

Targeting cardiovascular disease and Alzheimer's disease

BEYOND REGULATING CHOLESTEROL LEVELS, NEW DRUG TARGETS EMERGE FROM LIPID RESEARCH.

The nexus of heart and brain disease

Once considered very separate medical maladies, diseases of the brain and of the circulatory systems are now leading researchers to converge on a common set of drug targets. Extensive evidence is accumulating that these two broad categories of diseases share many common risk factors. In autopsy studies of Alzheimer's disease (AD) patients, for example, counting microvascular lesions was as predictive of the extent of the disease as counting amyloid plaques;¹ and the prevalence of AD pathology was 11 times higher in women who had died of stroke.²

A significant risk factor for cardiovascular disease (CVD) and AD is metabolic syndrome, characterized by a cluster of attributes including elevated waist circumference, blood pressure, triglycerides, and fasting glucose, along with reduced levels of high-density lipoproteins (HDL). Diabetes alone increases the risk for AD by 30 to 65%, and patients with metabolic syndrome have a nearly 4-fold greater probability of

developing AD.^{3,4} The pathophysiological basis of metabolic syndrome is complex, but it is apparent that disruption of critical regulators of lipid metabolism has dire consequences, not just for CVD^{5,6} but for AD,⁷ Huntington's disease, multiple sclerosis, diabetes, and more.

To investigate these critical lipid trafficking pathways, Invitrogen provides an array of Molecular Probes® reagents and assays that can be used to attack the questions from multiple entry points, from the lipid transporters themselves to the enzymes that regulate their function and levels in circulation (Table 1).

Cholesterol: Here, there, and everywhere

It is well established that blood plasma levels of cholesterol and their carrier particles—low-density lipoproteins (LDL) and HDL—play a critical role in CVD. Treatment of atherosclerosis in particular has focused on strategies for lowering overall cholesterol levels using HMG-CoA reductase inhibitory therapies or statins. Disruptions in cholesterol regulation, however, can also have consequences for the functioning of the human brain, which contains 25% of the body's cholesterol. The association of lipids with neural degeneration was first indicated by studies showing that apolipoprotein E (ApoE)-containing HDL particles were dramatically upregulated following nerve injury.⁸ Later, identification of the ApoE4 allele as the first qualified risk factor for AD began the search for understanding how lipoproteins and cholesterol retention influence the consequences of amyloid burden on brain function.⁹⁻¹²

Researchers are now shifting attention from the cholesterol-lowering statin drugs to the regulation of phospholipases that line endothelial vessels, float in plasma, and complex with lipoproteins. Of the four distinct phospholipase families, phospholipase A1 (PLA1) and phospholipase A2 (PLA2)—which cleave at the SN1 site and SN2 site, respectively, in the phospholipid head group—are drawing the most interest because members of these two families modulate both the levels and toxicities of plasma HDL and LDL particles (see sidebar). These proatherogenic phospholipases are thus prime targets for controlling and monitoring the progression of many disease states. →

The tale of two phospholipases

Endothelial lipase (EL) is a cell-surface PLA1 enzyme that lines arterial blood vessel walls. Upregulated by cytokines and proinflammatory drugs, EL lowers HDL levels while elevating free fatty acids and reactive cholesterol in plasma. In extreme cases, endothelial lipase completely metabolizes HDL particles, activating peroxisome proliferator-activated receptor^{1,2} (PPARalpha) and releasing the HDL-mediated repression of leukocyte adhesion.³ Anti-inflammatory drugs can downregulate EL and reverse its effects by elevating intact healthy plasma HDL.

Lipoprotein lipase (LpL), a secreted PLA2 enzyme, is the primary enzyme acting on circulating triglycerides and oxidized LDL to form the proinflammatory lysophosphatidylcholine and oxidized nonesterified fatty acids that can lead to patchy foci of plaques on endothelial walls, which are the hallmark of nascent disease. The overexpression of this secreted PLA2 has been found to be an accurate predictor of CVD, with many researchers suggesting that PLA2 will soon replace C-reactive protein as the go-to patient diagnostic test.

References

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Selective phospholipase A1 and phospholipase A2 substrates

Invitrogen has introduced highly selective fluorogenic substrates for the PLA1 and PLA2 families of phospholipases. A recent paper has established the robustness and scalability of earlier less-specific versions of these compounds for ultrahigh-throughput screening of lipase inhibitors.¹⁴ In our own studies, the new PED-A1 and Red/Green BODIPY® PC-A2 substrates show dramatically improved signal-to-noise ratios and significantly increased enzyme selectivity. Cleavage of these PLA1 and PLA2 substrates by their corresponding phospholipases produces a 20-fold increase in fluorescence (Figure 1). In addition, phospholipase

assays with these new substrates can be run in real time for true kinetic analysis of enzyme rates (Figure 2).

Available separately or as part of an EnzChek® Phospholipase Assay Kit (Table 1), the PED-A1 and Red/Green BODIPY® PC-A2 substrates provide true feed-and-read assays: simply add the substrates in liposomes to the microplate wells, wait 30 minutes, and read the increase in fluorescence (or decrease, for candidate inhibitor compounds). These assays are ideal for large screens, but sensitive and selective enough for downstream lead characterization. Although these substrates are designed for microplate-based assays, studies have indicated their potential utility in whole-cell or even whole-animal studies.

Table 1—A selection of Invitrogen™ products for studying lipid dysregulation in CVD and AD.

	Quantity	Cat. no.	Notes
Fluorogenic phospholipase substrates			
PED-A1	100 µg	A10070	PLA1 activity releases intramolecular quenching of the BODIPY® FL dye by DNP, yielding an increase in emission at 530 nm.
Red/Green BODIPY® PC-A2	100 µg	A10072	PLA2 activity releases a BODIPY® FL dye, resulting in decreased quenching by FRET of a BODIPY® 558/568 dye and an increase in emission detected in the range 515–545 nm.
bis-BODIPY® FL C ₁₁ -PC	100 µg	B7701	PLA1 or PLA2 activity releases intramolecular quenching of the two BODIPY® FL dyes, yielding an increase in emission at 512 nm.
PED6	1 mg	D23739	PLA2 activity releases intramolecular quenching of the BODIPY® FL dye by DNP, yielding an increase in emission at 511 nm.
Cholesterol assay and enzyme assays			
Amplex® Red Cholesterol Assay Kit	1 kit, 500 assays	A12216	Cholesterol is readily quantitated using this enzyme-coupled assay and a fluorescence microplate reader or fluorometer, with a detection limit of ~80 ng/ml.
Amplex® Red Monoamine Oxidase Assay Kit	1 kit, 500 assays	A12214	This enzyme-coupled assay detects as low as 12 µU/ml purified monoamine oxidase activity <i>in vitro</i> using a fluorescence microplate reader or fluorometer.
Amplex® Red Sphingomyelinase Assay Kit	1 kit, 500 assays	A12220	This enzyme-coupled assay detects ≥80 µU/ml sphingomyelinase activity <i>in vitro</i> using a fluorescence microplate reader or fluorometer.
EnzChek® Paraoxonase Assay Kit	1 kit, 100 assays	E33702	This assay detects ≥50 mU/ml paraoxonase activity <i>in vitro</i> using a fluorescence microplate reader or fluorometer.
EnzChek® Phospholipase A1 Assay Kit	1 kit, 2-plate size 1 kit, 10-plate size	E10219 E10221	This assay employs the PED-A1 substrate to detect ≥0.04 U/ml PLA1 activity <i>in vitro</i> using a fluorescence microplate reader or fluorometer.
EnzChek® Phospholipase A2 Assay Kit	1 kit, 2-plate size 1 kit, 10-plate size	E10217 E10218	This assay employs the Red/Green BODIPY® PC-A2 substrate to detect ≥0.05 U/ml PLA2 activity <i>in vitro</i> using a fluorescence microplate reader or fluorometer.
EnzChek® Myeloperoxidase (MPO) Activity Assay Kit	1 kit, 400 assays	E33856	This kit provides assays for the determination of both chlorination and peroxidation activities of MPO in solution and in cell lysates.
Zen™ Myeloperoxidase (MPO) ELISA Kit	1 kit, 200 assays	Z33857	This assay detects MPO over a broad dynamic range (0.2 to 100 ng/ml) <i>in vitro</i> using a fluorescence microplate reader or fluorometer.
Unlabeled and fluorescent low-density lipoprotein (LDL) and acetylated LDL (AcLDL) particles			
Unlabeled LDL from human plasma	200 µl, 2.5 mg/ml	L3486	Fluorescent LDL is labeled with Dil or BODIPY® FL dye, highly fluorescent lipophilic dyes that diffuse into the hydrophobic portion of the LDL complex without affecting the LDL-specific binding of the apoprotein. Fluorescent LDL has been used to quantitate LDL receptors, analyze their motion and clustering, and follow their internalization.
Dil LDL	200 µl, 1 mg/ml	L3482	
BODIPY® FL LDL	200 µl, 1 mg/ml	L3483	
Unlabeled AcLDL from human plasma	200 µl, 2.5 mg/ml	L35354	AcLDL no longer binds to the LDL receptor, but instead is taken up by macrophage and endothelial cells that possess scavenger receptors specific for the modified LDL. Once the AcLDL complexes accumulate within these cells, they assume an appearance similar to that of foam cells found in atherosclerotic plaques. For some applications, the BODIPY® FL and Alexa Fluor® conjugates of AcLDL may be the preferred probes because these fluorophores are covalently bound to the modified apoprotein portion of the LDL complex and, unlike the Dil label, are not extracted during subsequent cell manipulation.
Dil AcLDL	200 µl, 1 mg/ml	L3484	
BODIPY® FL AcLDL	200 µl, 1 mg/ml	L3485	
Alexa Fluor® 488 AcLDL	200 µl, 1 mg/ml	L23380	
Alexa Fluor® 594 AcLDL	200 µl, 1 mg/ml	L35353	
Recombinant human apolipoprotein E (ApoE)			
ApoE2, rHuman	50 µg	P2002	Recombinant human isoforms of apoE are produced by baculovirus-mediated expression in the <i>Spodoptera frugiperda</i> (Sf) insect cell line, purified by affinity chromatography, and supplied in solution.
ApoE3, rHuman	50 µg	P2003	
ApoE4, rHuman	50 µg	PV2004	

Other enzymes that regulate lipid metabolism

Phospholipases are not the only players in regulating the toxicity of lipoprotein particles. Paraoxonase, made in the liver as part of lipoprotein particles, protects against the detrimental effects of oxidative damage from any source. The EnzChek® Paraoxonase Assay Kit provides a homogeneous fluorometric assay for paraoxonase's organophosphatase activity and is >10 times more sensitive than colorimetric assays.

Historically linked to immune system function, myeloperoxidase (MPO)—which produces pathogen-killing hypochlorous from chloride anion and hydrogen peroxide—has recently been identified as a key marker in coronary artery disease, where it can act as a source of reactive oxygen species contributing to increased pools of oxidized or carbamylated LDL.^{15,16} The EnzChek® MPO Activity Assay Kit provides continuous assays for the determination of MPO's chlorination and peroxidation activities in solution and in cell lysates; the Zen™ MPO ELISA Kit provides accurate and sensitive quantitation of MPO's peroxidation activity in a variety of biological samples, including human serum.

In addition to these kits, Table 1 lists microplate-based assays for cholesterol, monoamine oxidase, and sphingomyelinase. The Amplex® Red Cholesterol Assay Kit was recently employed in a study of the effect of cholesterol levels on A β peptide production.¹³ Sensitive ELISA kits for A β peptides as well as primary antibodies to peroxisome proliferator-activated receptor and many other key proteins can be found at our website at www.invitrogen.com.

Looking forward

The emergence of drug candidates that regulate not only lipid levels but also lipid toxicity may have broader implications than merely

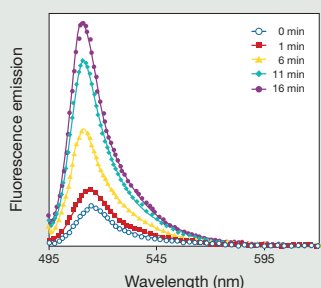


Figure 2—Real-time measurements of PLA1 activity. Fluorescence emission spectra of PED-A1 incorporated in liposomes are shown at time points after the addition of PLA1 at room temperature.

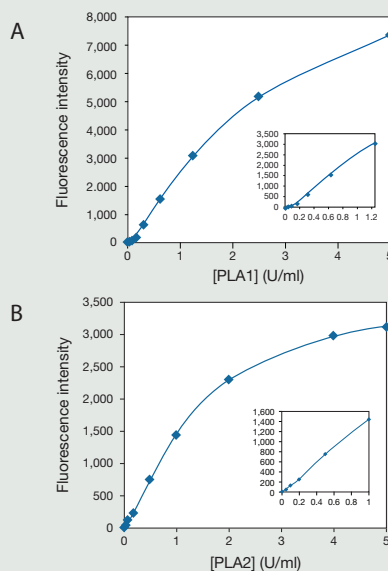


Figure 1—Fluorescence emission intensities of PLA substrates versus PLA concentration. (A) Plot of fluorescence emission of PED-A1 incorporated into liposomes vs. PLA1 concentration at 30 min at room temperature. (B) Plot of fluorescence emission of Red/Green BODIPY® PC-A2 incorporated into liposomes vs. PLA2 concentration at 10 min at room temperature. In both cases, fluorescence was measured using 460 nm excitation and 515 nm emission on a SpectraMax® M5 reader (MDS Analytical Technologies). Background fluorescence determined for the no-enzyme control reaction has been subtracted for each value.

treating vascular disease.^{5,6} It is conceivable that there may someday exist a single treatment regime for brain and heart diseases; statins have already been suggested as such a treatment.⁷ Equally promising is the prospect that brain disease may be amenable to drugs that, while unable to cross the blood-brain barrier, can nonetheless treat the extensive vascular system in the brain. ■

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