Bioorganic & Medicinal Chemistry Letters 23 (2013) 2093–2097

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Fluorescent probes designed for detecting human serum albumin on the basis of its pseudo-esterase activity

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ARTICLE INFO

Article history:
Received 5 January 2013
Accepted 29 January 2013
Available online 7 February 2013

Keywords:
Activity-based probe
Human serum albumin
Esterase

ABSTRACT

We developed activity-based fluorescent probes for detecting human serum albumin (HSA) on the basis of its pseudo-esterase activity. These probes could also detect HSA in blood-contaminated tissue samples.

Human serum albumin (HSA) is the most abundant protein in human blood plasma, with a concentration of 0.7 mM.[1] It is a 66-kDa protein that is produced in the liver and helps maintain the osmotic pressure of body fluids. HSA works as an extraordinary molecular sponge because it can bind many endogenous and exogenous compounds, including hormones, fatty acids, vitamins, heme, metabolites, and drug molecules at different sites in 3 homologous subdomains.[1] This sponge-like ability of HSA significantly influences the pharmacokinetic and pharmacodynamic effects of many exogenous drugs.[2,3]

Interestingly, HSA exhibits esterase-like activity and hydrolyzes drugs having an ester group, such as acetylsalicylic acid (aspirin).[4–6] Lys199, Lys402, Lys519, and Lys545, which form the pseudo-esterase active site of HSA, are acetylated by aspirin, which allows these acetylated lysine residues to remain unhydrolyzed for over 3 weeks under normal physiological conditions.[6] Drug-induced acetylation of HSA may inhibit the transport of endogenous biomolecules[7] and the natural esterase activity of HSA.[8] Therefore, sensitive monitoring of aspirin-induced acetylation of HSA is crucial in early drug development and clinical drug dosing.

Calorimetric or chromatographic techniques have been developed for analyzing the binding of drugs to HSA,[2] but these techniques are rather laborious to implement and are not adaptable to high-throughput screening. In a recent study, Chang and co-workers used a combinatorial approach to develop fluorescent probes that can non-covalently bind to HSA; these probes proved useful in identifying drugs that bound more strongly to HSA than did the probes.[9,10] However, these non-covalently binding HSA probes cannot be used for detecting HSA in denaturing conditions, such as in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Herein, we report the in-gel fluorescence detection of HSA by using activity-based probes (ABPs) that could covalently bind to HSA on the basis of its esterase-like activity.[11,12] These probes could selectively detect blood contamination in patient-derived protein samples.

ABPs were originally developed using mechanism-based inhibitors (suicide inhibitors)[13] and designed for catalysis by enzymes and covalent binding to the active sites of enzymes. Some researchers have developed ABPs for selective detection of a certain class of enzymes in whole-cell proteomes for proteome profiling[14,15] or to diagnose cancer progression by imaging.[16,17] Some ABPs have been developed on the basis of the formation of a quinone methide as a reactive intermediate by the action of carboxylesterase;[18–21] however, these probes have not been tested until now for detecting HSA.

Because HSA shows pseudo-esterase activity, we attempted to develop ABPs that could be activated by both HSA and normal hydrolases. Our results showed that HSA activated the ABPs (probes **1** and **4**) to a greater degree than normal hydrolases did. HSA hydrolyzes the reactive acyl moiety of the probe, cleaves the carbamate bond to release 4-nitroaniline and carbon dioxide, and eventually produces quinone methide as a temporarily unstable intermediate. Subsequently, a covalent bond is formed between the quinone methide and a nucleophile near the active site of the enzyme, resulting in the formation of a probe–protein complex (HSA-**1**; Scheme 1).

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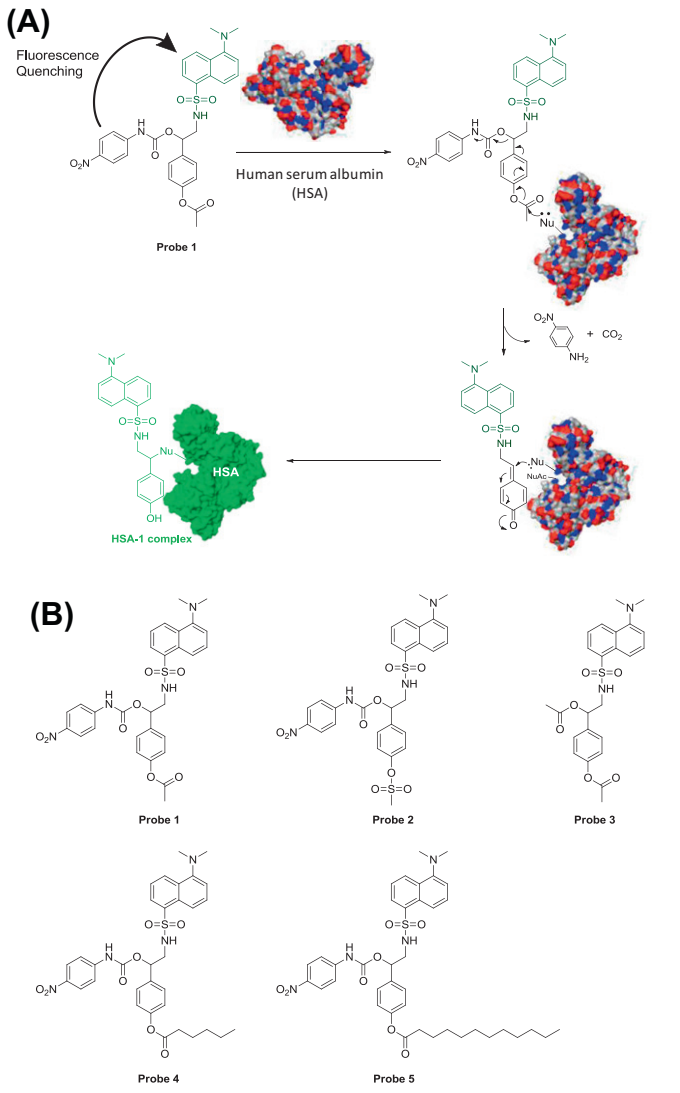
http://dx.doi.org/10.1016/j.bmcl.2013.01.124



Scheme 1. (A) Mechanism underlying the binding of probes to human serum albumin (HSA). (B) Chemical structures of activity-based probes for HSA.

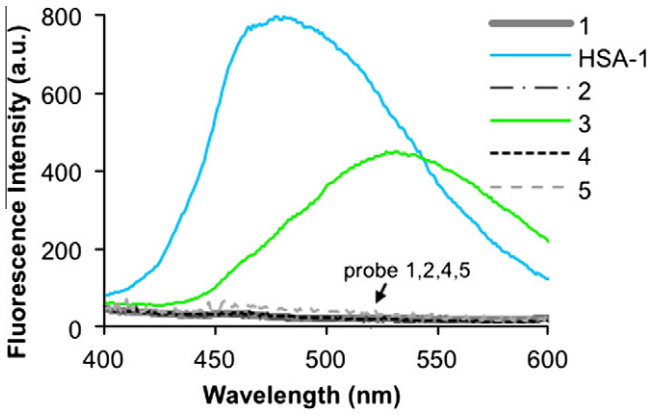


Figure 1. Fluorescence emission spectra (excitation at 340 nm) of each probe (10 μM) and the purified HSA protein–probe **1** complex (HSA-**1**) (10 μM) in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 10 mM) buffer (pH 7.4).

We synthesized 5 probes having the following acyl groups of various lengths: probe **1**, acetyl group; probe **2**, methanesulfonyl (mesyl) group, which is not normally hydrolyzed by esterases; probe **3**, acetyl group in place of 4-nitrophenylcarbamate in the benzylic position; probe **4**, hexanoyl group; and probe **5**, dodecanoyl group.

All these probes have a dansyl group as the fluorescent reporter and 4-nitrophenylcarbamate as the fluorescent quencher for the dansyl fluorophore.

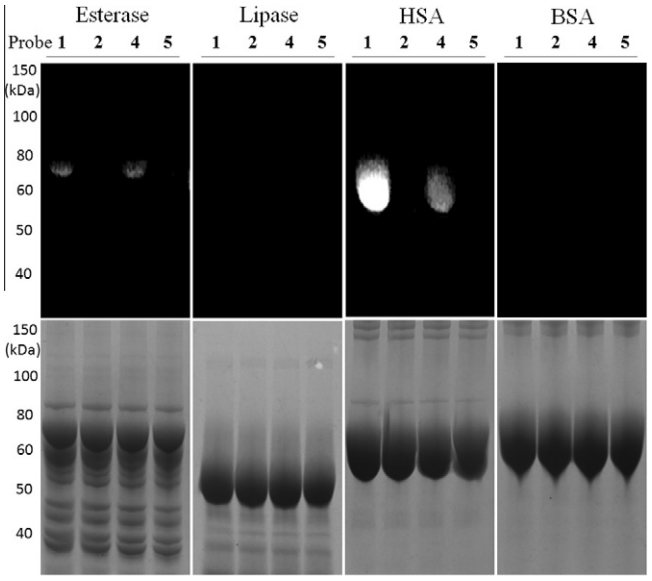


Figure 2. In-gel (8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE]) fluorescence (upper) and Coomassie Brilliant Blue stain (below) gel images of esterase (from porcine liver), lipase, HSA and BSA (each 20 μg) after a 12-h incubation with probes **1**, **2**, **4**, and **5** (each 50 μM) at 30 °C.

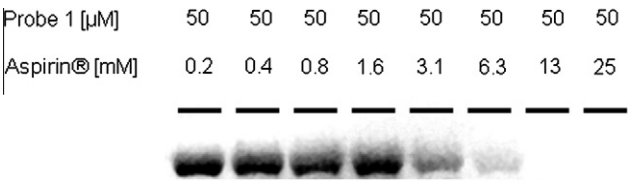


Figure 3. In-gel (8% SDS–PAGE) fluorescence showing loss of esterase activity of HSA (5 mg/mL) after co-incubation with aspirin at various concentrations of aspirin and probe **1** (50 μM).

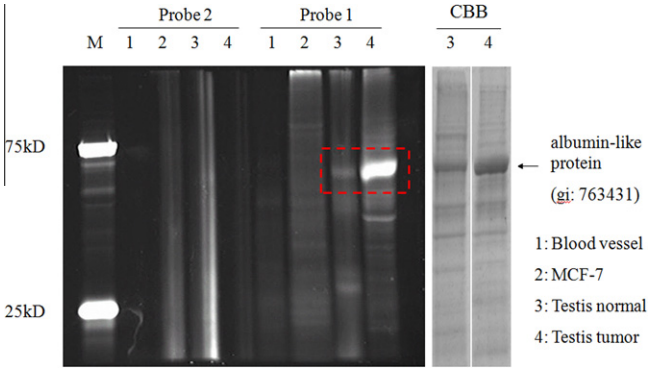


Figure 4. In-gel (12% SDS–PAGE) fluorescence (left) and Coomassie Brilliant Blue (CBB, right) gel staining of lysate samples of blood vessel cells, Michigan Cancer Foundation-7 (MCF-7) breast cancer cells, normal testicular cell lysate, and testicular tumor cell lysate after incubation with probes **1** and **2** (50 μM). A strong fluorescent band was identified as an albumin-like protein (gene identification [GI] number: 763431) by tandem-MS spectrometry (see SI).

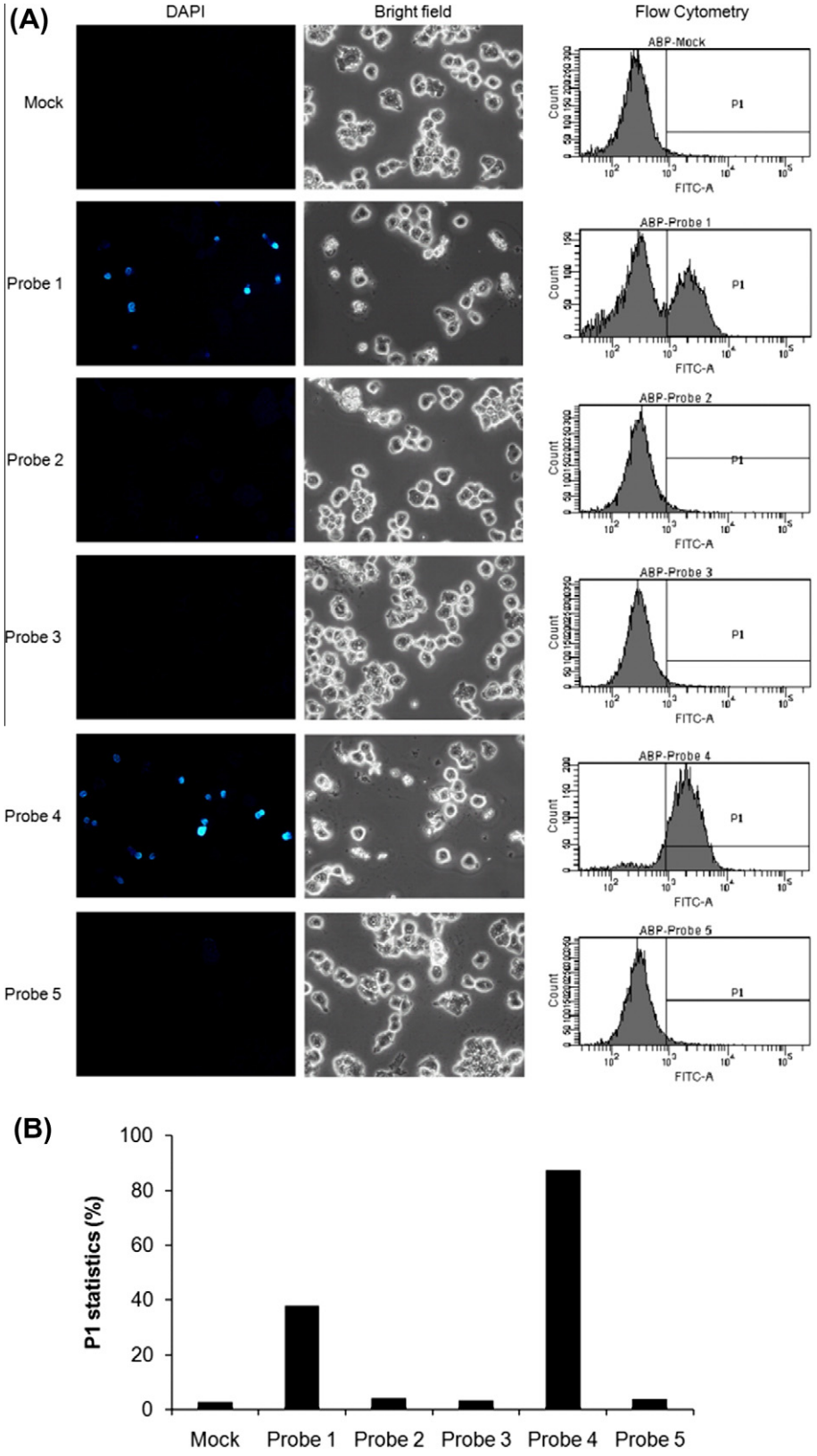


Figure 5. Activity-based labeling of human acute monocytic leukemia (THP-1) cells. (A) Fluorescence detection by fluorescence microscopy and flow cytometry. (B) Fluorescence intensity (P1, fluorescence intensity region in the flow cytometry results) shown by each probe for activity-based staining of THP-1 cells. The fluorescence was measured after 72 h incubation of the probes with the cells at 35 °C.

The fluorescence of probes **1**, **4**, and **5** was completely quenched by 4-nitrophenylcarbamate, whereas that of probe **3** remained unquenched (Fig. 1). As expected, the fluorescence emission intensity of probe **1** (10 μM) was greatly enhanced (~35-fold at 477 nm)

on incubation with HSA (10 μM). HSA-**1** was detected as a fluorescent band on SDS–PAGE when the gel was photographed under ultraviolet (UV) light (Fig. 2). HSA-**1** was successfully purified by size-exclusion/gel-filtration column chromatography (see supplementary data [SI]). All experimental data indicated that probe **1** was covalently bound to HSA.

It is noteworthy that, compared to the emission intensity of probe **3** (maximum wavelength [λ_{max}] = 531 nm), the emission intensity of HSA-**1** (10 μM) was enhanced with a blue-shift (54 nm) in its λ_{max} (477 nm), which indicates that the dansyl fluorophore in HSA-**1** could be surrounded by the hydrophobic environment of HSA.[22,23]

In order to compare the activities of the probes toward various enzymes, 4 probes (**1**, **2**, **4**, and **5**; each of 50 μM) were incubated with porcine liver esterase, lipase, HSA, and BSA at 30 °C for 12 h. As shown in Figure 2, probes **1** and **4** showed a higher covalent binding with HSA than with the other hydrolases, including esterase (Fig. 2). This could be because the binding site of HSA is larger than that of esterase, which makes it easier for a substrate to bind to HSA. Only the HSA proteins were labeled when probes **1** and **4** were incubated with human plasma. No fluorescence was observed in BSA on being labeled with these probes. The results of our study were in agreement with those of previous studies in which BSA had considerably weaker esterase-like activity than HSA.[24] We also tested the probes with other potential ester hydrolases (cholesterol esterase, alkaline phosphatase, phosphodiesterase, proteinase K) but these enzymes showed no labeling with our probes (**1**, **2**, **4**, and **5**; unpublished data).We found that HSA could be labeled much faster by using probe **1** than by using probe **4** (see SI).

We noted that probe **2** did not react with HSA or any other ester hydrolase, although its structure was similar to that of probe **1**, except for substitution of the mesyl group with the acetyl group. Because the mesyl group was not susceptible to hydrolysis by HSA or hydrolases, no labeling reaction or protein bands were observed. This result indicated that hydrolysis of the acetyl group in probe **1** by HSA plays a crucial role in triggering the covalent attachment of the probe to HSA. Further, HSA was not labeled when probe **5** was used, possibly because the entry of probe **5** into the reactive core of HSA was prevented by the dodecanoyl group of probe **5**. In-gel fluorescence showed that probe **3** was covalently bound to HSA (data not shown); this implies that the acetyl group in the benzylic position of the probe acted as the leaving group during the enzymatic reaction with HSA. Incubation of HSA with probe **3** resulted in a smaller change in fluorescence intensity than did its incubation with probe **1**, because of the lack of a fluorescence-quenching moiety. Thus, we selected probe **1** for further studies. Further, we determined whether probe **1** could detect the aspirin-induced loss of pseudo-esterase activity of HSA. Because acetylation of the active site of HSA results in loss of the pseudo-esterase activity of HSA, HSA would not react further with our probes. Thus, acetylated HSA could be identified by the diminished fluorescence labeling intensity of the probe. As expected, the fluorescence labeling intensity of the probe distinctly decreased in the presence of 1 mM aspirin, and the fluorescence intensity of the labeled band was negligible after co-incubation with probe **1** and 13 mM aspirin at 30 °C for 12 h (Fig. 3).

Furthermore, we used the ABPs for selective detection of blood contamination in patient-derived protein samples. Patient-derived samples are commonly contaminated by blood, therefore, assessing the level of blood contamination in these samples is important for estimating the performance of medical experiments or diagnostic tests (e.g., immunoblotting). Abundant HSA can compromise the accurate analysis of less-abundant blood proteins that could be analytical biomarkers for human diseases.[25,26] Hence, depletion of highly abundant albumin from the plasma sample is necessary for the mass analysis of human blood plasma proteome and can be performed using several processes.[27] Therefore, direct in-gel detection of HSA can be very useful for checking HSA (or blood) contamination level in human-derived samples. If a 66-kDa fluorescent protein is detected in SDS–PAGE after sample incubation with our probes, it can be assumed to be HSA, and we could confirm that the sample is contaminated with blood or HSA.

We tested different kinds of human-tissue lysates and cultured-cell lysates (each 20 μg) by incubating them with probes **1** and **2** (each 50 μM) at 30 °C for 12 h, and then performed SDS–PAGE. A fluorescent band strongly corresponding to a 66-kDa protein was observed for testicular tumor tissue lysate with probe **1** (Fig. 4). No fluorescent proteins were detected with probe 2. We identified the fluorescent band as an albumin-like protein (gene identification [GI] number: 763431) after performing protein sequence analysis using liquid chromatography–tandem mass spectrometry (LC/MS/MS). Therefore, we concluded that among different samples tested, testicular tumor-tissue lysate was more contaminated with blood than other samples were. Media-grown cell lysate of MCF-7 cells was used as the negative control and showed negligible labeled protein bands in its whole proteome.

To check the reactivity of probes toward the whole proteome of media-grown living cells, we also performed imaging experiments with the quenched probes in a medium containing acute monocytic leukemia (AMoL) cells, which are known to have strong esterase activity.[28,29] Each probe (5 μM) was incubated with live THP-1 cells (human AMoL model cell line) at 35 °C. The fluorescence was assessed by fluorescence microscopy and flow cytometry. We found that fluorescence could be detected after 72 h, which was a much longer reaction time than that observed for HSA (3 h). These findings were similar to those obtained in in vitro study, where only probes 1 and 4 reacted with the cells showing bright fluorescence (Fig. 5). Furthermore, distinct cell labeling was observed on using probe 4 than on using probe **1**. Probes **2**, **3**, and **5** did not show any fluorescence staining.

Quinone methide intermediate can diffuse away from an enzyme active site and react with any protein that it comes in contact with.[30] However, there was no loss of sensitivity or selectivity during the detection of HSA in tissue lysates and esterase activity in THP-1 cells.

In conclusion, we developed selective probes for detecting HSA on the basis of its pseudo-esterase activity. By using these probes, we were able to observe the drug-induced loss of pseudo-esterase activity of HSA. Furthermore, these probes could detect blood contamination in human-derived tissue lysates.

Acknowledgments

This work was supported by the National Research Foundation (NRF) Grant funded by the Ministry of Education, Science and Technology (MEST; grant number, 2012-0000159). H.-W.R. is recipient of the POSCO TJ Park Postdoctoral Fellowship. We also wish to acknowledge the BK21 fellowship grants received by H.-W.R. and H.-J.P.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.124.

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