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Sulfotransferase 2B1b, Sterol Sulfonation, and Disease

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Running Title: SULT2B1b Regulation of Sterol Signaling

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Abstract

The primary function of human sulfotransferase 2B1b (SULT2B1b) is to sulfonate cholesterol and closely related sterols. SULT2B1b sterols perform a number of essential cellular functions. Many are signaling molecules whose activities are redefined by sulfonation — allosteric properties are switched "on" or "off," agonists are transformed into antagonists, and vice versa. Sterol sulfonation is tightly coupled to cholesterol homeostasis and sulfonation imbalances are causally linked to cholesterol related diseases including certain cancers, Alzheimer's disease and recessive X-linked ichthyosis — an orphan skin disease. Numerous studies link SULT2B1b activity to disease-relevant molecular processes. Here, these multifaceted processes are integrated into metabolic maps that highlight their interdependence and how their actions are regulated and coordinated by SULT2B1b oxysterol sulfonation. The maps help explain why SULT2B1b inhibition arrests the growth of certain cancers, and make the novel prediction that SULT2B1b inhibition will suppress production of amyloid beta (Aβ) plaques and tau fibrils while simultaneously stimulating Aβ plaque phagocytosis. SULT2B1b harbors a sterol-selective allosteric site whose structure is discussed as a template for creating inhibitors to regulate SULT2B1b and its associated biology.

Significance Statement Human sulfotransferase 2B1b (SULT2B1b) produces sterol-sulfate signaling molecules that maintain the homeostasis of otherwise pro-disease processes in cancer, Alzheimer's disease and X-linked ichthyosis – an orphan skin disease. The functions of sterol sulfates in each disease are considered and codified into metabolic maps that explain the interdependencies of the sterol-regulated networks and their coordinate regulation by SULT2B1b. The structure of the SULT2B1b sterol-sensing allosteric site is discussed as a means of controlling sterol sulfate biology.

Table of Contents

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I. Introduction

Thirteen cytosolic sulfotransferases (SULTs) are encoded in the human genome. The substrate specificities of the isoforms are broad, somewhat overlapping, and centered in distinct metabolic areas. Collectively, the family presents a robust, redundant catalytic network that regulates the activities of hundreds, perhaps thousands, of metabolites. SULT2B1b is distinguished from other human SULT isoforms by its atypically long N- and C-termini, and by its substrate specificity, which centers on cholesterol and related sterols. The majority of human SULT isoforms (nine of thirteen) consist of a ~290-residue long protein whose structure is remarkably well conserved. In SULT2B1b, this core structure is sandwiched by a 25-residue N-terminus and a proline-rich, 53-residue C-terminus. The C-terminal sequence (32% proline) is reminiscent of highly proline enriched proteins (Williamson, 1994) — a class of proteins whose structures and functions remain largely undefined. Phosphorylation of the C-terminus at Ser347 is linked to SULT2B1b transport into the nucleus (He and Falany, 2006; Falany and Rohn-Glowacki, 2013). The N-terminus forms a sterol-sensing allosteric pocket whose structure has recently been determined. The pocket is unique to SULT2B1b and offers a structural template that can be targeted to inhibit the enzyme and control its related biology — a similar strategy has suppressed SULT1A1 activity in humans (Cook et al., 2019).

SULT2B1b is the primary enzyme responsible for the sulfonation of cholesterol and its closely related sterols (Fuda et al., 2002). Cholesterol sulfate is among the most abundant circulating steroid sulfates (Strott and Higashi, 2003). It is a critical signaling molecule in multiple pathways (Strott and Higashi, 2003) and has been linked to diseases ranging from cancer (Eberlin et al., 2010; Zhu et al., 2010; Chen et al., 2016; Vickman et al., 2016; Vickman

et al., 2019; Tatsuguchi et al., 2022b) to cardiovascular (Merten et al., 2001), skin (Heinz et al., 2017), metabolic (Shi et al., 2014; Bi et al., 2018), and Alzheimer's disease (AD) (González-Domínguez et al., 2015; Elbassal et al., 2016). SULT2B1b has been detected *via* immunoreactivity in brain (Salman et al., 2011), lower intestine (Riches et al., 2009), lung (He et al., 2005), tonsil (Chen et al., 2016), oral mucosa (human protein atlas) (Uhlén et al., 2015), platelets (Yanai et al., 2004), sex tissues (testis and ovary) (Uhlén et al., 2015) and skin keratinocytes (Higashi et al., 2004) — the single largest source of cholesterol sulfate in the body (Geese and Raftogianis, 2001; Higashi et al., 2004; He et al., 2005; Dumas et al., 2008; Salman et al., 2011).

Clinical studies find that elevated SULT2B1b levels, as detected by immunoreactivity, in tumors strongly correlate with patient mortality in breast (Dumas et al., 2008), liver (Vickman et al., 2016; Vickman et al., 2019), liver (Yang et al., 2013), colorectal (Hu et al., 2015) stomach (Chen et al., 2016) and androgen-independent prostate (Vickman et al., 2016; Vickman et al., 2019) cancers. Further, SULT2B1b siRNA knockdown prevents tumor growth in rodents (Hu et al., 2015; Chen et al., 2016). In contrast, elevated SULT2B1b levels slow growth of androgen-dependent cancers (He and Falany, 2007). Despite strong evidence linking SULT2B1b to cancer, the molecular basis of this linkage remains elusive. The Liver-X Receptor β (LXRβ), a key regulator of cholesterol transport in the body, is a target of considerable interest for treating AD (Riddell et al., 2007; Bogie et al., 2019) and heart disease (Schultz et al., 2000; Joseph et al., 2002). In brain, SULT2B1b controls LXRβ activation by adjusting the balance of 24(*s*)-hydroxycholesterol (24HC) and 24(*s*)-hydroxycholesterol-3-sulfate (24HCS). 24HC, a brain-specific LXRβ agonist, is transformed by sulfonation into an antagonist with ~ 13-fold enhanced affinity (Cook et al., 2009). Consequently, SULT2B1b inhibition favors LXRβ

activation by preventing formation of the antagonist. LXRβ activation dramatically improves short-term memory and decreases amyloid beta (Aβ) plaque levels roughly 80% in AD rodent models (Bogie et al., 2019). X-linked ichthyosis is an orphan skin disease (Webster et al., 1978; Hernández-Martín et al., 1999) that affects ~ 1/3000 males and is associated with dark hexagonal scaling of the skin surface. The disease is caused by hyper-accumulation of cholesterol sulfate in the two outermost layers of skin, the corneum and granulosum. SULT2B1b resides primarily at the interface between the layers, a mere 10 microns below the surface, and might well prove an accessible, viable target for controlling the disease.

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II. Disease Correlates

A. Cancer

1. Overview. SULT2B1b levels in tumors correlate with patient mortality in stomach (Chen et al., 2016), colon (Hu et al., 2015), breast (Falany et al., 2006) and androgen independent prostate (Vickman et al., 2016) cancer. *In vitro* studies demonstrate that SULT2B1b plays a central role in multiple cancer cell functions, including immune system evasion, inhibition of apoptosis, stimulating angiogenesis, cell growth and migration (Falany et al., 2006; Lo Sasso et al., 2010; Hu et al., 2015; Chen et al., 2016; Vickman et al., 2016; Yang et al., 2019). SULT2B1b siRNA knockdown greatly attenuates tumor growth, and its overexpression dramatically accelerates tumor growth in several mouse cancer models (Yang et al., 2013; Hu et al., 2015; Yang et al., 2019). Levels of cholesterol sulfate, a major *in vivo* product of SULT2B1b activity, are elevated 5-500 fold in tumors (Kiguchi et al., 1998; Eberlin et al., 2010; Hu et al., 2015; Tatsuguchi et al., 2022b) relative to surrounding tissue and strongly correlate with patient mortality (Eberlin et al., 2010).

Cholesterol sulfate regulates a number of important signaling networks related to cancer cell growth. These systems are integrated into a metabolic linkage map, seen in Fig 1, which reveals that the networks, and their numerous cellular consequences, are interdependent and coordinately regulated by the actions of SULT2B1b. The following three paragraphs outline cardinal features of these processes and their dependence on sterol sulfonation.

2. Immune Suppression. The elevated levels of cholesterol sulfate in cancer cells protects them from killer T cells by modulating the signaling behavior of the Tumor Necrosis Factor Receptor 1 (TNFR), the so-called “death receptor” (Schall et al., 1990). TNFR is ubiquitously

expressed in human cells and is activated by the binding of Tumor Necrosis Factor alpha (TNF-α), a major cytokine released by activated killer T cells and other lymphocytes. TNFR is the central receptor in a massive complex with numerous partners, including FADD (Fas-Associating Protein with Death Domain, (Hsu et al., 1996)) and IKK (Inhibitor of KB Kinase) (Devin et al., 2001). Normally, TNFα binding activates FADD, which leads to proteolytic generation of caspase 8, and ultimately apoptosis (Watters and O'Connor, 2011). At elevated cholesterol sulfate, TNFα binding instead activates IKK, which phosphorylates IκB (Inhibitor of Nuclear Factor-κB) and triggers transport of NF-kB (Nuclear Factor–κB) to the nucleus, where it alters gene expression to promote cell survival. Hence, cholesterol sulfate "titrates" the complex from apoptotic to pro-survival signaling. The mechanism of the shift is not fully understood but may have to do with cholesterol sulfate induced changes in lipid raft properties (Bacia et al., 2005) that are critical for proper TNFR1 function (Legler et al., 2003).

In addition to its role in preventing apoptosis, cholesterol sulfate protects cancer cells from killer T cells by binding to and inhibiting two key activators of the anti-tumor immune response — DOCK-2 (Dedicator of Cytokinesis 2) (Sakurai et al., 2018; Tatsuguchi et al., 2022b) and the T-Cell receptor (TCR) (Wang et al., 2016a). ***DOCK-2*** is a guanine nucleotide exchange factor that operates in conjunction with Ras-related C3 botulinum toxin substrate GTPases (RAC1&2) to stimulate lymphocyte migration into tumors (Terasawa et al., 2012). Cholesterol sulfate binds tightly (~ 0.9 μM (Sakurai et al., 2018)) to the DOCK-2 DHR-2 domain, inhibiting its nucleotide-exchange functions and greatly reducing the ability of killer T cells to infiltrate and attack the tumor (Tatsuguchi et al., 2022b). ***TCR*** binds the Class 1 Major Histocompatibility Complex (MHC-1) — the major antigen presenting complex in human cells. TCR recognition of pro-cancer MHC-1•antigen complexes triggers release of cytokines, including TNFα, which

leads to apoptosis (Brehm et al., 2005). The trans-membrane domain of TCR harbors a cholesterol/cholesterol-sulfate binding site. The affinity of cholesterol sulfate for the site is 300-fold greater than that of cholesterol and its binding inhibits TCR dimerization, which prevents formation of the MHC:TCR Complex (Wang et al., 2016a; Chen et al., 2022). In normal lymphocyte development, SULT2B1b mediated control of cholesterol sulfate levels retards TCR activation until the later stages of maturation (Wu et al., 2016), and reduced SULT2B1b leads to a hyper-immune response (Wang et al., 2016a) and autoimmunity (Kostarnoy et al., 2017; Sakurai et al., 2018).

3. Angiogenesis and LXR Activation. In addition to protecting cancer cells from the immune system by "cloaking" them in high levels of cholesterol sulfate, SULT2B1b simultaneously promotes cell growth through at least two mechanisms — increased tumor vascularization, *via* upregulation of VEGF-A (Vascular Endothelial Growth Factor A) (Verheul and Pinedo, 2000), and increased cellular cholesterol levels, *via* inhibition of LXR (Liver-X-Receptor). VEGF-A binding to the endothelial VEGF receptor stimulates a range of angiogenic pathways critical for forming blood vessels in the tumor. The precise mechanism of SULT2B1b enhanced vascularization is not known; however, among seven distinct transcriptional VEGF activators, the SULT2B1b effects are CS independent and require only SP1 (Specificity Protein 1) and AP2 (Activating Protein 2) (Chen et al., 2016). LXR activation inhibits cancer cell growth by promoting cholesterol efflux (Fukuchi et al., 2004; Ju et al., 2017) and inhibiting cholesterol biosynthesis (Pfeifer T, 2016). SULT2B1b sulfonation of endogenous LXR ligands (oxysterols) transforms them from activators to potent inhibitors and, as expected, SULT2B1b inhibition increases LXR activity and slows cancer cell growth (Fukuchi et al., 2004; Pfeifer T, 2016; Ju et al., 2017).

The *in vitro* and *in vivo* findings outlined above demonstrate that SULT2B1b upregulation promotes tumorigenesis *via* multiple pathways, and that its inhibition profoundly retards cancer cell growth — the 2B1b isoform seems a highly promising anti-cancer target (Eberlin et al., 2010; Chen et al., 2016; Vickman et al., 2016; Vickman et al., 2019; Tatsuguchi et al., 2022b).

B. Alzheimer's Disease

1. Overview. Alzheimer's Disease (AD) is the most common cause of dementia worldwide and currently affects approximately three million people in the United States. To date, there are no effective AD therapeutics. Studies over the last thirty years suggest that cholesterol and its metabolites are critical in AD development (Fan et al., 2001; Puglielli et al., 2003; Björkhem and Meaney, 2004; Rebeck, 2004; Rahman et al., 2005; Leoni and Caccia, 2011). High cholesterol levels in the brain promote accumulation of Aβ plaques (Puglielli et al., 2003; Björkhem and Meaney, 2004; Rebeck, 2004; Leoni and Caccia, 2011) and tau protein aggregates (Fan et al., 2001; Rahman et al., 2005). Cholesterol cannot cross the blood brain barrier (Vitali et al., 2014) and must be synthesized in the brain (Björkhem and Meaney, 2004). In the AD brain, both cholesterol and cholesterol sulfate are significantly elevated (Avdulov et al., 1997; Puglielli et al., 2003) and the cholesterol-sulfate/cholesterol ratio increases ~5 fold (González-Domínguez et al., 2015). Precise regulation of the cholesterol levels in brain is maintained through a complex series of steroid sensing proteins, including the amyloid-β protein precursor (APP) (Beel et al., 2010), that are tuned to maintain the concentration and distribution of cholesterol in a very narrow physiological window (Theofilopoulos et al., 2013).

Sulfonated oxysterols allosterically regulate cholesterol homeostasis, Aβ plaque formation and tau protein aggregation. The central aspects of this regulation, which coordinates actions across multiple cell types, are presented in the metabolic map given in Fig. 2.

2. CS Stimulated Aβ Plaque and Tau Fibril Formation. The two main molecular characteristics of AD are Aβ plaques and tau fibrils (Spires-Jones and Hyman, 2014) and cholesterol sulfate has been implicated in formation of both (Spires-Jones and Hyman, 2014). The amyloid precursor protein (APP) is a cholesterol-sensing protein expressed selectively in brain astrocytes and neurons (Beel et al., 2008). The APP cholesterol-binding site is located in the transmembrane domain (Beel et al., 2010) and cholesterol binding shifts APP cleavage from production of AP40 peptide to the pro-disease AP42 (Roher et al., 1999; Barrett et al., 2012). Following cleavage, cholesterol binding to AP peptides (~1:1 stoichiometry) (Yanagisawa and Matsuzaki, 2002; Kandel et al., 2019) stimulates peptide aggregation into neurotoxic plaques (Elbassal et al., 2016; Kandel et al., 2019). Notably, relative to cholesterol, cholesterol sulfate binds more tightly to AP peptide, accumulates ~50-fold in plaques over cell membranes, and accelerates the rate of Aβ plaque formation ~10-fold (Elbassal et al., 2016). Moving to tau fibrils, cholesterol sulfate, but not cholesterol, activates protein kinase C (PKC) in an isoform specific manner (α, δ, ε, η and ζ isoform activities increase 5.0, 1.5, 12, 6.0, 1.2-fold fold, respectively) (Denning et al., 1995). In the neuron, only α and δ phosphorylate tau protein, and their upregulation leads to tau protein hyper-phosphorylation and tau fibril aggregation (Fan et al., 2001; Rahman et al., 2005; Martin et al., 2013).

2. LXRβ Activated Aβ Plaque Clearance. The LXRβ receptor regulates sterol efflux from the brain (Whitney et al., 2002) and plays a critical role in regulating AD progression (Riddell et al., 2007; Theofilopoulos et al., 2013; Sodhi and Singh, 2014). In AD mouse models, LXR

activation reduces plaques and recovers short-term memory (Riddell et al., 2007; Bogie et al., 2019). In support of the *in vivo* findings, LXR activation stimulates Aβ plaque macrophage phagocytosis (Mailleux et al., 2018) and inhibits AB peptide production in neurons and astrocytes (Riddell et al., 2007; Sodhi and Singh, 2014). Cholesterol cannot cross the blood brain barrier and cholesterol levels in brain are reduced by LXRβ stimulated efflux of cholesterol-derived sterols. 24HC is the major LXR activator in brain (Schultz et al., 2000; Wang et al., 2002). 24HC is synthesized exclusively in brain *via* CYP46A catalyzed oxidation of cholesterol and constitutes roughly 80% of the total cholesterol efflux from healthy brain, approximately 20% of which is sulfonated at the 3-OH. 24HC sulfonation by SULT2B1b potently inhibits LXR activation in a wide variety of cells (Bai et al., 2011; Bai et al., 2012); likewise, siRNA knockdown of SULT2B1b increases LXR activation (Bai et al., 2011). Sulfonation of 24HC transforms it from an agonist into an antagonist with ~13-fold higher affinity (Song et al., 2001; Cook et al., 2009), and thus it is the balance of 24HC and 24HCS that determines LXR activation and the rate of sterol efflux. A SULT2B1b inhibitor is likely to inhibit tau fibril formation *via* reducing PKC activity and reduce Aβ plaque formation *via* LXRβ activation.

The findings outlined above predict that SULT2B1b inhibition will have multiple, synergistic effects on metabolic processes that underlie AD, and thus may prove a fruitful avenue for treatment and perhaps even reversal of AD symptoms.

C. X-linked Ichthyosis

1. Overview. The normal human epidermis consists of four layers, or strata (Fig 3), each defined by a particular type of keratinocyte (Sokol et al., 2015; Moreci and Lechler, 2020) — the

predominant cell type in each layer (Proksch et al., 2008; Moreci and Lechler, 2020). Keratinocytes in the basal, or proliferative layer are the progenitors of virtually all epidermal keratinocytes (Watt, 1989). Basal cell differentiation initiates a cellular program in which keratinocytes are fated to migrate upward toward the surface of the epidermis. *En route* to the surface, keratinocytes migrate through signaling gradients (Menon et al., 1985; Moreci and Lechler, 2020) that continually transform them ultimately into corneocytes — enucleated "wax-coated" cells that form the protective outermost layer of skin.

Corneal cell interiors are laden with intermediate-length (~70 Å) keratin fibrils bundled into thick filaments by the binding of filaggrin (Hoober and Eggink, 2022) — a 38-kDa protein shed from secretory granular cells (Elias et al., 1998) into the interface between the corneum and granulosum. Corneal cells are encased in lipids that along with other constituents are actively transported to the corneum in lamellar vesicles formed in the granular layer (Wood et al., 2004). The major lipids of corneum are ceramides, free fatty acids, cholesterol and its derivatives, including cholesterol sulfate (Proksch et al., 2008).

2. Etiology. Cholesterol sulfate comprises approximately 0.2% of total cholesterol in non-keratinized epithelia on a molar basis (Strott and Higashi, 2003). In normal skin epithelia, the percentage rises to ~7% in the corneum (Strott and Higashi, 2003) and further, to ~ 50%, in the corneum of individuals afflicted with X-linked ichthyosis (Strott and Higashi, 2003) (OMIM #308100) — a rare, recessive disease afflicting ~1 in 3000 individuals (Elias et al., 2014; Xie et al., 2020). The disease manifests clinically as widespread, adherent, dark brown, polygonal scales (Hernández-Martín et al., 1999; Heinz et al., 2017). X-linked Ichthyosis is caused by mutations in steroid sulfatase (STS) that lead to a loss of the enzyme's activity (Webster et al., 1978) and the consequent accumulation of cholesterol sulfate.

Steroid sulfatase is compartmentalized along with lipids in lamellar bodies that are delivered from the granulosum to the lower corneum (Elias et al., 2004), where its activity is highest (Elias et al., 1984). As is evident in the staining of normal epithelium (Fig 4A-C (Sokol et al., 2015)) SULT2B1b is expressed at low levels throughout the granulosum and concentrated at the granulosum/corneum interface. Hence, SULT2B1b and steroid sulfatase co-localize at the interface and the imbalance in their activities due to the loss of sulfatase results in excess cholesterol sulfate at-or-near the interface, from which it enters the corneum.

3. Treatment. Diseased epithelial strata are distinguishable from those of normal tissue by a ~ 4-fold thickening of the corneal layer (Elias et al., 1984) caused by a slowing of the rate at which corneal cells detach from the surface (i.e., *desquamation*) (Candi et al., 2005). Topical application of cholesterol sulfate induces pathological scaling on the back-skin of nude mice that can be modulated by addition of cholesterol (Elias et al., 1984). While the mechanism by which cholesterol sulfate slows desquamation is not fully understood (Strott and Higashi, 2003; Elias et al., 2004) it may be due in part to cholesterol sulfate inhibition of the proteases that degrade corneal desmosomes (Sato et al., 1998) — the cell-cell adhesion proteins that link corneal keratinocytes into a 3-D cellular matrix (Green and Simpson, 2007).

Given its etiology, it may well prove possible to treat X-linked ichthyosis with a topically applied SULT2B1b inhibitor to decrease cholesterol sulfate levels. In certain diseases, including ichthyosis, it may prove desirable to decrease rather than eliminate SULT2B1b turnover; if so, it has proven possible to design SULT inhibitors that inhibit to a desired degree at saturation (Cook et al., 2021). These non-competitive inhibitors are not expected to require assiduous dosing protocols; they slow, rather than stall turnover, which should lower, rather than eliminate cholesterol sulfate levels at saturation.

III. Targeting SULT2B1b

1. Overview. Over the past decade it has become clear that most, perhaps all SULTs harbor an allosteric pocket crafted to respond to metabolites specific to their domains. To date, mefenamic acid is the only such inhibitor to enter a clinical trial. A recent study has identified a natural product, SULT2B1b inhibitor that targets the active site of the enzyme (Tatsuguchi et al., 2022a). The SULT allosteric sites share certain fundamental similarities — they form a cleft beneath the so-called active site cap, shown in red in Fig 5A, which encapsulates substrates and must open and close to bind and release nucleotide (Cook et al., 2013; Leyh et al., 2013; Cook et al., 2015; Darrah et al., 2019). Release is the rate limiting step in all SULTs studied to date (Wang et al., 2014; Wang et al., 2016c; Wang et al., 2016b; Wang et al., 2017; Cook et al., 2021). Allosteres directly interact with and stabilize the closed conformation(s) of the cap and thus slow turnover to an extent given by the magnitude of the stabilization, which is allostere specific. It appears natural selection has produced a system in which allosteres physically interact with the protein at a point in space, and moment in time when it is arguably catalytically most vulnerable — the rate limiting step. SULT allosteric pockets, like their active sites, are somewhat promiscuous. Their allosteres tend to be structurally and chemically similar, and exhibit variable affinities and cap stabilization. In a cellular *milieu* in which related allosteres compete for the allosteric site, the site will multiplex numerous input signals into a single variable output — turnover.

2. The SULT2B1b Allosteric Pocket Structure. The SULT2B1b allosteric pocket consists primarily of three structural elements, the cap, base and N-terminus — highlighted in red, teal and blue in Fig 5A (Cook and Leyh, 2022). The structure of the pocket was determined with quercetin (Qu) bound at the allosteric site, and PAP and cholesterol (CH) at the active site. It is a

solution structure arrived at using a combination of NMR-distance measurements and molecular-dynamics modelling, and was confirmed using site-directed mutagenesis. As is evident, Qu interacts with one aromatic residue from each of the elements, and these appear to be the only direct-contact interactions with the enzyme (Cook and Leyh, 2022). A close-up view of the pocket, presented in Fig 5B, suggests Qu acts to bind the three elements to one another (Cook and Leyh, 2022).

3. The Pocket as a Unique Target. Whether the SULT2B1b allosteric site is sufficiently distinct to allow it to be targeted without also inhibiting other SULT isoforms has yet to be determined. It is the only member of the SULT2 family (SULTs 2A1, 2B1a and 2B1b) who's N-terminus is long enough to contribute to the allosteric pocket; hence, the site is unique among SULT2's. The sequence of SULT2B1a, a SULT2B1b splice variant (Ji et al., 2007), is identical to that of 2B1b except for its N-terminus, which is 15 residues shorter. The N-terminal truncation removes a structural element that is both critical to the function of the allosteric-pocket (Cook and Leyh, 2022) and substantially redirects substrate specificity (Fuda et al., 2002). The pockets of SULT1 family members contain a fourth structural element that contacts allosteres. The additional element is proline rich, 6-residues in length, and forms a small stable loop that is absent from the SULT2 family. The structural consequences of the fourth loop are considerable. As seen in Fig 5C and D, the long axis of quercetin points inward toward PAPS when bound to 2B1b, and laterally along the perimeter of the protein in the 1A1 structure. All catalytically active human SULT isoforms, 12 in all, are members of either the SULT1 or SULT2 family. Given the considerable differences between the SULT2B1b allosteric sites and those of all other SULT1 and SULT2 isoforms, it seems quite feasible to create potent, allosteric small-molecule inhibitors that are highly selective for SULT2B1b.

4. Targeting SULT Allosteric Pockets In Vivo. Isoform-specific allosteric SULT inhibition has been used to enhance acetaminophen (APAP, Tylenol) efficacy in humans (Cook et al., 2019). The major APAP metabolites are the sulfate and glucuronide conjugates, which are inactive, form at comparable rates, and clear rapidly to urine. SULT1A1, which catalyzes APAP-S synthesis, is present at high levels in liver, and is allosterically inhibited by NSAIDS (non-steroidal anti-inflammatory drugs) (Wang et al., 2017). SULT2A1, also at high levels in liver, is not NSAID sensitive, and is highly selective for DHEA (dehydroepiandrosterone), which clears to urine as DHEA-S. Mefenamic acid (an NSAID) binds and inhibits SULT1A1 with high affinity (~27 nM), and ~520-fold more weakly to SULT2A1 (~14 μM). When taken orally at therapeutic doses, Mef virtually completely inhibits first-pass sulfonation of APAP and has no detectible effect on the DHEA-S formation. These findings establish the feasibility of the approach, and bear on the long-standing question of why the analgesic effects of aspirin (a high-affinity NSAID inhibitor of SULT1A1, K_i 260 nM (Wang et al., 2017)) and APAP are synergistic (Diener et al., 2005).

An evaluation of SULT2B1b inhibitors as potential therapeutics requires an assessment of possible adverse side effects. CS is a negative feedback signal for gluconeogenesis (Bi et al., 2018) and lipogenesis (Shi et al., 2014); hence, SULT2B1b inhibition might upregulate these pathways to an extent that it promotes insulin insensitivity (Bi et al., 2018) and eventual type II diabetes (Bi et al., 2018). The toxicity of any SULT2B1b inhibitor, like any potential therapeutic, will have to be carefully evaluated. To-date, numerous mouse cancer studies have used SUTL2B1b inhibitors (Yang et al., 2013; Hu et al., 2015; Yang et al., 2019; Tatsuguchi et al., 2022a) to restrict tumor growth and none have reported a serious adverse reaction.

III. Conclusions

In the three diseases considered, cancer, AD and X-linked ichthyosis, elevated SULT2B1b activity substantially promotes disease. The cancer and AD metabolic maps catalogue numerous disease relevant cellular processes whose actions and interactions are regulated by SULT2B1b. Multiple laboratories have identified SULT2B1b as a target of interest in cancer biology because of its role in promoting tumor growth and immune evasion. In mouse models, SULT2B1b inhibition dramatically slows tumorigenesis (Hu et al., 2015; Chen et al., 2016; Vickman et al., 2016; Vickman et al., 2019; Tatsuguchi et al., 2022b) and enhances immune susceptibility of tumors by increasing their macrophage penetrance (Hu et al., 2015; Vickman et al., 2019; Tatsuguchi et al., 2022b). It is generally agreed that AD is a consequence of dysregulated cholesterol homeostasis. LXR is the master regulator of cholesterol metabolism in brain (Björkhem and Meaney, 2004) where its activity is largely regulated by the balance of 24HC (agonist) and 24HCS (antagonist). In *ex vivo* studies, downregulating SULT2B1b potently upregulates LXR, presumably by preventing 24HCS synthesis (Cook et al., 2009; Bai et al., 2011; Zhang et al., 2020) and *in vivo* work with AD mouse models demonstrate that diet-induced sterol activation of LXR causes a remarkable decrease in Aβ plaque load (~80%, hippocampus) and reversal of short-term memory loss. Finally, it is well established that X-linked ichthyosis, an orphan skin disease, is a consequence of elevated cholesterol sulfate in the epidermis. In each scenario, SULT2B1b inhibition is expected to substantially delay onset, lessen severity and perhaps reverse disease symptoms. In closing, the SULT2B1b allosteric pocket offers an excellent target for the design of isoform-specific allosteric inhibitors that can be used to regulate the multifaceted biology of SULT2B1b and ultimately to mitigate disease.

Authorship Contribution

Performed data analysis: Ian T. Cook and Thomas S. Leyh

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Footnotes

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No author has an actual or perceived conflict of interest with the contents of this article.

Figure Legends

Figure 1. *SULT2B1b Functions in Cancer Biology.* The levels of SULT2B1b and cholesterol sulfate (CS) increase in many cancers (see, *Main Text*). SULT2B1b (2B1b, aqua) is shown converting cholesterol (C) to CS in the upper right quadrant of the cancer cell. CS is transported to the extracellular environment by a wide range of potential transporters including members of the organic anion transporters family (OAT3, OAT4, OAT5, OAT6, OAT7, and/or OAT9) (Nigam et al., 2015) and ATP-binding cassette transporters from the sub-family C (ABCC1, ABCC3, and/or ABCC4) (Konig et al., 1999). CS is transported into mature killer T cells *via* ABCG2 transporter (Thurm et al., 2021). Upon entering a mature killer T cell, which does not express SULT2B1b, CS binds DOCK2 (Dedicator of Cytokinesis 2), a guanine nucleotide exchange factor (DOCK2, brown), which prevents it from binding and activating RAC (RAC, green) — a GTPase critical for T-cell migration and tumor penetration. CS also binds the monomer form of the T-Cell Receptor (TCR, orange) transmembrane domain, which inhibits its dimerization and prevents it from forming a functional complex with Major Histocompatibility Complex 1 (MHC, green; antigen, red). Formation of the MHC:TCR complex initiates the immune response, resulting in the export of cytokines, including Tumor Necrosis Factor alpha (TNFα, blue trimer). The Tumor Necrosis Factor alpha Receptor (TNFR) is the central receptor of a massive complex (TNFR Complex) who's signaling switches between cell survival and apoptosis in response to CS levels, which regulate the activities of certain components of the complex, notably, FADD (FAS-Associated Death Domain protein) and IKK (Inhibitor KappaB Kinase). The TNFR Complex is shown in the lower-right segment of the cancer cell membrane in three signaling states (I – III) that differ in the activation status of FADD and IKK —

activation is indicated by a color change to red. The addition of TNFα to Complex I, which forms Complex II, activates the pro-apoptosis pathway by enabling the so-called death domain of FADD to bind pro-caspase 8 (P8, purple oval/red square) causing it to auto-cleave to caspase 8 (Casp 8, purple), which, in turn, cleaves more caspases and commits the cell to apoptosis. Through an as yet unknown mechanism, CS shifts Complex II toward Complex III, and thus biases signaling toward survival and away from apoptosis. The pro-survival pathway is initiated by the activation of IKK, which phosphorylates Inhibitor KappaB (IκB, orange), causing release of Nuclear Factor Kappa B (NF-κB, grey) and exposing its nuclear-localization signaling peptide. As a result, NF-κB translocates to the nucleus and alters gene expression to inhibit apoptosis and promote survival.

Oxysterols (small concentric red and yellow circles, cancer cell upper-right quadrant) produced either by the cancer cell or obtained from systemic circulation can bind the LXR receptor (LXR, teal), causing it to heterodimerize with the RXR receptor (R, purple) and enter the nucleus. In the nucleus, LXR upregulates expression of proteins related to C export, including ABCA1 (lime green) and APOE, a key component of chylomicrons (multicolored brown circle). In addition, the dimer can bind and inactivate SREBP (Sterol Regulatory Element Binding Protein-2, teal) a major transcriptional activator of C biosynthesis. The slowed synthesis and enhanced elimination of C inhibits cell growth. SULT2B1b sulfonates oxysterols that bind tightly and inhibit LXR (grey LXR dimer), which increases C and stimulates growth of the cancer cell. Finally, SULT2B1b expression upregulates two transcription factors, Specific Protein 1 and Activating Protein 2 (not shown), that enhance expression of VEGF (Vascular Endothelial Growth Factor alpha, orange) which stimulates vascularization of the tumor. The mechanism linking SULT2B1b and VEGF is not known — perhaps SULT2B1b sulfonates an as-

yet unidentified molecule (blue) that signals upregulation of SP1 and/or AP2. This figure was created using *BioRender* (BioRender.com).

Figure 2. *SULT2B1b Functions in Alzheimer's Disease.* Cholesterol, which cannot cross the blood brain barrier, is oxidized prior to transport into systemic circulation. 24-hydroxycholesterol (24HC, labeled red sphere, top of neuron) is produced by the CYP46A-catalyzed oxidation of cholesterol. CYP46A (lavender symbol) is found exclusively in the neuron where it associates with the smooth ER. Upon entering the astrocyte, 24HC can be uploaded onto chylomicrons and transported into an adjoining vessel, or bind the LXRβ receptor (LXR, teal) causing it to heterodimerize with the RXR receptor (R, purple). The heterodimer enters the nucleus where it regulates scores of activities including stimulating 24HC export by upregulating the oxysterol transporter (ABCA1, lime green) and APOE, a key structural component of chylomicrons. The LXR dimer can also bind and inactivate SREBP, the Sterol Regulatory Element Binding Protein-1. SREBP (teal symbol, nucleus) is shown upregulating cholesterol biosynthesis and forming an inactive (grey) complex with the LXR dimer. SULT2B1b (aqua, nucleus) converts 24HC to 24HCS, which slows cholesterol biosynthesis by binding to and inactivating LXR (grey complex); thus, the 24HC/24HCS balance regulates both synthesis and export of cholesterol from the astrocyte. In the astrocyte cytosol, cholesterol is seen binding to the amyloid precursor protein (APP), a cholesterol sensor located in the cell membrane (purple and orange structures). Cholesterol binding shifts the APP cleavage-product peptide from a 40-mer to the pro-disease 42-mer, fostering formation of Aβ plaques (circular brown aggregate). Microglial phagocytosis of Aβ plaques is upregulated by LXR and inhibited by 24HCS binding and inactivation of LXR. SULT2B1b in the astrocyte cytosol converts cholesterol to CS, both of which are provided by the astrocyte to the mature neuron *via* the ATP-

binding cassette transporter-2 (ABCA2) (Ahmad et al., 2019), which does not synthesize cholesterol. Once in the neuron, CS binds Protein Kinase C (PKC, brown) causing it to hyper-phosphorylate (red dots) tau protein fibrils, which aggregate into toxic neurofibrillary tangles. Elevated cholesterol levels in the neuron upregulate CYP46A resulting an increase in 24HC, which, once transported into astrocytes, increases 24HC export into systemic circulation and downregulates cholesterol biosynthesis. This intracellular circuitry allows a neuron to monitor its cholesterol levels and signal to the astrocyte, *via* 24HC, the need to adjust the levels. The AD metabolic map predicts that SULT2B1b inhibition will significantly decrease cholesterol levels, Aβ plaques and tau protein aggregates. This figure was created using *BioRender* (BioRender.com).

Figure 3. *Strata of Normal Human Epithelium*. Strata are labeled and color coded: Corneum (blue/black), Granulosum (green), Spinosum (purple), and Basale (red). This figure was adapted from (Sokol et al., 2015).

Figure 4. *Immunofluorescent Localization of SULT2B1b in Normal Human Epithelium*. **Panel A**. Localization of filaggrin (green), a marker for the corneum/granulosum interface. Granulosum keratinocytes are shown in blue. **Panel B.** Localization of SULT2B1b (red). **Panel C**. Superposition of Panels A and B. This figure was adapted from (Sokol et al., 2015).

Figure 5. *SULT2B1b and SULT1A1 Allosteric Pockets*. **Panel A.** *The SULT2B1b oxysterol-binding site*. The binding site consists primarily of three structural elements, the cap (red), base (teal) and N-terminus (blue), and is bound to quercetin (Qu). PAPS and cholesterol (CH) are bound at the active site. **Panel B**. *Close-up of the oxysterol-binding site.* Quercetin directly contacts one residue from each structural element — these appear to be the only direct contacts between the allostere and enzyme. **Panels C and D**. *Comparison of the SULT2B1b and*

SULT1A1 allosteric sites. Unlike SULT2B1b, the N-terminus of SULT1A1 (small blue sphere, *Panel C*) is not long enough to contact quercetin, and SULT1A1 contacts quercetin at four (rather than three) points — two in the cap, one in the base, and one in a small loop (yellow) that is not present in any member of the SULT2 family. As is evident, quercetin orients quite differently in the two enzymes — inward toward PAPS in the SULT2B1b structure (*Panel D*), and along the perimeter of SULT1A1.

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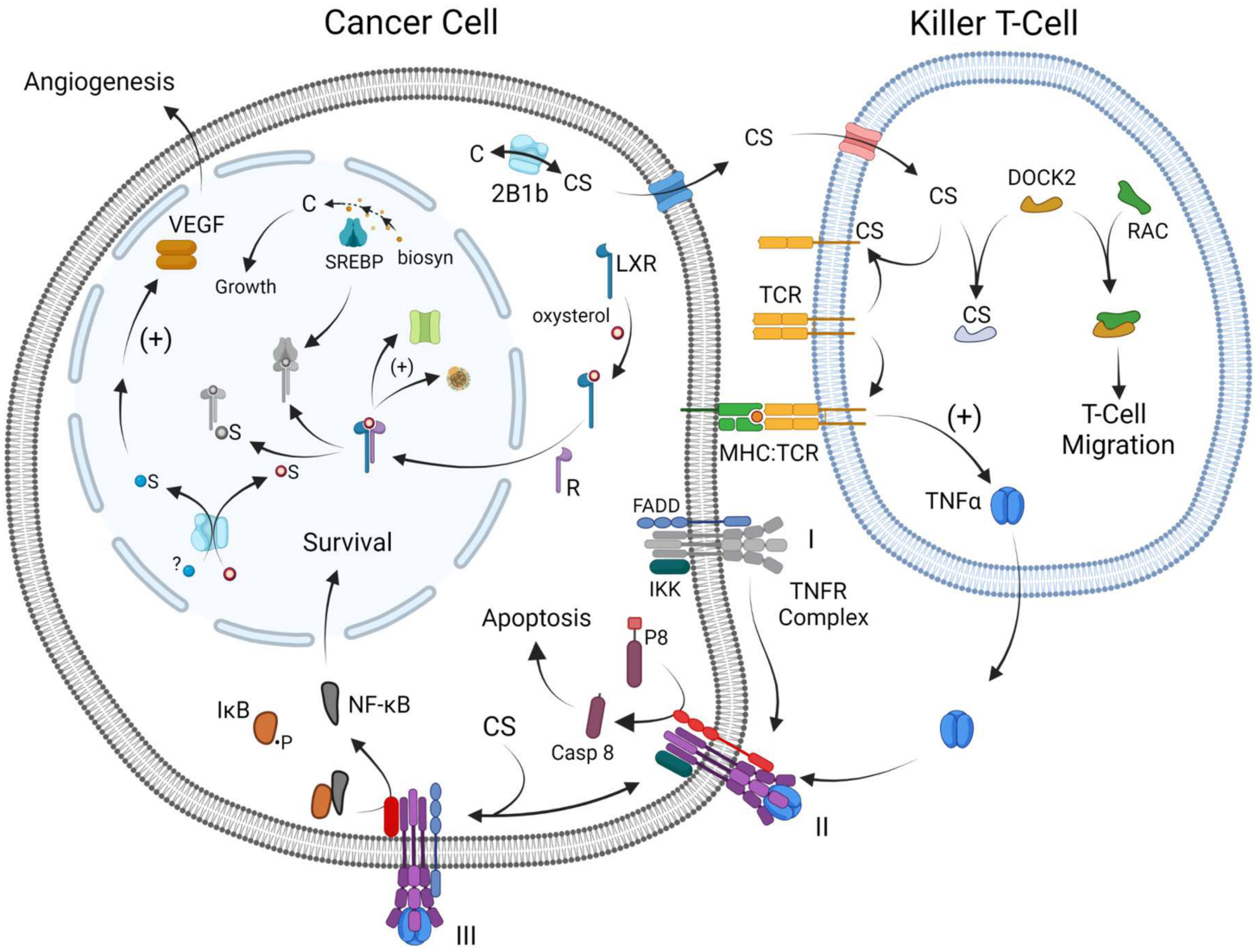
Fig. 1



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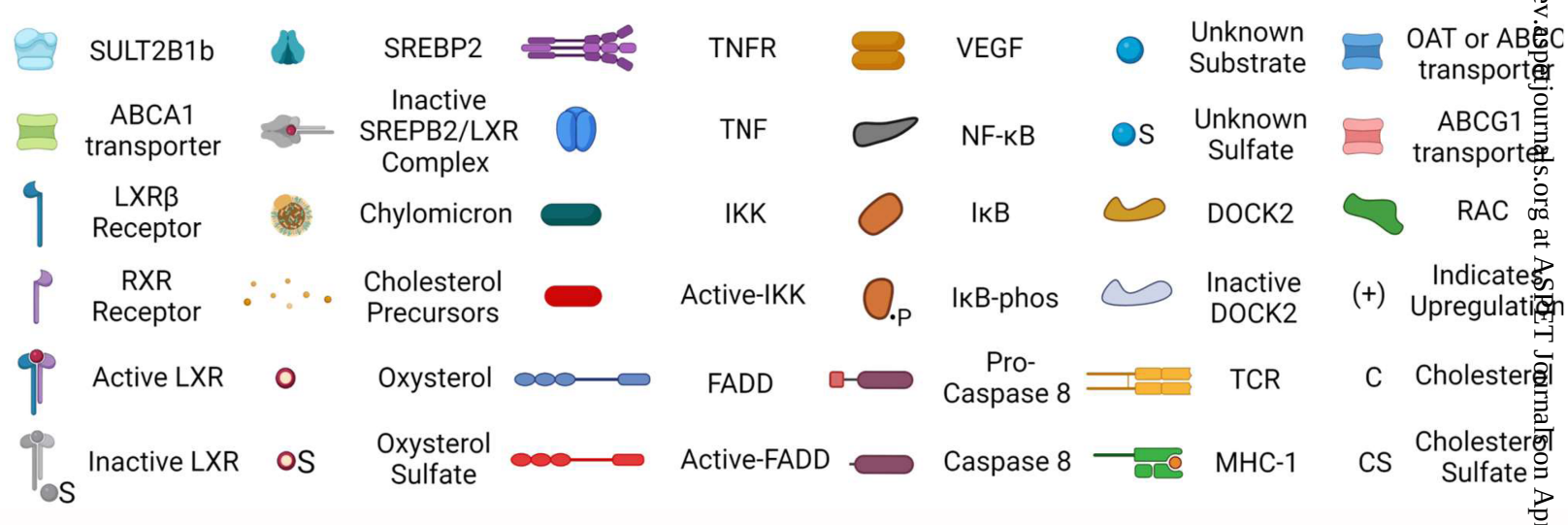

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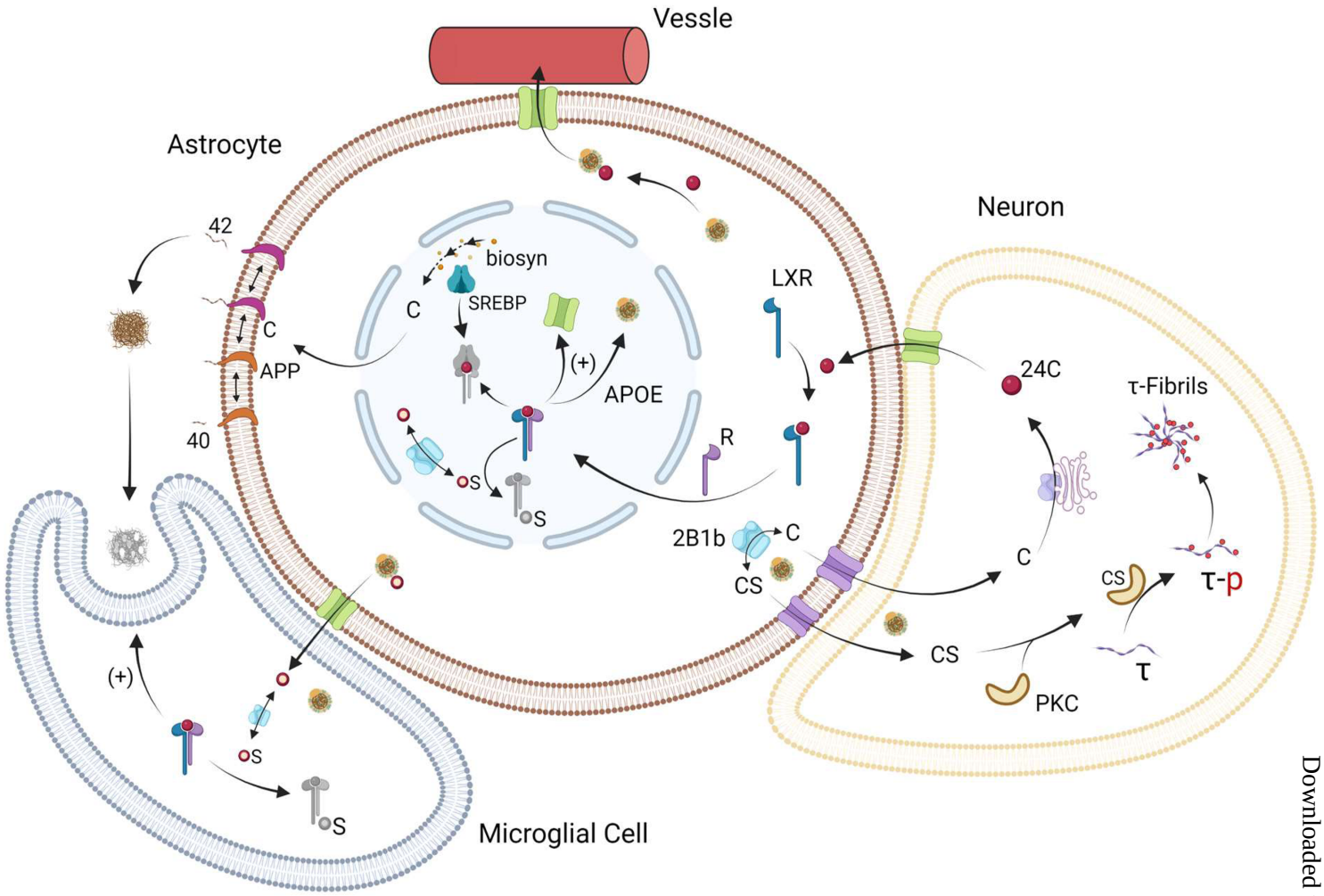
Fig. 2



Legend

- SULT2B1b
- CYP46A
- ABCA1 transporter
- Endoplasmic Reticulum
- Amyoid Preprotein
- ABCA2 transporter
- LXRβ Receptor
- RXR Receptor
- Active LXR
- Inactive LXR
- 40 42 Amyoid Peptides 40 and 42
- SREBP1
- Inactive SREPB1/LXR Complex
- Chylomicron
- Aβ Plaque
- Deteriorating Aβ Plaque
- (+) Indicates Upregulation
- C cholesterol
- CS cholesterol sulfate
- 24-OH cholesterol
- S 24-OH cholesterol sulfate
- oxysterol
- S oxysterol sulfate
- Protein Kinase C
- Tau Fibril
- Phosphorylated Tau Fibril
- Tau Fibril Aggregates
- Cholesterol Precursors

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Fig. 3

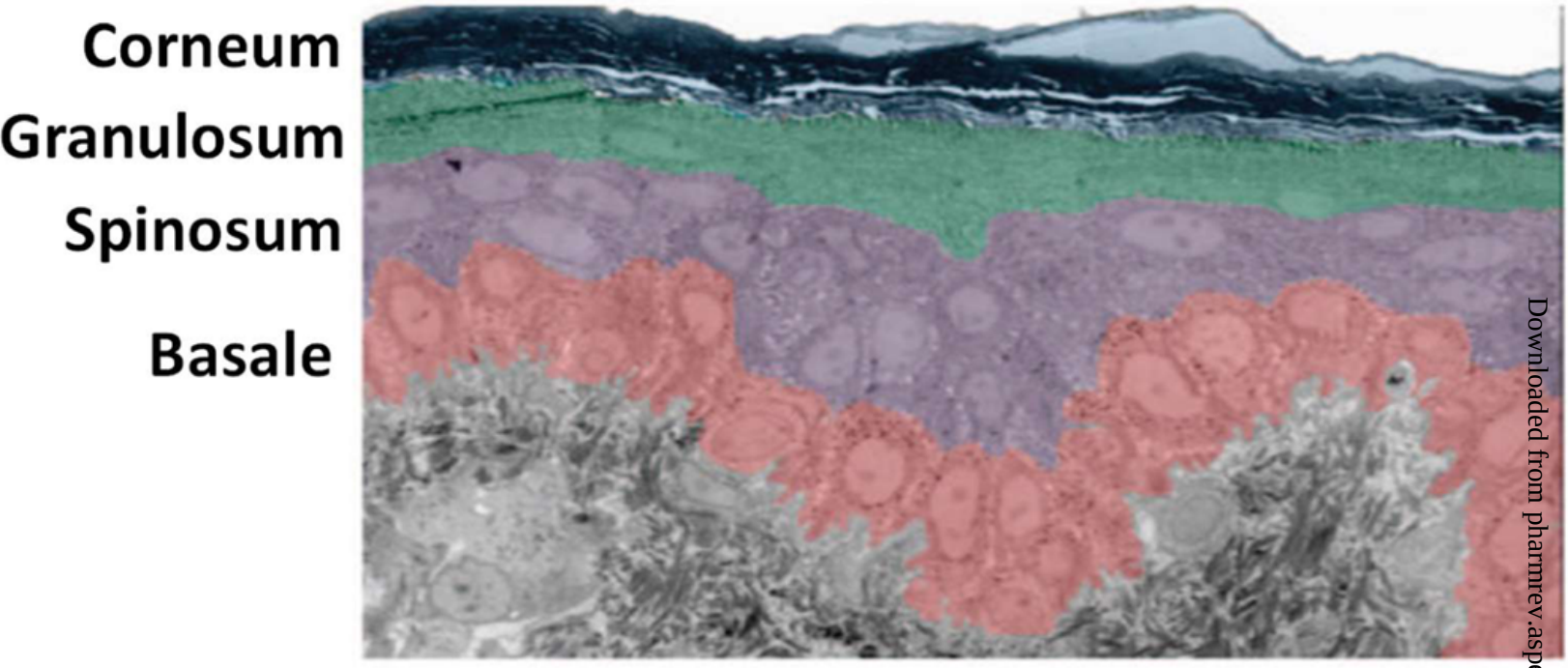

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Fig. 5