An Unbiased Chemical Biology Screen Identifies Agents That Modulate Uptake of Oxidized LDL by Macrophages

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Abstract—Macrophage-derived foam cells are thought to play a major role in atherosclerotic lesion formation and progression. An automated assay was established to evaluate the uptake of fluorescently labeled oxidized low-density lipoprotein (oxLDL) by a monocyte/macrophage cell line. The assay was used to screen 480 known bioactive compounds. Twenty-two active compounds were identified. Efficacy studies in peritoneal macrophages demonstrated a high rate of concordance with the initial screening results. Inhibitory compounds confirmed important previous findings and identified new drugs of interest including: 3 blockers of nuclear factor κB activation, 2 protein kinase C inhibitors, a phospholipase C inhibitor, and 2 antipsychotic drugs. In addition, an opioid receptor agonist was found to increase the oxLDL uptake of macrophages. The involvement of nuclear factor κB in oxLDL uptake was validated in peritoneal macrophages in vivo. The results support a model in which oxLDL uptake is dependent on the activation of multiple intracellular signaling pathways that culminate in actin-mediated lipoprotein internalization. (Circ Res. 2009; 105:148-157.)

Key Words: atherosclerosis  ■  foam cells  ■  oxidized LDL  ■  chemical screening

Macrophage-derived foam cell formation is an important aspect of atherosclerotic lesion development. Foam cells are thought to develop when monocytes enter the subintimal space of arteries, differentiate into macrophages, and take up lipid from extracellular particles such as oxidized low-density lipoprotein (oxLDL). The accumulation and retention of lipoproteins, such as LDL, in the subintimal space of arteries likely plays a role in initiating the development of atherosclerotic lesions, and most therapy for humans with atherosclerosis acts to reduce the concentration of circulating LDL particles in the bloodstream, thereby inhibiting the accumulation of LDL in the subintimal space. In contrast, no therapy is currently used to directly block foam cell formation in the face of elevated circulating LDL.

oxLDL is a potent mediator of foam cells formation. In addition, oxLDL binds to a variety of macrophage surface scavenger receptors such as CD36 and SR-A, leading to the internalization of the oxLDL particle by an undefined mechanism. The relative role of clathrin-coated pit-mediated endocytosis, phagocytosis, pinocytosis, or other internalization mechanism in foam cell formation remains to be determined. In addition, the crucial role of CD36 and SR-A in foam cell formation has recently been challenged.

Evidence for the involvement of various signaling cascades in oxLDL uptake into macrophages is accumulating. Targeted disruption of the c-Jun NH2-terminal kinase (JNK)2 gene was shown to inhibit macrophage oxLDL uptake and atherosclerotic lesion formation in apoE−/− mice maintained on a high fat diet. This study also found that pharmacological inhibition of JNK activity with SP600125 efficiently reduced oxLDL uptake and plaque formation. The role of p38 mitogen-activated protein kinase (MAPK) in macrophage foam cell formation was also suggested by the recent finding that systemic deficiency of the MAPK2, a downstream effector of p38 MAPK, reduced foam cell formation and plaque formation in hypercholesterolemic mice. A recent study from our laboratory demonstrated that macrophages obtained from mice haploinsufficient for the intracellular scaffolding protein Grb2 exhibit reduced JNK and p38 MAPK activation and are also deficient in the uptake of oxLDL. Accordingly, Grb2+− apoe−/− mice maintained on a high-fat Western diet were found to be highly resistant to atherosclerotic lesion formation. Another new study indicated a role for protein kinase (PK)Cβ in oxLDL uptake of phorbol myristate acetate (PMA)-activated human macrophages. Taken together, these results suggest that the activation of many intracellular signaling cascades is important for macrophage foam cell formation. However, the relative role of different cascades as well as the sequence of events that link these pathways to the binding, uptake, and metabolism of oxLDL is still poorly understood.
To investigate the molecular mechanisms underlying foam cell formation and thereby to identify new therapeutic targets for the treatment of atherosclerosis, a simple automated assay of oxLDL uptake by macrophages was developed. In this study, the usefulness of this method as a screening tool was demonstrated by applying it to screen the ICCB library of chemicals with known biological activity. Twenty-two compounds with known mechanisms of action were found to affect oxLDL uptake by macrophages, and the majority of these compounds are known inhibitors of signaling proteins. Although various detected compounds confirmed previous findings, others are predicted to inhibit novel molecular targets that are involved in oxLDL uptake.

Materials and Methods
All animal protocols were approved by the Division of Comparative Medicine at Washington University in St Louis. An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results
Screening Design and Positive Control Selection
Preliminary experiments to optimize the screening conditions indicated that wells of J774 cells incubated with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-oxLDL for 2 hours demonstrated a strong and uniform DiI-oxLDL fluorescence signal following PBS washing. However, proper calibration of the PBS washing was mandatory to extrude the majority of extracellular DiI-oxLDL without causing extensive cell washout (see detailed description in the Online Data Supplement). The short-term exposure to DiI-oxLDL and the absence of extracellular lipoproteins responsible for reverse cholesterol transport enabled focused assessment of oxLDL influx under these experimental conditions.

We next sought a reliable, functionally characterized compound as a positive control. The role of JNK2 in atherosclerotic lesion formation was previously demonstrated by use of JNK2-/- mice and the pharmacological blocker of JNK activity SP600125.8 Several reports indicated that inhibition of p38 MAPK also reduces oxLDL uptake.9,12 We therefore assessed the effects of the JNK pathway inhibitor SP600125 (10 μmol/L) and the p38α/β inhibitor SB203580 (10 μmol/L) on DiI-oxLDL uptake by J774 cells. Both SP600125 and SB203580 reduced DiI-oxLDL uptake when compared to the DMSO by 17.7±3.1% (P=6.91×10⁻⁵, n=8), and 21.6±2.9% (P=3.61×10⁻⁶, n=8), respectively. The JNK inhibitor SP600125 was finally selected for use as a positive control because of its previously established ability to block atherosclerotic lesion formation in vivo.8

We next established that the assay was sensitive enough to detect compounds that were at least as potent as SP600125. 96-well plate wells were loaded with SP600125 and compared to control wells exposed to DMSO only. Using a cutoff of ±3 median absolute deviation (MAD), all wells exposed to SP600125 could be easily differentiated from the DMSO wells following PBS washout of the cells (Figure 1).

ICCB Library Screening
The ICCB library of “known bioactives” is a collection of 480 chemical compounds with known mechanisms of action at a molecular level. This library of compounds was tested in the robotic assay of DiI-oxLDL uptake by J774 cells. Figure 2 provides an example of the data that was gathered for one of the 6 drug plates that was tested in duplicate to screen the entire ICCB library. Of 480 tested compounds, 67 (~14%) substantially affected the prewash fluorescence. These com-
pounds were excluded from further analysis. From the remaining 413 compounds, 22 compounds were found to affect DiI-oxLDL and also passed a secondary confirmation (defined as a total of 4 of 5 positive tests on different cell plates).

Of the 22 detected compounds, most were inhibitors of intracellular signaling proteins (Online Table I). The JNK inhibitor SP600125 is among the compounds identified in the screen independent of its role as a positive control. Other identified compounds included 3 potent suppressors of nuclear factor (NF)-κB activation (TPCK, Gliotoxin and Bay 11-7082), 2 protein kinase (PK)C inhibitors (Go6976 and GF-109203X), 2 src family tyrosine kinase inhibitors (phosphatase [PP]1 and PP2), 2 inhibitors of protein phosphatases (cyclophilin A and cantharidin), a broad-spectrum kinase inhibitor (K252A), and an inhibitor of phospholipase (PL)C (U73122). In addition, 2 antipsychotic antidopaminergic medications (clozapine and trifluoperazine), 1 antagonist of platelet-activating factor named PCA4248, and a μ-opioid receptor agonist (loperamide) were found to modulate DiI-oxLDL uptake. Of note, loperamide was the only compound identified as an enhancer of oxLDL uptake.

Additionally, several chemicals known to regulate endocytosis and endocytic pathways were identified. These include an inhibitor of clathrin coated pit-mediated endocytosis (ikarugamycin), a vacuolar ATPase inhibitor (bafilomycin A1), an inhibitor of ER-Golgi transport (brefeldin A), and 2 actin polymerization inhibitors (cytochalasin D and latrunculin B). Finally, a calcium ionophore (ionomycin) was also identified as an active compound.

Confirmation of oxLDL Uptake Modulation
Because repeated washings were intrinsic to the primary screening protocol, it was important to confirm that the reduced postwash fluorescence signal resulted from the inhibition DiI-oxLDL uptake rather than a washout of cells. To address this issue in a systematic way, the cells were stained with Hoechst dye and the fluorescence was recorded for all active compound wells and compared to the DMSO controls (Figure 3). For 14 of the active compounds, no change in Hoechst fluorescence was detected, and this confirmed that these drugs were bona fide modifiers of oxLDL uptake. Of the remaining 8 drugs, 4 active compounds (PCA 4248, U73122, gliotoxin, and Bay 11-7082) showed a “moderate” washout effect (Hoechst signal between −3 to −6 MAD), whereas the other 4 drugs (calyculin A, cantharidin, ikarugamycin, and ionomycin) caused a severe reduction of the Hoechst signal to levels lower than −6 MAD. Figure 4 demonstrate that several active compounds affected cell morphology and the distribution of the DiI-oxLDL signal inside cells. However, compounds that caused decreased Hoechst signal (Figure 3) also reduced the number of observed cells. In 2 cases, ionomycin and calyculin A, clear signs of cell lysis were also noted microscopically (Figure 4, bottom).

Active compounds causing a reduced Hoechst signal were designated as unconfirmed compounds and were subjected to further analysis to determine whether they were bona fide inhibitors of DiI-oxLDL uptake aside from their effect on cell

Figure 2. High-throughput robotic screening identified several compounds that modulate oxLDL uptake by J774 cells. An example of total fluorescence signals obtained from 2 cell plates that were used in duplicate to assess 80 compounds from the ICCB drug library. Each column (dash verticals line) represents a single drug that was applied to the 2 different cell plates (marked as closed square and open circle for each drug). Top, Prewash DiI-oxLDL fluorescence readings. Bottom, postwash DiI-oxLDL fluorescence readings. Values are normalized to MAD and presented as deviations from the DMSO median. Horizontal dash bars mark the ±3 MAD range. B indicates blank controls (no DiI-oxLDL added); DMSO, control wells exposed to DMSO only; JNK inh., positive control wells exposed to SP600125; Q, drugs for which fluorescence quenching was detected (excluded from further analysis as denoted by gray vertical bar in the upper panel); H, drugs identified as true modifiers of postwash DiI-oxLDL signal.
survival and adherence. However, ikarugamycin was not subjected to further analysis, because its ability to block oxLDL uptake in J774 cells was previously demonstrated by another group. To test the 7 remaining unconfirmed compounds, J774 cells were exposed to the drugs on 12 well plates followed by DiI-oxLDL application. All cells (adherent and nonadherent) were collected and subjected to flow cytometric analysis. Interestingly, all 7 unconfirmed compounds did in fact inhibit oxLDL (Figure 5). Samples exposed to ionomycin demonstrated increased amount of cell debris and lower density of intact cells, consistent with cell lysis (data not shown).

Dose–Response Analysis in Peritoneal Macrophages

The potencies of 16 confirmed active compounds were further evaluated by dose–response analyses. In these assays, the uptake of DiI-oxLDL by murine peritoneal macrophages was evaluated after exposure to drugs by use of the automated plate reader to measure whole well fluorescence. The results of these analyses indicated a good correlation with the potency of the compounds in J774 cells and also showed typical dose–response curves for 10 of the drugs (Figure 6), including all 3 that block NF-κB activation (Bay 11-7082, TPCK and gliotoxin), the PLC inhibitor U73122, the broad-spectrum kinase inhibitor K252A, the vacuolar ATPase inhibitor bafilomycin A1, the inhibitor of endoplasmic reticulum (ER)-Golgi transport brefeldin A and the 2 phosphatase inhibitors calyculin A and cantharidin. Ionomycin demonstrated relatively low potency and was only effective at dosed higher than that used in the J774 cell chemicals screen. In addition, its effect was associated with a 10% to 20% reduction in Hoechst fluorescence and a microscopic morphology consistent with cell death. Of note, none of the other compounds that were tested in the dose–response analysis affected the Hoechst signal of postwash DiI-oxLDL fluorescence. Although the effect of clozapine on the postwash signal was more potent than on the prewash signal, both followed quite closely together, indicating that a direct effect of clozapine on the DiI-oxLDL fluorescence cannot be excluded.

Atypical dose–response curves were observed for 4 active compounds (Figure 7). Loperamide was found to have a complex effect: it increased DiI-oxLDL uptake by murine peritoneal macrophages at low doses but inhibited uptake at higher doses, consistent with a multimode mechanism of action (see Discussion). Clozapine appears to quench DiI-oxLDL fluorescence. Although the effect of clozapine on the postwash signal was more potent than on the prewash signal, both followed quite closely together, indicating that a direct effect of clozapine on the DiI-oxLDL fluorescence cannot be excluded. Trifluoperazine, the second antipsychotic active compound, was found to be effective only at high concentrations in which it also affected the prewash fluorescence to some extent. The PKC inhibitor GF-109203X was also unexpectedly found to reduce the prewash fluorescence. However, the postwash signal for GF-109203X-treated cells did not correlate with the prewash effect, and the drug was clearly effective at low concentrations when there was no effect on the prewash fluorescence. The second PKC inhibitor, Go6976, was found to be ineffective at blocking DiI-oxLDL update by murine peritoneal macrophages. Because of the complex results noted above with the PKC inhibitory compounds in peritoneal macrophages, both PKC inhibitors were additionally tested with a flow cytometry-based technique in J774 cells. Go6976 was tested at the original dosage used for the primary screen and was found to reduce the DiI-oxLDL signal to 52.8±22% of DMSO control (P=0.003, n=4). GF-109203X was tested at a concentration of 3 μmol/L, at which no effect was observed on prewash
fluorescence (Figure 7). GF-109203X at this dose still reduced the DiI-oxLDL signal to 72.2±6.2% of DMSO control (P=0.006, n=5).

Viability Studies in Peritoneal Macrophages
To systematically evaluate the possible role of drug-induced cytotoxicity in macrophages, all compounds for which a dose–response analysis was performed were reevaluated by use of an Alamar Blue viability assay (Online Figures II and III). The majority of compounds demonstrated some degree of cytotoxicity at higher drug concentrations. However, minimal cytotoxicity in the effective dose range (for inhibiting oxLDL uptake) was observed for the NF-κB inhibitors TPCK and Bay 11-7082, the PKC inhibitors Go6976 and GF-109203X, the broad-spectrum kinase inhibitor K252A, the JNK inhibitor SP-600125 and bafilomycin A1. In contrast, severe cytotoxicity was noted for ionomycin, calyculin A, cantharidin, and PCA 4248 in their effective dose ranges. For the remaining drugs, an intermediate effect on cytotoxicity was noted. Of note, the inhibitory effect of high concentrations of loperamide on oxLDL uptake was correlated with increased cytotoxicity (Online Figure III).

In Vivo Efficacy of Bay11-7082
Because Bay11-7082 was previously found to be well tolerated in vivo at a dose range of 5 to 20 mg/kg body weight,13,14 we sought to determined whether it can prevent oxLDL uptake in vivo. To that end, mice with thioglycollate-elicited macrophages were treated with Bay11-7082 or with DMSO by intraperitoneal injection (IP) for 2 hours followed by IP
administration of Dil-oxLDL. Two hours following Dil-oxLDL administration, macrophages were collected, fixed, and analyzed by flow cytometry. Whereas 5 mg/kg Bay11-7082 did not exhibit significant effect (n = 3, not shown), the Dil-oxLDL fluorescence using 20 mg/kg Bay11-7082 was reduced to 8.4 ± 4.8% of that observed in macrophages from mice treated with DMSO (n = 4 for each group, P = 0.014). In addition, when 20 mg/kg Bay11-7082 was administered subcutaneously 12 hours before Dil-oxLDL administration, Dil-oxLDL uptake by macrophages was also reduced compared to DMSO-treated animals, but to a lesser extent. Indeed, the median Dil-oxLDL fluorescence of macrophages from mice treated with Bay11-7082 by subcutaneous injection was 83.6 ± 4.0% that observed in macrophages from mice treated with DMSO by subcutaneous injection (P = 0.06). Of note, Trypan blue staining of elicited macrophages from animals treated with IP injection of 20 mg/kg Bay11-7082 showed that the majority of cells are viable 4 hours following drug application (not shown). Moreover, flow cytometry analysis of nonfixed cells indicated similar efficacy of 20 mg/kg Bay11-7082 when only viable cells were subjected to analysis based on costaining with 7AAD (Online Figure IV).

**Discussion**

Atherosclerotic lesion formation is an inflammatory process in which macrophages play a major role. Although oxLDL uptake is only one of the components affecting the transition of macrophages to foam cells, the concept of attenuating atherosclerosis by blocking the uptake of oxLDL by macrophages is supported by several recent in vivo studies. Interestingly, it appears that some modes of inhibiting the short-term uptake of oxLDL, such as blockade of the JNK and p38 MAPK signaling pathways, blocks atherosclerotic lesion development in long-term in vivo models. These results support the hypothesis that oxLDL uptake by macrophages in vitro is an informative model of the formation of foam cells, hallmarks of atherosclerotic lesion formation and an attractive target for the development of new therapeutics. The application of such methodology to the clinical setting, in which established atheromatous lesions are mostly encountered, is not clear at the present time. However, it is possible that foam cells may be formed throughout the history of lesions. Furthermore, inhibition of cholesterol influx in the presence of continued cholesterol efflux may cause regression of established foam cells within lesions.

In general, the majority of compounds identified in the present high-throughput screen affected signaling proteins or proteins involved in endocytosis. Although several signaling proteins were previously found to be important for oxLDL uptake by macrophages, the unbiased results of the present screen provide compelling evidence that signaling proteins, in particular protein kinases, regulate oxLDL uptake by macrophages.
Confirmation of Previous Findings

Apart from the JNK inhibitor SP600125, which was shown to affect atherosclerosis in vivo and was identified by the present screen independent of its role as a positive control, several other active compounds confirmed important previously published results.

Oxidized LDL was recently shown to induce CD36-dependent activation of JNK in macrophages, and the carboxy-terminal cytoplasmic tail of CD36 was found to interact with a signaling complex containing MEKK2 as well as the Src tyrosine kinase Lyn. Accordingly, a pharmacological blocker of Src-family kinases AG1879 (PP2) was found to block CD36-dependent uptake of oxLDL into macrophages. PP2 and an additional Src-family inhibitor (PP1) were both identified in an unbiased fashion by the present high-throughput chemical screen as active compounds.

Two PKC blockers (Go6976 and GF-109203X) were identified in the present high-throughput chemical screen as inhibitors of oxLDL uptake by J774 cells. Many, if not all, PKC family members are expressed in J774 cells and in primary mammalian macrophages. In addition, 2 inhibitors of PKC, staurosporine and H-7, were previously shown to inhibit oxLDL uptake by cultured peritoneal macrophages. Recently, specific inhibition of PKCβ1 in PMA-activated human macrophages was found to attenuate oxLDL uptake and foam cell formation in vitro. The results of the present study are therefore concordant with previously published data supporting a role for PKC family members in foam cell formation. It is notable that both Go6976 and GF-109203X were found to inhibit oxLDL uptake in J774 cell, whereas Go6976 was found to be ineffective in peritoneal macrophage (Figure 7). Similar inactivity of Go6976, an inhibitor of classic PKC isoforms, was previously described in thioglycollate-elicited peritoneal macrophages. Although the mechanism underlying this differential response of J774 cells and peritoneal macrophages to Go6976 is not clear at the present, it is worth noting that an important difference between these 2 cell types is the absence of apoE expression in J774 cells, which may affect some of the processes of oxLDL internalization.
Scavenger receptor–mediated endocytosis is one possible route of oxLDL uptake by macrophages. Although clathrin-coated pit-mediated endocytosis was implicated in one study, other modes were also implicated specifically in oxLDL uptake and cellular trafficking by class B scavenger receptors. Previous studies showed that actin polymerization inhibitors blocked oxLDL uptake by macrophages. In addition, ER-Golgi transport may also be involved in oxLDL uptake. The detection of ikarugamycin, latrunculin B, cytochalasin D and brefeldin A as active compounds by the present high-throughput chemical screen confirm and extend these results and suggest that actin polymerization and clathrin-coated pit mediated endocytosis are important in oxLDL uptake by macrophages.

The vacuolar ATPase inhibitor bafilomycin B1 was previously found to block lipid accumulation of J774 cells exposed to oxLDL. Bafilomycin A1 is a close derivative of bafilomycin B1 with similar pharmacological properties. The present high-throughput screen detected this drug and additional experiments confirmed it to be a potent inhibitor of oxLDL uptake by murine peritoneal macrophages (Figure 6).

New Mechanistic Insights
The identification of Bay 11-7082, a potent blocker of NF-κB activation, as a potent inhibitor of macrophage oxLDL uptake is a new finding from the present study. In agreement with the activity of Bay 11-7082, the protease inhibitor TPCK and the 20S proteasome inhibitor gliotoxin, 2 chemicals also known to block NF-κB activation, were identified as active compounds that block oxLDL uptake (Online Table I). Bay 11-7082 was also tested and found active in vivo at inhibiting oxLDL uptake by macrophages. The common mechanism governing all 3 drugs is their ability to prevent IκBα from being degraded, thereby preventing NF-κB activation and translocation. Although the potential role of NF-κB as a key regulator of macrophage oxLDL uptake is a new finding of the present study, previous work implicated the NF-κB pathway in the pathogenesis of atherosclerosis and block of NF-κB activation was also found to inhibit atherosclerotic lesion formation in vivo. In addition, murine macrophages overexpressing a transdominant, nondegradable form of IκBα were resistant to lipid loading following 5 days of treatment with ox-LDL. However, because mRNA levels of CD36 were increased in these cells, the effect of NF-κB inhibition was postulated to result from augmented cholesterol efflux rather than reduced oxLDL influx (although influx was not directly tested in this study). Interestingly, although oxLDL was previously shown to activate NF-κB in a CD36-dependent manner, the present finding that inhibitors of NF-κB potently block oxLDL influx has not been previously reported.

The present finding that U73122, an inhibitor of PLC, blocked oxLDL uptake by macrophages is also novel. However, given the increasing evidence that PKC family members regulate oxLDL uptake, and the fact that PLC generates diacylglycerol and inositol 1,4,5-trisphosphate, which are direct and indirect activators of some PKCs, a role of PLC is not surprising. It should be noted that U73122 had a moderate effect on cell viability which may contribute to its ability to block oxLDL uptake whereas the PKC inhibitors had no such effect on viability (Online Figure II).

The role of G-coupled receptors in macrophage oxLDL uptake has not been previously established. The finding that Loperamide, a μ-opioid receptor agonist, increased oxLDL uptake by J774 cells is of interest and may complement experiments suggesting a role for opioid receptors in atherosclerosis. Although the activity of loperamide on oxLDL uptake became inhibitory at high concentrations (Figure 7), this was correlated with decreased cell viability at these high doses (Online Figure III). The detection of 2 antipsychotic
dopamine antagonists as active compounds is also interesting given recent studies linking the long term clinical use of L-dopa with an accelerated rate of atherosclerosis. However, trifluoperazine reduced oxLDL uptake only at high concentrations in which the drug may also act as a calmodulin inhibitor. Therefore, more work is needed to determine whether dopamine receptors regulate oxLDL uptake.

Modified forms of LDL are taken up by macrophages via scavenger receptors including SR-A and CD36. Therefore, the mechanistic relationship between identified drug targets and these scavenger receptors is an important issue. As already stated, CD36 is known to bind to Src family members, and presumably liganding of CD36 results in Src activation and JNK2 activation. Because NF-κb seems to play a significant role in oxLDL uptake according to our unbiased screen, and because NF-κb was previously shown to be activated in a CD36-dependent manner, it seems likely that CD36 liganding triggers NF-κb activation that is required for oxLDL uptake. The relationship between NF-κb activation and Src-JNK2 activation following CD36 liganding is an important issue for future investigation. There is no known direct signaling activity of SR-A, but JNK2-dependent phosphorylation of SR-A may modulate LDL uptake.

In summary, in the present study a novel, high-throughput chemical screening technique was developed to investigate foam cell formation. This technique provides a simple, potent and reliable method to identify active compounds that regulate oxLDL uptake by macrophages. This technique may also give important insights into the pathogenesis of atherosclerotic lesion formation and may identify novel therapeutic agents for the treatment of patients with atherosclerotic vascular disease.

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Disclosures

None.

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Materials and Methods

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written. All animal protocols were approved by the Animal Studies Committee of the Division of Comparative Medicine at Washington University in St. Louis.

Cells

J774 murine monocyte/macrophage cells were grown in Medium A (Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-Glutamine, 100 U/ml penicillin and 0.1mg/ml streptomycin supplemented with 10% fetal bovine serum). Peritoneal macrophages were collected from C57Bl6/J adult mice 4 days after an IP injection of 4% (W/V) BBL Thioglycollate (BD, France) by use of peritoneal washings with cold Hank's Buffered Salt Solution. Cells were resuspended in Medium A, for plating as described below. All research involving the use of mice was performed in strict accordance with protocols approved by the Animal Studies Committee of Washington University School of Medicine.

Reagents

Human Dil-oxLDL (*Autogen Bioclear UK*) was diluted in Medium B (serum free Medium A) to create a stock solution, which was added to each well to reach a final concentration of 10 µg protein per ml of the Dil-oxLDL. The 480 compounds of the ICCB
known bioactive library (BIOMOL) were diluted in DMSO to create six 96 well drug screening plates (for details on master stock concentration of individual drugs see http://htc.wustl.edu). Final application included 1:100 dilutions of the drugs so that DMSO reached a concentration of 1%. The JNK II inhibitor SP600125 (10µM in 1% DMSO, Calbiochem, USA) served as positive control. The positive "hits" selected for dose response studies in peritoneal macrophages were prepared in DMSO at master stock concentrations.

High-throughput screen

One day before the screening, sub-confluent J774 cells were resuspended in Medium A and plated at 25000 cells per well in twelve 96-well plates (140 µL per well) with an automated cell plater (Wellmate, Matrix, USA). On the day of screening, drugs were added to 96 well plates that were loaded with Medium B by automated liquid handling (Sciclone ALH 3000, Caliper, USA). Thereafter, cell plates were washed once with PBS (ELX405, BioTek, USA), and each well was loaded with 125 µl of the drugs diluted in Medium B. Cell plates were incubated for 2 hours. All compounds were tested in duplicate plates that included 8 wells containing DMSO (negative control) and 8 wells containing SP600125 (positive control). After 2 hours, the Dil-oxLDL was added (20 µl per well) and plates were incubated for an additional 2 hours. At the end of the incubation period, fluorescence intensity was measured with a multi-mode microplate reader (Synergy-2, BioTek, USA, excitation; 525 nm; Emission; 580 nm) before removing the incubation medium (pre-wash reading, to detect autofluorescence and quenching). Fluorescence was then measured a second time after the Dil-oxLDL-
containing medium was removed and the cells were washed 3 times with PBS in order to determine the amount of cellular uptake of Dil-oxLDL (post-wash reading). Next, cells were fixed with 4% paraformaldehyde (Sigma, St. Louis MO) and stained with 3 µg/ml Hoechst 33342 (Sigma, St. Louis MO). Images of cell morphology (transmission), Hoechst fluorescence and Dil-oxLDL fluorescence were obtained with a 20X objective using a high content imager (ImageXpress Micro, Molecular Devices, USA).

Proper calibration of the post-wash reading was found to be a crucial issue for obtaining reliable and uniform data about oxLDL uptake. In preliminary studies, microscopic evaluation often revealed small areas of extracellular Dil-oxLDL that were non-specifically retained in the extracellular space following post-incubation cell washes. However, the use of extensive washing protocols (by increasing the number of washing cycles or the washing intensity) often resulted in increased cell loss. Therefore, the wash protocol that was eventually adopted was one that resulted in maximal extracellular Dil-oxLDL washing without causing cell loss. While this approach resulted in highly consistent results (see Figures 1, 2), some of the Dil-oxLDL signal was a consequence of retained extracellular Dil-oxLDL and this caused an underestimation of the actual efficacy of compounds to inhibit Dil-oxLDL uptake (Online Figure I). Since flow cytometry evaluates cell-specific fluorescence, retained extracellular Dil-oxLDL was not present in flow cytometry experiments and the absolute effect of drugs was probably more accurately determined by this method (Figure 5, Online Figure IV).

Flow cytometry analysis
J774.A1 cells were plated on 12 well plates at a concentration of 260,000 cells per well in Medium A. On the following day, the wells were incubated with media B containing selected compounds for 2 hours followed by addition of Dil-oxLDL (10µg/ml) for an additional 2 hours. Adherent and non-adherent cells were washed with PBS and resuspended in 4 % paraformaldehyde. Flow cytometry analysis of Dil-oxLDL fluorescence (excitation; 530 nm, emission; 590 nm) was done with a MoFlo high speed cell sorter (Cytomation, USA).

**Dose response and viability studies in murine peritoneal macrophages**

Peritoneal macrophages were plated at 25,000 cells per well in 96-well plates and incubated overnight. Selected compounds were serially diluted in DMSO on 96 well plates. Drug plates were robotically mixed with Medium B and applied to the cell plates. All other procedures were performed as described for the primary chemical screen. For viability studies, similar cell plates of peritoneal macrophages (25,000 cells/well) were exposed to the drugs of interest at different dilutions and incubated for 2 hours. Thereafter Alamar blue dye (Resazurin, 440µM, Sigma-Aldrich, USA) was added to all wells (10% volume) and plates were incubated for additional 1 hour. Fluorescence measurements were than applied (Synergy-2, BioTek, USA, excitation; 545 nm; Emission; 590 nm) and the results were compared to DMSO control wells.

Computerized assessment of cell-specific Dil-oxLDL fluorescence in peritoneal macrophages (Online Figure I,B) was done on images obtained following cell fixation using a 20X objective on a high content imager (ImageXpress Micro, Molecular
Devices, USA). For each well nine standard fields were obtained (each containing an average of ~115 cells). Hoechst fluorescence and Dil-oxLDL fluorescence were used for analysis, with MetaExpress 2.0 software (Multiwavelength cell Scoring application). Dil-oxLDL signal in all locations was related to the closest nuclei detected in Hoechst image. A threshold for Dil-oxLDL signal was determined from observations in DMSO control fields so that cells were positive if at least $50 \mu m^2$ of the cell area was at least 300 gray scale levels above local background. Using this criteria analysis was done on all wells of the plate and the % of Dil-oxLDL positive cells was determined for each well. While patches of extracellular Dil-oxLDL were still related to cells by the described methodology, it largely attenuated the effect of such patches on the overall results, since such patches affected the results of a small minority of cells in each field. Positively detected cells were averaged on each well and compared to the average results obtained in the 8 DMSO wells (Online Figure I,B).

**In vivo assessment of Bay11-7082 activity on oxLDL uptake**

C57BL/6 mice were injected with thioglycollate in order to elicit macrophages. After 4 days, mice were injected intra-peritoneally with Bay11-7082 at a tolerated dose of 20 mg/kg body weight $^{1,2}$ in 50% DMSO. Control mice were injected with equivalent doses of 50% DMSO only. Two hours later, Dil-oxLDL (2 µg/gm body weight diluted in PBS to a total volume of 1 ml), was injected intra-peritoneally. After two hours of exposure to the oxLDL, animals were killed and peritoneal macrophages were collected. Cells were resuspended in red blood cell lysis buffer for 10 minutes. Thereafter, collected cells were washed, fixed and Dil-oxLDL uptake was analyzed by flow cytometry. In a
different set of experiments, animals were treated subcutaneously with a similar dose of Bay11-7082 or DMSO that were applied overnight before Dil-oxLDL application for 2 hours and cell collection. For the evaluation of plasma membrane integrity, non-fixed macrophages elicited from control and Bay11-7082-treated animals following 4 hours of drug exposure (2 hours of drug exposure followed by 2 hours of Dil-oxLDL application) were diluted 1:1 with 4% Trypan blue dye (Sigma-Aldrich, USA) and assessed by bright-field microscopy.

In addition, non-fixed peritoneal macrophages elicited from control and Bay11-7082-treated animals co-stained with 7-AAD viability staining solution (eBioscience, USA), and flow cytometry measurements of Dil-oxLDL fluorescence (excitation; 530 nm, emission; 590 nm) and 7-AAD fluorescence (excitation; 488 nm, emission; 647 nm) were performed. Evaluation of Dil-oxLDL fluorescence was limited to 7AAD-negative, and therefore viable, macrophages

Statistical analysis

Median ± 3 median absolute deviation (MAD) $^3$ was used as a standard cutoff in analyzing the high-throughput chemical screening results. Dose response curves for compounds applied to peritoneal macrophages are presented as the mean ± SD of 3-4 independent readings at each dose of compound. Flow cytometry measurements (Fig 5) were statistically compared to DMSO control with a Student’s T-test with a Bonferroni correction for repeated measures. In vivo comparison of Dil-oxLDL fluorescence
between Bay 11-7082 treated animals and DMSO controls was done using unpaired Student's T-test.

References


Figure legends

**Online Figure I.** Underestimation of drug effect due to retained extracellular Dil-oxLDL.

A. Microscopic view of peritoneal macrophages that were exposed to Bay11-7082 in the dose response experiments summarized in Figure 6. In each photograph, an overlay of transmission and Dil-oxLDL fluorescence signal is shown. Bay11-7082 concentration is marked for each photograph. Black arrows indicate nonspecific Dil-oxLDL that was retained in the extracellular space after PBS washings. Note that Bay11-7082 administered in concentrations higher than 1.5 μM caused almost complete inhibition of Dil-oxLDL uptake consistent with the photographs of J774 cells from the ICCB screen (Figure 4), the flow cytometry results obtain for J774 cells (Figure 5) and the flow cytometry results obtain for peritoneal macrophages in vivo (Online Figure 4 below). B. Computerized analysis of cell-specific Dil-oxLDL uptake for 4 of the drugs that were evaluated in the dose response experiments in Figure 6 of the manuscript. Note that this analysis revealed substantial underestimation in the absolute effect of drugs using the high-throughput methodology. Nevertheless, IC50 results were very similar with both the image-based analysis and the whole cell fluorescence readout results, respectively (Bay 11-7082: 3.45 μM vs. 4.15 μM; TPCK: 4.13μM vs. 7.03 μM; U73112: 11.58 μM vs. 16.9 μM; K252A: 0.27 μM vs. 0.48 μM).

**Online Figure II.** Evaluation of peritoneal macrophage viability after treatment with compounds found to inhibit Dil-oxLDL uptake. Drugs previously evaluated for dose response characteristics measured by inhibition of Dil-oxLDL uptake in Figure 6 were reevaluated for their ability to reduce cell viability. Serial dilutions of drugs were applied
to peritoneal macrophages on 96-well plates. Following 2 hours of incubation with drugs and an additional 1 hour incubation with resazurin, Alamar blue fluorescence was evaluated. Values are normalized to the DMSO control wells (dash horizontal line). Each point represents the mean ± SD of 2-3 different plates. The vertical dot lines represent the IC50 values that were obtained for Dil-oxLDL uptake (Figure 6). The vertical bar on the X axis represents the dose of the compound used in the original high-throughput screen.

**Online Figure III.** Evaluation of peritoneal macrophage viability after treatment with compounds found to inhibit Dil-oxLDL uptake (continued). Drugs previously evaluated for dose response characteristics measured by inhibition of Dil-oxLDL uptake in Figure 7 were reevaluated for their effect on cell viability as described for Online Figure 2.

**Online Figure IV.** Flow cytometry analysis of Dil-oxLDL uptake by peritoneal macrophages that were elicited from animals exposed to Bay11-7082 at a dose of 20 mg/kg body weight for 2 hour followed by intraperitoneal application of Dil-oxLDL for an additional 2 hours. Cells were incubated with red blood cells lysis solution and thereafter they were resuspended in HBSS and incubated with 7AAD for 10 minutes before flow cytometry assessment. Dil-oxLDL fluorescence was analyzed in cells that were negative for 7AAD staining (viable cells). Upper histogram (gray): a sham mouse exposed to DMSO only. Lower histograms (black): 3 animals injected with Bay11-7082.
Online Table I: Summary of active compounds identified in the initial high-throughput screen.

<table>
<thead>
<tr>
<th>Chemical Agent</th>
<th>Putative Mechanism of Action</th>
<th>Drug Con. (µg/l)</th>
<th>Drug Con. (µM/l)</th>
<th>Hit intensity (MADs from DMSO)</th>
<th>Dil-oxLDL signal (% of DMSO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNK II Inh. (Pos. cont.)</td>
<td></td>
<td>10</td>
<td>4.34</td>
<td>67.9</td>
<td>123.04</td>
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<tr>
<td>Loperamide</td>
<td>Opioid receptor agonist, Calcium channel blocker</td>
<td>10</td>
<td>19.4</td>
<td>-3.57</td>
<td>123.04</td>
</tr>
<tr>
<td>Tosyl-Phe-CMK (TPCK)</td>
<td>Serine protease inhibitor, blocks NF-κb activation</td>
<td>10</td>
<td>28.4</td>
<td>5.52</td>
<td>64.81</td>
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<tr>
<td>Calyculin A</td>
<td>PP1, PP2A inhibitor</td>
<td>1</td>
<td>0.99</td>
<td>10.74</td>
<td>25.38</td>
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<tr>
<td>Cantharidin</td>
<td>PP2A inhibitor</td>
<td>10</td>
<td>50.9</td>
<td>7.69</td>
<td>44.93</td>
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<tr>
<td>Ikarugamycin</td>
<td>inhibits clathrin coated pit-mediated endocytosis</td>
<td>10</td>
<td>20.8</td>
<td>12.57</td>
<td>12.49</td>
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<tr>
<td>Bafilomycin A1</td>
<td>Vacuolular ATPase inhibitor</td>
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<td>1.6</td>
<td>8.57</td>
<td>40.26</td>
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<tr>
<td>Clozapine</td>
<td>Dopamine antagonist</td>
<td>10</td>
<td>30.5</td>
<td>4.93</td>
<td>64.56</td>
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<tr>
<td>Brefeldin A</td>
<td>ARF GEF inhibitor</td>
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<td>35.6</td>
<td>4.14</td>
<td>71.15</td>
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<tr>
<td>Cytochalasin D</td>
<td>Inhibitor of actin polymerization</td>
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<td>19.7</td>
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<td>51.57</td>
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<td>Go6976</td>
<td>PKC inhibitor</td>
<td>10</td>
<td>26.4</td>
<td>4.43</td>
<td>65.12</td>
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<td>Latrunculin B</td>
<td>Actin polymerization inhibitor</td>
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<td>25.2</td>
<td>6.52</td>
<td>48.6</td>
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<tr>
<td>Ionomycin</td>
<td>Calcium ionophore</td>
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<td>14.1</td>
<td>11.56</td>
<td>8.89</td>
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<td>Bay 11-7082</td>
<td>Inhibitor of IKK kinase</td>
<td>10</td>
<td>48.2</td>
<td>11.71</td>
<td>7.69</td>
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<tr>
<td>Gliotoxin</td>
<td>Inhibitor of 20S-proteasome, blocks NF-κb activation</td>
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<td>30.6</td>
<td>9.67</td>
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<td>K252A</td>
<td>Kinase inhibitor (Broad spectrum)</td>
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<td>2.1</td>
<td>5.86</td>
<td>53.75</td>
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<td>U73122</td>
<td>Lipid biosynthesis, PLC inhibitor</td>
<td>10</td>
<td>21.5</td>
<td>5.12</td>
<td>53.93</td>
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<td>PCA 4248</td>
<td>PAF antagonist</td>
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<td>PP1</td>
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<td>PP2</td>
<td>Src family tyrosine kinase inhibitor</td>
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<td>33.1</td>
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<td>SP-600125</td>
<td>JNK inhibitor</td>
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<td>45.4</td>
<td>3.16</td>
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<td>Trifluoperazine</td>
<td>Dopamine antagonist, Calmodulin inhibitor in high doses</td>
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<td>20.8</td>
<td>3.27</td>
<td>70.1</td>
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<tr>
<td>GF-109203X</td>
<td>PKC inhibitor</td>
<td>10</td>
<td>24.2</td>
<td>6.93</td>
<td>39.59</td>
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</tbody>
</table>

The name, putative mechanism of action, concentration used (µg/l), concentration used (µM/l), normalized efficacy (number of MAD deviations from the median of DMSO) and % efficacy (% of detected post-wash Dil-oxLDL signal compared to DMSO controls) are listed for each compound. Values obtained for the positive control wells (exposed to 10µM SP600125) are presented at the top of the table (marked with gray).
Online Figure I

A

Bay 11-7082 dose response

DMSO

1.5 µM

12 µM

24 µM

B

Bay 11-7082

U73122

TPCK

K252A

Drug effect (% of DMSO)

Drug Concentration (µM)

Drug effect (% of DMSO)

Drug Concentration (µM)
Online Figure II

Bay 11-7082

TPCK

Gliotoxin

U73122

K252A

Brefeldin A

Bafilomycin A1

Ionomycin

Cantharidin

Calyculin A

PCA 4248

SP-600125

Fluorescence (% of DMSO)

Drug Concentration (µM)
Online Figure III

Drug Concentration (µM)

Fluorescence (% of DMSO)

Loperamide

Clozapine

Trifluoperazine

GF-109203X

Go6976
Online Figure IV

Cell count (7AAD negative cells) vs. Dil-oxLDL fluorescence

- DMSO
- Bay 11-7082