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Discovery of the First Environment-Sensitive Fluorescent Probe for GPR120 (FFA4) Imaging

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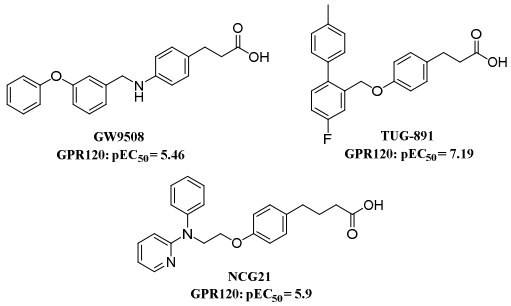
KEYWORDS. GPR120, fluorescent probes, cell imaging, subcellular localization.

ABSTRACT:GPR120, which is activated by long-chain free acids (FFAs), has been recognized as a new attractive target for the treatment of type 2 diabetes and metabolic disease. The visualization and location of GPR120 in native cells can provide powerful information for guiding the physiological and pathological studies of GPR120. We report herein the first potent fluorescent probes that sensitively detect GPR120. We designed and synthesized a series of novel environment-sensitive probes with suitable fluorescence property, high biological activity on the GPR120, and acceptable cytotoxicity. These fluorescent probes targeting GPR120 are expected to expand the toolkit for further studies on GPR120.

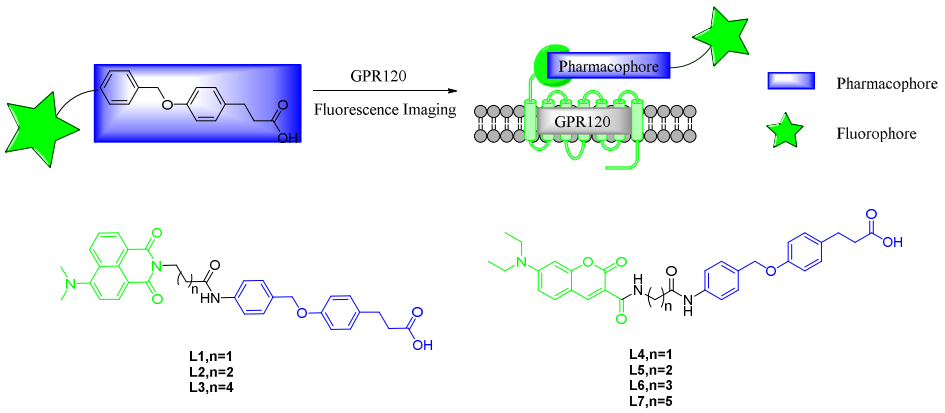
G protein-coupled receptor 120 (GPR120), as the prominent members of G protein-coupled receptors (GPCRs), is abundantly expressed in various types of organs, tissues, and cells, which mediate GLP-1 secretion, insulin sensitization, anti-inflammatory, and anti-obesity effects.[1,2] It should be noted that GLP-1, a potent incretin hormone, has been reported to play a pivotal role in insulin sensitivity, appetite, and gastric emptying.[3] Thus, GPR120 activated by long-chain free fatty acids (FFAs) has been recognized as a new potential target for treatment of type 2 diabetes and obesity.[4,5] So far, a series of potent agonists have been discovered for the treatment and diagnosis of GPR120-related diseases, such as GW9508, TUG-891, and NCG21 (Scheme 1).[6] GW9508, a small-molecule agonist of the fatty acid receptors GPR40, also possesses moderate GPR120 agonist activity.[7] TUG-891 was recently discovered as a potent and selective agonist for GPR120.[8] NCG21, derived from PPARγ ligands, turned out to be a potent and selective GPR120 agonist.[9] We have previously reported a series of excellent agonists for GPR120 based on pharmacophore modeling and virtual screening.[10] However, we still face many challenges on investigating the physiological and pharmacological functions of GPR120. The similarity between GW9508 and TUG-891 sparkled our interest in exploring fluorescent probes to detect GPR120. Here we report the first potent environment-sensitive fluorescent probe on GPR120.

During the past decade, fluorescent GPCR ligands have been widely applied to localize the distribution of receptors and enable real-time monitoring of processes triggered by ligand-receptor interactions, such as internalization, trafficking, sequestration, and recycling.[11,12] Environment-sensitive fluorescent ligands are very sensitive to the change in the physicochemical properties of the surrounding environment, which exhibit weak fluorescence in aqueous solution but emit bright fluorescence in low polarity and high viscosity solvents or when bound to hydrophobic domains in cells (Scheme 2).[13-15] Regarding GPR120, there has been thus far no report on the detection of GPR120 on the cell surface with small molecule fluorescent probes. Given that small molecule fluorescent probes exhibit high sensitivity, selectivity, visualization, and fast response, there is an urgent need for developing a convenient fluorescent ligand toolbox to trace GPR120 for understanding the physiological and pathological studies.

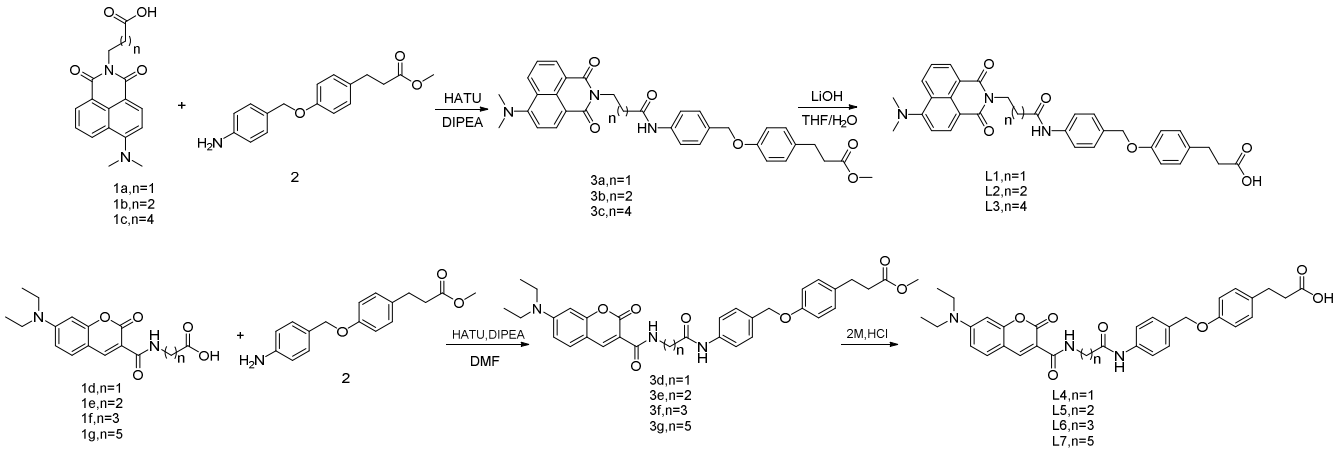


Scheme 1. Current GPR120 agonists.

In general, a small-molecule fluorescent probe for a specific biotarget contains two parts: a fluorophore group that can tag the target with fluorescent properties, as well as a pharmacophore moiety that can recognize the biotarget through the receptor-ligand interaction.[16] The essential components of TUG-891 and GW9508 were chosen as the pharmacophore moiety for its high potency to GPR120. In addition, naphthalimide and coumarin groups are selected as fluorophore group due to their favorable fluorescent properties, which were linked to the pharmacophore moiety by an aliphatic spacer. Subsequently, a series of fluorescent probes were well designed and synthesized as small molecule fluorescent probes for GPR120 (Scheme 2).



Scheme 2. The strategy of small-molecule fluorescent probes for GPR120.



Scheme 3. The synthetic schemes of fluorescent probes.

The two synthetic routes of fluorescent probes were shown in Scheme 3. Briefly, coupling the pharmacophore moiety with fluorophore group under HATU/DIPEA condition yielded our key ester intermediates. All final probes were obtained via ester hydrolysis. Further synthesis details can be found in the Supporting Information.

Table 1. Photophysical parameters of synthesized probes.

Compd	λ_{max}/nm	λ_{ex}/nm	λ_{em}/nm	Φ^a/%		
				PBS	EA	DCM
L1	430	415	535	4.0	10.8	32.4
L2	435	435	545	1.1	147.2	39.0
L3	430	415	535	2.6	83.9	69.2
L4	424	415	490	20.1	159.0	244.7
L5	429	415	470	11.6	174.5	275.0
L6	434	435	475	6.7	162.5	252.6
L7	430	420	475	8.6	192.6	316.1

a Fluorescence quantum yields of these probes were calculated in reference to fluorescein in 0.1 M NaOH as a standard (Φ = 0.92).

The absorption and fluorescence spectroscopy of these probes were measured in methanol, ethyl acetate, dichloromethane and PBS buffer with Thermo Varioskan microplate reader (Figures S1-3). In addition, the fluorescence quantum yields of these probes were measured in a different solvent of ethyl acetate, dichloromethane, and PBS buffer. The influence of solvents on fluorescence quantum yield and fluorescence spectroscopy was evident (Table1). The results showed that their fluorescence emissions were quenched in methanol and PBS buffer, and the quantum yields in PBS are low. In particular, when the polarity of a solvent decreases, fluorescence intensity increases significantly. It indicated that the bright fluorescence is released when these probes bind to GPR120 active site as the hydrophobic domain with low polarity. Herein, we discover these compounds could serve as environment-sensitive fluorescent probes for GPR120.

Table 2. Cytotoxicity results of synthesized probes.

Compd	IC_{50} (μM)		
	HEK293	HT-29	STC-1
L1	>100	>100	>100
L2	>100	>100	93±3
L3	>100	>100	50±0.7
L4	49±3	92±2	55±0.7
L5	41±4	28±5	75±1
L6	>100	>100	>100
L7	>100	>100	>100

It is important that the reasonable probes should possess low cytotoxicity while labeling the GPR120. Additionally, the cytotoxicity of these probes was evaluated by an MTT cytotoxicity assay with STC-1, HT-29 and HEK-293 cells. As shown in Table 2, all compounds had acceptable cytotoxicity that

were biocompatiable to cells. Overall, our results demonstrated that these fluorescent probes have acceptable cell toxicity for use in labeling and imaging of GPR120 in living cells at the nanomolar concentration.

These fluorescent probes were evaluated on GPR120 transfected HEK293 cells by β-arrestin 2 interaction bioluminescence resonance energy transfer (BRET) assay.[17] In this assay, TUG-891 was chosen as the positive control. The results revealed that these probes have a high activity for the GPR120 and are close to the positive control (Table3). When the fluorophore group is changed from naphthalimide to coumarin, the activity to GPR120 has no obvious effect. In all fluorescent probes, probe L3 with a linker of six carbon atoms showed the best activity, while the other probes, which have the spacer shorter than six carbon atoms, displayed lower and similar activity to GPR120. It indicated that the probes with a longer carbon chain as the linker would possess better activity.

Table 3. Bioactivity of synthesized probes onGPR120.

Compd	GPR120 (BRET)[a] pEC_{50} (% E_{max})[b]
TUG-891	7.17±0.02 (100)
L1	5.89±0.03 (110)
L2	5.90±0.02 (98)
L3	6.62±0.05 (108)
L4	5.65±0.01 (111)
L5	5.88±0.02 (115)
L6	6.01±0.06 (106)
L7	5.84±0.04 (135)

[a] GPR120 (BRET) pEC_{50} determined using β-arrestin 2 interaction bioluminescence resonance energy transfer (BRET) assay. [b]E_{max} expressed as % of TUG-891.

The fatty acid receptors GPR120 (FFA4) and GPR40 (FFA1), which bind the same broad group of fatty acids, have very similar pharmacological properties. The GPR120 agonists described hitherto have poor or no selectivity overGPR40 receptor. Therefore, further investigation has been done to detect whether the potent compound L3 has the selectivity. As a result, compound L3 has a weak activity for the GPR40 in this calcium assay and the $[Ca^{2+}]_i$ response is low (Figure S4). We next examined the medium chain length fatty acid receptor GPR84 and the short chain length fatty acid receptor GPR43, which would be standard for an new chemical series targrting a member of this receptor family. In brief, the result revealed that the compound L3 also has a weak $[Ca^{2+}]_i$ response for the GPR84 and the GPR43. Moreover, the pEC_{50} value of probe L3 for GPR120 was 6.72±0.18, which is close to that of TUG-891 (pEC_{50}=7.26±0.04) in this calcium assay (Figure S4). These probes displayed the similar molecular mechanism to the TUG-891 at GPR120 across various assay endpoints, including β-arrestin 2 recruitment and stimulation of Ca^{2+} mobilization. In conclusion, compound L3 is a potent and selectiveGPR120 agonist.

Overall, appropriate fluorescent properties, high bioactivity to GPR120 and acceptable cell toxicity were observed, and thus, laid a solid foundation for the following fluorescence imaging in living cells. Thus, cell imaging potential was further evaluated. So far, few reports are available on GPR120 subcellular localization in native cells. Our preliminary study with fluorescent probes indicated the varying distribution of GPR120 in different cells. In subcellular localization, the GPR120 was reported to be localized on the cell surface using HEK293 cells stably (and also transiently) expressing the GPR120-GFP fusion protein.[5] In order to extend the application of fluorescent probes, HEK293 cells were firstly chosen for fluorescence imaging. The imaging results showed that its fluorescence mainly distributed on the cell surface (Figure1A). Furthermore, it is a known fact that GPR120 are highly expressed in HT-29 and STC-1 cells.[18,19] After successful staining of GPR120 with fluorescent probes in HEK293 cells, we tested these probes on HT-29 and STC-1 cells that endogenously express GPR120. In the case of HT-29 cells, we found the strong fluorescence aggregation mainly distributed on the cell surface, as well as slight decoration on plasma membrane (Figure1B). These probes exhibited the distribution of fluorescence, in agreement with those reported previously using a GPR120-specific antibody.[20] An observation that is further supported by fluorescent probes label GPR120 in STC-1 cells that endogenously express GPR120. As shown in Figure 1C, its fluorescence was not merely partitioning in the cell membrane and are highly consistent with Thompson's conclusion using ^{14}C-labelled dodecanoic acid (one type of FFA) to probe the site of fatty acid action.[21] Accordingly, we choose PC-3 cells as a negative control which expresses lower levels of GPR120 mRNA.[22] The results showed that feeble fluorescence was detected in negative PC-3 cells (Figure1D). These findings confirm that these probes can label the target for visualization in GPR120 overexpressing cells. The other six probes present similar results in fluorescence imaging (see Supporting Information).

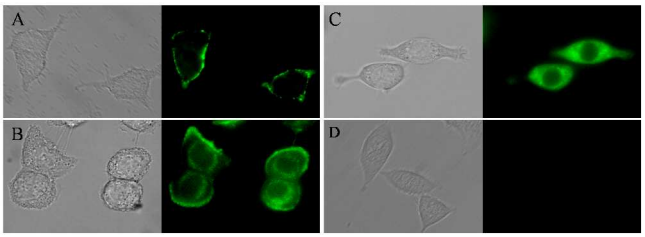


Figure 1. Fluorescence microscopic imaging of mammalian cells incubated with probe **L3**. All cells are incubated with **L3** at 37 °C for 5 min and washed immediately, and the exposure time remained the same among all groups. The background was adjusted by Image J software. Performed in Zeiss Axio Observer A1, GFP channel, objective lens, 63×.(A) Representative images of HEK293 cells incubated with **L3** (20 nM); (B) Image of HT-29 cells incubated with **L3** (20 nM); (C) Image of STC-1 cells incubated with **L3** (20 nM); (D) Image of PC-3 cells incubated with **L3** (20 nM).

As a result, these cells endogenously expressing GPR120 could be labeled by fluorescent probes at the nanomolar concentration(Figure 1), which would be a new milestone for locating the position of GPR120 with fluorescent probes. Subsequently, we want to define whether the observed fluorescence actually represents specific binding. For this to be the case it should be outcompeted by ligands from distinct chemical series that also have affinity for GPR120. The HEK293 cell stably expressing the GPR120 protein was used and the selective diarylsulfonamide GPR120 agonist GSK137647A was chose as a negative control, which might make a good choice for a structurally orthogonal GPR120 active agonist to figure out this issue. These results exhibited that the fluorescence intensi-

ty was significantly decreased by incubating the cells with GSK137647A (30 μM) together with probe L3 and indicated that the labeling with probe L3 can be outcompeted by a non fluorescent GPR120 ligand (Figure 2). The implication of a development of a labeled GPR120 agonist indicated that these probes can be used as labeling tools for binding to this receptor and be expected to guide the physiological and pathological studies of GPR120. Moreover, we expect that these probes could be employed as fluorescent competitive substrates in the GPR120 ligand activity screening.

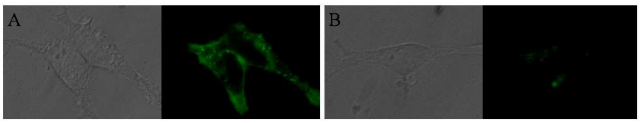


Figure 2. Fluorescence microscopic imaging of mammalian cells incubated with probe **L3**. All cells are incubated with **L3** at 37 °C for 15 min and washed immediately, and the exposure time remained the same among all groups. The background was adjusted by Image J software. Performed in Zeiss Axio Observer A1, GFP channel, objective lens, 63×. (A) Representative images of HEK293 cells stably expressing the GPR120 protein incubated with **L3** (200 nM); (B) Representative images of HEK293 cells stably expressing the GPR120 protein incubated with GSK137647A (30 μM) and probe **L3** (200 nM).

It should be noted that the selectivity and specifity of such a probe still needs to be enhanced even if it could label GPR120 at the cellular level. Moreover, its instrinic short wavelength of fluorescence greatly limited the utility of the probe in vivo experiments, which have weak light penetration and can't effectively pass through deep tissue. Therefore, the near-infrared probe with high selectivity and specifity for the GPR120 should be designed for in vivo study, and the proposed fluorescent probe should be further studied as a competitive substrate to construct a rapid screening agonist platform.

In conclusion, we herein designed and synthesized a series of small-molecule fluorescent probes with excellent fluorescent properties for tracking and detecting GPR120 in living cells at real time. These probes have been successfully used in localization and visualization of GPR120 in cellular imaging, in cell lines such as HEK 293, STC-1 and HT-29 at the nanomolar level. Moreover, these fluorescent probes exhibited high biological activity on the GPR120 and low toxicity in cells. The preparation of these fluorescent probes is also convenient and affordable by starting from inexpensive organic materials. Therefore, we reason that these probes can be used as powerful tools for drug screening and cell staining, as well functional studies on GPR120.

ASSOCIATED CONTENT

Supporting Information.
Full experimental procedures, analytical and spectral characterization data of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

GPR120, G protein-coupled receptor 120; FFA4, free fatty acid receptor 4 (GPR120); FFA, free fatty acid; GPCRs, G protein coupled receptors; GLP-1,glucagon-likepeptide-1; BRET, bioluminescence resonance energy transfer.

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