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An activity-based probe for high-throughput measurements of triacylglycerol lipases

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ABSTRACT

Modulating the activity of lipases involved in the metabolism of plasma lipoproteins is an attractive approach for developing lipid raising/lowering therapies to treat cardiovascular disease. Identifying small molecule inhibitors for these membrane-active enzymes, however, is complicated by difficulties associated with measuring lipase activity and inhibition at the water–membrane interface; substrate and compound dynamics at the particle interface have the potential to confound data interpretation. Here, we describe a novel ELISA-based lipase activity assay that employs as "bait" a biotinylated active-site probe that irreversibly binds to the catalytic active-site serine of members of the triacylglycerol lipase family (hepatic lipase, lipoprotein lipase, and endothelial lipase) in solution with high affinity. Detection of "captured" (probe–enzyme) complexes on streptavidin-coated plates using labeled secondary antibodies to specific primary antibodies offers several advantages over conventional assays, including the ability to eliminate enzyme–particle and compound–particle effects; specifically measure lipase activity in complex mixtures *in vitro*; preferentially identify active-site-directed inhibitors; and distinguish between reversible and irreversible inhibitors through a simple assay modification. Using EL as an exemplar, we demonstrate the versatility of this assay both for high-throughput screening and for compound mechanism-of-action studies.

Members of the triacylglycerol lipase family, including endothelial lipase (EL),[1] lipoprotein lipase (LPL), and hepatic lipase (HL) are *sn-1* lipases that play a central role in triglyceride and/or phospholipid hydrolysis within plasma lipoproteins. Despite the high sequence homology among EL, LPL, and HL [1], they have been shown to display distinct preferences both for their lipid substrates and for the particles on which they act: EL predominantly hydrolyzes phospholipids in HDL particles; LPL predominantly hydrolyzes triglycerides in ApoB-containing particles; and HL readily hydrolyzes both triglycerides and phospholipids in all classes of lipoproteins [2]. Extensive knockout and overexpression studies in mice have confirmed the importance of EL, HL, and LPL mass on the overall lipid profile [3–7]. Recently, a double EL:HL knockout mouse was constructed, both of which demonstrated the complementary effects of each lipase on the metabolism of HDL and apoB-containing lipoproteins, and highlighted the potential importance of developing EL inhibitors that are exquisitely selective against HL, in addition to LPL [8].

Assays designed to measure lipase activity and identify inhibitors or activators thereof typically use radiolabeled or fluorescently labeled lipid substrates emulsified in glycerol and/or non-ionic detergents such as Triton X-100 [9]. More sophisticated lipase assays using synthetic- or native-HDL particles have been developed recently and are amenable for screening large sample sets in high-throughput mode [10,11]. While each of these formats offers unique capabilities, the requirement for inclusion of lipid particles in these assays introduces potential artifacts related to compound–particle interactions, which could dramatically alter the apparent potency of a particular compound, through either perturbation of the particle/substrate or sequestration of compound within particles. One approach to circumvent such lipid particle effects is to measure lipase activity using a soluble substrate, usually a short alkyl chain linked through an ester linkage to a chromophore that fluoresces on cleavage, which does not require emulsification. The general utility of the small, soluble substrate approach, however, is limited by the promiscuity of such substrates to esterases in general, precluding lipase-specific measurements in complex mixtures. Moreover, care must be taken to

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[1] *Abbreviations used:* ABP, activity-based probe; DMSO, dimethyl sulfoxide; EL, endothelial lipase; HL, hepatic lipase; HUVEC, human umbilical vein endothelial cells; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; SEM, standard error of the mean; TNF, tumor necrosis factor.

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ensure that substrates remain "soluble", and do not form mixed micelles in the presence of certain compounds.

Here, we describe the development of a particle-free, lipase active-site occupancy assay using a biotinylated activity-based probe that forms an irreversible covalent bond with the lipase active-site catalytic serines through a designed sulfonyl fluoride warhead. Detection of streptavidin-captured probe:lipase complexes with antibodies specific to either EL or LPL engenders the ability to differentially and specifically measure the activity and inhibition of different lipases simultaneously, and in complex mixtures. Through simple modifications of this assay, we further demonstrate the utility of this approach for critical inhibition mechanism-of-action studies.

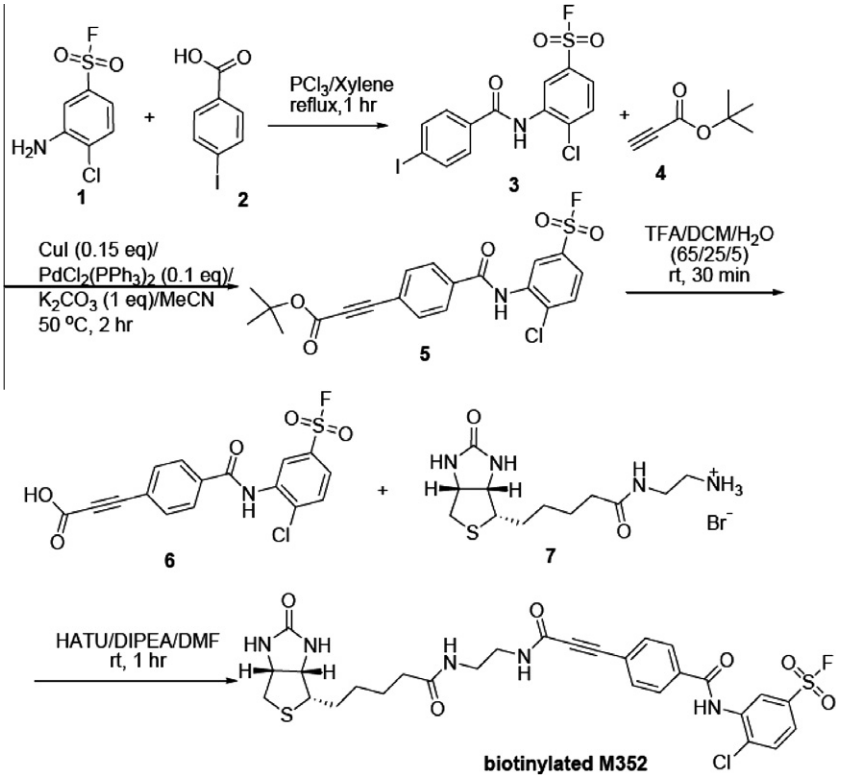
Materials and methods

Materials

Plasticware used for cell culture and assays was purchased from Fisher Scientific (Pittsburgh, PA). Streptavidin Hi-bind black plates, Superblock buffer, and Quantablue peroxidase substrate were purchased from Pierce (Rockford, IL). Polyclonal anti-EL antibodies ab14797 and ab14796 and anti-LPL antibody 21356 were purchased from Abcam (Cambridge, MA), and anti-rabbit peroxidase antibody RPN4301 was purchased from GE Healthcare (Baie d'Urfe, QC). Human umbilical vein endothelial cells (HUVEC) and EGM media (containing a final concentration of 2% FBS) were purchased from Lonza (Basel, Switzerland). Expression plasmids encoding wild-type EL and active-site S169A mutant EL were prepared by inserting the coding sequence of the respective genes into the pcDNA3 vector (Invitrogen, Carlsbad, CA). DNA for transfections was prepared by maxiprep (Promega, Madison, WI).

Chemical synthesis of the activity-based probe (ABP)

The scheme for the synthesis of the ABP is shown below. All reagents and solvents used in the synthesis were from a commercial source and used as received. The newly synthesized compounds were all characterized by HNMR and FNMR and agreed with their assigned structures.



The synthesis of biotinylated M352 was achieved in four steps with commercially available compounds **1** as the staring material. By refluxing equimolar amounts of **1**, **2**, and PCl3 in xylene, compound **3** was obtained. Under the cocatalyzation of CuI (0.15 eq) and PdCl2(PPh3)2 (0.1 eq) and in the presence of 1 eq of K_2CO_3 as base, the coupling between **3** and **4** led to the formation of compound **5**. The treatment of compound **5** with a mixture of TFA/DCM/H_2O at rt for 30 min yielded the corresponding acid **6**. In the presence of HATU (1 eq) and DIPEA (1 eq), equimolar amounts of compound **6** and compound **7** were coupled to formed the final biotinylated M352.

Compound inhibitors

The ABP is a biotinylated derivative of a sulfonyl fluoride molecule that covalently and irreversibly binds to the active site of lipases (M-352). The proprietary test compounds M-352, M-217, M-890, M-792, M-973, and M-809 were prepared at Merck. The pan-lipase inhibitor orlistat [12–14] is from Roche Pharmaceuticals (San Francisco, CA). Compounds being tested were first serially diluted in DMSO before adding to the enzyme reaction mixture.

Cell culture

Human umbilical vein endothelial cells were grown in EGM media containing FBS until confluency. EL protein expression was induced by replacing the growth media with serum-free EGM containing 10 ng/mL TNFα (R&D Systems, Minneapolis, MN). The next

day heparin (Sigma, St. Louis, MO) was added to a final concentration of 10 U/mL to dissociate EL from the cell surfaces, and the media were collected and concentrated 250-fold by ultrafiltration prior to storage at −20 °C. The concentration of active enzyme was estimated by measuring the hydrolysis of Bodipy-labeled phospholipid particles, with 1 unit defined as the amount of enzyme able to generate a 10-fold increase in fluorescence after a 20-min reaction time [10].

Alternatively, recombinant EL protein was obtained by transient transfection of HEK293-T cells with plasmid vectors encoding the full-length wild-type or S169A active-site mutant EL sequences. Cells were plated in 225-cm tissue culture flasks and cultured in DMEM media containing 10% FBS until they reached 90% confluency. Transfection of 40 μg of plasmid DNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and the media were replaced 6 h post transfection with Optimem media (Invitrogen). The EL was dissociated and collected 72 h later by the addition of heparin to a final concentration of 10 U/mL for 30 min. The media were then concentrated 15-fold by ultrafiltration prior to storage at −20 °C. The concentration of active recombinant enzyme was estimated by a comparison with the activity of a known quantity of HUVEC-derived EL. The concentration of the S169A active-site mutant was normalized to the wild-type enzyme by measuring Western blot band intensity.

Active-site ELISA

The final assay conditions after optimization were as follows: For each well in a polypropylene 96-well plate, 100 μL of assay buffer (PBS/0.01% TX100) containing 3 nM EL enzyme was mixed with 0.5 μL of compound or vehicle (DMSO), and incubated for 60 min. The ABP was added in a volume of 0.5 μL for a final concentration of 50 nM, and incubated for 10–15 min or 120 min for standard and reversibility conditions, respectively. Next, the reaction was quenched using an excess (1 μM) of nonbiotinylated probe M-352 before transferring to a prewashed (PBS/0.1% Tween) Hi-bind streptavidin multiwell plate. The streptavidin plate was incubated while shaking for 30 min to allow binding of the enzyme–biotin probe complexes to the immobilized streptavidin. The plate was then washed four times with wash buffer (PBS/0.1% Tween) using a Skatron plate washer (Molecular Devices, Toronto, ON) to remove unbound enzyme. Bound EL was detected by the combined addition of a 1/1500 dilution of the ab14797 and ab14796EL antibodies in PBS/0.1X Superblock, and incubation for 60 min, followed by another plate wash step. Alternatively, the detection of bound LPL was performed using a 1/5000 dilution of ab21356. The sandwich ELISA was completed by incubating PBS/0.1X Superblock containing a 1/8000 dilution of anti-rabbit (for anti-EL polyclonal antibodies) or anti-mouse (for anti-LPL monoclonal antibody) horseradish peroxidase for 60 min. After a final plate wash step, peroxidase activity was measured by adding 100 μL/well Quantablue substrate for 10 min followed by 100 μL/well Quantablue stop solution, and the fluorescence signal was measured in a Gemini plate reader (Molecular Devices) at an ex/em of 325/420 nm. Note that the entire assay was performed at room temperature.

Assay optimization was performed by varying the concentration of the ABP, the incubation time, the anti-EL antibodies, and the detergent concentration of the assay buffer. The 96-well assay was expanded to 384-well format using Biomek FX robotics (Beckman, Mississauga, ON), with assay conditions as described above, except for an assay volume of 50 μL and the use of 384-well plasticware. The Z' value was calculated using the equation $Z' = 1 - [(3s_f + 3s_b)/|\mu_b - \mu_f|]$, where s = standard deviation, μ = average, f = background control, and b = positive control. There was no significant change in assay performance between 0% and 2% DMSO final concentration.

Immunoprecipitation and Western blots

In order to detect the interaction of the ABP with EL by immunoblot, EL pretreated with vehicle (DMSO) or 5 μM orlistat for 60 min was incubated with 100 nM ABP for 60 min before quenching with 1 μM nonbiotinylated probe M-352. For each condition, sufficient material corresponding to 12 wells of the active-site ELISA was prepared. Streptavidin Sepharose (GE Healthcare) was added and the reaction was incubated on a rocking platform mixer for 60 min. The resin was then pelleted by centrifugation, and washed with assay buffer. The unbound material was concentrated 10-fold using Microcon YM-50 ultrafiltration devices (Millipore, Billerica, MA) and retained for Western blot analysis. The Sepharose resin was boiled for 5 min with Laemmli loading buffer plus β-mercaptoethanol (Bio-Rad, Mississauga, ON) to solubilize and release the ABP:EL complexes, and the samples were loaded on a 4–20% gradient SDS polyacrylamide gel (Invitrogen). Following electrophoresis, samples were transferred to nitrocellulose using an iBlot device (Invitrogen), blocked with 5% milk/TBS, and probed with a 1/3000 dilution of the ab14797 anti-EL antibody. Following an overnight incubation with the primary antibody, the blot was washed with TBS/0.1% Tween 20 and incubated with a 1/5000 dilution of anti-rabbit horseradish peroxidase (GE Healthcare) for 60 min. After the final washes in TBS/Tween 20, chemiluminescent detection was carried out using Supersignal West Femto substrate (Pierce) and exposing to Biomax MR film (Kodak, Rochester, NY).

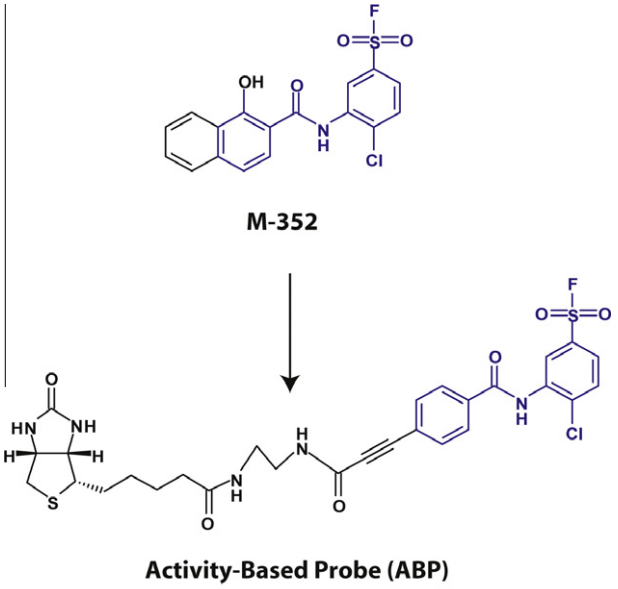
Fluorescence-based micellar lipase assay

To test lipase activity and inhibition in a more standard micelle-based assay, we employed a fluorescence-based micellar lipase assay, described by Mitnaul et al. [10]. Briefly, test compounds were diluted in DMSO, added to 96-well plates containing the lipase enzyme in assay buffer (PBS, 10% DMEM, 1.5% glycerol, 0.5% BSA), and incubated for 30 min prior to adding the phospholipid particles (Mono-Bodipy-TG or Bis-Bodipy-PC in 0.1% Triton X-100) to a final concentration of 20 μM. The increase in fluorescence over time was read in a plate reader at ex/em of 550/590 nm.

Results and discussion

Design of a lipase-directed activity-based probe

In designing a prototypic lipase-specific ABP, we sought a molecular scaffold that satisfied the following criteria: straightforward chemical synthesis; stable in aqueous solution; similar potency against EL, HL, and LPL; and containing a warhead specific for serine-hydrolases. We focused on compounds with greater ligand efficiency containing a sulfonyl fluoride warhead—a widely studied rapid and irreversible inhibitor of serine hydrolases [15]. M-352 (Fig. 1) emerged as a promising parent scaffold molecule, based on its overall chemical properties and potency profile. M-352 potently inhibited EL, HL, and LPL in micellar assays with low nanomolar potency in a time-dependent manner that was unchanged by rapid dilution, consistent with the expected irreversible mode of inhibition (see below). In order to convert M-352 into a prototypic activity-based probe, we incorporated a biotin moiety distal to the sulfonyl fluoride warhead separated by a flexible linker (Fig. 1). The linker was included to provide sufficient distance for the biotin to "snorkel" out of the deep lipase active sites to bind streptavidin unhindered. The fully elaborated ABP retained significant potency against all three lipases when tested in a standard micellar lipase assay: EL IC_{50} = 47 nM; HL IC_{50} = 73 nM; and LPL IC_{50} = 73 nM.



Lipase	ABP Potency (IC50)
Endothelial Lipase	47 nM
Hepatic Lipase	73 nM
Lipoprotein Lipase	73 nM

Fig.1. Chemical structure of the activity-based probe (ABP) and potency against triacylglycerol lipases. The ABP was based on a potent pan-selective, irreversible triacylgycerol lipase inhibitor, M-352.

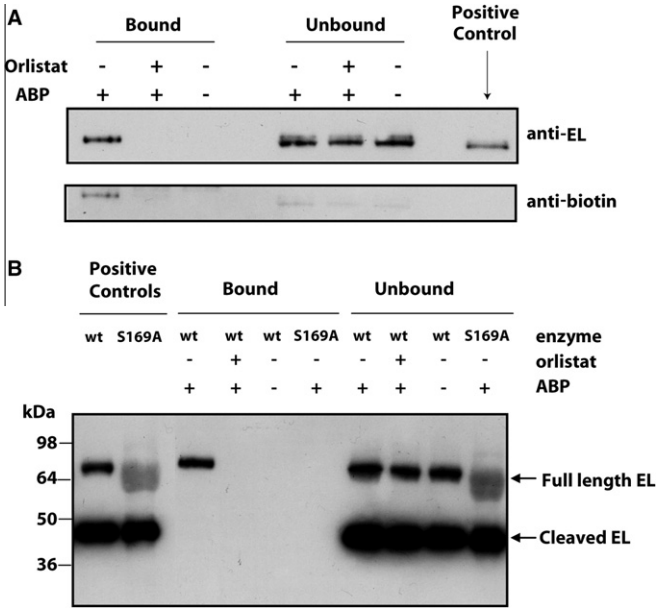


Fig.2. Western blot detection of EL–ABP complexes pull-down by streptavidin beads. (A) Recombinant EL was visualized by immunoblot following incubation with ABP, capturing to streptavidin Sepharose, and elution with Laemmli sample buffer. EL-ABP complexes were detected using an EL-specific polyclonal antibody or an anti-biotin antibody. Where indicated, EL was pretreated with 5 μM orlistat, or not incubated with ABP. SDS-resistant EL–ABP complexes were detected in the streptavidin-bound fraction only when incubated with the ABP, and this effect was abolished by pretreatment with orlistat. The results are representative of three experiments. (B) The ABP binds exclusively to wt full-length EL. Recombinant wild-type (wt) or serine 169 to alanine mutant EL was visualized by immunoblot following incubation with ABP, capturing to streptavidin Sepharose, and elution with Laemmli sample buffer. EL was detected using an EL-specific polyclonal antibody. Where indicated, the wild-type EL was pretreated with 5 μM orlistat, or not incubated with ABP. Arrows indicate full-length and cleaved EL. The full-length wild-type EL, but not the cleaved EL or active-site mutant EL, was labeled by the ABP.

Characterization of the ABP

As an initial proof-of-principle set of experiments, we first tested the ability of the ABP to recognize and bind EL in a complex heterogeneous mixture. Primary HUVEC cells were treated with TNF-α to specifically induce the expression of EL [16,17,10]. Cells were then treated with heparin to release heparin sulfate-associated EL into the medium. EL-containing cell supernatants were then pretreated for 30 min either with DMSO or with the pan-lipase inhibitor tetrahydrolipstatin (THL, or orlistat), followed by a 30-min incubation with the ABP, after which the streptavidin Sepharose was added to "capture" EL:ABP complexes. After extensive washing EL was released from streptavidin by boiling the resin in SDS-Laemmli sample buffer, followed by SDS–PAGE separation and visualization by immunoblot using both an EL-specific polyclonal antibody and an anti-biotin antibody (Fig. 2). As shown in Fig. 2A, EL was detected in the streptavidin-bound fraction when incubated with the ABP, and this band was not detected on pretreatment with orlistat. The ability of the irreversible lipase inhibitor orlistat to prevent binding of the ABP is consistent with their expected overlapping binding sites (i.e., the active site). On probing with an anti-biotin antibody, a weak, but reproducible signal was detected at the expected molecular weight of EL, consistent with an irreversible covalent ABP-EL interaction. To further confirm that binding of the ABP to EL was via the expected irreversible trapping of the ABP to the active-site catalytic serine, we measured the ability of the ABP to bind to the active-site mutant recombinant S169A as compared with wild-type EL. The ABP did not label the S169A active-site mutant of EL (Fig. 2B), confirming both active-site engagement and previous findings that this construct is catalytically inactive [18].

Also observed in the loading control lanes (Fig. 2B) was that the recombinant EL stocks used for these experiments contained both the full-length and catalytic domain-only truncated form, which arises from cleavage immediately downstream of the RNKR site of the full-length enzyme by endogenous proprotein convertases [19]. Interestingly, the ABP bound to the full-length form of EL, and not the truncated catalytic domain, suggesting that the complete enzyme, including the carboxy terminus lipid-binding domain is required for substrate/inhibitor engagement. The apparent absence of enzyme activity in the truncated EL observed in the present study is in agreement with the findings of Gauster et al. [20], who reported that the full-length form of EL is cleaved by endogenous proprotein convertases to a truncated form, leading to loss of HDL hydrolyzing activity. Cleavage of EL appears to be a common occurrence in native endothelial cell lines as well [19]. Moreover, cleavage of EL is not an exclusive feature of mammalian cells; it was noted that expression of recombinant EL in a baculovirus/insect cell system yielded a similar sized cleavage product (A. Auger, unpublished results).

Development and optimization of an ABP-based ELISA

We next sought to use the ABP to develop an ELISA-based assay to quantitatively measure lipase activity and inhibition in a format that could potentially be used for high-throughput measurements of activity and inhibition. To determine the optimal probe binding conditions in the context of a particle-free, ELISA-based binding assay, a time course was performed using different concentrations of the probe with a fixed concentration of EL enzyme (3 nM). Whereas no signal was observed in the absence of probe, the mag-

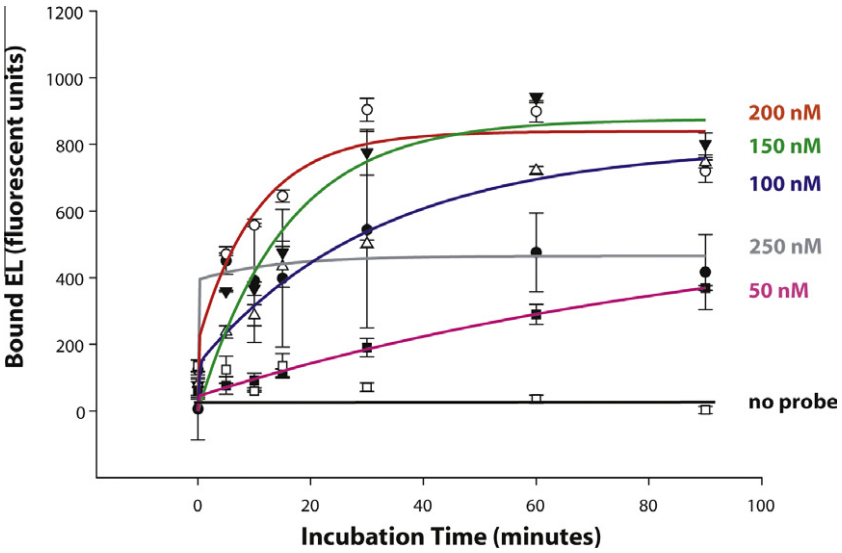


Fig.3. ABP time course and titration. EL from HUVEC conditioned media was incubated with 0–250 nM ABP for 0–90 min before quenching with 1 μM M-352. Binding of biotinylated EL to the streptavidin plate and detection by ELISA was as described under Materials and methods. Bars represent SEM of duplicate wells.

nitude of ELISA fluorescent signal associated with probe-bound EL increased with increasing probe concentration up to 200 nM, but began to display diminished signal at a concentration of 250 nM (Fig. 3). Furthermore, when the probe was tested at concentrations beyond 150 nM, assay signal amplitude loss became apparent at the 90-min time point. The diminishing returns at higher probe concentrations reflect interference by increasing concentrations of free probe, which begins to compete with probe–enzyme for binding sites on the streptavidin plate, thereby reducing observed signal. Based on the data presented in Fig. 3, we used a probe concentration and incubation time of 50 nM and 10 min, respectively, for subsequent experiments. Such conditions were chosen to ensure that sufficient assay signal is achieved, while minimizing the displacement of enzyme-bound compounds over time, and avoiding conditions that lead to loss of signal. Using such optimized conditions produced a >10-fold window above mock-transfected background that was dose dependently decreased by orlistat (Fig. 4).

To further improve the sensitivity of the assay, we combined two commercially available antibodies directed against mutually exclusive regions of EL. The antibodies raised against the amino-terminal and carboxy-terminal EL were tested both individually and as a pooled mixture; it was found that pooling the two antibodies provided up to 60% greater signal compared to using either antibody alone (data not shown). Finally, we measured the effect of increasing concentrations of Triton X-100 to the assay protocol. The use of sub-CMC concentrations of non-ionic detergents has previously been shown to be beneficial to the general performance of enzyme assays, by reducing the incidence of compound or enzyme aggregation [21]. We found that sub-CMC concentrations of Triton X-100 were tolerated, and in fact improved; addition of 0.001% (v/v) and 0.01% (v/v) Triton X-100 increased assay signal by 33& and 100%, respectively. As expected, above the CMC of Triton X-100 (CMC ~0.033%) signal loss was evident: 45% signal loss at 0.1% Triton X-100. The use of Tween 20 and heparin was also evaluated, but was not found to significantly improve the assay signal (data not shown).

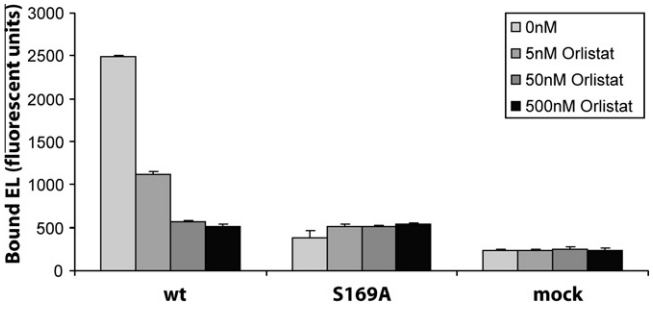


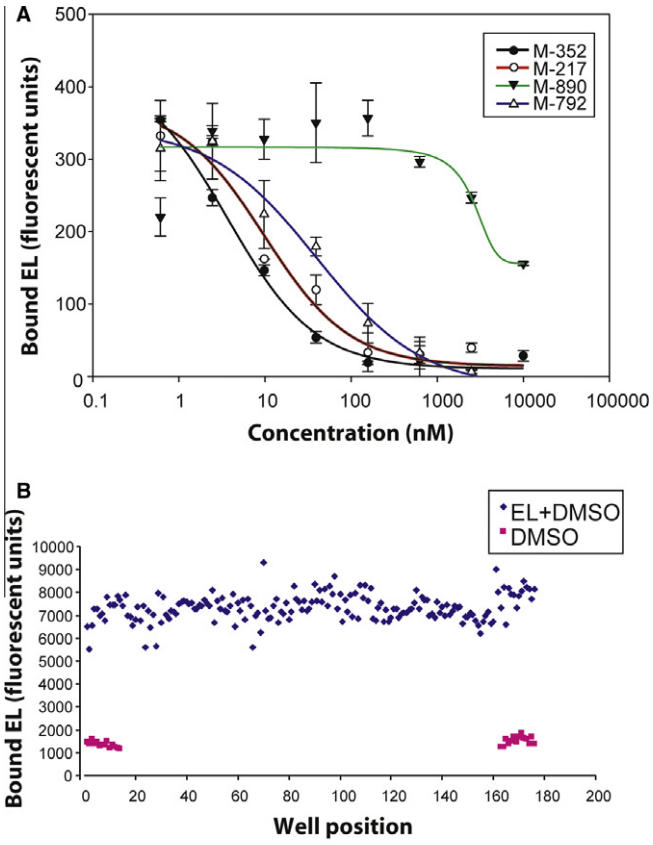
Fig.4. The wild-type but not the catalytically dead S169A mutant was labeled by the ABP and detected by ELISA. Orlistat was serially diluted in DMSO and added to 3 nM recombinant wild-type sequence (wt) or serine 169 to alanine active-site mutant (S169A) EL from HEK293 culture media for 60 min. As a negative control, culture media from mock-transfected cells were used. The ABP was added to a final concentration of 50 nM for 10 min before quenching with 1 μM M-352 and applying to a 96-well streptavidin plate. Detection of bound EL by ELISA was as described under Materials and methods. Bars represent SEM of duplicate wells.

Inhibitor titrations and optimization of the ABP-ELISA to 384-well format

Initially, the performance of the ABP-ELISA assay was tested in a dose–response format in 96-well plates. We selected a panel of structurally diverse inhibitors of EL with a range of potencies, as measured in standard *in vitro* micelle-based lipase assays. As shown in Fig. 5A, test compounds yielded classic dose–response curves with IC50 values that agreed well with their potencies in micellar assays (i.e., M-352 was <2-fold shifted and orlistat was <3-fold shifted from the micellar assay to the EL ELISA). To facilitate automated high-throughput screening, the 96-well assay was miniaturized to a 384-well format and liquid dispensing was automated using robotics. Enzymes with DMSO were added to replicate wells of a 384-well plate in columns 1, 2, 3, 5, 9, 13, 16, 19, 22, 23, and 24, and compared to the basal signal in the DMSO plus buffer-only controls in columns 4 and 21 (Fig. 5B). The measured average fluorescence in the enzyme-containing wells was 7335 units, compared to 1435 average fluorescent units measured in the control wells, while the Z' was calculated to be 0.62 (Fig. 5C).

Reversibility mode

To develop a format of the assay that could potentially measure the reversibility of test compounds—a desirable property for a potential small molecule lead—we took advantage of the time-depen-



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	EL+DMSO	DMSO
Mean	7335	1435
Stdev	583	174
%CV	8.0	12.1

Assay Zí = 0.62

Fig.5. (A) Titration of compounds by active-site ELISA. Each compound was serially diluted in DMSO and added to 3 nM EL from HUVEC conditioned media. After 60 min ABP was added to a final concentration of 100 nM and incubated for 15 min. The reaction was quenched with 1 μM M-352, and added to a streptavidin plate. The ELISA was performed using anti-EL polyclonal antibodies and detection was by a fluorogenic peroxidase substrate as described under Materials and methods. Bars represent SEM of duplicate wells. (B) Adaptation of the active-site ELISA from 96- to 384-well format. Enzyme + DMSO was added to all vertical columns 1, 2, 3, 5, 9, 13, 16, 19, 22, 23, and 24 of a 384-well plate. DMSO alone was added to columns 4 and 21. (C) Assay statistics.

dent inhibition of the ABP to develop a reversibility assay. We reasoned that longer ABP incubation would allow for displacement of reversibly bound inhibitor–enzyme complexes, leading to greater probe binding at a given concentration of inhibitor. A reversible compound would thus appear less potent on increased probe incubation, whereas an irreversible inhibitor would maintain its potency. As seen in Fig. 6, a time course of enzyme labeling at a concentration of 50 nM probe and 3 nM recombinant EL resulted in time-dependent labeling of EL with a peak signal after 120 min. To test this concept, three classes of test compounds were tested: a known irreversible inhibitor (M-352—the parent scaffold to the ABP); a known reversible-covalent inhibitor (M-973—a close

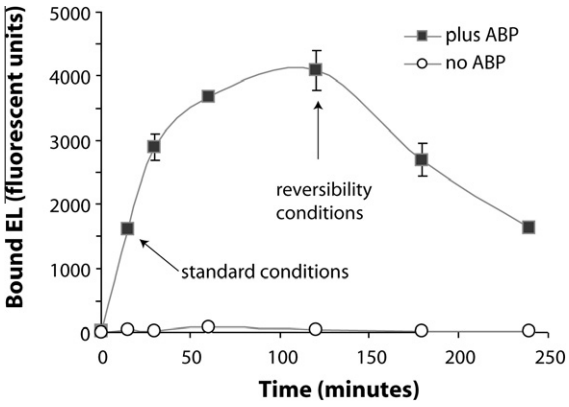

Fig.6. Extending probe incubation time to identify reversible compounds. Recombinant EL from HEK293 media was incubated with 50 nM ABP (squares) or no probe (circles) for 0, 10, 30, 60, 120, 180, and 240 min before quenching with 1 μM M-352 and applying to a streptavidin plate. Detection of bound EL by ELISA was performed as described under Materials and methods. Arrows indicate the time points chosen to test compounds under standard and reversibility conditions, respectively. Bars represent SEM of duplicate wells.

analog of M-352 with a Boronic acid warhead in place of a sulfonyl fluoride); and a suspected reversible compound (M-809—a close analog of M-352 with a methyl in place of the warhead). A rightward shift in the titration curves for M-809 and M-973 was observed with the extended probe incubation time, consistent with their expected reversible binding mode (Fig. 7). In contrast, the measured potency of the nonbiotinylated sulfonyl fluoride M-352 was not affected by the ABP incubation time, thus indicating that this compound could not be displaced by the probe once bound to the active site, and is therefore an irreversible compound.

Utility of the ABP against other lipases

To demonstrate that ABP could be used to measure a different triglyceride lipase, we modified the assay for LPL activity measurements. Orlistat was serially diluted in DMSO and added to diluted culture media containing 3 nM recombinant EL or LPL. As a negative control, culture media from mock-transfected cells were used. The mixture was then incubated with ABP before applying to the streptavidin plate. When an anti-LPL-specific antibody was used, bound LPL could be detected, and this signal was dose dependently attenuated by preincubation with the pan-lipase inhibitor orlistat (Fig. 8). The LPL-specific antibody did not cross-react with EL or with proteins from mock-transfected cell culture media.

Conclusion

Assays of lipase activity typically employ fluorescent or radioactive lipid-like substrates that are embedded in lipid emulsions. Though generally robust, when applied to the identification of inhibitors in high-throughput screens, these assays are often prone to artifacts arising from signal interference and/or compound–lipid interactions. Here, we describe the development and optimization of a particle-free ELISA-based assay that is capable of measuring active-site engagement of triglyceride lipase family members in complex mixtures through the use of a designed activity-based probe. The assay has excellent statistics in high-throughput screening mode and can be used to distinguish reversible from irreversible inhibitors. Given the versatility and uniqueness of this approach, relative to existing assays, we anticipate that this assay and probe will be very useful for researchers studying lipases, and in particular for those attempting to identify inhibitors of triglyceride lipase family members.

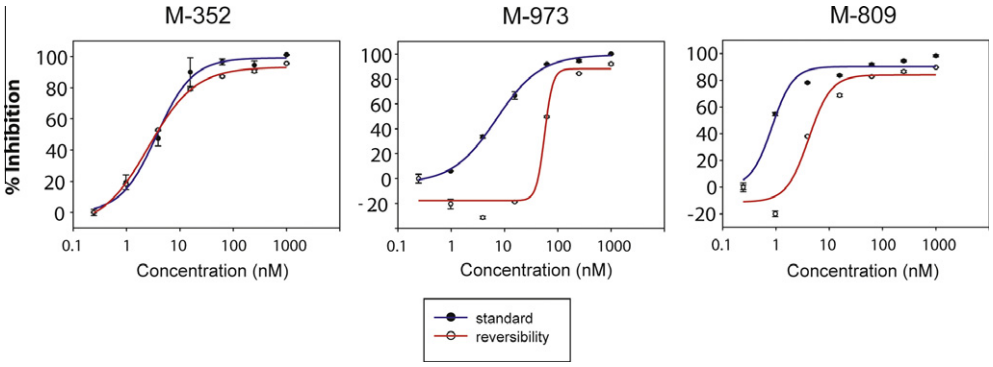

Fig.7. The active-site ELISA performed under long vs short probe incubation times can identify reversible compounds. Each compound was serially diluted in DMSO and added to 3 nM recombinant EL from HEK293 conditioned media. After 60 min ABP was added to a final concentration of 100 nM and incubated for 10 min (filled circles) or 120 min (open circles). The reaction was quenched with 1 μM M-352, and added to a streptavidin plate. The ELISA was performed using anti-EL polyclonal antibodies and detection was by a fluorogenic peroxidase substrate as described under Materials and methods. Bars represent SEM of duplicate wells.

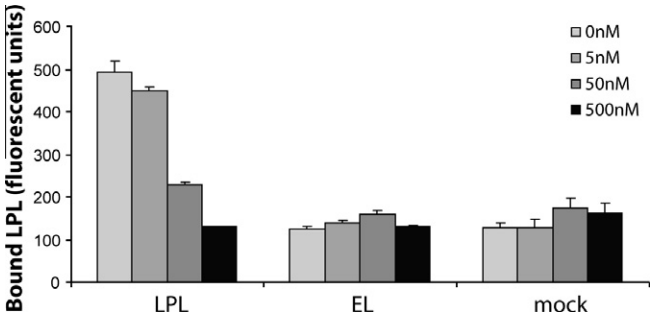

Fig.8. The ABP can be used to measure the activity of other lipase family members. Orlistat was serially diluted in DMSO and added to 3 nM recombinant EL or LPL from HEK293 culture media for 60 min. As a negative control, culture media from mock-transfected cells were used. The ABP was added to a final concentration of 50 nM for 10 min before quenching with 1 μM M-352 and applying to a 96-well streptavidin plate. Detection of bound LPL by ELISA was as described under Materials and methods using a LPL-specific antibody. The results are representative of two experiments, and the bars represent SEM of duplicate wells.

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