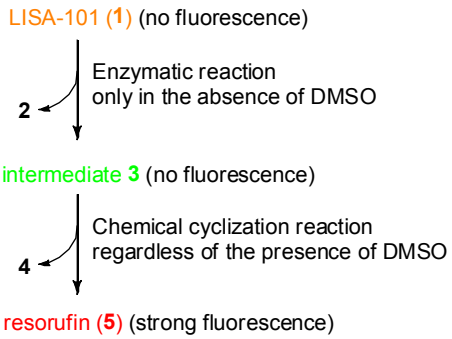
On the assumption of applying the probe to cell-based imaging, the fluorophore was changed to resorufin (emission λ_{max}: 590 nm) which has a longer fluorescence wavelength than the methylumbelliferone (emission λ_{max}: 445 nm) used in LISA-4. Consequently, we designed two probes, LISA-101 [γ-Glu-A_2bu(N^{γ}-Et-N^{γ}-CO-resorufin)] and LISA-102 [γ-Glu-A_2bu(N^{γ}-*i*Pr-N^{γ}-CO-resorufin)]. Both LISA-101 and LISA-102 were readily synthesized (Scheme 2). As expected, both compounds were sufficiently stable under neutral conditions. Decomposition of LISA-101/102 in pH 8 buffers[9] was under the detection limit (< 0.1 mol%) over 24 h at room temperature. We also confirmed that LISA-101/102 has virtually no fluorescence compared with the strong fluorescence of resorufin itself (Fig. 1). That is, the fluorescence intensity of resorufin was more than hundredfold that of LISA-101/102 (excitation at 530 nm). The digestion progress of these probes with recombinant GGCT in the buffer (pH 8) was monitored by RP-HPLC and fluorometry. While both probes were processed by GGCT to liberate the fluorophore, LISA-101 was found to be a better substrate for GGCT because the fluorophore could be released faster from LISA-101 than from LISA-102 (Fig. 2). This difference might be attributed to the bulkiness of the *N*-alkyl structure. Additionally, non-enzymatic release of resorufin was not obserbed from LISA-101 under this condition. In view of this, we employed LISA-101 for additional examinations.

In the enzyme reaction with LISA-101, there was a time lag between the decrease of the probe and the increase of resorufin in the RP-HPLC detection. Thus, fluorescence intensity measurement with a fluorometer could not be used to interpret the enzyme reaction at that moment. This was due to the slow

Table 1 Half-lives of cyclization reaction of the intermediate amino acid **3** to afford intact fluorophre **5**.[a]

buffer	hepes	tris	phosphate	tris	tris[b]
DMSO (%)	0	0	0	50	50
half-life (min)	33	24	27	25	8.3

[a] The intermediate amino acid **3** derived from LISA-101 was synthesized and dissolved in pH 8.0 buffered solutions at room temperature. Cyclization reaction (*i.e.* release of resorufin) was monitored using HPLC. [b] Reaction at 37 °C.



Scheme 3 GGCT reaction of LISA-101 from the viewpoint of fluorescence. An intermediate amino acid is a product of GGCT treatment, but does not possess fluorescence. Thus, the intermediate amino acid should be converted to intact resorufin after addition of DMSO, under which condition the enzyme reaction has been stopped.

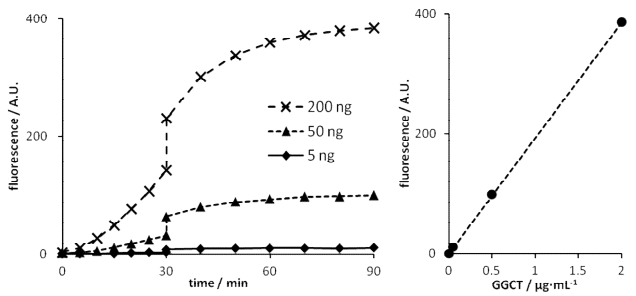


Fig. 3 GGCT reaction in pH 8 tris buffer at 37 °C was monitored by fluorometry with LISA-101. A) The enzyme reaction was stopped at 30 min by addition of DMSO, and the conversion of the intermediate amino acid was completed by ca. 60 min. (Fluorescence intensity was raised by the addition of DMSO.) B) Using the fluorescence intensity at 90 min, GGCT could be quantified. Dashed line is a regression line ($R^2 > 0.99$).

conversion of the non-fluorescence intermediate **3** to the intact fluorophore **5** in the assay medium. In fact, a significant amount of intermediate **3** was detected in the course of enzyme digestion by HPLC. This hampered direct monitoring of the enzyme reaction with the guidance of fluorescence spectroscopy because the enzyme digest, *i.e.* intermediate **3**, has no fluorescence due to its *O*-acylated linkage. To quantify the enzyme activity precisely by measuring fluorescence intensity, intermediate **3** has to be completely converted to the cyclic compound **4** and resorufin **5** via the cyclization reaction. In general, cessation of the enzymatic reaction is achieved by temperature elevation, pH change or denaturant addition. Taking into account the stability of the undigested probe **1** and the conversion propensity of intermediate **3** in the media after terminating the enzyme activity, we tried adding DMSO to the reaction mixture to stop the enzymatic reaction but not prevent the subsequent chemical reaction (Scheme 3). We examined the conversion efficiency of

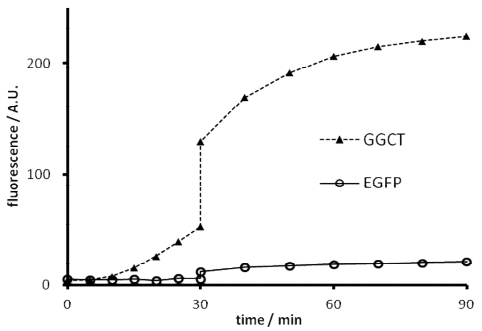


Fig. 4 LISA-101 was applied to the cell-based assay in pH 8 tris buffer at 37 °C with lysates of NHDF cells, which stably expressing GGCT or EGFP. EGFP-expressing cell lysate was prepared as a negative control.

intermediate **3**, which had been synthesized separately, under neutral conditions with or without organic solvent. The results are summarized in Table 1. In the buffer at pH 8, regardless of its salt, intermediate **3** spontaneously cyclized and simultaneously released resorufin with half-lives of around 30 min at room temperature. The conversion rate was not affected even when DMSO was added to the buffer. Raising the temperature of the buffer containing 50% DMSO to 37 ºC accelerated the conversion rate, leading to completion of the conversion within 1 h. On the other hand, we confirmed that GGCT was deactivated and LISA-101 itself remained unchanged in this buffer containing 50% DMSO (Fig. S2). From these observations, we concluded that the addition of DMSO to the GGCT reaction mixture stops only the enzyme reaction, and after an additional 1 h, GGCT activity can be quantified from the fluorescence intensity.

According to the protocols thus established, we applied LISA-101 for further GGCT assays (Fig. 3A). First, we validated that GGCT activity can be quantitatively monitored using a fluorometer. After LISA-101 had been treated with GGCT at 37 °C for 30 min, addition of DMSO to stop the enzyme activity uninterruptedly promoted the chemical cyclization reaction to increase the fluorescence. An hour later at 37 ºC, the fluorescence intensity reached a plateau, suggesting that the cyclization reaction was completed. By measuring the fluorescence at this point, we could quantify GGCT activity. The fluorescence intensities observed at these points were in proportion to the respective amounts of GGCT used (Fig. 3B). Next, we confirmed the applicability of LISA-101 for cell-based assays using the NHDF cell line, which is known to natively express GGCT in a small quantity (Fig. 4). We compared two transgenic cell lines: GGCT-expressing line and EGFP-expressing line (negative control). The fluorescence increased when the lysate of GGCT-expressing NHDF cells was applied, while a limited rise was detected when that of the negative control cells was applied. These results suggested that LISA-101 is a specific probe for GGCT applicable for cell-based assays.

Finally, we compared the GGCT activities of MCF-7 and NHDF cell lines. MCF-7 was adopted as a representation of human cancer cell, and NHDF was adopted as a representation of human normal cell. GGCT activities of cell lysates were compared using LISA-101, and we confirmed that MCF-7 showed 100-times higher activity than NHDF. This result supports that knock down of GGCT have an efficacious effect against a drug-resistant MCF-7 breast cancer without affecting normal tissues.[5b]

Conclusions

We designed and synthesized LISA-101 as a stable GGCT fluorogenic probe. LISA-101 could be readily prepared and did not possess fluorescence. When LISA-101 was treated with GGCT, resorufin was released, and the fluorescence was regained. Using the DMSO-stopped method, GGCT activity can be quantified by fluorescence intensity. Moreover, LISA-101 could be applied to cell-based assay using a plate reader. Much more GGCT activity could be detected in the cancer-related cell compared with a normal cell line. These results suggested that LISA-101 should be useful for GGCT-related cancer studies including those for GGCT inhibitor development. Analyses of GGCT activity in plasma from patients with several kinds of cancers are now in progress.

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Notes and references

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† Electronic Supplementary Information (ESI) available.

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9 In this paper, every assays were conducted at pH 8, which is in the optimum pH range of GGCT.[2b]