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Small Molecule Colorimetric Probes for Specific Detection of Human Arylamine *N*-Acetyltransferase 1, a Potential Breast Cancer Biomarker

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Probes to enable the specific detection of biomacromolecules have been widely used in cellular biology and pathology. Selective detection of native proteins using antibodies or recombinant proteins using affinity tags is now well established.[1] Small molecule probes[2] to selectively label native[3] or engineered[4] proteins have been reported, but the majority involve covalent tagging of the target. We describe here a specific, noncovalent sensor to detect a potential protein biomarker in its native form without the need for a tag or an antibody.

Arylamine *N*-acetyltransferases (NATs) are found in a range of species and catalyze the transfer of an acetyl group from acetyl coenzyme A (AcCoA) to arylamines.[5] Human NATs were originally identified as drug metabolizing enzymes,[5a] but more recently human NAT1 (hNAT1) has been implicated in cancer[6] and, along with its homologue mouse Nat2 (mNat2), in development.[7] Human *NAT1* (*hNAT1*) is one of the 10 most highly overexpressed genes in estrogen-receptor-positive (ER+ve) breast tumors, in contrast with ER-ve tumors, and a strong association exists between *hNAT1* overexpression and breast tumor grade.[8] This suggests that hNAT1 plays a role in breast cancer progression and is a candidate biomarker in ER+ve breast cancer.[8] To identify hNAT1 inhibitors to probe its endogenous role,[9] we screened 5000 compounds against 5 different NATs.[10] Among the specific hNAT1 inhibitors identified in the screen was naphthoquinone **1**, which was observed to exhibit some striking properties, described herein (Figure 1).

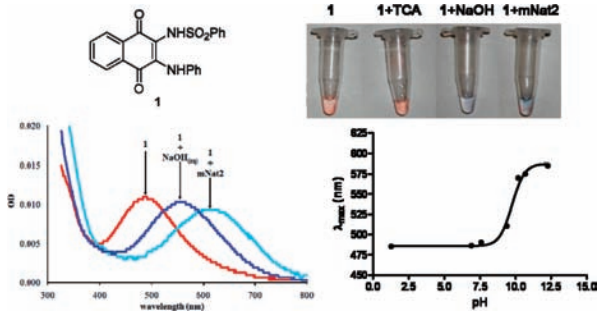


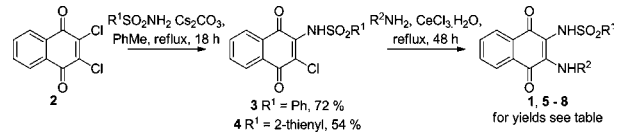
Figure 1. Top right: **1** (400 μM) in aq. Tris·HCl (pH 8.0); 20% trichloroacetic acid (TCA); 4 M aq. NaOH; or mNat2 in aq. Tris·HCl (pH 8.0). Bottom left: Visible spectra of **1** (10 μM) in aq. Tris·HCl (pH 8.0, red line, λ_{max} = 489 nm), **1** in aq. NaOH solution (dark blue line, λ_{max} = 561 nm), and **1** with 2 equiv of mNat2 in aq. Tris·HCl (pH 8.0, light blue line, λ_{max} = 610 nm). Bottom right: pH titration curve for **1** (pK_a ~9.5).

1 was shown to be a selective inhibitor of hNAT1 and mNat2, with IC_{50} = 1.65 and 1.86 μM, respectively, but showed no inhibition against the other human and mouse NAT enzymes. **1** was also shown to be a competitive inhibitor of mNat2 (K_i = 520 nM). Moreover, a solution of **1** was observed to undergo a distinctive color change, observable by eye, from red to blue in the presence of either hNAT1 or mNat2 but not the other isozymes. This is consistent with the established functional similarity between hNAT1 and mNat2: they have been shown to share a substrate specificity profile[9b] and biological properties.[7] The observed differences between substrate and inhibitor specificity profiles in this isozyme family exist even though all the isozymes share over 70% identity in the amino acid sequence (Supplementary Table 1).[5b,9b,11]

When an acid−base titration was carried out, the color change was shown to be a pH-dependent event with a pK_a of ~9.5[12] (Figure 1). *Thus, we hypothesized that* **1** *was undergoing a deprotonation within the active site of hNAT1 or mNat2 upon selective binding, but no deprotonation was occurring with other NAT isoforms.*[13]

To probe the scope of the proposed recognition event between **1** and hNAT1 and mNat2, a series of analogues of **1** were next prepared *via* a two-step protocol from dichloronaphthoquinone **2**. Intermediates **3** and **4** were first prepared from **2** in 72% and 54% yields, respectively. Optimal conditions for the second substitution were determined to be treatment of **3** or **4** with the requisite aniline and cerium chloride[14] to afford **1** and **5**−**8** in 10−88% yield (Table 1). Analysis of the colorimetric properties of **1** and **5**−**8** and their inhibitory activities against hNAT1 and mNat2 were next undertaken. All the compounds showed, to varying degrees, inhibitory activity against the enzymes (Figure 2, Table 2), and many of the compounds again showed a distinctive color change upon basification or in the presence of mNat2.[13]

Table 1. Preparation and Assessment of Compounds **1**, **5**−**8** As Inhibitors of mNat2[a]



entry	substrate	R^1	R^2	product	yield (%)	IC_{50} (μM)
1	**3**	Ph	Ph	**1**	63	1.86
2	**3**	Ph	4-BrC_6H_4-	**5**	10	0.88
3	**3**	Ph	4-^{t}BuC_6H_4-	**6**	60	5.86
4	**3**	Ph	3,5-$Me_2C_6H_3$-	**7**	53	0.99
5	**4**	2-thienyl	Ph	**8**	88	3.12

[a] mNat2 activity was determined as hydrolysis of AcCoA in the presence of *para*-amino benzoic acid (*p*ABA).

Compound **7** was identified as showing moderately improved potency (IC_{50} 0.99 μM vs mNat2), inhibition of hNAT1 in breast cancer cell lysates (90% inhibition at 5 μM), and a markedly increased absorption at 498 and 610 nm (Figure 2), and subsequent studies therefore focused on **7**.

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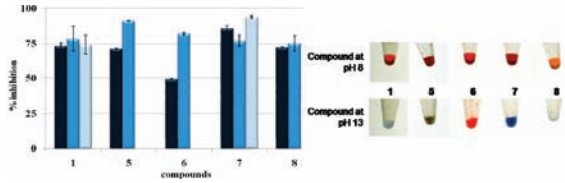

Figure 2. Left panel: Screening of **1**, **5**−**8** (5 μM) against pure mNat2 (dark blue columns), hNAT1 in *Escherichia coli* cell lysate (blue columns), and hNAT1 in breast cancer cell lysate (ZR-75-1 cells, **1** and **7** only, light blue columns). NAT activity was determined as acetylation of *p*ABA in the presence of AcCoA. Right panel: Compounds **1**, **5**−**8** (2 mM) in Tris·HCl buffer (pH 8.0, top) or aq. NaOH (bottom).

Table 2. Color Change of **1**, **5**−**8** (10 μM) with aq. NaOH or mNat2

Compound	**1**	**5**	**6**	**7**	**8**
λ_{max} (nm) in buffer	489	487	484	498	490
$\Delta\lambda_{max}$ with NaOH (nm)	+72	+49	0	+74	+69
$\Delta\lambda_{max}$ with mNat2 (nm)	+121	+123	0	+127	+125

As an alternative explanation, the susceptibility of the naphthoquinone function toward reduction led us to investigate whether any reducing agents may give rise to the color change of **7**, especially DTT which is present in the recombinant protein solution at 34 μM. Treatment of **7** with DTT at 34 μM gave no reduction and did not affect its color, but at high concentration (100 mM) DTT led to a decolorization of a solution of **7**, which was reversed upon exposure to air.[15] Other reducing agents such as NAD(P)H gave no visible change, and only treatment with base gave the characteristic red shift in the visible spectrum of **7**.

To assess specificity more broadly, **7** was tested for inhibitory activity and colorimetric properties with a range of other mammalian and bacterial NAT enzymes (Table 3). Although partial crossreactivity was observed with mNat1, the color change was only observed with mNat2 and hNAT1 (Figure 3).

Table 3. Effect of **7** (10 μM) on the Activity of Different NATs, Determined As Hydrolysis of AcCoA in the Presence of 5-Aminosalicylate, and λ_{max} Determined from the Visible Spectra of **7** in the Presence of Different NATs (See Supporting Information)

Enzyme	hNAT1	mNat2	mNat1	STNAT	PANAT	MMNAT	MSNAT
Inhibition (%)	91.7 ±1.8	92.8 ±0.4	55.9 ±6.4	< 5	19.6 ±3.0	< 5	< 5
λ_{max} (nm)	585	625	498	498	498	498	498

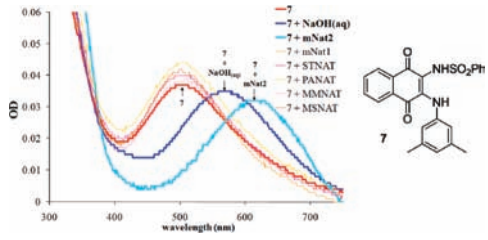

Figure 3. Visible spectra of **7** (10 μM) with different NATs (20 μM).

Having confirmed the specificity of **7** for mNat2, the colorimetric properties of **7** were used to assess the dissociation constant (K_d) and the number of binding sites. The molar extinction coefficients (ε) at 514 nm of the red and blue species were determined to be 14 336 and 7215 M^{-1} cm^{-1}, respectively. The optical density of varying concentrations of **7** with mNat2 at 514 nm allowed an estimate of the concentration of free **7**. Michaelis−Menten and Scatchard analysis supported a single binding site and gave a K_d value of 4.9 μM (Figure 4).

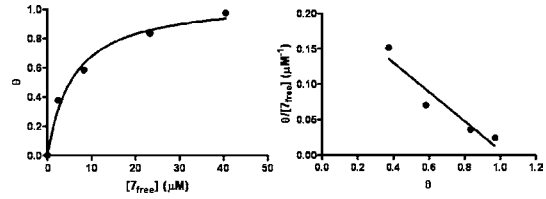

Figure 4. Assessment of the number of binding sites and K_d value for **7** with mNat2 by Michaelis−Menten curve (left) and Scatchard plot (right).

In summary, we have identified a family of reagents which bind selectively to human NAT1 and its murine homologue mouse Nat2 with a concomitant color change driven by a proton transfer event. As the expression of hNAT1 is related to tumor type, this property may be subsequently exploited for diagnosis.

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Supporting Information Available: All experimental procedures; compound characterization data; complete ref 6a. This material is available free of charge via the Internet at http://pubs.acs.org.

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