An electroaffinity labeling platform for chemoproteomic-based target identification

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ABSTRACT:
Target identification involves deconvoluting the protein target of a pharmacologically active small molecule ligand, which is critical for early drug discovery yet technically challenging. Photoaffinity labeling strategies have become the benchmark for small molecule target deconvolution, but covalent protein capture requires the use of high energy UV light, which can complicate downstream target identification. Thus, there is a strong demand for alternative technologies that allow for controlled activation of chemical probes to covalently label their protein target. Here, we introduce an electroaffinity labeling platform that leverages the use of a small, redox-active diazetidinone functional group to enable chemoproteomic-based target identification of pharmacophores within live cell environments. The underlying discovery to enable this platform is that the diazetidinone can be electrochemically oxidized to reveal reactive intermediate useful for covalent modification of proteins. This work, for the first time, demonstrates the electrochemical platform to be a functional tool for drug-target identification.

Improving the quality of target selection in the drug discovery process is one of the most important factors to reducing drug attrition in the clinic.[1–4] The selection of potential drug targets is aided by target identification and validation efforts that link a biologically active pharmacophore directly to the protein(s) it modulates. However, while the search for bioactive pharmacophores have been greatly enabled by disease relevant phenotypic assay screens, downstream identification of the actual protein target remains a challenging technical hurdle.[1,3,5] This hinders our ability to deconvolute therapeutically relevant targets and their involvement in disease biology. Thus, technologies that can facilitate the detection of a pharmacophore binding target within complex biological environments, such as the use of covalent capture methods, are in high demand to expedite commonly used downstream isolation and proteomic-based analytical efforts to determine target protein identity (Figure 1A).

Conventional affinity-based covalent capture technologies rely on the use of chemical- or photochemical-based cross-linker groups that, when appended to a pharmacophore of interest, enhance identification of their binding targets and molecular mechanisms (Figure 1B).[5,6] In particular, photoaffinitiy labeling (PAL) through use of the diazirine, a unique three-membered ring containing N=N bond, has emerged as a highly useful option due to its small size and high reactivity (Figure 1B).[7–10] Comparatively, the relatively inert nature of diazirine until photoactivation makes it far preferrable to chemical cross-linkers that are always activated and cause background labeling issues. However, despite its widespread adoption, the activation of diazirines relies on the use of high energy UV light, generating multiple reactive moieties such as carbenes and diazo species with differing bio-reactivity profiles that can complicate downstream proteomic analysis and target identification.[11–13]

Here we present an electroaffinity labeling (ECAL) platform technology in live cells (Figure 1C). The method relies on a similarly small functional group rarely explored in organic synthesis, a diazetidinone (DZE), that enables chemoproteomic-based target identification of pharmacophores within complex cell environments (Figure 1C). To showcase ECAL, we profiled DZE analogs of well-validated ligands as well as a previously unexplored REV-ERBα [also known as *NR1D1* (nuclear receptor subfamily 1 group D member 1)] agonist in live cells. Recent studies have revealed REV-ERBα as a potential drug target due to its broad role in pathologies such as sleep disorder, metabolic syndrome, heart failure, cancer, and inflammation.[14,15] Its putative ligands,[16] SR-9009 and SR9011, have been utilized to elicit various pharmacological activities in multiple *in vitro* and *in vivo* disease-phenotypes.[15] However, despite the wide-ranging biological implications, chemoproteomic validation of REV-ERBα as the target for these ligands has not been reported.

Results and Discussion

A suitable electrochemically active functional group

As with photochemistry, electrochemistry is one of the oldest methods for facilitating redox reactions, which still remains the most direct means for accomplishing oxidation and reduction. Presumably due to its heterogeneous nature and lack of protocols that reliably proceed in water, the application of electrochemistry to biomolecule functionalization in complex biological systems is limited.[17] Several recent successful applications in this context rely on the anodic oxidation of triazolinediones,[18] phenothiazines[19] or *N*-oxyls[20] to capture tyrosine or tryptophan residues in small oligopeptides and *in vitro* protein labeling. To our knowledge, there is no precedent for

electrochemical protein labeling in complex cellular environments. To begin these studies, several criteria were set for a useful functional group for ECAL. To allow minimally invasive placement of a reactive group on drug binding motifs,[9] the desired functional group must be as small as possible, redox-active, stable under physiological conditions, and readily accessible (Figure 2A). It must also be easily incorporated into a trifunctional monomer that includes orthogonally reactive functional groups such as an alkyne and a carboxylic acid to enable modular incorporation of desired small molecule ligands as well as reporter groups. Most importantly, a highly reactive species should be generated through mild electrochemical activation that can be performed bioorthogonally in a complex cellular environment.

Satisfying these criteria is challenging, since redox-active compounds are usually reactive or possess large π-systems, deviating from the ideal probe design. Moreover, the redox potential necessary for activation needs to be ideally within ± 1 V (vs Ag/AgCl), considering the cathodic limit by H_2O reduction (around –1 V) and anodic limit by oxidatively labile amino acid residues (Trp 1.02 V, Tyr 0.93 V).[17] On top of these difficulties, generating highly reactive species which rivals to carbenes (reactive species from photochemical activation of diazirines) through electrochemical redox under aqueous conditions in a biorthogonal manner is a demanding requirement. Not surprisingly, there is no such small motif known in the literature to satisfy all of these criteria. Accordingly, our explorations began by surveying a wide range of redox-active small molecules that could generate reactive species (Figure 2A). Some of them were inspired by existing literature, and others were designed based on first principles. For example, reductive means of activation on structures **1**-**3** were hypothesized to lead to reactive species such as vinylidene carbenes,[21] quinone methides,[22] and carbenes,[23] respectively. Unfortunately, these strategies were barely successful from the standpoint of stability, size, accessibility, and modularity. Oxidative modes of activation were also explored on structures **4**-**9** wherein reactive carbenes, diazonium,[24] benzynes,[25] sulfeniums, and acyl pyridiniums[26] could potentially be formed. Yet, these precursors showed no promising reactivity in a model study and were not further optimized or explored due to similar issues facing the reductive approach. Nevertheless, the oxidative approach was further pursued considering the fact that reactive species generated under oxidative conditions would likely be electrophilic, suitable for targeting peptides or proteins in which reactive amino acid residues are usually nucleophilic. The lesson from these exploratory studies and literature precedent was that hydrazine-containing species might be a promising motif due to their ease of oxidation under mild conditions.

Returning back to the drawing board, we considered the smallest functional group possible analogous to the diazirines enlisted for photoaffinity labeling. The insertion of a single carbonyl group into a diazirine with the reduction of N=N double bond led to the proposal of the diazetidinone (DZE) **10** (Figure 2B). This small, strained ring system was designed based on the intuitive notion that the embedded hydrazine motif would be readily oxidized to release a ketene upon nitrogen extrusion. Indeed, it is predicted that thermolysis of analogous structure could lead to ketene formation by cycloreversion,[27] further supporting the notion that DZEs are prone to fragment into ketenes upon suitable activation. Of note, DZEs are a rarely utilized functional group in organic synthesis. Their main use is as a bioisostere of β-lactams,[28] and acylated variants have been identified as serine-selective probes.[29] Many methods for their preparation have been reported,[28] yet their value as intermediates in synthesis is underexplored, and the electrochemical behavior of DZEs is not reported. Thus, the model DZE **11** was prepared, and detailed analysis of **11** was conducted by X-ray crystallography as well as cyclic voltammetry (CV) (Figure 2B,

Supplementary Figure 1-2). Interestingly, X-ray crystallographic analysis revealed that the amide nitrogen is considerably pyramidalized due to the highly strained four-membered ring, a rare example of twisted amides.[30] Furthermore, a cyclic voltametric study clearly suggested that **11** is indeed redox-active, although the oxidation peak around 1 V is ill-defined under the cyclic voltametric conditions. Bulk electrolysis of **11** in the presence of benzylamine afforded an amide product **12** in moderate yield. Only a trace amount of **12** was observable without electrolysis, indicating that **11** is mostly unreactive during the timescale of the control experiment (2.5 h). The formation of amides as well as deuterium incorporation at the α-position to the carbonyl support the hypothesis of a ketene as the reactive intermediate. Having demonstrated electrochemical activation of this latent functional group, we next designed a minimalist trifunctional monomer (**14**) consisting of a carboxylic acid group for ligand attachment, a reporter handle, and a DZE warhead.[9] The key DZE intermediate **14** can be conveniently accessed from the carboxylic acid **13** in one step by [2+2] cycloaddition between the corresponding ketene and di-*t*-butyl azodicarboxylate (Figure 2C).[31] Amide bond formations and click reactions can be used for attaching various ligands and reporter tags as exemplified through the synthesis of nine small molecule ligand-based DZE probes (see SI for complete listing).

Targeted protein labeling using DZE

With the DZE probes in hand, we first evaluated if a carbonic anhydrase (CA)-DZE-desthiobiotin trifunctional probe (sulfa-DZE, **15)** can label free CA in an electrochemically dependent and selective manner. Accordingly, **15** was incubated in the presence of 1:1 CA and bovine serum albumin (BSA) in PBS followed by 1-minute electrolysis (Figure 3A). Visualization via western blot revealed desthiobiotinylation of CA in an electrochemically dependent manner with highly selective labeling of CA over BSA (Figure 3B and Supplementary Figure 3). Importantly, this biotinylation effect could be competed away with free CA-inhibitor.

Next, the covalent labeling nature of the DZE probe was explored by performing peptide mapping of CA after electrolysis with **15**. Labeled protein was digested by LysC/Trypsin and desthiobiotinylated peptides were enriched by streptavidin agarose beads. The digested peptide samples were first analyzed by LC-MS to identify peptides containing the expected DZE adduct product (Supplementary Figure 4). The same samples were then submitted to a follow-up, targeted LC-MS analysis of the DZE-peptide adducts to sequence and quantify DZE-peptide adduct peptide spectral matches. From this analysis we observed modification of a diverse set of amino acids surrounding the entrance of the zinc binding pocket that include aspartate, glutamate, lysine, and tyrosine residues, as well as minimally detected labeling of valine, and phenylalanine (Figure 3C and Supplementary Table 1). As a comparison, we performed an identical proteomic analysis of CA exposed to photoaffinity labeling (PAL) via a broadly utilized, fully functionalized dialkyl diazirine probe containing design features similar to our DZE probe **15** (Figure 3C and Supplementary Table 2). UV irradiation of the CA-diazirine probe **16** in the presence of CA resulted in labeling near the active site entrance similar to the DZE probe but with different residue labeling patterns that primarily includes glutamate and aspartate residues (Figure 3C). This carboxylate side chain preference achieved by **16** was consistent with recent studies using a dialkyl diazirine probe,[11,12] highlighting the restricted amino acid residue reactivity profile for this commonly used PAL reagent. While the increased detection of peptides labeled by the UV/diazirine method suggest a higher labeling efficiency for PAL over the ECAL approach, the

broader amino acid labeling coverage achieved by the DZE probe can potentially facilitate increased opportunities for covalent protein capture within complex environments.

As further demonstration of the versatility of this electrochemical labeling approach, four additional small molecule ligand scaffolds (dasatinib, VX680, JQ-1, and PD0325901) were studied.[9,32] The DZE functional group was appended to each of the inhibitors to prepare **17**-**20**, and subsequently incubated in the presence of their respective binding proteins ABL, AURKA, BRD4, and MEK1. Electrochemically dependent desthiobiotinylation was detected for each of these protein/DZE-ligand pairs that could be competed away in the presence of excess free ligand (Figure 3D and Supplementary Figure 5). Importantly, similar to the CA labeling experiment in Figure 3B, the labeling effect was specific to the target protein as no off-target protein labeling was observed in the presence of a bystander protein (Supplementary Figure 6). A similar labeling trend could also be achieved in HCT116 cell lysates using MEK1 and AURKA inhibitors (Supplementary Figure 7).

ECAL in live cells

Having demonstrated the compatibility of ECAL with a wide range of ligand-protein pairs, ECAL was field-tested within the context of chemoproteomic profiling of known ligands in live cell culture. Considering that this is the first instance of ECAL in live cell systems, the biocompatibility of the electrochemical system on cell viability was evaluated with the finding that short reaction times employed within our labeling experiments do not impact cell viability (Supplementary Figure 8). Using dasatinib or JQ1 equipped with DZE and desthiobiotin (compounds **17** or **19**) we set out to identify endogenous proteins labeled within HCT116 colorectal cancer cells (Figure 4A). Cells were incubated with the DZE probes followed by electrochemical activation to initiate covalent protein labeling followed by enrichment for subsequent proteomic analysis (Figure 4B). Targeted labeling using JQ1-DZE-desthiobiotin probe **19** in HCT116 cells resulted in the significant enrichment of the known binding partners BRD4 and BRD2 (Figure 4C, Supplementary Figure 9, and Supplementary Tables 3-6). In contrast, when Dasatinib-DZE-desthiobiotin **17** was used for targeted protein labeling, SRC kinase (a known dasatinib ligand) was identified as a highly enriched target (Figure 4D and Supplementary Table 7). Comparing the 25 most enriched proteins detected from JQ1-DZE-desthiobiotin **19** and Dasatinib-DZE-desthiobiotin **17** targeted labeling led to no observed direct protein overlap (Figure 4E) as well as distinct differences in enriched GO-terms for biological and molecular functions (Figure 4F and 4G).

The ECAL technology was further showcased through CA-DZE **15** targeted labeling of CA in HT29 cells that resulted in increased CA enrichment in both hypoxic and normoxic conditions (Supplementary Figure 10 and Supplementary Tables 8-9).[33] These results, along with other alkyne- and desthiobiotin-based probe examples (Supplementary Figure 11, 12 and Supplementary Tables 10-11), highlight the ability of the ECAL technology to achieve selective chemoproteomic detection of ligand targets in complex biological systems. Finally, detection of cellular labeling using the ECAL method was done via confocal imaging of HCT116 cells labeled with Dasatinib-DZE. For this experiment, Dasatinib-DZE containing a desthiobiotin handle was added to cells followed by electrochemical activation. The cells were then imaged by confocal microscopy for desthiobiotinylation signal where we observed evidence of biotinylation only in cells to which electrochemical stimulation was applied and not in cells containing excess competitive ligand or in the absence of electrochemical stimulation (Supplementary Figure 13). As an additional control,

we performed labeling using a Dasatinib-biotin probe without the DZE group and failed to detect evidence of cellular biotinylation showing that labeling is dependent on presence of the DZE group (Supplementary Figure 13).

Regarding how the labeling of endogenous proteins was achieved using an exterior electrode, at this moment we consider that both direct and mediated oxidation pathways are possible. It is known that direct exchange of electrons is possible through cell membranes such as the case in red blood cells.[34] On the other hand, mediated oxidation is also feasible considering the myriad of redox-active co-factors as well as redox-active proteins that participate within various electron-transfer pathways in and through a cell.[35] However, unveiling the detailed mechanism of the probe activation is outside the focus of the current study, and could be a subject of future investigation. Regardless of DZE activation mechanism, we must admit that ECAL inevitably induces certain degree of redox perturbation in a cell. This aspect could be a potential caveat of this technology and could limit conditions that ECAL could be performed in some cases. In addition, it is noteworthy to mention that this method might not work on all target/probe pair labeling examples. Possible labeling variation based on target/probe pairs cannot be ruled out, and further optimization to electrochemical instrumentation and labeling methods will be required.

ECAL for chemoproteomic profiling of REV-ERB ligands

Having established our ECAL technology as a robust approach for target identification, chemoproteomic profiling of two structurally similar tertiary amine (TA) pharmacophores, SR9009 and SR9011 was pursued (Figure 5A).[15] These compounds were initially developed at Scripps as synthetic agonists of transcription factors REV-ERBα and REV-ERBβ for the regulation of circadian rhythm,[16] and have attracted further interest for their involvement in a number of important cell functions through suspected REV-ERB interactions.[15] This is due, in part, to the connection of REV-ERB to biological processes under control of the circadian cycle that includes immunological and metabolic pathways.[36,37] In an *in vivo* study, cellular treatment with SR9009 was linked to REV-ERBα-mediated boosting of exercise capacity via increased mitochondrial production in skeletal muscle.[38] This observation has led to the illicit usage of these SR9009-based agonists as exercise supplements and performance-enhancers within the body-building community.[39] SR9009 and SR9011 have also shown the ability to effect other biological processes that include the negative regulation of proinflammatory Th17 cell-mediated autoimmunity[40] and tumor cell killing,[41] all presumably through REV-ERB modulation. However, despite the illicit use in humans and the multiple *in vitro* and *in vivo* based activities of SR9009 and SR9011, direct identification of REV-ERB as the protein target is lacking. Thus, we set out to validate REV-ERB binding of these important biologically relevant molecules via chemoproteomic analysis using our ECAL technology.

TA-DZE probe **21** was rationally designed based on our minimalist trifunctional monomer **14** and the required TA pharmacophore of the SR9009 agonist (Figure 5B). Amide coupling of SR9009 synthetic agonist core with DZE trifunctional monomer **14** followed by Boc-deprotection delivered the desired probe **21** in high yields (49% for two steps). To confirm that the newly designed and synthesized TA-DZE probe **21** retains desired pharmacological properties, the probe was screened along with parent compounds SR9009 and SR9011 in a previously reported Th17 differentiation assay on primary cells.[40] Primary human CD4 T cells were cultured under Th17 differentiation conditions with or without compounds (DMSO, SR9009, SR9011 and TA-DZE probe **21**). Similar

to parent compounds SR9009 and SR9011, TA-DZE probe **21** treatment inhibits Th17 differentiation (Figure 5C).

With the desired TA-DZE probe in hand, chemoproteomic analysis using ECAL was performed in HEK293T cells overexpressing REV-ERBα, the reported target of this molecular scaffold (Figure 5D and Supplementary Figure 14). Accordingly, HEK293T-REV-ERBα cells were incubated with TA-DZE probe **21** with or without electrolysis. Following the electrochemical treatment step, labeled proteins were then conjugated to biotin-azide through CuAAC click reaction and affinity purified on streptavidin beads. Enriched proteins were subjected to tryptic digestion and downstream TMT-based quantitative LC-MS/MS analysis. Volcano plot analysis identified proteins from our ECAL method showing significant enrichment over the no electrolysis controls (Figure 5E and Supplementary Table 12). Gene ontology analysis of the detected proteins showed enrichment of biological terms associated with both SR9009/SR9011 and REV-ERB activity that include metabolic and gene expression processes (Figure 5F). However, to our surprise, REV-ERBα was not enriched with our TA-DZE probe **21** despite overexpression of this protein in 293T cells (Figure 5E, Supplementary Figure 15, 16, and Supplementary Tables 13-15). A similar lack of REV-ERBα protein enrichment in HEK293T-REV-ERBα cells was observed when a diazirine analog of the SR9009 probe (TA-Diazirine) was used for covalent protein capture via UV activation (Supplementary Figure 17, and Supplementary Table 16). Additionally, we performed labeling with TA-DZE in PBMCs and CD4+ T cells where the TA probes displayed functional activity and failed to detect enrichment of REV-ERBα (Supplementary Figure 18, and Supplementary Tables 17-18). These results, in conjunction with a recent study reporting that SR9009 modulates cellular metabolism, viability, proliferation, and gene expression in REV-ERB knockout systems,[42] suggests that these TA scaffolds potentially operate independently of REV-ERB.

Given the broad pharmacological properties of these TA scaffolds and failure to detect REV-ERB protein enrichment, the gene expression pattern of protein hits from ECAL targeted labeling method across various cell types was studied using a single-cell RNA sequencing (scRNAseq) BioTuring Database comprising 255 gene studies. Through the analysis of ranked expression, we observed that the majority of these genes are most prominently expressed within lymphoid, T and B cell populations (Figure 5G). In addition, a subset of 19 genes were detected that shared similar expression patterns across cell subsets to RORγ and REV-ERBα (*NR1D1*) suggesting shared function or biology (Supplementary Figure 19). The reported role of SR9009 in modulating Th17 cell differentiation[40] led us to next examine the gene expression profile of enriched protein hits within naïve CD4+ T cells and differentiated Th17 cells, as annotated in the BioTuring resource. From this analysis we identified genes that are abundantly and commonly expressed in cells representing Th17 and naïve CD4 T cells (>70%), including *FAU, YBX1, SUB1* and *APRT* (Figure 5H). We also observed some genes that are more abundantly found in Th17 cells as compared to naïve CD4 T cells (e.g. *TPI1, PPIB, LYAR, ELOVL5* and *GYG1*). Collectively, these results suggest that the Th17 differentiation effects of these compounds potentially occur in a REV-ERB binding independent manner and further highlight their possible pleiotropic nature.

Conclusion

This disclosure reports the development of an electrochemical-based targeted protein labeling platform within live cells. This technology relies on the use of a minimalist DZE functional group that leverages its intrinsic electrochemical activation properties to generate a reactive intermediate for covalent protein labeling. By appending appropriately functionalized DZE to small molecule ligands of interest, targeted labeling of proteins can be achieved in an electrochemically dependent fashion in both free protein and complex cellular environments. The biocompatibility and reaction tunability afforded by electrochemical activation will likely open up new possibilities for chemical biology-based applications within biological environments.

ACKNOWLEDGMENT

The work was supported by National Science Foundation Center for Synthetic Organic Electrochemistry CHE-2002158 (Exploration and development of electrochemically active functional group), National Institutes of Health grant GM-118176 (Synthesis of elaborated probes for biological studies), and gifts from Merck & Co., Inc., Kenilworth, New Jersey, USA (Synthesis of elaborated probes for biological studies). G.N.H was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 419055018 / HE 8427/1-1. A.F.S. was supported by Lundbeck Foundation (Grant No. R208-2015-3354), E.R.-C. was supported by the Galician Programme for Research, Innovation and Growth for 2018. We thank D.-H. Huang and L. Pasternack (Scripps Research) for assistance with NMR spectroscopy; J. Chen, B. Sanchez, and E. Sturgell (Automated Synthesis Facility, Scripps Research) for purification of compounds and acquisition of HRMS data. We thank T. Wyche (Merck & Co., Inc.) for assistance with HRMS data, S. Ingale (Merck & Co., Inc.) for assistance with peptide synthesis, and J. Oh (Merck & Co., Inc.) for helpful discussions.

Author Contributions

RCO, OOF and PSB conceptualized the study, YK, GNH, AFS, JCV, ERC, PSB designed and performed chemical experiments, KAR, LAA, AKO, LRR, RCO, OOF designed and performed biological experiments. All the authors contributed to data analysis. YK, KAR, RCO, OOF, PSB wrote the manuscript.

Competing Interests

KAR, LAA, AKO, LRR, RCO, and OOF are/were employees of Merck and Co., Inc. during the preparation of this manuscript.

Figure Legends and Captions

Figure 1. Electroaffinity labeling as a new labeling method for drug-target identification. A) Workflow highlighting role of target identification through covalent capture in the drug discovery process. B) Covalent labeling strategies have relied on the use of chemical and photochemical cross-linker functional groups. C) Schematic depiction of ECAL for the covalent capture of targeted proteins through electrochemical activation. DZE was identified as a small, electrochemically active, and synthetically accessible functional group that can be appended to a molecule of interest for ligand-directed covalent labeling.

Figure 2. Development of a redox-active small molecule for ECAL. A) Early exploration of various redox-active units to identify a small and reactive species that can be generated upon mild electrochemical activation. B) Diazetidinone was identified as a small, readily accessible motif. Importantly, electrochemical oxidation of diazetidinone was found to afford a ketene as a potent electrophile. C) Synthesis of functional probes. The diazetidinone motif was constructed in one step from a secondary carboxylic acid that can be readily synthesized by alkylation of a malonate ester.

Figure 3. Use of ECAL enables targeted covalent capture across a diverse set of protein-ligand pairs.. A) Schematic depicting Carbonic Anhydrase (CA)-targeted labeling using a DZE-containing arylsulfonamide probe (Sulfa-DZE,15; 100 uM) in the presence or absence of Bovine Serum Albumin (BSA) bystander protein. B) Western blot analysis of targeted labeling of CA shows covalent labeling over BSA where desthiobiotinylation of CA but not BSA was observed in the presence of electrolysis and could be competed away with excess free sulfonamide CA inhibitor (Sulfa; 1 mM). Data are representative of n=3 independent experiments with similar results. C) To profile amino acid labeling sites, LC-MS/MS-based peptide mapping of CA labeled with a Sulfa-DZE (15) or Sulfa-Diazirine (16) using 1 min of electrochemical-or UV-activation, respectively. Labeled residues are highlighted on the protein (pdb code: 1v9e) and by bar plot analysis that shows residue number (x-axis) and spectral counts (y-axis) of labeled peptide containing the modification. Compared to the diarine probe, DZE labeling via ECAL results in covalent labeling of a broader set of protein residues on CA. D) Electroaffinity labeling using a diverse set of known small molecule ligands linked with DZE against ABL, AURKA, BRD4, and MEK1 proteins. Western blot analysis shows that protein desthiobiotinylation of occurs in an electrochemically dependent manner in the presence of the DZE ligand (10 uM) that can be competed away with excess free ligand (100 uM). Data are representative of n=2 independent experiments with similar results..

Figure 4. ECAL-mediated enrichment of JQ1 and Dasatinib protein targets in live cells. A) Schematic depicting live cell labeling via ECAL using a DZE-based ligand followed by LC-MS/MS analysis for protein identification. B) Chemical structures of JQ1-DZE (19) or Dasatinib-DZE (17) probes used for targeted labeling in HCT116 cells. C) Volcano plot analysis showing enrichment of JQ1 protein targets in HCT116 cells treated with JQ1-DZE (20 uM) with or without excess free JQ1 (200 uM) followed by ECAL (1 min) resulting in enrichment of known JQ1 protein targets (BRD2, BRD4, red dots). This is visualized through volcano plots of significance (y-axis) vs. fold-enrichment (x-axis; electrolysis:electrolysis with competitor). D) Volcano plot analysis showing enrichment of a Dasatinib protein target in HCT116 cells treated with Dasatinib-DZE (20 uM) with or without excess free Dasatinib (200 uM) followed by ECAL (1 min) resulting in enrichment of a known Dasatinib protein target (SRC, red dot). This is visualized through volcano plots of significance (y-axis) vs. fold-enrichment (x-axis; electrolysis:electrolysis with competitor). E) Venn diagram analysis of top 25 enriched proteins from JQ1-DZE (light blue) or Dasatinib-DZE (dark blue) targeted labeling in panels C and D within the same cell line (HCT116) shows no overlap of enriched proteins. Gene ontology term analysis of F) biological and G) molecular functions for protein hits enriched from JQ-1-DZE (light blue) and Dasatinib-DZE (dark blue) targeted labeling show apparent differences between enriched proteins groups.

Figure 5. Application of ECAL for the chemoproteomic profiling of SR9009-based DZE probe in live cells. A) Chemical structures of SR9009 and SR9011 tool compounds and their implicated roles in REV-ERB biology have attracted interest as exercise supplements/performance-enhancers. B) Chemical structure of SR9009 and SR9011 ligand DZE probe design based on the tertiary amine (TA) pharmacophore (TA-DZE,21). C) Functional activity of TA-DZE is similar to parent compounds SR9009 and SR9011 (5 uM for each condition) in a Th17 cell differentiation assay demonstrating that TA-DZE retains desired pharmacological properties. All conditions run with n=3 (DMSO control n=2) biologically independent samples in a single experiment. Data presented as +/- standard deviation. D) Schematic showing ECAL of TA-DZE in 293T cells overexpressing REV-ERBα (NR1D1). E) Volcano plot analysis of HEK293T cells overexpressing REV-ERBα treated with TA-DZE (20 uM) with or without electrochemical activation does not enrich out REV-ERBα (NR1D1, indicated with green dot). This is visualized through volcano plots of significance (y-axis) vs. fold-enrichment (x-axis; electrolysis:no electrolysis). F) GO-term analysis of the top 100 enriched protein hits combined from two independent experiments of TA-DZE labeling in HEK293T cells overexpressing REV-ERBα results in the enrichment of biological terms associated with SR9009/SR9011 and REV-ERB. Log-scaled p-values were computed with Fisher's exact test with Benjamini-Hochberg multiple hypothesis correction. G) Gene expression profile analysis of the top 100 enriched protein hits (combined from duplicate analysis) across a single cell RNA sequencing database (BioTuring) showed prominent gene expression within lymphoid, T and B cell populations. Data are shown as a z-score heat map of genes (y-axis) across multiple cell types (x-axis). H) Circos plot of genes from the top 100 enriched protein hits (combined from duplicate analysis) across naïve CD4+ (blue) and Th17 (orange) T cells show abundant expression of enriched protein hits within these cell types highlighting a potential role of SR9009/SR9011 in modulating Th17 biology independent of REV-ERB. Line width represents percentage of cells with expression of the indicated gene.

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Data Availability

Data supporting the main findings of this work are available within the Article and Supplementary Information. Crystallographic data for the structures reported in this Article have been deposited at the Cambridge Crystallographic Data Centre, under deposition numbers CCDC 2093827 (**11**). Copies of the data can be obtained free of charge via https://www.ccdc.cam.ac.uk/structures/.