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Enzymatic profiling in prostate and breast cancer cells: phosphate hydrolysis and alcohol oxidation

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ABSTRACT

Enzyme activities in non-tumor and cancer cells, in different cell compartments, were visualized with two small fluorogenic probes: 7-(3-hydroxypropyl)-resorufin (**7**) and 7-(3-phosphatepropyl)-resorufin (**9**), with a primary alcohol and a phosphate group as enzymatic recognition sites. The designed probes were evaluated by in vitro assays, proving that both were biotransformed by their respective enzymes (probe **7** by alcohol dehydrogenases, ADHs, and probe **9** by a protein serine/threonine phosphatase, PSTP). Additionally, joint action of both enzymes (ADH and PSTP) revealed that probe **9** underwent a multi-enzymatic cascade and that phosphate hydrolysis is the rate-limiting step. These probes were evaluated in four human cell lines: prostate cancer cells (PC-3), prostate cells (RWPE-1), breast cancer cells (MCF-7 BUS) and breast cells (MCF-10A). Flow cytometry experiments showed distinct profiles for prostate and breast cells and assays with phosphatase inhibitors confirmed the proposed multi-enzymatic cascade in living cells.

1. Introduction

Cancer cells are insensitive to growth-inhibitory signals, have limitless replicative potential, invade tissues and cause metastasis.[1] These biological processes in tumor tissues are closely related to abnormal enzymatic activities, such as alcohol dehydrogenases (ADH) and protein phosphatases (PP).[2–10]

Alcohol dehydrogenases catalyze oxidation and reduction reactions with broad substrate tolerance. These enzymes protect living systems against excess endogenous alcohols, lipid peroxidation and some exogenous alcohols. The latter is the best characterized function of human ADHs.[11] Increased ADH activity in cancer cells when compared to healthy tissues may be related to carcinogenesis. The overexpression of highly active ADHs increases the amount of acetaldehyde in cancer cells, a highly toxic metabolite of alcohol metabolism, which may cause DNA damage. In this context, ADHs are promising candidates as tumor markers, and monitoring such activities in live cell assays would help rationalize the role of these enzymes in cancer.[7–10,12]

Protein phosphorylation is one of the most important reversible, post-translational modifications (PTMs) of proteins in eukaryotic cells. This type of reversible PTM plays a critical role in the regulation of a broad variety of cellular events, such as gene expression, cell division, differentiation, apoptosis, transport, cell locomotion, learning and memory.[6,13,14] The dynamic and reversible process of protein phosphorylation on specific serine (Ser), threonine (Thr) or tyrosine (Tyr) residues is performed by protein kinases (PKs) and PPs. Kinases catalyze protein phosphorylation, whereas PPs catalyze protein dephosphorylation. Aberrant phosphorylation profiles are directly connected to numerous human diseases, such as cancer, diabetes and neurodegenerative or inflammatory disorders.[15–17]

PP and ADH activities are commonly detected with chromogenic or fluorogenic substrates, which exhibit significant signal changes of their spectroscopic properties in association with structural changes.[18–23] However, these methodologies have to be adapted to real-time live-cell imaging experiments due to cell background interference ($\lambda_{ex(NADH)}$=350 nm and $\lambda_{em(NADH)}$=460 nm; $\lambda_{ex(FAD)}$=360 and 450 nm and $\lambda_{em(FAD)}$=520 nm).[24]

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In this context, we synthesized two fluorogenic probes derived from resorufin and evaluated their ability to detect ADH and PP activities in human non-tumor and cancer cells, as determined by flow cytometry and confocal microscopy.

2. Results and discussion

2.1. Fluorogenic probes design

Enzymes related to cancer onset and development stages are present in various compartments and in crowded environments in living cells; thus, the understanding of these processes requires the design of molecular probes to monitor real-time enzymatic activities.[25] In our first attempt to design such probes, umbelliferone (**1**) was selected as the fluorogenic moiety because the alkylation of its 7-hydroxyl group produces an almost non-fluorescent compound.[26,27] Additionally, an alkyl linker was inserted between **1** and the hydroxyl (**2**) or phosphate group (**3**), with **3** mimicking a protein phosphoserine residue (Fig. 1).

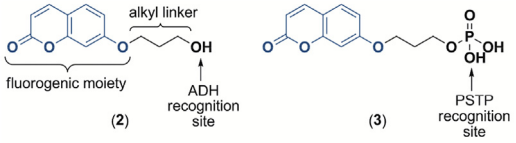
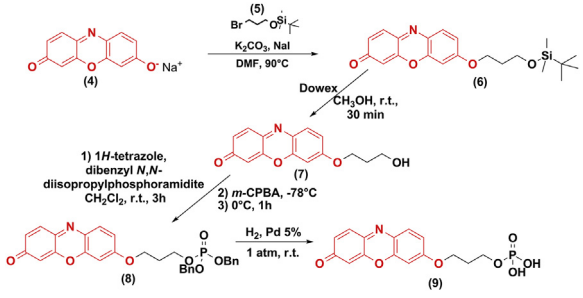


Fig. 1. Structure of fluorogenic ADH (**2**) and PSTP (**3**) probes derived from umbelliferone.

However, initial live-cell imaging experiments revealed that the superposition of the cell background fluorescent signals (i.e., λ_{ex}(NADH)=350 nm and λ_{em}(NADH)=460 nm)[24] and umbelliferone (**1**) (λ_{ex}=360 nm and λ_{em}=460 nm) hindered accurate data acquisition and rendering. Therefore, the umbelliferone moiety (**1**) was substituted by resorufin (**4**), which has an excitation wavelength at 572 nm and emission at 585 nm, which are more appropriate for live-cell assays.

Resorufin-based probes designed to detect phosphatases (**9**) and alcohol dehydrogenases (**7**) were synthesized as described in Scheme 1. Briefly, alkylation of resorufin (**4**) with (3-bromopropoxy)(*tert*-butyl)dimethylsilane (**5**) in DMF, followed by deprotection of TBS ether **6**, gave probe **7** in 90% yield. Finally, treatment of alcohol **7** with dibenzyl *N*,*N*-diisopropylphosphoramidite and 1*H*-tetrazole, followed by oxidation with *m*-CPBA and hydrogenolysis to remove the benzyl (Bn) protective groups, gave probe **9** in 38% overall yield as an orange amorphous solid.



Scheme 1. Synthesis of fluorogenic probes **7** and **9**.

The products and intermediates were characterized by high-resolution mass spectrometry (HRMS) and by ^{1}H and ^{13}C NMR, as described in Section 4.2.

The stability and fluorescence latency of probes **7** and **9** was analyzed in Dulbecco's modified Eagle's medium (DMEM) by monitoring for eight hours. Both compounds exhibited low initial fluorescent signals, displayed good chemical stability under the experimental conditions and were only 1% (**7**) and 10% (**9**) as fluorescent as resorufin. These characteristics made both compounds promising candidates to investigate the enzymatic activities by on/off fluorescence methodologies.

Therefore, the efficiencies of molecular probes **7** and **9** to detect ADH and phosphatase enzymatic activities were evaluated, as described below, using in vitro enzymatic assays and flow cytometry and confocal microscopy live-cell experiments.

2.2. In vitro enzymatic assays

Probe **7** was evaluated using three commercial ADHs (Sigma–Aldrich 16892, 79854 from horse liver, and A7011 from *Saccharomyces cerevisiae*), taking into consideration that analogous alcohols were oxidized by isolated ADH.[28] All evaluated enzymes catalyzed the oxidation of **7** to aldehyde **10**, which spontaneously underwent β-elimination, releasing the fluorescent resorufin anion (Scheme 1 and Table 1, line 1). The best performance was observed using ADHs from horse liver, which is homologous to human ADH and converted **7** into **4** in 90% yield within 24 h. Similar results were obtained with ADH from *S. cerevisiae*, attesting to the probe's efficiency.

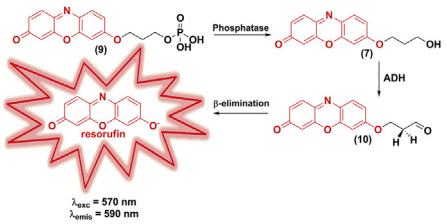
Table 1
Summary of the in vitro enzymatic assays with probes **7** and **9**

	Enzymatic activity (% of conversion)[a]		
	HL ADH[b]	SC ADH[c]	16892 ADH[d]
Probe **7**	89	91	24
Probe **9**+Calcineurin[e]	34	23	7

[a] Data recorded in 24 h.
[b] Horse liver ADH.
[c] *Saccharomyces cerevisiae* ADH.
[d] 16892 Sigma ADH.
[e] Multi-enzymatic assay.

Probe **9**'s ability to reveal phosphatase activity was monitored with calcineurin, a Ca^{2+}/calmodulin-regulated PSTP encoded in the human genome, which plays a key role in Ca^{2+}-mediated signal transduction and T-cell activation. This phosphatase is overexpressed in various human cancers, making it a good target for activity monitoring with the proposed probe.[29,30] Calcineurin-catalyzed phosphate hydrolysis was detected using malachite green reagent, revealing that probe **9** produced alcohol **7** and inorganic phosphate (8% conversion in 24 h).

Additionally, probe **9** was applied to monitor the multi-enzymatic cascade involving phosphatase and alcohol dehydrogenase, a three-step cascade process shown in Scheme 2.[31,32] The multi-enzymatic experiments revealed that probe **9** underwent this cascade and that best results were obtained with



Scheme 2. Proposed enzymatic cascade and resorufin release (fluorescence signal).