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Endocannabinoid Transport Proteins: Discovery of Tools to Study Sterol Carrier Protein-2

Cecilia J. Hillard^{*,1}, Huan Huang², Caleb D. Vogt³, Beatriz E. Rodrigues³, Terrence S. Neumann^{3,4}, Daniel S. Sem³, Friedhelm Schroeder², Christopher W. Cunningham^{*,3}

¹Medical College of Wisconsin, Milwaukee, WI 53226;

²Texas A&M University, TVMC, College Station, TX 77843;

³Concordia University Wisconsin School of Pharmacy, Mequon, Wisconsin, 53097;

⁴Texas Wesleyan University, Fort Worth, TX 76105

Abstract

The endocannabinoid (eCB) neurotransmitter system regulates diverse neurological functions including stress and anxiety, pain, mood, and reward. Understanding the mechanisms underlying eCB regulation is critical for developing targeted pharmacotherapies to treat these and other neurologic disorders. Cellular studies suggest that the arachidonate eCBs, *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), are substrates for intracellular binding and transport proteins, and several candidate proteins have been identified. Initial evidence from our laboratory indicates that the lipid transport protein, sterol carrier protein 2 (SCP-2), binds to the eCBs and can regulate their cellular concentrations. Here, we present methods for evaluating SCP-2 binding of eCBs and their application to the discovery of the first inhibitor lead molecules. Using a fluorescent probe displacement assay, we found SCP-2 binds the eCBs, AEA ($K_i = 0.68 \pm 0.05 \mu\text{M}$) and 2-AG ($K_i = 0.37 \pm 0.02 \mu\text{M}$), with moderate affinity. A series of structurally diverse arachidonate analogues also bind SCP-2 with K_i values between 0.82 and 2.95 μM , suggesting a high degree of tolerance for arachidonic acid head group modifications in this region of the protein. We also report initial structure-activity relationships (SAR) surrounding previously reported inhibitors of *Aedis aegypti* SCP-2, and the results of an *in silico* high-throughput screen (HTS) that identified structurally novel SCP-2 inhibitor leads. The methods and results reported here provide the basis for a robust probe discovery effort to fully elucidate the role of facilitated transport mediated by SCP-2 in eCB regulation and function.

Keywords

cannabinoid; 2-arachidonoylglycerol; N-arachidonylethanolamine; AM-404; N-arachidonoyl-dopamine; virodhamine; N-arachidonoyl-serotonin

* Address correspondence to either: Cecilia J. Hillard, Ph.D., Neuroscience Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, 414-955-8493, chillard@mcw.edu, Christopher W. Cunningham, PhD, Concordia University Wisconsin School of Pharmacy, 12800 N. Lake Shore Drive, Mequon, WI 53097, 262-243-2792, chris.cunningham@cuw.edu.

1. Introduction

Two arachidonates, *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), have been identified as endogenous ligands of cannabinoid receptor subtype 1 (CB1R) and are called the endocannabinoids (eCBs) (Hillard, 2015). The CB1R is widely expressed throughout the body, with particularly high expression in brain neurons. The eCB/CB1R signaling paradigm underlies activity-dependent, retrograde forms of synaptic plasticity. In particular, 2-AG has been shown to be produced in post-synaptic neurons in response to increased neuronal activity and activation of receptors that couple to phospholipase C (PLC) from PLC-derived diacylglycerol through the actions of diacylglycerol lipase. CB1Rs are present in high density on axon terminals and their activation reduces the probability of neurotransmitter release. AEA can also regulate synaptic plasticity, but the mechanisms that regulate its synthesis are not well understood.

Among the outstanding questions regarding eCB signaling in the brain is whether there are proteins that act as carriers for the eCBs either in membranes or aqueous compartments, including the cytosol and extracellular space. Several lipid binding proteins have been identified that bind, sequester, and/or traffic eCBs that are hypothesized to contribute to their availability to activate cannabinoid receptors. For example, two members of the fatty acid binding protein (FABP) family, FABP5 and FABP7, have been shown to bind AEA *in vitro* (Kaczocha, Glaser, & Deutsch, 2009) and both albumin and heat shock protein 70 have been characterized as AEA binding proteins (Oddi, Fezza, Pasquariello, D'Agostino, Catanzaro & De Simone, et al., 2009).

Sterol carrier protein-2 (SCP-2) is a multi-purpose lipid binding protein that shuttles cholesterol and other lipids from the endoplasmic reticulum, where they are synthesized, to the cell surface (Gallegos, Atshaves, Storey, McIntosh, Petrescu & Schroeder, 2001). SCP-2 can bind many types of lipid, including branched fatty acids, fatty acyl CoA derivatives, and phospholipids (Schroeder, Atshaves, McIntosh, Gallegos, Storey & Parr, et al., 2007). Furthermore, there is evidence that SCP-2 is expressed in the brain and is particularly enriched in synaptosomal preparations (Avdulov, Chochina, Igbavboa, Warden, Schroeder & Wood, 1999; Myers-Payne, Fontaine, Loeffler, Pu, Rao & Kier, et al., 1996). We found that micromolar concentrations of AEA compete with cholesterol for SCP-2-mediated transfer between vesicles and cell membranes; and *in silico* docking studies predict that both AEA and 2-AG bind to SCP-2, but that AEA has higher predicted affinity (Liedhegner, Vogt, Sem, Cunningham & Hillard, 2014). These findings support the hypothesis that SCP-2 plays a role in the regulation of the concentrations of the eCBs available to activate the CB1R.

To further test this hypothesis, selective, high-affinity inhibitors of eCB binding to SCP-2 are required. In pursuit of that goal, we have utilized an *in vitro* SCP-2 binding assay to determine the affinities of a variety of head group-substituted fatty acids and a second series of compounds that had been shown previously to inhibit binding of lipids to the *Aedes aegypti* (mosquito) SCP-2 homologue. Finally, we applied computer-aided drug design (CADD) techniques toward the rational discovery of structurally unique, small-molecule inhibitor leads.

2. Materials and Methods-NBDS Displacement Assay

2.1 Materials

Human recombinant SCP-2 was prepared and purified as previously described (Matsuura, George, Ramachandran, Alvarez, Strauss 3rd & Billheimer, 1993). The fluorescent probe, 12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazo)aminostearic acid (NBD-stearate, NBDS) was purchased from Avanti Polar Lipids (Alabaster, AL). AEA and 2-AG, and fatty acid analogues **3–13**, were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Screening compounds were purchased from ChemBridge Corporation (San Diego, CA, USA). Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Compounds **19** and **20** were synthesized and characterized as reported elsewhere (Bursavich, Parker, Willardsen, Gao, Davis & Ostanin, et al., 2010).

2.2 NBDS Displacement Assay.

Binding of putative inhibitors to SCP-2 was determined by a fluorescent NBDS displacement assay at 24°C in 10mM phosphate buffer (pH=7.4). NBDS is weakly fluorescent in buffer, and becomes highly fluorescent upon binding to SCP-2. If a chemical binds to SCP-2, it would displace the bound NBDS, and NBDS fluorescence would decrease. NBDS fluorescence intensity was recorded with excitation wavelength of 490 nm while emission was scanned from 515 to 600 nm with a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., Palo Alto, CA).

2.2.1. Binding Affinity of NBDS ($K_{d,NBDS}$) to SCP-2 was Determined by Reverse and Forward Titrations.—First, in the reverse titration, NBDS (100 nM) was titrated with recombinant SCP-2 (0–3 μ M). The fluorescence intensity at each titration point was corrected by subtracting the background signals from NBDS (100 nM) without SCP-2, and the signals from SCP-2 (0–3 μ M) in the absence of NBDS. The reverse titration data were then plotted as fluorescence intensity (after correction) on the Y-axis vs SCP-2 concentration on the X-axis. The maximum fluorescence intensity was obtained by curve fitting using SigmaPlot (Systat Software, Inc., San Jose, CA). The maximum fluorescence was then used to calculate fluorescence intensity of NBDS (per nM) when fully bound to SCP-2. This number was used in the forward titration (below) to calculate the fractional saturation and free NBDS concentration.

Second, in the forward titration, SCP-2 (500 nM) was titrated with NBDS (0–1.8 μ M). Each data point was then corrected by subtracting the signals from SCP-2 without NBDS, and the signals from NBDS (0–1.8 μ M) in the absence of SCP-2. The forward titration data were plotted as the fractional saturation on the Y-axis vs free NBDS concentration on the X-axis, The Fractional Saturation = Fluorescence Intensity / (NBDS fluorescence intensity per nM when fully bound $\times$ 500 nM from the forward titration). Free NBDS concentration = [NBDS] total – 500 nM $\times$ Fractional Saturation. K_d and B_{max} were then calculated by fitting the forward titration curve to the single site saturation binding equation [$Y = B_{max} X / (K_d + X)$] using SigmaPlot software. B_{max} is the number of binding sites per molecule of protein.

Results from three independent reverse titration curves and four independent forward titration curves yielded the following parameters for SCP-2 binding NBDS: $K_d = 0.22 \pm 0.03 \mu\text{M}$, $B_{\text{max}} = 0.89 \pm 0.04$ binding sites per molecule of SCP-2.

2.2.2. Displacement Assays Were Used to Determine Max % Displacement, EC_{50} and K_i .—The solution of SCP-2/NBDS complex was titrated with putative inhibitors (0.05 to 0.5 μL aliquots from 10 mM stock solution in dimethylsulfoxide (DMSO) or ethanol, final DMSO or ethanol concentrations were less than 0.5%) until NBDS fluorescence no longer decreased. A complex was formed by incubating SCP-2 (500 nM) with NBDS (500 nM) in buffer until the fluorescence intensity was stabilized. NBDS fluorescence was recorded after each addition of putative inhibitor into the solution containing the SCP-2/NBDS complex. Each data point was corrected by subtracting the signals from SCP-2 only, and signals from NBDS plus inhibitor in the absence of SCP-2. At the end of the titration, UV absorbance of the mixture was measured to ensure there was no inner filter effect ($A_{490} < 0.1$). Displacement curves were constructed by plotting the percentage of NBDS fluorescence remaining (Y) after all the corrections were made (at emission maximum 528nm) versus ligand concentration (X). EC_{50} was obtained by curve fitting with SigmaPlot software to find the ligand concentration where there is 50% of the maximum displacement of NBDS.

The displacement dissociation constant K_i is determined according to the equation: $K_{i,\text{ligand}} = (K_{d,\text{NBDS}} \times EC_{50})/[NBDS]_{\text{total}}$, where $K_{d,\text{nbds}}$ is the binding affinity (dissociation constant) of NBDS to SCP-2 (obtained in Methods 1.1.), EC_{50} is the inhibitor concentration at which half of the maximum NBDS displacement Probes of Endocannabinoid Transport 103 occurred, and $[NBDS]_{\text{total}}$ (0.5 μM) is the total NBDS concentration used in the displacement assays.

2.3. Computational Methods.

The atomic coordinates of ligand-bound SCP-2 are not currently available, though coordinates for SCP-2 determined from an NMR study are available (1QND, <http://www.rcsb.org/pdb/home/home.do>; Garcia et al., 2000), as are the coordinates for a homologous protein, human peroxisomal multifunctional enzyme type 2 (MFE-2) bound to lipid substrate, Triton X-100 (1IKT, <http://www.rcsb.org/pdb/home/home.do>; Haapalainen, van Aalten, Merilainen, Jalonon, Pirila & Wierenga, et al., 2001). Before using the SCP-2 NMR structure as a basis for automated docking, we virtually removed the ligand from MFE-2 and docked Triton X-100 within MFE-2 and SCP-2 using AutoDock4.0 (for details, see Liedhegner et al., 2014). When the proteins were aligned (Pymol, Schrödinger, LLC), a high degree of overlap was seen between the lowest-energy docking states for Triton X-100 within MFE-2 and SCP-2. The lowest-energy docking pose for Triton X-100 within the MFE-2 (PDB 1IKT) structure was largely reproduced by this docking procedure. This affords confidence that automated docking using the SCP-2 NMR structure (PDB 1QND) provides a reasonable approximation to the biologic state of the SCP-2 binding domain.

Co-crystallized waters and bound ligands were removed from the 1QND protein structure file and a grid box of dimensions $22.5 \times 15 \times 18.75 \text{ \AA}$ surrounding the hydrophobic cavity

was generated using AutoGrid. Molecular structures for ligands were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) and converted to mol2 files using Open Babel (<http://openbabel.org>; O'Boyle, Banck, James, Morley, Vandermeersch & Hutchison, 2011). Amino acid side chains were maintained as rigid. AutoDock Tools (ADT) was used to prepare protein and ligands. In all cases, Gasteiger charges were applied for ligand and protein in their physiologically relevant ionization state at pH 7.4. Compounds were docked using 100 genetic algorithm optimization runs, clustered by lowest binding energy (LBE, G), and the LBE pose was selected as the predicted binding orientation. These general docking conditions were also applied to the molecular library of diverse small molecules housed at the CUW Center for Structure-Based Drug Design and Development (CSD³). The CSD³ houses a collection of 11,000 compounds compiled from a variety of sources (<https://pubchem.ncbi.nlm.nih.gov/>) and also compounds synthesized in-house. 3D coordinates are maintained for this collection in their physiologically relevant ionization state. Following automated high-throughput screen (HTS) *in silico*, compounds were scored (G , kcal/mol) and ranked, and the top-100 scoring compounds were evaluated for further studies.

3 Results and Discussion

3.1 Discovery of Inhibitor Probe Lead Compounds

Selective inhibitor probes of human SCP-2 are lacking, as only a single report of HTS against mosquito (*A. aegypti*) SCP-2 is in the literature (Kim, Wessely, & Lan, 2005). This necessitates the use of various early lead discovery approaches to inhibitor development. To this end, we employed three complementary techniques frequently utilized in hit and lead identification: 1) generation of structure-activity relationships (SAR) describing known substrates; 2) optimization and pharmacophore identification of known lead inhibitors; and 3) computer-aided drug design (CADD) and HTS *in silico* to identify hits from diverse small-molecule libraries.

3.2 SAR of Head Group-Substituted Fatty Acids

The first approach to lead discovery involves investigation of the SAR governing known, endogenous SCP-2 substrates. Sterols and amphiphilic fatty acid derivatives represent the most understood classes of SCP-2 substrates described in the literature (selected representative examples: Schroeder, Myers-Payne, Billheimer & Wood, 1995; Dansen, Westerman, Wouters, Wanders, van Hoek, Gadella & Wirtz, 1999; Atshaves, Jefferson, McIntosh, Gallegos & McCann et al., 2007). The structural features supporting binding to SCP-2 of the lipid portion of fatty acids and sphingolipids have been well-characterized (Gadella & Wirtz, 1994; Stolowich, Frolov, Atshaves, Murphy, Jolly & Billheimer, et al., 1997; Stolowich, Frolov, Petrescu, Scott, Billheimer & Schroeder, 1999), though it was not until recently that carboxylate-substituted fatty acid amides and esters were reported to be transported by SCP-2 (Liedhegner et al., 2014). A large number of head group-modified arachidonate analogues are commercially available, representing an extensive library of AEA and 2-AG analogues from which to generate SAR (Fig. 1A). In addition to AEA and 2-AG, a sample of structurally diverse, head group-modified analogues **3–11** were purchased and evaluated for the ability to compete with NBDS for binding to SCP-2 (Fig. 1A). As a test of the tolerance of the SCP-2 binding site to alternate head group-modified lipids,

oleamide (**12**) and docosaheptaenoyl ethanolamide (DHEA, **13**) were also evaluated (Fig. 1A). All tested compounds displaced SCP-2 bound NBDS to varying extent.

Table 1 provides the structures of compounds **1–11**. Analysis of multiple displacement curves (n=4) for each compound (Table 1) shows the relative potencies (EC₅₀, K_i) and efficacies as given in % displacement of NBDS. The eCBs, AEA (**1**) and 2-AG (**2**) were the most potent competitors of NBD binding (K_i < 1.0 μM), though only produced ~55–60% maximal displacement. One possible explanation for the partial displacement of NBDS by **1** and **2** may be poor solubility in buffer under these conditions.

The 2-AG regioisomer, 1-arachidonoyl glycerol (1-AG, **3**) behaved similarly to **2**, although exhibited a small reduction in potency. Replacement of the ester functionality of **2** with an amide bioisostere (arachidonoyl serinol, **4**) resulted in an 8-fold reduction in potency. This suggests that the ester oxygen of **2** engages in a hydrogen bond-accepting interaction that stabilizes binding within the SCP-2 active site. The diminished SCP-2 binding potency of arachidonoyl-2'-fluoroethylamide (2'-fluoro-AEA, **5**) compared to **1** is notable in light of reports that this structural change enhances affinity for CB1 receptors (Lin, Khanolkar, Fan, Goutopoulos, Qin, Papahadjis & Makriyannis, 1998). This indicates a point of divergence between arachidonate SAR at SCP-2 and cannabinoid receptors.

The tolerance of SCP-2 for charged groups in this region was examined using O-AEA (**6**) and *N*-arachidonoyl glycine (**7**). O-AEA, which was termed “virhodamine” by Felder and colleagues (Porter, Sauer, Knierman, Becker, Berna & Bao, et al., 2002), possesses an *O*-arachidonoyl ethanolamine in opposition to the *N*-arachidonoyl ethanolamine of **1**. This reveals a cationic primary amine that occupies similar space as the terminal alcohol of **1** and **2**. This space is occupied by an anionic carboxylate in **7**. Both **6** and **7** displaced NBDS with slightly lower affinity compared to **1**. That **6** and **7** both bind SCP-2 is an important finding for probe discovery, because it suggests that the head group binding region is capable of recognizing diverse charged groups that can be formulated as aqueous-soluble salts.

The lipid head group binding site is tolerant of aromatic substitutions (**8–10**). *N*-Arachidonoyl serotonin (**8**) and *N*-arachidonoyl dopamine (**9**) exhibited slightly lower affinity but higher efficacy (max displacement > 80%) compared to AEA in the NBDS competition assay. These agents have been reported to be endogenous arachidonate conjugates of their respective monoamine neurotransmitters and inhibit fatty acid amide hydrolase at concentrations in the same concentration range in which they compete with NBDS for SCP-2 binding (Bisogno, Melck, DePetrocellis, Bobrov, Gretskaya & Bezuglov et al., 1998; Bisogno, Melck, Bobrov, Gretskaya, Bezuglov, De Petrocellis, & Di Marzo, 2000). Compound **9** has been isolated in high quantities in the striatum and is a potent vanilloid receptor agonist (Huang, Bisogno, Trevisani, AlHayani, De Petrocellis & Fezza et al., 2002). As SCP-2 is expressed in brain (Myers-Payne et al, 1996), the data shown here suggest a potential role for SCP-2 in the pharmacologic actions of **9**. *N*-Arachidonoyl-*p*-aminophenol (AM404, **10**) is a synthetic analogue of an active metabolite of acetaminophen (Ottani, Leone, Sandrini, Ferrari & Bertolini, 2006). In the present study, AM404 displaced NBD binding at a maximum of 89% at a K_i of 1.41 μM. AM404 has been shown to inhibit the uptake and accumulation of AEA in cells and is considered to be an inhibitor of a theoretical

membrane transport protein (Hillard, Edgemond, Jarrahan, & Campbell, 1997; Beltramo, Stella, Calignano, Lin, Makriyannis & Piomelli, 1997). The speculation that such a transporter exists has been the subject of much debate (Glaser 2003, Hillard and Jarrahan 2003). However, if uptake and sequestration are linked as suggested (Hillard and Jarrahan 2003), the ability of AM404 to compete for SCP-2 binding of the eCBs would be predicted to inhibit their cellular accumulation and reduce uptake.

The influence of lipid modification on SCP-2 binding was examined using oleamide (**11**) and DHEA (**12**). Oleamide is an 18:1 *cis*-9 fatty acid amide analogue of **11** (20:4(ω -6)) and DHEA is a 22:6(ω -3) analogue of AEA (**2**). Comparison of the displacement data for **11** and **12** indicates a threefold drop in potency when arachidonate is substituted with an oleate chain. In this test, **12** is the least potent endogenous substance tested in the series. Replacement of the ω -6 arachidonate with an ω -3 docosahexaenoic acid group also decreased potency, though to a lesser extent than from **11** to **12**.

The results described here indicate that the active site of SCP-2 is capable of recognizing structurally diverse fatty acid head group modifications. In several cases, the potency of these analogues to displace NBDS binding is comparable to that for binding eCB targets, e.g., **8** and **9**, suggesting that inhibition of eCB binding SCP-2 could contribute to the observed pharmacologic effects of these agents.

3.3 Hit-To-Lead Optimization

The second approach to lead discovery is optimization of hits identified from physical HTS (Hann & Oprea, 2004; Howe, Costanzo, Fey, Gojobori, Hannick & Hide et al., 2008). Use of HTS is advantageous when the structural features of substrates required for protein binding are poorly understood, or when structurally diverse substrates are lacking. For probe development, hit optimization revolves around pharmacophore identification, generation of SAR, and improvement of target selectivity.

A 2005 report described application of HTS to SCP-2 inhibitor hit identification (Kim et al., 2005). This resulted in a set of five, structurally diverse small-molecule ligands (**14**–**18**, Fig. 2) that were found to inhibit *A. aegypti* SCP-2 with IC₅₀ values between 0.042 and 0.347 μ M. Of these hits, two stand out as being potentially advantageous for lead development. SCPI-1 (**14**) is a 2-aminophenyl-4-phenylthiazole, a core scaffold that is present in sphingosine kinase inhibitors (Gustin, Li, Brown, Min, Schmitt & Wanska, et al., 2013) and probes of opioid receptor allosteric binding sites (Burford, Wehrman, Bassoni, O'Connell, Banks, Zhang & Alt, 2014). SCPI-5 (**18**) was the most potent inhibitor of mouse SCP-2 from this preliminary study. In addition to the ease of generation of a diverse analogue library (Bursavich, Parker, Willardsen, Gao, Davis & Ostanin, et al., 2010), **18** does not contain structural features typical of pan-assay interference compounds (PAINS), a frequent issue with probes identified from HTS (Baell & Holloway, 2010; Baell & Walters, 2014). A small series of analogues of **14** and **18** were purchased or synthesized, and examined using the SCP-2 displacement assay (Fig. 1B). The results of these tests are shown in Table 2 and Figure 1B.

Compounds **19–21** were generated as described (Bursavich et al., 2010) or purchased to determine the influence of the dichlorophenyl group on SCP-2 binding activity (Fig. 1B). The parent (**14**) produced the highest affinity competitor of NBDS binding to SCP-2 of all compounds tested with a K_i value of 1.0 μM (Table 2). Results from iterative removal of the 3'-Cl (**19**) and 4'-Cl (**20**) suggests that both chloro groups are beneficial for SCP-2 binding, and removal of the 3'-Cl had the more pronounced detrimental effect on SCP-2 binding affinity. Removal of both chloro groups (**21**) resulted in an additive effect on SCP-2 binding (EC_{50} 17.7 μM , K_i 7.8 μM). Compound **22** has replaced the acetanilide group of **14** with an ethoxy group that would occupy similar chemical space. Though this change resulted in a 5-fold loss of affinity for SCP-2 compared to **14**, compound **22** no longer contains an *N*-acetylaniline function, which is a potential precursor to redox-reactive aniline species (Smith, 2011). Understanding the structural features of this potential toxicophore required for SCP-2 inhibitory activity is critical to developing safe pharmacologic probes. The most potent hit identified by Lan and colleagues against AeSCP-2, SCPI-5 (**18**), was found to be approximately twofold less potent of an SCP-2 inhibitor than **14**. This may be due to subtle species differences between *A. aegypti* SCP-2 and *Homo sapiens* SCP-2, and may also be impacted by differences in screening methodology.

3.4 Hit Discovery by HTS *in Silico*

A third approach to hit identification is the use of computational screening (Kitchen, Decornez, Furr & Bajorath, 2004). Advances in CADD have greatly expanded the capacity to screen large libraries of small molecules *in silico* prior to *in vitro* assay screening (Shoichet, 2004). This is advantageous as a resource-sparing approach to hit prioritization, though target-based drug design requires understanding of the protein tertiary structure prior to screening.

Achieving success in hit discovery through virtual HTS requires selection of a structurally diverse small-molecule library. A number of virtual “drug-like,” “lead-like,” and fragment libraries can be found in the ZINC database (zinc.docking.org; Irwin, Sterling, Mysinger, Bolstad & Coleman, 2012). The ZINC database contains approximately 90 million commercially available compounds that can be virtually screened, purchased, and tested; this process can be streamlined further when a library is cultivated to possess desirable drug-like properties and stored in-house. The CSD³ primary screening collection (www.csddd.org) contains over 10,000 compounds that generally conform to “Lipinski’s Rule of Five” (RO5; Lipinski, Lombardo, Dominy & Feeney, 1997). Agents that adhere to the RO5 may be predicted to not suffer from poor solubility or permeability, and represent a useful guideline for conducting lead discovery and development efforts. Further iterations of the RO5 guidelines have been reported (see for example Veber, Johnson, Cheng, Smith, Ward, & Kopple, 2002; Lipinski, 2004; Leeson, 2012) that impose limitations on the number of rotational bonds and physicochemical properties such as topological polar surface area (TPSA) for “drug-like” or “lead-like” compounds. Statistics describing the CSD3 screening collection can be seen in Figure 3 and Table 3.

In the absence of an X-ray crystal structure showing SCP-2 bound to a fatty acid analogue, the 3D coordinates of SCP-2 predicted by NMR were used to conduct target-based drug

design (1qnd, <http://www.rcsb.org/pdb/home/home.do>, Garcia et al, 2000). Automated docking (AutoDock 4.0, Morris 2009) was conducted with parameters described earlier (Liedhegner et al., 2014); but, specifically with 50 separate docking runs and 1,750,000 evaluations per run, and a root mean square deviation (rmsd) of 2.0 angstroms, for entry into a cluster. Docking was done using a primary screening collection of 10,586 compounds (www.csddd.org). This collection was developed using routine cheminformatic filters to guide both the design (in-house synthesized) and purchase of drug-like compounds. In particular, the library was designed to comply with the RO5, where compounds typically have molecular weight ≤ 500 g/mol, A LogP ≤ 5 , the number of hydrogen bond acceptors ≤ 10 , and the number of hydrogen bond donors ≤ 5 (Figure 3 and Table 3). Over 95% of compounds in the collection satisfy all 4 RO5 criteria, and also lack the presence on nondrug-like motifs like Michael acceptors. Docked compounds were scored and rank-ordered using the default scoring function and the top 100 hits with binding energy < 9.0 kcal/mol were prioritized for further analysis. From these 100 hits, a sample of four compounds was selected and evaluated as described earlier for the ability to displace NBDS binding from SCP-2. Structures of these compounds are shown in Figure 4, the displacement curves in Figure 1C, and the individual displacement parameters in Table 4.

Compounds **23–26** were selected from the top 100 scoring hits for further evaluation due to their structural diversity and accessibility to further optimization. Virtual screening of these compounds suggested a high degree of complementarity within the lipid binding cavity of SCP-2 in a rank-order of **23–26**. When these compounds were tested *in vitro*, the rank order of inhibition was actually **26–23**, with **23** producing no displacement of NBDS. Though the potencies for NBDS binding inhibition is lower than those seen with the arachidonates (Table 1), compounds **24–26** are generally equipotent with analogues of other small-molecule SCP-2 inhibitor leads (Table 2). This supports continued investigation into targeted library screening for novel SCP-2 inhibitor leads.

There are several possible reasons for the discrepancy between predicted and observed affinities for SCP-2 binding. It is clear from the nuclear magnetic resonance data (Garcia et al., 2000) and recent review of SCP-2 domains (Burgardt, Gianotti, Ferreyra & Ermacora, 2017) that the lipophilic binding cavity of SCP-2 is highly variable and may even change shape in order to accommodate diverse substrates. Indeed, SCP-2 contains a C-terminal type 1 peroxisomal targeting signal sequence that extends away from the lipid binding cavity when engaging protein targets such as the peroxisomal receptor Pex5p (Stanley, Filipp, Kursula, Schuller, Erdmann & Schliebs, et al., 2006). For these reasons, future rational probe development must be aided by dynamic modeling studies to elucidate the molecular mechanisms by which SCP-2 recognizes substrates.

The results described here can be rationalized in a general way using rigid protein modeling. As described earlier, AutoDock 4.0 and AutoDock Vina 4.2 (Morris et al., 2009) were used to dock all compounds described herein within the SCP-2 substrate binding cavity. Figure 5 shows representative low-energy binding poses of the arachidonate AM-404 (10, Figure 5A) and small molecule leads **24–26** (Figure 5B). Figure 5A shows two, equal-energy binding poses for AM404 that differ most substantially in the orientation of the head group extended toward the surface of SCP-2. In Pose I (blue), the *p*-OH of AM404 engages in a beneficial

hydrogen bonding interaction with the backbone carbonyl of Lys100. This group is rotated approximately 90° in Pose II (magenta) to engage an exposed backbone carbonyl of Phe94. The lipophilic arachidonate tail portion for both poses extends deep within the binding cavity defined by the side chains of Ile73, Leu107, Phe35, Ile16, and Leu83 and Phe80. The cavity is defined by hydrophobic amino acid side chains that do not appear to engage in dipolar or electrostatic interactions with substrates. In Figure 5B, the lipophilic naphthyl functions of **24** and **26**, and the 2,6-dichlorobenzamide of **25**, are buried within the hydrophobic cavity of SCP-2 occupying similar chemical space as the arachidonate tail of AM404. Different from AM404, the surface-exposed portions of **24–26** do not contain hydrogen bond-donating phenolic substituents that could engage surface-exposed backbone carbonyl groups. Of these compounds, it is noteworthy that **26** has the highest affinity for SCP-2 and is also predicted to extend the furthest of this series into the hydrophobic cavity.

4. Conclusions

Our understanding of the basic mechanisms that surround eCB function requires discovery of selective probes of eCB synthesis, transport, and degradation. The methods and results described herein will facilitate development of probes to study the role of SCP-2 in eCB transport. The SAR for arachidonic acid analogues suggests that the region of SCP-2 that recognizes the polar head group of fatty acids is tolerant of diverse structural changes, whereas the hydrophobic cavity generally prefers arachidonate lipids over oleic and docosahexadienoic acids. Lead optimization and lead discovery efforts have also resulted in small-molecule SCP-2 inhibitors that may form the basis for selective probe development. Once the details surrounding substrate binding are revealed, we can begin to fully recognize the “druggability” of this protein as a potential new target for manipulating the endocannabinoid signaling system.

Abbreviations:

1-AG	1-arachidonoylglycerol
2-AG	2-arachidonoylglycerol
AEA	<i>N</i> -arachidonylethanolamine
AM404	<i>N</i> -arachidonoyl- <i>p</i> -aminophenol
CADD	computer-aided drug design
CB1R	subtype 1 of the cannabinoid receptor
DHEA	docosahexaenoyl ethanolamide
DMSO	dimethylsulfoxide
eCB	endocannabinoid
FABP	fatty acid binding protein
HTS	high throughput screen

LBE	lowest binding energy
NBDS	12- <i>N</i> -methyl-(7-nitrobenz-2-oxa-1,3-diazo)aminostearic acid
O-AEA	<i>O</i> -arachidonylethanolamine
PAINS	pan-assay interference compounds
PLC	phospholipase C
SAR	structure activity relationships
SCP-2	sterol carrier protein-2

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Fig. 1. Displacement curves of SCPI displacing SCP-2 bound NBDS.

After SCP-2 (500 nM) was equilibrated with NBDS (500 nM), it was titrated with increasing amount of SCPI. Panel A, arachidonates; Panel B, SCPI-1 and -5 analogs; Panel C, HTS cpds. NBDS fluorescence was recorded ($E_x = 490$ nm, $E_m \text{ max} = 528$ nm) and corrected as described in Methods. Data were presented as mean $\pm$ SE ($n=4$).

Fig. 2. Structures of SCPII-5 (14–18).

A lead set of five SCP-2 inhibitors identified from HTS against *A. aegypti* SCP-2 was prioritized for initial optimization. Of these leads, compounds **14** and **18** were prioritized due to their ease in analogue synthesis (**14**), and lack of “PAINS” attributes (**18**).

Fig. 3. Lipinski RO5 analysis of the CSD3 compound collection.

Charts show the breakdown of compounds in the 10,586-member collection by A) number of hydrogen bond donors, B) number of hydrogen bond acceptors, C) atomic logP (ALogP), and D) molecular weight (Da). The upper-limit RO5 values are shown as black arrows.

Fig. 4. New lead SCP-2 inhibitors identified from HTS *in silico*.

Of the top-100 scoring SCP-2 inhibitors, compounds **23–26** were prioritized for evaluation *in vitro*. These agents occupy unique chemical space, including weakly acidic, weakly basic, and neutral functional groups.

Fig. 5. Representative images from automated docking within SCP-2.

Low-energy binding poses for representative series of SCP-2 inhibitors determined using AutoDock4.2 (Morris et al., 2009). Atomic coordinates were downloaded from PDB entry 1QND ([rscb.pdb.org](https://www.rcsb.org/entry/1QND), Garcia et al., 2000). The “gate” separating the hydrophobic cavity from the aqueous-exposed surface is lined by amino acids Leu83, Met75, and Leu107 in the middle of the image. The hydrophobic cavity is lined by Ile73, Leu107, Phe35, Ile16, Leu83, and Phe80. The protein surface of interacting amino acid residues within 5 Å of AM404 is shown in grey at 40% transparency. Interacting side chains are shown as lines. Ligands are shown in tube display. A) Two binding poses for AM404: Pose I (blue) and pose II (magenta). B) Low-energy binding poses for **24** (pink), **25** (green), and **26** (blue).

Table 1. Displacement of SCP-2-Bound NBDS by Arachidonate Compounds: Maximum % Displacement and K_i Values

Compound	Common Name	R	Max % Displacement	EC ₅₀ (μM)	K _i (μM)
1	AEA		59.3 ± 1.2	1.54 ± 0.10	0.68 ± 0.05
2	2-AG		55.1 ± 1.0	0.84 ± 0.04	0.37 ± 0.02
3	1-AG		57.7 ± 0.7	1.87 ± 0.05	0.82 ± 0.02
4	N-arachidonoyl serinol		69.2 ± 1.7	6.70 ± 0.28	2.95 ± 0.12
5	2'-Fluoro AEA		64.4 ± 2.2	6.33 ± 0.48	2.78 ± 0.21
6	O-AEA		94.7 ± 1.6	3.64 ± 0.04	1.60 ± 0.02
7	N-arachidonoyl glycine		82.0 ± 0.8	2.78 ± 0.06	1.22 ± 0.03
8	N-arachidonoyl serotonin		84.8 ± 0.8	3.33 ± 0.05	1.46 ± 0.02

Compound	Common Name	R	Max % Displacement	EC ₅₀ (μM)	K _i (μM)
9	N-arachidonoyl dopamine		94.9 ± 0.1	2.54 ± 0.05	1.12 ± 0.02
10	AM404		89.2 ± 0.8	3.21 ± 0.07	1.41 ± 0.03
11	Arachidonoyl amide		71.1 ± 1.3	4.09 ± 0.23	1.80 ± 0.10
12	Oleamide	N/A	68.3 ± 1.7	13.1 ± 2.3	5.8 ± 1.0
13	DHEA	N/A	73.9 ± 1.3	4.33 ± 0.10	1.91 ± 0.04

Maximum % displacement and *K_i* values were determined from multiple displacement curves for each compound in Fig. 1A as described in Methods.

Table 2.

Displacement of SCP-2-Bound NBDS by SCPI-1 and -5 Analogues: Maximum % Displacement and K_i values

Compound	R_1	R_2	R_3	Max % Displacement	EC_{50} (μM)	K_i (μM)
14 (SCPI-1)	NHAc	Cl	Cl	96.6 ± 0.7	2.2 ± 0.2	1.0 ± 0.1
19	NHAc	H	Cl	61.6 ± 1.2	8.5 ± 1.0	3.7 ± 0.4
20	NHAc	Cl	H	71.1 ± 1.0	5.5 ± 0.2	2.41 ± 0.08
21	NHAc	H	H	86.4 ± 2.5	17.7 ± 0.6	7.8 ± 0.3
22	OEt	Cl	Cl	80.4 ± 0.9	11.4 ± 0.4	5.0 ± 0.2
18 (SCPI-5)	N/A	N/A	N/A	49.3 ± 1.9	5.4 ± 0.2	2.4 ± 0.1

Maximum % displacement and K_i values were determined from multiple displacement curves for each compound in Fig. 1B as described in Methods.

Table 3.

Statistical Evaluation of the CSD3 Primary Screening Collection for Lipinski RO5 Criteria

RO5 Criteria	Mean ($\pm$ St. Dev.)
MW (Da)	322.4 ($\pm$ 87.1)
<i>AlogP</i>	2.63 ($\pm$ 1.51)
HBA	4.54 ($\pm$ 2.18)
HBD	1.45 ($\pm$ 1.12)
nrot	4.00 ($\pm$ 2.20)
TPSA	91.90 ($\pm$ 41.94)
Number of Rules Passed	Compounds (%)
4	10134 (95.69)
3	10158 (95.92)
2	10308 (97.34)
1	10321 (97.46)
0	10586 (99.96)

The original RO5 (Lipinski et al., 1997) consisted of molecular weight ($MW < 500$ Da), $\log P < 5.0$, number of hydrogen bond acceptors ($HBA < 10$), and number of hydrogen bond donors ($HBD < 5$). Subsequent guidelines (Veber et al., 2002) include limits for number of rotational bonds ($nrot < 10$) and topological polar surface area ($TPSA < 140$) for orally bioavailable drug candidates.

Table 4.

Displacement SCP-2-Bound NBDS by HTS Compounds

Compound	Library ID	LBE (kcal/mol) ^a	Max % Displacement	EC ₅₀ (μM)	K _i (μM)
23	CSDDD 6534	-11.18	N/D	N/A	N/A
24	CSDDD 9368	-10.49	52.3 ± 4.9	12 ± 2	5.4 ± 0.8
25	CSDDD 3327	-10.38	45.2 ± 3.1	10 ± 2	4.6 ± 0.8
26	CSDDD 9551	-10.28	38.6 ± 4.3	5.3 ± 0.5	2.3 ± 0.2

^aLBE, lowest binding energy.

Maximum % displacement and K_i values. Maximum % displacement and K_i values were determined from multiple displacement curves for each compound in Fig. 1C as described in Methods. LBE=lowest binding energy for docking poses calculated from AutoDock.