



Determination of carboxylesterase 2 by fluorescence probe to guide pancreatic adenocarcinoma profiling

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ARTICLE INFO

Keywords:

Carboxylesterase 2
Fluorescent probe
Inhibitor
Pancreatic adenocarcinoma
Highthroughput screening

ABSTRACT

Carboxylesterase 2 as a critical hydrolase, current studies are limited to its role in prodrug metabolism while functional studies found that pancreatic adenocarcinoma (PAAD) is associated with expression differences of this enzyme. However, it lacks of organelle level assessment of carboxylesterase 2 activity in the fluorescent tools. Herein, we develop a fluorescent probe (NI-MO) that achieves selective visualization of human lysosomal carboxylesterase 2 in living cells to distinguish PAAD and normal cells, which provide a new avenue to guide pancreatic adenocarcinoma profiling. Notably, we constructed a high-throughput screening approach to rapidly screen the potential inhibitors through carboxylesterase 2 activity.

1. Introduction

Human carboxylesterase (hCEs) is the main metabolic enzyme, participating in the biotransformation of endogenous and exogenous substances. For example, it is involved in the hydrolysis process of endogenous cholesterol esters in body, meantime, it play an important role in the metabolism, activation and detoxification of drugs *in vivo* [1]. It belongs to the esterase family, which is used to catalyze hydrolysis of ester bond, amide bond, thioester bond and so on, it has widely substrates [2]. The expression and gene polymorphism of CEs were closely related to drug metabolism. Their significance in the biological process has been well studied, especially human carboxylesterase 2(hCE2), the abnormality of carboxylesterase 2 is also correlated with cancers, such as pancreatic cancer [3]. Pancreatic adenocarcinoma(PAAD) is one of the deadliest human malignancies with genetically complex, even a single tumor mass from a given patient contains up to 63 genetic alterations and 12 core signal pathway abnormalities [4], to say nothing of other tumor biomarker expression among different cancer patients. These markers are important in disease diagnosis and fluorescent probes have been developed to identify these markers [5,6]. It is especially worth noting that carboxylesterase 2 was mentioned in PAAD, the mainly studied is its activity changes and effects in the treatment of prodrug Irinotecan [7]. However, its own value of carboxylesterase 2 in

pancreatic cancer has not been concerned. Because it is inconvenient to detect carboxylesterase 2, and the analysis of carboxylesterase 2 activity at organelle level is incomplete, so it lacks of suitable analytical tools to monitor activity of carboxylesterase 2 selectively at organelle level in living biological systems. Fluorescent probes, with simplicity, convenience, and non-invasive nature, might be a good tool for sensing carboxylesterase 2, cancer cell recognition and inhibitor evaluation.

Currently, existing fluorescent probes of carboxylesterase 2 show optical changes after cleavage by carboxylesterase 2 at the active site, and they have been widely used to measure carboxylesterase 2 activity *in vitro* [8–9]. But positioning is rarely involved. It may be an urgent need to develop a fluorescent probe to analyze lysosomal carboxylesterase 2 activity. A novel fluorescence probe of carboxylesterase 2 was proposed. To simplify the probe structure, we synthesized a multi-functional fluorescent probe (NI-MO), including a lysosomal carboxylesterase 2 targeting and detection structure. Selectivity studies showed that probe NI-MO was catalyzed carboxylesterase 2 specifically and it is a good fluorescence substrate by kinetic studies. Then, Ni-MO was used for biological imaging and monitoring of lysosomal carboxylesterase 2 levels in living cells. Ni-MO demonstrated the ability to distinguish PAAD cells from normal cells based on their lysosomal carboxylesterase 2 levels. It is suggested that NI-MO could be used to guide the early diagnosis of pancreatic cancer. Meanwhile, the probe has been used as a

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simple tool for high-throughput drug screening. Therefore, we consider the fluorescent probe NI-MO to be a valuable tool for practitioners of lysosomal carboxylesterase 2 detection in this field.

2. Results and discussion

Different from the well-studied other esterase in tumors, its own value of carboxylesterase 2 is not getting attached, and only its role in prodrug metabolism has been focused on. To generally explore the association between carboxylesterase 2 and the tumor, we excavated the carboxylesterase 2 expression level in neoplastic and normal tissues by using The Cancer Genome Atlas (TCGA) database and Genotype-Tissue Expression (GTEx) Portal [10–11]. The results showed that pancreatic adenocarcinoma has different carboxylesterase 2 expression level compared with normal pancreas samples (Fig. 1a). Due to its potential in pancreatic adenocarcinoma and its treatment, we speculated that the carboxylesterase 2 could be used as a potential target for diagnosing that offers important insights into PAAD.

It is well known that an enzyme, as a catalyst with specificity, has a preferred substrate. Due to the substrate characteristics of carboxylesterase 2, here we designed a fluorescent probe (NI-MO, Fig. 1b). The structure started with the naphthalimides scaffold due to its flexibility in structural modification and steric hindrance adjustment around the enzyme recognition group, NI-MO is synthesized by coupling morpholine unit to the hydroxyl (details in supporting information). The probe was designed with bifunctional site in one unit—with both active site and located site. For the active site, a morpholineacetyl chloride unit is linked to the phenolic oxygen of naphthalimide as the enzyme-triggered moiety. Upon addition of carboxylesterase 2, enzyme-triggered cleavage of the carboxylic ester bond exposes the free hydroxyl group of naphthalimide, it is the carboxylate bond cleaved product (NBOH). For NBOH, based on our previous study, its first excited state (S1) is an intramolecular charge transfer (ICT) state [12]. The ICT state can lead to a redshift in both absorption and emission spectrum. In order to certify an ICT process, we calculated the frontier molecular orbitals. As shown

in Figure S3, one can note that S0 of NBOH possesses relatively more charge separation character as compared with that of NI-MO. This leads to lower vertical excitation energy of NBOH compared to that of NI-MO of their S0 to S1 transition (Table S1). We would expect the redshift absorption and emission spectrum of NBOH compared to NI-MO. Furthermore, to investigate the possible binding of NI-MO to carboxylesterase 2 active pocket, a low energy complex was constructed by molecular docking. The simulation result indicated that the probe could successfully enter the catalytic pocket of carboxylesterase 2, and hydrogen bonds are formed between the NI-MO and the residue of carboxylesterase 2 (Fig. 1c). One can notice hydrogen bonds were formed between the morpholine group and carboxylesterase 2, which is beneficial for stabilizing the complex.

To confirm the predicated carboxylesterase 2 sensing mechanism of NI-MO, we carried out spectroscopic study. NI-MO showed a maximal absorption peak at 340 nm and fluorescence peak at 412 nm in Boric acid borax(BB) buffer (Figure S4). The absorption and emission spectrum were recorded for NI-MO before and after reaction with carboxylesterase 2 under physiological condition (Fig. 2a-b). Carboxylesterase 2 was added, the original absorption and emission intensity of NI-MO decreased, and a new red-shifted absorption and fluorescence emission spectrum emerged with peak located at 450 nm and 560 nm, respectively. The new absorption and emission peaks were consistent with that of NBOH (Figure S5). To further verify this response, we monitored the reaction by liquid chromatography–mass spectrometry (LC-MS). The probe (10 μ M) was incubated with carboxylesterase 2 (50 μ g/ml), a new peak was observed at \sim 11 mins. And the same peak was observed for NBOH (Figure S6). The generation of a mass peak at m/z 270.1151 [$M + H$] $^{+}$ suggested the formation of a hydrolysis product (NBOH) as the major product (Figure S6).

To check the selectivity of NI-MO, the fluorescence of NI-MO was evaluated with a series of possible interfering biological enzymes and molecules (Fig. 2c, S9). For other evaluated biological interferences, NI-MO exhibited the highest response to carboxylesterase 2. Meantime, bis-*para*-nitrophenylphosphate (BNPP) and loperamide [13,14], as the

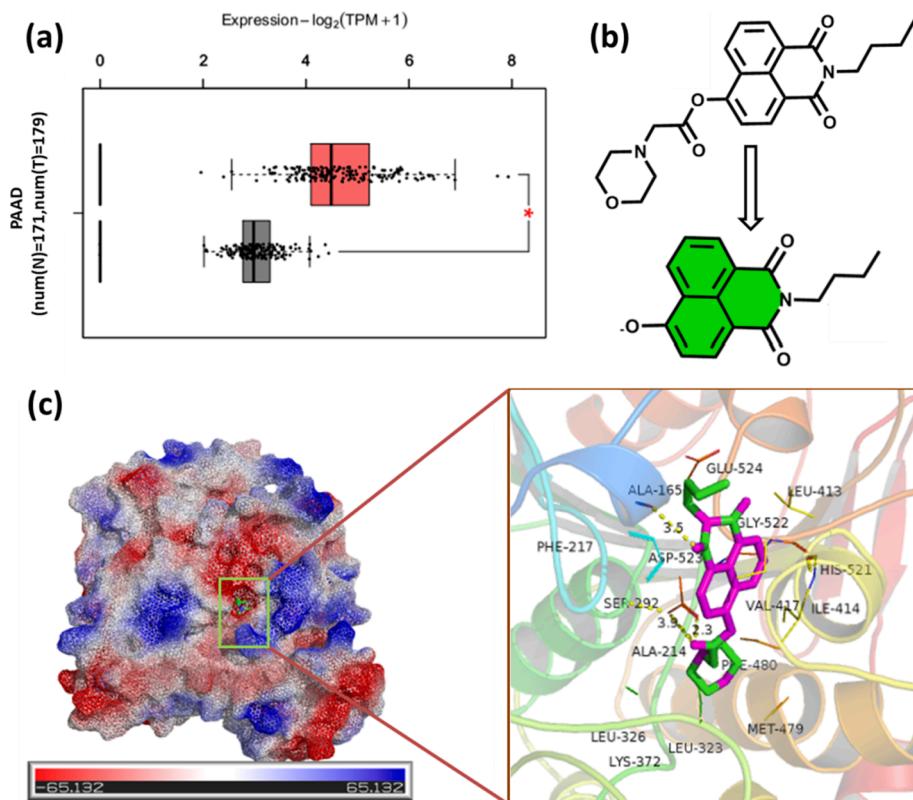


Fig. 1. (a) The expression distribution of carboxylesterase 2 gene in tumor tissues and normal tissues, where the vertical axis represents different groups of samples, the horizontal axis represents the gene expression distribution, where red represent tumors and grey represent normal groups. For the 179 patients of The Cancer Genome Atlas (TCGA) database, tumoral RNA-seq data were downloaded from the Genomic Data Commons (GDC) data portal (TCGA) and 171 of the tumors also had mRNA expression data of paired normal tissue samples. (b) Molecular design to NI-MO response. (c) The docking of carboxylesterase 2 with NI-MO by molecular dynamics simulation (H-Bonds between the NI-MO and amino acid residues of carboxylesterase 2 are indicated with yellow dotted lines).

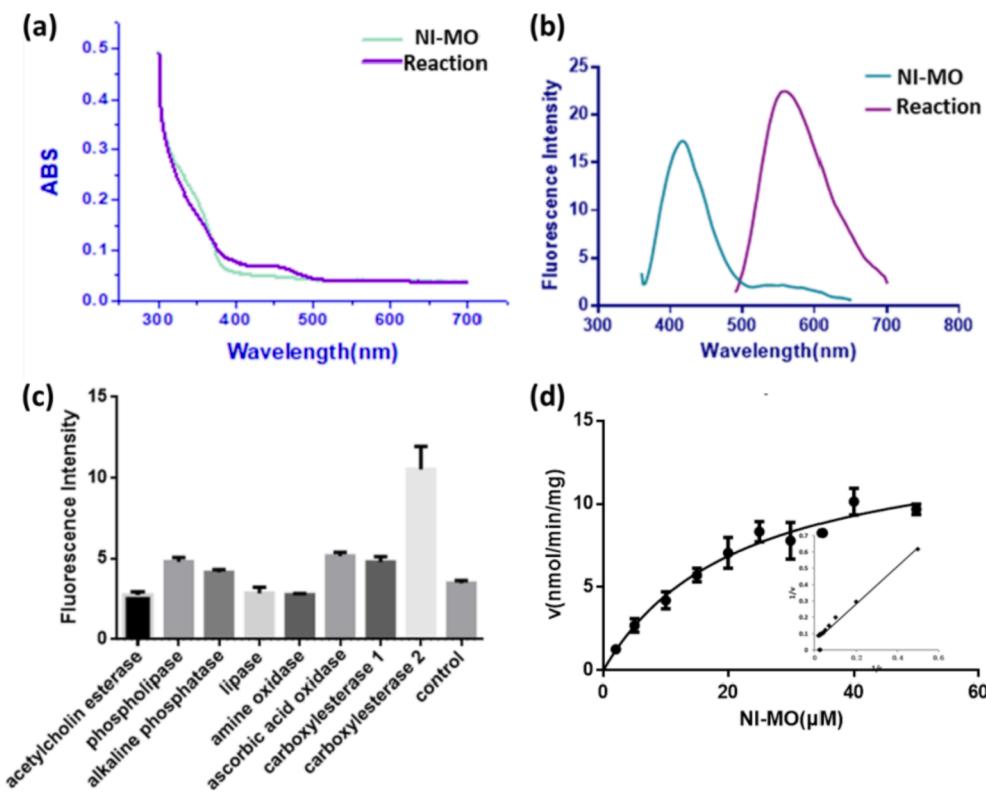


Fig. 2. (a-b) The absorption and emission spectrum of NI-MO ($10 \mu\text{M}$) and its response to carboxylesterase 2 ($50 \mu\text{g}/\text{ml}$) after 30 min incubation ($\text{Ex} = 450 \text{ nm}$). (c) The fluorescence enhancement of NI-MO ($10 \mu\text{M}$) toward various analytes: Acetylcholin esterase, Phospholipase, Alkaline phosphatase, Lipase, Amine oxidase, Ascorbic acid oxidase, Carboxylesterase 1 and Carboxylesterase 2 ($50 \mu\text{g}/\text{ml}$). The measurement was performed after incubating at 37°C for 30 min in BB buffer(pH 5.0) ($\text{Ex}/\text{Em} = 450 \text{ nm}/560 \text{ nm}$). (d) Determinations of kinetic parameters of NI-MO towards carboxylesterase 2.

commercial inhibitors of carboxylesterase, were chosen to verify the fluorescence attribution (Figure S7). It showed that the fluorescence decreased when the enzyme activity was inhibited, indicating that the fluorescence came from the response of carboxylesterase 2. These results proved that NI-MO has high selectivity to carboxylesterase 2. Then, the effect of pH was assessed to further determine the property of the probe toward variations of pH microenvironments, and stability was also verified (Figure S8). The fluorescence changed obviously at pH 5.0 suggesting that the probe could work in lysosome under physiological contexts. The enzyme kinetics information showed that the Michaelis constant (K_m) and V_{\max} were calculated to be $21.72 \mu\text{M}$ and $17.45 \text{ nmol}/\text{min}/\text{mg}$, respectively, indicating that the carboxylesterase 2 exhibits its affinity toward NI-MO (Fig. 2d). To test if the probe is suitable for quantitative carboxylesterase 2 detection, the reaction kinetics was monitored by measuring the fluorescence intensity at 560 nm . There is a good linear relation between fluorescence intensity and carboxylesterase 2 concentrations (up to $50 \mu\text{M}$). The detection limit is $1.986 \mu\text{g}/\text{ml}$ (Figure S10). The results confirmed that NI-MO can detect carboxylesterase 2 qualitatively and quantitatively.

Carboxylesterase 2 was considered as a drug target for cancer detection and treatment. Nonetheless, some effective inhibitors based on the carboxylesterase 2 have not been assessed in clinical trials. The major challenge in screening inhibitors for carboxylesterase 2 is the lack of robust detection assays. Considering the performance of the NI-MO as a fluorescence probe for carboxylesterase 2, we evaluated whether NI-MO could serve as an effective identification tool for screening potential drugs against carboxylesterase 2. We firstly explored a simple and effective fluorescence-based high-throughput screening through 560 drug compounds from commercially libraries purchased from MedChemExpress. Those compounds with significantly decreased

fluorescence might be effective inhibitors and were submitted for further verification. The results found that parecoxib, erdosteine and nitisinone could be new carboxylesterase 2 inhibitors and the inhibitory activities of three drugs towards carboxylesterase 2 were identified by inhibition assay. The IC_{50} value of for parecoxib, erdosteine and nitisinone are $30.73 \mu\text{M}$, $31.55 \mu\text{M}$ and $34.41 \mu\text{M}$ respectively, and parecoxib has a relatively good effect (Fig. 3a-c). The results indicated that the three drugs maybe have a promising for cancer treatment towards carboxylesterase 2, and NI-MO can be an excellent tool for high-throughput drug screening in seeking potential carboxylesterase 2 inhibitors.

Based on the high selectivity and sensitivity of the probe, the feasibility of the probe to visualize carboxylesterase 2 was evaluated in living cells. We chose human pancreatic cancer cells (Panc-1) and human normal pancreatic duct epithelial cells (HPDE6-C7 cells) as models for pancreatic cancer, and the cytotoxicity of the probe was tested (Figure S11), demonstrating low toxicity of the probe. The probe targeting was examined by cellular fluorescence, as shown in Fig. 3d-e, the cells were non-fluorescent before incubating with NI-MO (Fig. 3 control images). After incubation with NI-MO, fluorescence can be observed for both cells indicating NI-MO entered inside the living cells. It illustrated that the probe easily infuses within intact live cells and confined within the live cell providing a fluorescence signal. To further demonstrate new inhibitors, Panc-1 cells and HPDE6-C7 cells were pretreated with the three inhibitors which we have discussed above. Then the pretreated cells were incubated with NI-MO. The confocal imaging revealed the fluorescence in the cell was suppressed (Fig. 3e). The decreased fluorescence intensity indicated the inhibitors can efficiently blunt the fluorescence signals. Furthermore, the same experiment was performed in HPDE6-C7 cells and it also found that the fluorescence intensity

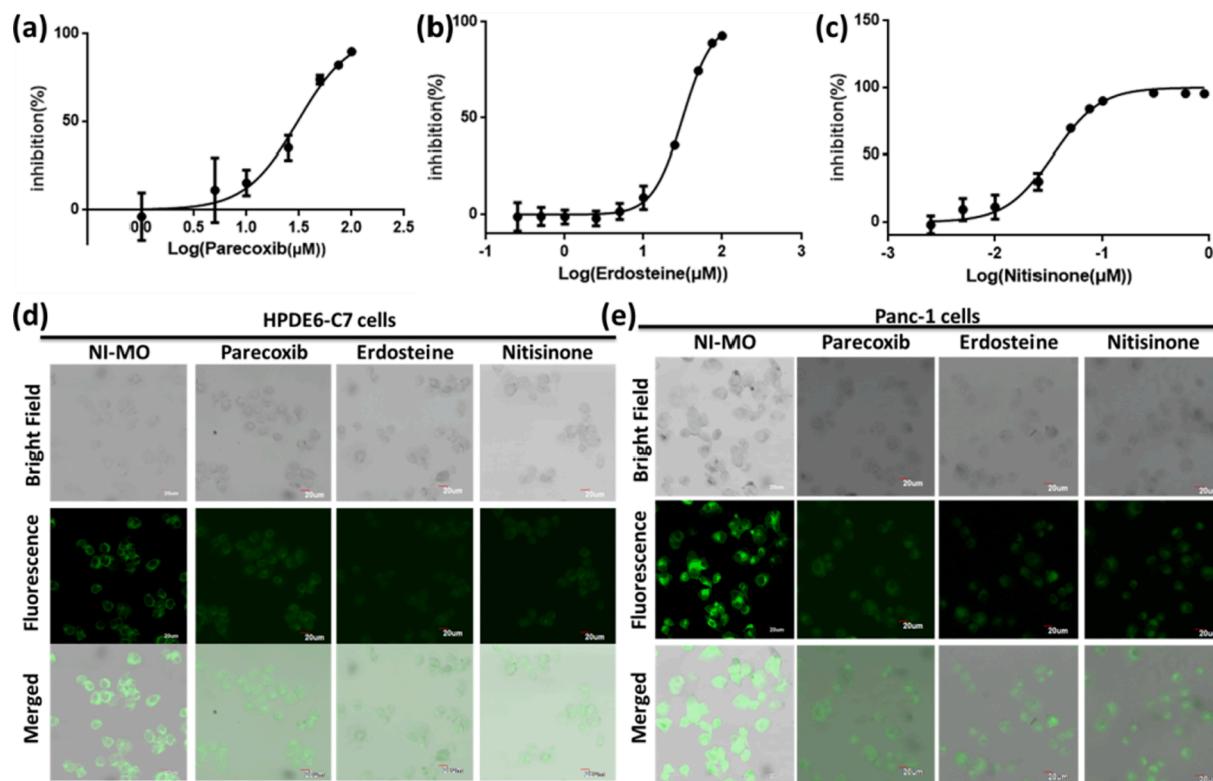


Fig. 3. (a-c) Inhibition values ratios of parecoxib (a), erdosteine (b) and nitisinone (c) on carboxylesterase 2(20 μ g/ml) by NI-MO(10 μ M). Inhibition value for assay is given as IC_{50} (μ M). (d-e) Confocal fluorescence images of NI-MO(10 μ M) in Panc-1 cells (d) and HPDE6-C7 cells. From left to right: cells only, NI-MO only, Parecoxib(100 μ M), Erdosteine (100 μ M) and Nitisinone(100 μ M).

differences were observed between inhibitor pretreated and untreated cells (Fig. 3d). These observations confirmed that the NI-MO has high selectivity towards carboxylesterase 2 and three inhibitors have an effect to inhibit carboxylesterase 2 activity, while the inhibitory effect in the cell were not as good as that in vitro, it is possible that these inhibitors have the properties of prodrugs or are not specific inhibitors, so it reduced the inhibitory effect of carboxylesterase 2. The living cell imaging results suggested NI-MO has the potential to be used as an efficient fluorescence probe for tracking carboxylesterase 2 in complex biological samples.

Subsequently, to investigate the subcellular localization capacity, the intracellular localization of the NI-MO in Panc-1 cells and HPDE6-C7 cells were evaluated through co-staining with commercially available Lyso-Tracker (Lyso-Red). The green channel fluorescence from NI-MO overlaps with the red channel fluorescence from Lysosomal-Tracker. The correlation mapping of the green and red fluorescence channel exhibited excellent colocalization (Fig. 4a) with Pearson's correlation coefficient (PCC) values of 0.90 and 0.84 in Panc-1 cells and HPDE6-C7 cells, respectively. Co-localization of the fluorescent signals from NI-MO and Lysosomal-Tracker indicated that NI-MO was primarily localizing in lysosome. The high co-localization demonstrates that NI-MO has excellent targeting ability to the lysosomal carboxylesterase 2, once again verifying the advantage of our innovative design for the probe targeting and detecting. Overall, it is visualized to note that the intracellular fluorescence was result from the carboxylesterase 2 and the probe could be employed as a simple tool to visualize carboxylesterase 2, especially lysosomal carboxylesterase 2 in living cells, as well as for inhibitor screening in the field of drug development, underscoring the (pre-)clinical promise of the screened drugs as potential agents for

inhibiting carboxylesterase 2 activities in live cells.

In addition, in the context of establishing the suitability of the probe in cells with lysosomal carboxylesterase 2, we investigated the potential of the probe for cells detecting the lysosomal carboxylesterase 2 activity at native levels. To test if NI-MO could be used to distinguish cancer cells and normal cells by their lysosomal carboxylesterase 2 level, we incubated Panc-1 cells and HPDE6-C7 cells with NI-MO at the same condition. We assumed that NI-MO has similar penetrating ability for Panc-1 cells and HPDE6-C7 cells. From the confocal fluorescence microscopy images (Fig. 4b) one can see that the cell lines show different fluorescence intensities, therein the Panc-1 cells with NI-MO show stronger fluorescence intensity than HPDE6-C7 cells with NI-MO. The fluorescence data further confirmed that the fluorescence intensity of Panc-1 cells is stronger than that of HPDE6-C7 cells (Fig. 4c). It is due to the carboxylesterase 2 expression of Panc-1 cells is higher than that in HPDE6-C7 cells. So it's not just carboxylesterase 2, it is lysosomal carboxylesterase 2 activity. That is, it is consistent with the design expectation that it is encouraging that probe targeting lysosomal carboxylesterase 2 could recognize pancreatic cancer cells. In terms of application, the probe is not limited to the value of inhibitor screening, it was also clear that NI-MO is able to distinguish pancreatic cancer cells and normal pancreas cells by monitoring their different lysosomal carboxylesterase 2 expression levels.

3. Conclusion

In summary, we developed a new fluorescent probe NI-MO to detect the activity of carboxylesterase 2. Better still, NI-MO can be used as a tool in living cell by which the selective visualization of lysosomal

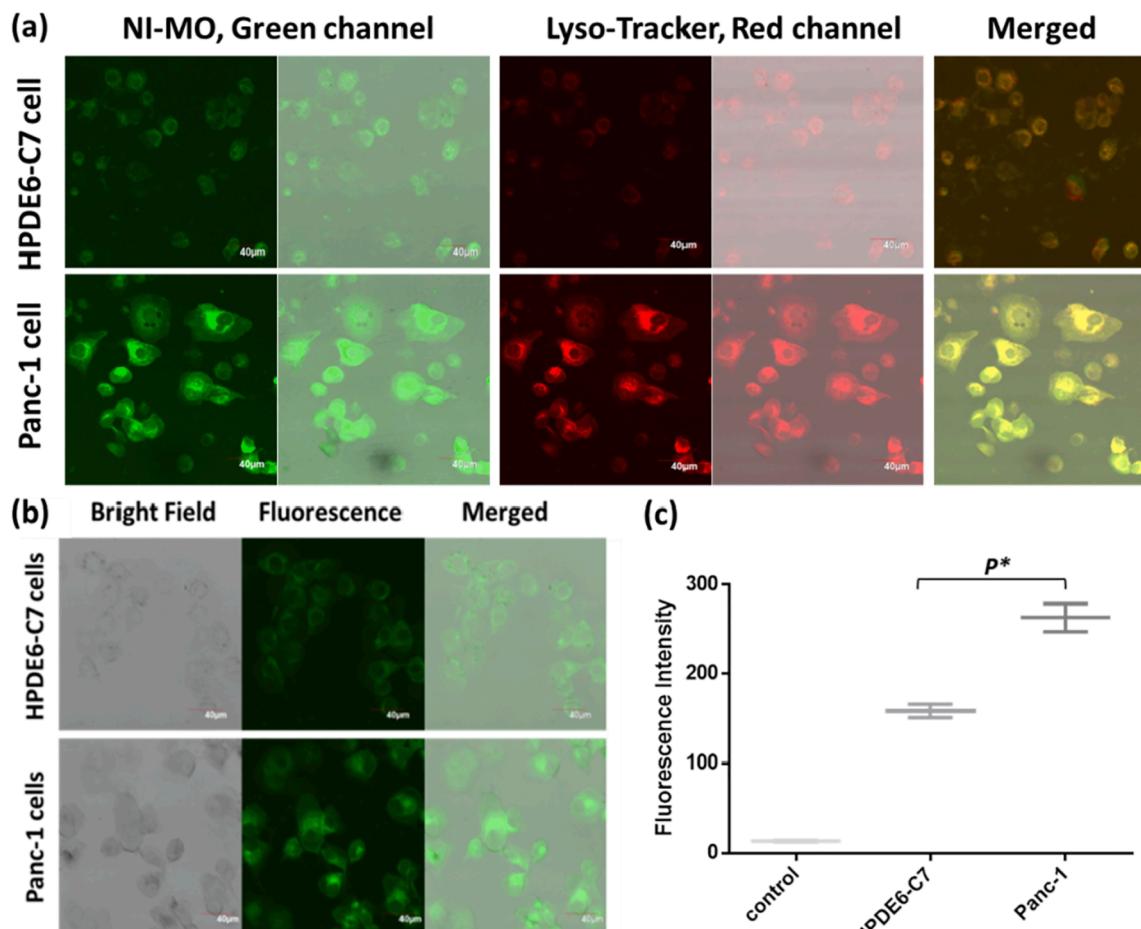


Fig. 4. (a) Confocal fluorescence images of Panc-1 cells and HPDE6-C7 cells treated with NI-MO (10 μ M, green channel) and Lyso-Tracker Red(50 nM, red channel) for 30 min at 37 °C, and fluorescence images were captured. (b) Confocal fluorescence images of NI-MO(10 μ M) in Panc-1 cells and HPDE6-C7 cells. (c) Fluorescence intensity of Panc-1 and HPDE6-C7 cells were treated with NI-MO(10 μ M). Values are expressed as the mean SEM($p^*<0.05$).

carboxylesterase 2. Our studies provided supporting for the notion that probe has a role to play in the identification of cancerous cells by assessment expression level of carboxylesterase 2, especially lysosomal carboxylesterase 2. It indicates NI-MO may help answering fundamental questions concerning carboxylesterase 2 expression in diseases. Furthermore, a simple high-throughput screening for drugs was constructed by NI-MO to screen drugs as carboxylesterase 2 inhibitors. This work not only proposed a selective visualization probe for human lysosomal carboxylesterase 2 to improve the reliability of probing cancer, but also illustrates a versatile tool for accelerating the discovery of drugs for cancer diagnosis and treatments.

Associated content

Supporting Information. Detailed experimental and theoretical procedures as well as schemes, supported figures and tables were included in the SI upon reading.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (grant numbers 21703245).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cplett.2021.139143>.

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