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An optimized ratiometric fluorescent probe for sensing human UDP-glucuronosyltransferase 1A1 and its biological applications

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ABSTRACT

20 This study aimed to develop a practical ratiometric fluorescent probe for highly selective and sensitive
detection of human UDP-glucuronosyltransferase 1A1 (UGT1A1), one of the most important phase II
enzymes. 4-Hydroxy-1,8-naphthalimide (HN) was selected as the fluorophore for this study because it
possesses intramolecular charge transfer (ICT) feature and displays outstanding optical properties. A series
24 of N-substituted derivatives with various hydrophobic, acidic and basic groups were designed and
synthesized to evaluate the selectivity of HN derivatives toward UGT1A1. Our results demonstrated that
the introduction of an acidic group to HN could significantly improve the selectivity of UGT1A1. Among
the synthesized fluorescent probes, NCHN (N-3-carboxy propyl-4-hydroxy-1,8-naphthalimide) displayed
28 the best combination of selectivity, sensitivity and ratiometric fluorescence response following
UGT1A1-catalyzed glucuronidation. UGT1A1-catalyzed NCHN-4-O-glucuronidation generated a single
fluorescent product with a high quantum yield ($\Phi = 0.688$) and brought remarkable changes in both color
and fluorescence in comparison with the parental substrate. The newly developed probe has been
32 successfully applied for sensitive measurements of UGT1A1 activities in human liver preparations, as well
as for rapid screening of UGT1A1 modulators, using variable enzyme sources. Furthermore, its potential
applications for live imaging of endogenous UGT1A1 in cells have also been demonstrated.

Keywords: Human UDP-glucuronosyltransferase 1A1; Ratiometric fluorescent probe; Real activity; Biological applications

1. Introduction

Uridine-disphosphate glucuronosyltransferases (UGTs) are a superfamily of drug-metabolizing enzymes located in the lipid bilayer of the endoplasmic reticulum, which catalyze the conjugation of potentially harmful hydrophobic compounds with glucuronic acid using UDP-glucuronic acid (UDPGA) as the donor (Tukey and Strassburg, 2000). Up to date, more than 20 UGTs have been discovered in human body and found most abundant in the liver. UGTs play important roles in disposition of many endobiotics and xenobiotics (Di, 2014; Rowland et al., 2013). UGT1A1, a member of the UGT1A subfamily, is of particular importance for human health due to its essential role in preventing bilirubin accumulation to toxic levels (Kiang et al., 2005). Polymorphism may be a genetic risk for low endogenous UGT1A1 protein abundance and consequently low bilirubin glucuronidation rate (Skierka et al., 2013). Genetic mutations in the UGT1A1 gene are commonly associated with bilirubin-related diseases, including mild Gilbert's syndrome, potentially fatal Crigler-Najjar syndrome and various cancers (Strassburg, 2010). Alongside bilirubin glucuronidation, UGT1A1 is involved in the conjugation of other endogenous compounds such as β -estradiol, bile acids and inflammatory mediators, as well as in the metabolism of many drugs and xenobiotics, such as SN-38, etoposide and diethylstilbestrol (Guillemette et al., 2014). Furthermore, patients with UGT1A1 dysfunction have an increased risk of adverse drug-drug interactions (DDI) (Stingl et al., 2014), such as in the case of the anticancer agent irinotecan which has evoked large concerns (O'Dwyer and Catalano, 2006). Additionally, co-administration of UGT1A1 inhibitors with drugs that are metabolized by UGT1A1 may cause severe side-effects, such as hyperbilirubinemia or acute liver failure (Singer et al., 2007; Strassburg et al., 1997; Zucker et al., 2001). Hence, there is a clear need to develop useful tools for the real-time assessment of enzyme activity of UGT1A1 in complex biological systems.

In recent years, fluorescent probes for functional enzymes have attracted considerable attention due to their inherent advantages, such as high sensitivity, cost-effectiveness and applicability to high-throughput screening (HTS) (Feng et al., 2015; Fischbach et al., 2014; Huang et al., 2011; Ma et al., 2015; Mu et al., 2014; Tan et al., 2012). However, the development of a practical fluorescent probe for a given UGT enzyme remains challenging for the following two reasons. First, human UGTs within a subfamily share high homology in amino acid sequence ($> 65\%$) and usually exhibit broad and overlapping substrate specificity. Second, the fluorescence properties of many fluorophores are often "turned-off" following O-glucuronidation at the hydroxyl group. Recently, Terai and co-workers reported the first intensity-based fluorescent probe for UGT1A1 with high sensitivity, which made a significant landmark in UGT-related fields (Terai et al., 2013). In contrast to the intensity-based fluorescent probes, ratiometric fluorescent probes allow the measurement of target by the ratio of two distant fluorescent signals, thus exhibiting some advantages, including minimization of the external interference and alleviation of the shortcomings of intensity-based probes in quantitative detection (Liu et al., 2014; Wan et al., 2014). It is thus highly desirable to develop a more accurate and sensitive ratiometric fluorescent probe to measure the real activity of UGT1A1 in human biological samples.

In this study, a ratiometric fluorescent probe has been designed and developed for rapid and highly sensitive detection UGT1A1 for the first time, based on its vital capacity for catalyzing O-glucuronidation towards polycyclic phenolic compounds. 4-hydroxy-1,8-naphthalimide (HN) was selected as the basic fluorophore due to its outstanding optical properties, including high photostability, large Stokes

shift(Silvers et al., 2013) and high fluorescence efficiency (Srikun et al., 2008; Zhu et al., 2011). The phenolic group at the C-4 site of HN serves as an electron donor, which triggers a strong intramolecular charge transfer (ICT) effect upon photon excitation (Hettiarachchi et al., 2014). Interfering with the electron donating ability of the C-4 phenolic group can result in significant changes in both absorption and emission spectra (Hou et al., 2014).The ICT characteristics made HN and its derivatives good candidates for the ratiometric fluorescent probes (Jin et al., 2014). Herein, we describe the discovery and development of the first ratiometric fluorescent probe N-(3-carboxy propyl)-4-hydroxy-1,8-naphthalimide (NCHN) for human hepatic UGT1A1 and its potential applications in complex biological samples, including tissue and cell preparations, as well as living cells.

2. Experimental

2.1. Synthesis of probe and structure modification

The detailed synthesis and structural characterization of these HN derivatives are described in the supplementary data (Scheme S1, Fig. S15-S26). In order to find a highly selective probe for UGT1A1, various substitutes differing in hydrophobicity, acidity and basicity are introduced into the N-site on the HN skeleton for exploring the potential structure-UGT selectivity relationship (Fig. S1). Given that the N-terminal of UGT1A1 is rich in basic amino acids and they probably play important roles in substrate binding or catalysis, several derivatives with acidic groups are deliberately synthesized.

2.2. UGT1A1 selectivity assays

The selectivity of NCHN toward UGT1A1 was evaluated by the following two experiments, namely isoform selectivity assay using recombinant human UGTs in hepatic tissue and chemical inhibition assay. For the isoform selectivity assay, a mixture of NCHN (20 μ M), UDPGA (5 mM), $MgCl_2$ (10 mM), and the different UGT isoforms (0.06 mg/mL) in Tris-HCl buffer (50 mM, pH 7.4) with 1% DMSO (total volume 100 μ L) was incubated in a 1.5 mL centrifuge Tubes at 37°C. In chemical inhibition assays, a series of chemical inhibitors for UGTs were selected, including protopanaxatriol (PPT) and nilotinib (NB) for UGT1A1 (Ai et al., 2014; He et al., 2013), magnolol for UGT1A9 (Zhu et al., 2012), and fluconazole for UGT2B7 (Uchaipichat et al., 2006). The concentration of these selective inhibitors were used to ensure that almost all of the glucuronidation activity (> 90%) could be selectively inhibited. Then each inhibitor was incubated with the mixture solution mentioned above with HLM (0.25 mg/mL) as an enzyme source for 40 min at 37°C. The IC_{50} value of PPT and NB for UGT1A1 was obtained for both recombinant human UGT1A1 (0.06 mg/mL) and HLM (0.25 mg/mL). Fluorescence measurements were performed using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio Tek). Inhibition percentage was calculated by comparing to control samples that were incubated under identical conditions, except in the absence of the inhibitors.

2.3. Inter-individual variability in UGT1A1 activity and correlation analysis

To investigate the inter-individual difference in glucuronidation of NCHN, the formation rates of NCHN-4-O-glucuronide (NCHNG) were measured in a panel of 14 individual HLMs. NCHN (final concentration, 20 μ M) was performed using HLM (final protein concentration, 0.2 mg/mL) for 30 min. Ethinyloestradiol (EE, final concentration, 25 μ M) was incubated with HLM (final protein concentration, 0.2 mg/mL) for 30 min. Bilirubin (final concentration, 1 μ M) was incubated with HLM (final protein concentration, 0.02 mg/mL) for 20 min. The concentrations of UGT1A1 in HLMs were determined by liquid chromatography-tandem mass spectrometer (LC-MS/MS), using multiple reaction monitoring (MRM)

mode and isotope labeled peptide as the internal standards. Specific peptides of T78YPVPFQR85 (for UGT1A1) were selected for its quantification using transition ion of 504.4/547.3. The correlation parameters were expressed by the linear regression coefficient (R^2) and $P < 0.05$ was considered statistically significant.

2.4. UGT1A1 induction assays

HepG2 cell microsomes were prepared by slight modification of a procedure described previously (Xu et al., 2014). Cells were cultured in 100 mm dishes for 1 to 2 days, and induced for 3 days with different concentrations of chrysin. The medium was changed every 24 h during the induction time and at the end of the 3rd day the cell were first washed twice with PBS (5 mL), then scraped off the dishes in the presence of PBS (5 mL). Following centrifugation for 5 min at $200 \times g$, the cell pellets were gently suspended in 10 mM sodium phosphate buffer (pH 7.4 containing 0.15 M KCl) containing protease inhibitor cocktail (1%). The cells were disrupted by sonication, on ice, for 5×5 sec pulses, with 30 sec intervals between the pulses, with the sonifier (JYD-650, Shanghai Zhixin Instrument Co., China) power setting at 150 W. Following sonication the cells suspension was centrifuged at $9,000 \times g$ for 20 min at 4°C and the supernatant fraction (S9) was further centrifuged at $100,000 \times g$ for 70 min at 4°C to precipitate the microsomes fraction. The total protein concentration of the cells microsomes was measured using the BCA protein assay kit.

2.5. Cells culture and bioimaging

The human cell line HepG2 was cultured in high glucose Dulbecco's modified Eagle medium (DMEM, 4.5 g/L glucose), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2 . The cells ($3 \times 10^8 \text{ L}^{-1}$) were seeded on 20-mm glass polylysine-coated confocal cell culture dish and allowed to adhere for 2 to 4 days. Then the cells were treated with 50 μM N-4-methyl butyrate-4-hydroxy-1,8-naphthalimide (NMHN) and incubated for 2 h for microscopic imaging, using an Olympus FV1000 confocal laser scanning microscopy.

3. Results and discussion

3.1. Selectivity optimization

Among these HN derivatives, ten of them could be efficiently glucuronidated to form the corresponding mono-glucuronides in HLM in the presence of UDPGA. The enzyme selectivity of these 10 HN derivatives toward human UGTs was then evaluated to explore the potential structure-UGT selectivity relationship. As shown in Fig. S3, most of the HN derivatives were glucuronidated by multiple UGTs. However, NCHN bearing a carboxylic substituent at the N-site exhibited excellent specificity towards human UGT1A1. This prompted us to rigorously characterize the features of NCHN as a probe substrate for UGT1A1 in complex biological systems.

3.2. General fluorescence properties of NCHN-4-O-glucuronidation

Upon addition of HLM or UGT1A1, NCHN was efficiently converted to a single mono-glucuronide in the presence of UDPGA (Fig. S4). To gain insight into the sensing mechanism of NCHN toward UGT1A1, the product was biosynthesized using pooled liver microsomes from monkey and human. Subsequently, its chemical structure was well-characterized as NCHNG by high-resolution mass spectral (HRMS) and nuclear magnetic resonance (NMR) (Fig. S2, S27-S31 and Table S1). UGT1A1 mediated NCHN-4-O-glucuronidation resulted in a highly sensitive and selective emission response with significant changes in both color and fluorescence (Fig. 1). NCHN displayed a moderate UV-Vis absorption around

450 nm, and a moderate fluorescence emission spectrum around 564 nm ($\Phi = 0.082$). NCHNG exhibited a different UV-Vis absorption around 362 nm, and a maximum emission spectrum at 450 nm with an extremely high fluorescence intensity ($\Phi = 0.688$) (Fig. S5 and Table S2). The effects of organic solvent and pH on the fluorescence response of NCHN and NCHNG were also studied. DMSO at low concentration ($\leq 2.5\%$) did not affect the formation rate of NCHNG (Fig. S6). In addition, NCHN was stable at pH 7.0 to 10.5, NCHNG was also stable in a much wider pH range between 2.0 and 11.0 (Fig. S7). These results demonstrated that NCHN could be used as a practical probe for UGT1A1 within the physiological pH range.

Scheme 1. The chemical structures of NCHN and its glucuronide NCHNG.

3.3. UGT selectivity screening of NCHN

As shown in Fig. 1a, there was more than 25-fold difference in the fluorescence intensity ratio between UGT1A1 and other human hepatic UGT enzymes. Among the extrahepatic human UGT isoforms, UGT1A8 and UGT1A10 displayed rather limited activities with NCHN-4-O-glucuronidation, while other UGTs did not participate in this glucuronidation reaction (Fig. S8). In the chemical inhibition assays, NCHN-4-O-glucuronidation activity was strongly inhibited by protopanaxatriol (a potent inhibitor of UGT1A1 and UGT2B7) or nilotinib (a potent and selective inhibitor of UGT1A1). In contrast, other chemical inhibitors including magnolol (a potent and selective inhibitor of UGT1A9) and fluconazole (a potent and selective inhibitor of UGT2B7) exhibited minor inhibitory effects on this glucuronidation reaction (Fig. 1b). These results demonstrated that NCHN was a highly selective fluorescent substrate for human UGT1A1.

Fig. 1. Isoform specificity of NCHN-4-O-glucuronidation. (a) The fluorescence intensity ratio responses of NCHN (10 μ M) toward various human hepatic UGT isoforms (0.06 mg/mL). (b) The inhibitory effects of selective UGTs inhibitors protopanaxatriol (100 μ M), nilotinib (10 μ M), fluconazole (500 μ M) and magnolol (1 μ M) on NCHN-4-O-glucuronidation in HLMs. Data are shown as mean $\pm$ S.D. (n = 3).

3.4. Fluorescence sensing behavior of NCHN towards UGT1A1

Fig. 2a denotes the fluorescence sensing behavior of NCHN in the presence of UGT1A1 at different concentrations. Increasing enzyme concentrations resulted in a remarkable enhancement of fluorescence at 450 nm, while the original emission peak of NCHN at 564 nm decreased gradually. There was a good linear relationship between the fluorescence intensity ratio ($I_{450 \text{ nm}}/I_{564 \text{ nm}}$) and UGT1A1 concentration in the range of 0 to 0.09 mg/mL (Fig. 2b). Moreover, there was approximately an increase in the fluorescence intensity ratio by 155-fold (from 0.024 to 3.724) in response to increasing concentrations of UGT1A1, indicating a highly sensitive response. The corresponding detection limit of the fluorescence assay was found to be 0.125 μ g/mL (Fig. S9), such sensitivity is much higher than the currently used UGT1A1 probes (Zhou et al., 2010, 2011). The feasibility of using NCHN for real-time monitoring the enzyme activity of UGT1A1 was also investigated. As expected, good linear relationships between the reaction time of UGT1A1 and the fluorescence intensity ratios were readily obtained upon addition of UGT1A1 (Fig. S10). Accordingly, the fluorescence intensity was used to characterize the enzymatic kinetics of NCHN-4-O-glucuronidation in different enzyme sources, including HLM and recombinant human UGT1A1. NCHN-4-O-glucuronidation in both HLM and UGT1A1 displayed substrate inhibition kinetic behavior, with K_m values of 364.6 μ M in HLM and 126.7 μ M in UGT1A1 (Fig. S11 and Table S3). These results indicated that NCHN was a sensitive ratiometric fluorescent probe that can be used to accurately measure the enzyme activity of UGT1A1 in complex biological systems.

Fig. 2. NCHN was incubated with increasing concentrations of UGT1A1 (0 to 0.09 mg/mL) for 2.5 h at 37°C. (a) Concentration-dependent fluorescence responses of NCHN toward UGT1A1 at 37°C. (b) Plot of fluorescence intensity ratio (I_{450} nm/ I_{564} nm) of NCHN versus increasing concentrations of UGT1A1.

3.5. Quantification of UGT1A1 in human liver microsomes

NCHN was applied to evaluate of UGT1A1 activities in 14 individual HLMs. As depicted in Fig. 3a, the enzyme activity ranged from 18 and 180 pmol/min/mg protein, with appropriately 10-fold individual variation. The coefficient of variation in the rate of NCHN-4-O-glucuronidation was 62%, which agreed well with the high variability of UGT1A1 activity in individual HLMs as reported previously (Katoh et al., 2007). The absolute concentration of UGT1A1 in individual HLMs was also determined by a mass spectrometry-based proteomic technique. As shown in Fig. 3b, there was a remarkably strong correlation ($R^2 = 0.93$, $p < 0.0001$) between the formation rates of NCHNG and the contents of UGT1A1 in 14 individual HLMs. The activities of NCHN-4-O-glucuronidation in these individual HLMs were also compared with those detected by two commonly used non-fluorescent probes of UGT1A1, including ethinyloestradiol and bilirubin. As expected, strong correlations ($R^2 = 0.91$, $p < 0.0001$) between the formation rates of NCHNG and that of ethinyloestradiol-3-O-glucuronide or bilirubin glucuronides were presented (Fig. 3c and 3d). These results demonstrated that NCHN could effectively measure the enzyme activity of UGT1A1 in liver samples with good sensitivity and high selectivity.

Fig. 3. (a) The formation rates of NCHNG in 14 individuals HLMs. (b) Correlation studies between the formation rates of NCHNG and the protein level of UGT1A1 in 14 individual HLMs. (c) Correlation studies between the formation rate of NCHNG and that of EE-3-O-glucuronide of UGT1A1 and (d) that of bilirubin total glucuronide of UGT1A1 in 14 individuals HLMs.

3.6. High-throughput screening inhibitors and inducers of UGT1A1

NCHN also allows the application of a HTS of potential UGT1A1 inhibitors which might cause adverse DDI. To demonstrate this potential use, a HTS test using NCHN-4-O-glucuronidation as the probe reaction was performed, in which two chemical inhibitors of UGT1A1 (PPT and NB) were used to evaluate their inhibitory effects toward UGT1A1 in both HLM and recombinant UGT1A1. As shown in Fig. 4a and S13, the inhibitory tendency and IC_{50} values of these two inhibitors were similar between HLM and UGT1A1, indicating that HLM can be used as the enzyme source instead of the more expensive recombinant UGT1A1 in such assays. In addition, NCHN could also be used to find UGT1A1 inducers using fluorescence based HTS assays. It was previously reported that chrysin could induce the expression and function of UGT1A1 in HepG2 cells (Walle et al., 2000). Therefore, different concentrations of chrysin (0, 25 and 50 μ M) were used to stimulate the expression and/or activity of UGT1A1. As expected, after incubation with 50 μ M chrysin for three days, there was a nearly 9-fold increase in UGT1A1 activity in cell homogenate (Fig. 4b). Induction of UGT1A1 by synthetic or natural chemicals in a clinical setting could be used to treat UGT1A1 deficiencies and be effective in prevention of cases such as CPT-11 toxicity (Aoshima et al., 2014). The findings presented here strongly suggested that NCHN could serve as an appropriate tool to find efficient UGT1A1 inducer(s) as new drug candidate(s).

Fig. 4. (a) The inhibitory effects of PPT towards UGT1A1 in HLM and recombinant UGT1A1, by using NCHN-4-O-glucuronidation as the probe reaction. (b) NCHN-4-O-glucuronidation by cell homogenates prepared from HepG2 cells pre-treated with chrysin (0, 25, 50 μM). Data are shown as mean ± S.D. (n = 3).

3.7. Bioimaging application in living cells

We next evaluated the potential applications of NCHN for the fluorescence bioimaging of endogenous UGT1A1 in living cells. To increase cell permeability, we transformed NCHN into its formyl ester NMHN, which was rapidly de-esterified by endogenous esterases into NCHN and subsequently glucuronidated by UGT1A1 in HepG2 cells, using the cell-made UDPGA as a glucuronic acid donor. Prior to imaging, the cytotoxicity of all associated compounds used in the cell imaging experiment was assessed in HepG2 cells, to assure the low toxicity to living cells. As shown in Fig. S14, NCHN and its formyl ester NMHN displayed relatively low cellular toxicity. HepG2 cells remained in good conditions following treatment with a high dose of both substrates (200 μM) for 48 h at 37°C. As shown in Fig. 5, fluorescence images of living HepG2 cells upon addition of NMHN were recorded by confocal-laser scanning microscopy. As expected, the cells exhibited intense intracellular fluorescence in blue channel after co-incubation with NMHN (50 μM) at 37°C for 2 h (Fig. 5b). In sharp contrast, under the same incubation conditions, the blue fluorescence signal was almost completely blocked upon pre-treatment with PPT (a potent inhibitor of UGT1A1) (Fig. 5e). These results indicated that NCHN was a good probe for human hepatic UGT1A1 and enabled ratiometric imaging and reliable detection of UGT1A1 activity in living cells.

Fig. 5. Confocal fluorescence images of UGT1A1 in HepG2 cells loaded with NMHN (50 μ M) for 2 h (a, b, c), and images of UGT1A1 in HepG2 cell pre-treated with PPT (25 μ M) for 30 min followed by incubation with NMHN (50 μ M) for 2 h (d, e, f). (a, d) bright field images; (b, e) fluorescence images collected at 420 to 500 nm (blue channel) upon excitation at 405 nm; (c, f) fluorescence images were collected at 500 to 600 nm (green channel) upon excitation at 458 nm.

4. Conclusions

In summary, this study describes the design, synthesis, characterization, and biological applications of a practical ratiometric fluorescent probe for human UGT1A1. The activity-based probe NCHN is designed and optimized for the selective detection of UGT1A1 based on its substrate preference and the ICT mechanism. NCHN exhibits a specific response to human hepatic UGT1A1 and triggers remarkable changes in the colour and fluorescence spectrum. The newly developed ratiometric fluorescent probe allows the development of a sensitive HTS-compatible assay to quantify the enzyme activity of UGT1A1 in complex biological systems. Such assays have also been applied successfully for rapid screening of UGT1A1 inhibitors and inducers, using tissue or cell preparations or living cells as the enzyme sources. The application of NCHN to measure active UGT1A1 in biological samples thus opens new avenues for future studies on xenobiotics metabolism, toxicological assessments and other related areas, including protein-protein interactions, enzyme regulation and induction/inhibition assays. Moreover, the strategies employed in the design and optimization of this probe in the present study may shed new lights on the development of other highly specific fluorescent probes for a given UGT enzyme.

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