

Sulfation pathways

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1 **Insights into Steroid Sulfation and Desulfation Pathways**

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8 **Abbreviations used:**

9 25-OH-D3-S, 25-hydroxy-vitamin D3-3-sulfate; APS, adenosine-5'-phosphosulfate; DHEA,
10 dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; LC-MS/MS, liquid-
11 chromatography-tandem-mass-spectrometry; PAP, 3'-phospho-adenosine-5'-phosphate; PAPS, 3'-
12 phospho-adenosine-5'-phosphosulfate; PAPSS, 3'-phospho-adenosine-5'-phosphosulfate synthase;
13 STS, steroid sulfatase; SULT (1A1/1A3/2A1/1E1/18), sulfotransferase (1A1/1A3/2A1/1E1/18); TPST
14 (1/2), tyrosyl-protein sulfotransferase (1/2); XLI, X-linked ichthyosis

15 Abstract

16 Sulfation and desulfation pathways represent highly dynamic ways of shuttling, repressing and re-
17 activating steroid hormones, thus controlling their immense biological potency at the very heart of
18 endocrinology. This theme currently experiences growing research interest from various sides,
19 including, but not limited to, novel insights about PAPS synthase and sulfotransferase function and
20 regulation, novel analytics for steroid conjugate detection and quantification. Within this review, we
21 will also define how sulfation pathways are ripe for drug development strategies, which have
22 translational potential to treat a number of conditions, including chronic inflammatory diseases and
23 steroid-dependent cancers.

24 Introduction

25 Steroid sulfation and desulfation pathways represent fundamental routes which regulate steroid
26 circulatory transport and action. Whilst sulfated almost all steroids are inert and unable to bind to
27 and activate their specific nuclear receptors. Indeed, as they are no longer lipophilic, sulfated
28 steroids require active transport into cells via organic anion-transporters. Once intracellular, steroid
29 conjugates can be desulfated, a process catalyzed by the ubiquitously expressed steroid sulfatase
30 (STS) enzyme.

31 Over the past 50 years, scientific perspectives on why sulfated steroids exist have changed several
32 times, from it being a mere solubilization step for subsequent renal secretion to sulfated steroids
33 representing a dynamic pool of steroid precursors fueling peripheral steroid signaling (Reed, et al.
34 2005). Such dynamic sulfation/desulfation processes are highly relevant in the endocrine
35 communication between mother and fetus, a field that recently was reviewed elsewhere (Geyer, et
36 al. 2017). Another twist comes from recent evidence that sulfated steroids can still be substrates for
37 steroidogenic enzymes, suggesting they may act as hormonal precursors for a wide range of steroids.
38 We have previously provided a comprehensive review examining how sulfation and desulfation
39 impacts steroid action in normal physiology and in a multitude of disease states (Mueller, et al.
40 2015). Here we aim to give an update on the key advancements in this rapidly moving field.

41 Different PAPS synthases for different sulfation pathways?

42 PAPS synthases and a subset of sulfotransferases work together to ensure efficient sulfation of
43 steroid hormones. PAPS synthases provide high-energy sulfate in the form of 3'-phospho-adenosine-
44 5'-phosphosulfate (PAPS) that is then used for sulfuryl transfer to hydroxyl- or amino-groups of
45 acceptor molecules (Mueller and Shafiqat 2013). Several recent cell-based studies investigated the
46 function of PAPSS1. Small interfering RNA-mediated knockdown of PAPSS1 sensitizes non-small cell
47 lung cancer cells to DNA damaging agents (Leung, et al. 2015; Leung, et al. 2017). PAPSS1 further
48 seems to be essential for nuclear provirus establishment during retroviral (HIV) infection (Bruce, et
49 al. 2008). This was independent from tyrosine sulfation of the CCR5 co-receptor of HIV, but required
50 the sulfotransferase SULT1A1 for HIV-1 minus-strand DNA elongation (Swann, et al. 2016); however,
51 the authors left open what SULT1A1 substrate was responsible for this effect.

52 A different picture emerges for the functionality of PAPSS2, the only other PAPS synthase encoded in
53 the human genome. Transcriptional co-regulation of the *PAPSS2* genes with the *SULT2A1*
54 sulfotransferase has been reported in some cases (Kim, et al. 2004; Sonoda, et al. 2002). Generally,
55 *PAPSS2* is believed to be an inducible gene (Fuda, et al. 2002; Mueller et al. 2015); controlled by TGF-
56 β via p38 kinase phosphorylating Sox9 (Coricor and Serra 2016). Rare compromising mutations in the

57 *PAPSS2* gene present clinically with bone and cartilage mal-formations and an endocrine defect
58 (Noordam, et al. 2009). By performing a DHEA challenge test, we established that inactivating
59 *PAPSS2* mutations cause apparent *SULT2A1* deficiency (Oostdijk, et al. 2015). DHEA could no longer
60 be efficiently sulfated and was downstream converted to biologically active androgens; manifesting
61 with undetectable DHEA sulfate, androgen excess and metabolic disease (Oostdijk et al. 2015).

62 Mechanistically, it is difficult to explain why two highly conserved enzymes with an amino acid
63 identity of 78% could not compensate for each other. Both enzymes have similar APS kinase catalytic
64 activity (Grum, et al. 2010) and they both shuttle between cytoplasm and nucleus, controlled by
65 conserved nuclear localization and export signals (Schroder, et al. 2012). However, PAPS synthases 1
66 and 2 differ markedly in their protein stability, with *PAPSS2* being partially unfolded at physiological
67 temperature (van den Boom, et al. 2012). The natural ligand and substrate adenosine-5'-
68 phosphosulfate (APS) stabilizes the enzyme, making APS an efficient modulator of sulfation
69 pathways (Mueller and Shafqat 2013). For the sulfation pathways studied so far, the PAPS co-factor
70 is always rate-limiting (Kauffman 2004; Moldrup, et al. 2011); but the question remains how
71 specificity for one of the PAPS synthases is generated.

72 Substrate specificity and regulation of sulfotransferases

73 Sulfotransferases provide specificity to sulfation reactions by means of binding specific subsets of
74 acceptor molecules (Coughtrie 2016). Our understanding of their structure, regulation, and function
75 within different sulfation pathways has significantly increased in recent years. The first crystal
76 structure of a plant sulfotransferase in complex with substrate, *Arabidopsis* *SULT18/AtSOT18* with
77 the glucosinolate sinigrin bound to it, identified essential residues for substrate binding and
78 demonstrated that the catalytic mechanism may be conserved between human and plant
79 sulfotransferase enzymes (Hirschmann, et al. 2017). Further, the core elements including the 5'-PSB
80 and 3'-PB motifs, both involved in the binding of PAPS, are structurally conserved even in the
81 distantly related tyrosine-protein sulfotransferases, human *TPST1* and *TPST2* (Tanaka, et al. 2017;
82 Teramoto, et al. 2013). Protein substrates have to locally unfold and bind in a deep active site cleft
83 to *TPSTs* and the vicinity of the acceptor tyrosine residues adopts an intrinsically unfolded
84 conformation in order to facilitate this process (Tanaka et al. 2017; Teramoto et al. 2013). *TPSTs*
85 were known to fulfill different biological functions; shear stress applied to primary cultures of human
86 umbilical vein endothelial cells lead to downregulation of *TPST1* via protein kinase C, but to
87 upregulation of *TPST2* via a tyrosine kinase-dependent pathway (Goettsch, et al. 2006; Goettsch, et
88 al. 2002). However, there are no obvious differences in the substrate-binding site of *TPST1* and 2;
89 these need to be hidden in other non-conserved residues in the periphery. Similarly, substrate
90 specificity may be controlled outside of the active center for *Arabidopsis* *SULT18/SOT18*
91 (Hirschmann et al. 2017). The substrate specificity of human *SULT1A3*, on the other hand, is well
92 understood. A single amino acid substitution in the substrate binding site (glutamic acid at position
93 146) makes *SULT1A3* highly selective for catecholamines (both endogenous and xenobiotic) as
94 Glu146 forms a salt bridge with the nitrogen on the catecholamine side chain (Dajani, et al. 1999).
95 With this one exception, the molecular understanding of the isoform specificity of sulfotransferases
96 remains a challenge despite the wealth of structural information.

97 Recent insights into enzyme kinetics may be helpful here. It is well known that sulfotransferases can
98 show substrate-inhibition due to the formation of non-productive ternary complexes (Gulcan and
99 Duffel 2011; Mueller et al. 2015). More recent is the view that sulfotransferases may be allosterically
100 regulated by their cofactor PAPS: This allosteric regulation extended the dynamic range of *SULT1A1*'s
101 catalytic efficiency (Wang, et al. 2014). Certainly, a new concept is that sulfotransferases might be

102 allosterically regulated in an isozyme-specific manner; liver sulfotransferase *SULT1A1* for example is
103 modulated by catechins (naturally occurring polyphenols) and nonsteroidal anti-inflammatory drugs
104 (Wang, et al. 2016). All these modes of regulation of *SULTs* are illustrated in **Figure 1**. A better
105 understanding of sulfotransferase enzymes may have direct translational potential for drug
106 development (Cook, et al. 2016): Raloxifene is an approved selective estrogen receptor modulator
107 that is quickly sulfated, and thus inactivated, in human cells. Modulating this compound in a way
108 that prevented sulfation, but left its interaction with the estrogen receptor untouched, resulted in
109 an enormous increase in estrogen receptor-activation efficacy (Cook et al. 2016). It is likely that this
110 approach could also work with other compounds.

111 Finally, it is population genetics influencing steroid sulfation pathways and the interindividual
112 variability in drug response. Several coding single nucleotide polymorphisms in *SULT* genes influence
113 an individual's sulfation capacity (Louwers, et al. 2013), but also gene number variations have been
114 reported for *SULT2A1* (Ekstrom and Rane 2015) and other sulfotransferases (Marto, et al. 2017). In
115 fact, the *SULT2A1* gene seems to be more evolvable than, for example, PAPS synthases (Mueller et
116 al. 2015); Ensembl (<https://www.ensembl.org>) lists various expansions of this gene in different
117 lineages with an eight-genes-comprising gene cluster in mice (Zerbino, et al. 2018), while a set of
118 two PAPS synthase genes is highly conserved in vertebrates (van den Boom et al. 2012). A reverse
119 approach using metabolomics and pharmacogenomics indicated that acetaminophen use
120 phenocopied the effect of genetic variants of *SULT2A1* on sulfated metabolites of androstenediol,
121 pregnenolone, and DHEA (Cohen, et al. 2018). This study also challenges views on the mechanism of
122 action of acetaminophen in pain management as sulfated sex hormones can function as
123 neurosteroids and modify nociceptive thresholds.

124 Analytics of steroid conjugates

125 From the very beginning of steroid metabolomics, steroid mixtures were de-conjugated before
126 analysis, mainly by gas-chromatography-mass-spectrometry (Shackleton 2010). However, measuring
127 both free and conjugated steroids may give complementary information. Quantification of
128 conjugates could be laboriously carried out using biochemical separation techniques (Shackleton, et
129 al. 1968) or in multi-step differential de-conjugation measurements (Hill, et al. 2010).
130 Experimentally, detection of intact steroid conjugates was reported already in 1982 (Shackleton and
131 Straub 1982), using particle beam ionization; however this technique did not become standard in
132 analytical labs. Only recently, more and more reports describe the targeted measurements of steroid
133 sulfates and glucuronides using LC-MS/MS. Galuska et al. (Galuska, et al. 2013) reported a combined
134 targeted method for intact steroid sulfates and unconjugated steroids. Six steroid sulfates were
135 quantified by ESI-MS-MS in negative mode and, separately, 11 unconjugated steroids were analyzed
136 by atmospheric pressure chemical ionization (APCI)-MS-MS in positive mode. This combined method
137 could be used for different biological matrices including aqueous solutions, cell lysates and serum
138 (Galuska et al. 2013). Validated targeted LC-MS/MS assays for different sex steroid sulfates from
139 human serum are becoming available (Dury, et al. 2015; Poschner, et al. 2017; Sanchez-Guijo, et al.
140 2015b). Nevertheless, all these assays require separate runs for the conjugated and free steroids. An
141 integrated method for quantifying free and sulfated steroids in a single LC-MS/MS run was recently
142 described (Lee, et al. 2016). It used both SIM and MRM modes as well as polarity switching and was
143 capable of detecting eight free steroids and four sulfated ones. All methods described so far, were
144 targeted assays.

145 Noteworthy, low-energy collision-induced dissociation may be a way to discover new sulfo-
146 conjugates. Maekawa et al. (Maekawa, et al. 2014) used this technique not only to detect sulfate

147 adducts (–97 m/z), but also glycine (–74 m/z) or taurine conjugates. The group of Oscar Pozo
148 developed a modification of this idea to monitor disulfates (McLeod, et al. 2017); these doubly
149 sulfated steroids will be discussed further below. Constant-ion-loss monitoring of one of the sulfates
150 (–97 m/z) allowed untargeted detection of potentially all soluble bis-sulfates; with the caveat that
151 phosphates could also cause this signal (McLeod et al. 2017). This method was recently applied in
152 prenatal diagnostics [Pozo et al, *J Mol Endocr*, accepted 19-Feb-2018]. Further developments in
153 steroid conjugate analytics may involve ultra-high-performance supercritical-fluid chromatography
154 linked to mass spectrometry (Doue, et al. 2015) and mass spectrometry-imaging as established for
155 sulfated gluco-lipids (Marsching, et al. 2014) or for testosterone (Shimma, et al. 2016); allowing for
156 spatial resolution of sulfation ratios.

157 Measuring sulfation ratios of different enzymes precisely might help to expand what has been called
158 the “sulfated steroid pathway” (Sanchez-Guijo, et al. 2016). The concept that sulfation does not
159 prevent downstream conversion of steroids, but modulates it, is based on the side-chain cleaving
160 activity of cytochrome P450 CYP11A1 towards cholesterol sulfate (Tuckey 1990). This observation
161 was then extended to CYP17A1 that bound and metabolized pregnenolone sulfate (Neunzig, et al.
162 2014). It is STS that can then convert sulfated steroids to biologically active steroids (Sanchez-Guijo
163 et al. 2016). Steroid analysis of patients with steroid sulfatase deficiency suggests that other
164 enzymes partially can complement STS (Sanchez-Guijo et al. 2016). In such a pathway, the sulfo-
165 group acts as protection group, allowing downstream biochemical conversions on one side of the
166 steroid molecule, but not on the other.

167 Selected steroid species in sulfo-focus

168 Several steroid conjugates have been known for decades, but only recently have these forms been
169 thought to be biologically meaningful and worth studying. Here, we briefly review knowledge about
170 vitamin D-sulfates, steroid disulfates and 11-oxo-androgens.

171 Vitamin D

172 25-hydroxy-vitamin D3-3-sulfate (25-OH-D3-S) is a major metabolite of vitamin D3 found in the
173 systemic circulation (Axelson 1985). As circulating concentrations of 25OH-D3-3-O-sulfate seem not
174 to be rapidly secreted by the kidney, there is the possibility that this sulfate metabolite may serve as
175 a reservoir of 25OH-D3 in vivo, contributing indirectly to the biologic effects of vitamin D (Wong, et
176 al. 2018). Sulfotransferase *SULT2A1* was identified as the major vitamin D3-sulfating enzyme (Kurogi,
177 et al. 2017; Wong et al. 2018). *SULT2A1* showed activity towards several vitamin D3-related
178 compounds, whereas *SULT1A1* and *SULT2B1a/SULT2B1b* only showed sulfating activity for,
179 respectively, calcitriol and 7-dehydrocholesterol (Kurogi et al. 2017).

180 The relationship between vitamin D and sulfation pathways is reciprocal. The vitamin D receptor also
181 induces transcription of the steroid sulfotransferases *SULT2A1* (Echchgadda, et al. 2004) and
182 *SULT2B1b* (Seo, et al. 2013) as well as the phase I monooxygenase *CYP3A4* (Ahn, et al. 2016), among
183 other genes. Interestingly, the induction of steroid sulfatase by vitamin D3 and retinoids was
184 reported in HL60 promyeloid cells (Hughes, et al. 2001). As net effect, vitamin D transcriptional
185 regulation results in androgen inactivation (Ahn et al. 2016) and elevated sulfation activity that
186 might increase the levels of vitamin D sulfate metabolites.

187 Several analytical methods have been reported to detect and quantify vitamin D3 sulfoconjugates
188 (Abu Kassim, et al. 2018; Gao, et al. 2017; Higashi, et al. 2014). Axelson reported values of 35±14 nM
189 for 25-hydroxy-D3-3-sulfate in plasma from 60 patients (Axelson 1985), Gao measured 56±24 nM for

190 25-OH-D3-3-sulfate in serum from six healthy volunteers (Gao et al. 2017) and Abu Kassim found a
191 range of 9.52–43.8 nM for 25-OH-D3-3-sulfate in serum of 10 volunteers (Abu Kassim et al. 2018).
192 Concentrations of this vitamin D3 sulfoconjugate were consistently higher than its glucuronidated
193 counterparts. More importantly, the reported circulating concentrations for vitamin D3-3-sulfate
194 reach up to what is regarded as the normal level of circulating 25-OH-vitamin D3, 80-250 nM (Hollis
195 2010). Early studies described vitamin D3-3-sulfate as less biologically active than free vitamin D3 in
196 rodents (Cancela, et al. 1987; Nagubandi, et al. 1981). Considering the high circulating
197 concentrations of 25-OH-D3-3-sulfate in the human circulation, it should be taken into account when
198 determining a person's vitamin D status - it could be a reservoir for local generation of 25-OH-D3 and
199 the active 1,25-di-OH-D3.

200 Steroid disulfates

201 Several steroid-diols like estradiol or androstenediol can be doubly sulfated, most likely by the same
202 steroid sulfotransferases due to the pseudo-symmetry of those steroids (Mueller et al. 2015) and a
203 high degree of plasticity in the substrate binding sites (Berger, et al. 2011). As early as in 1962,
204 steroid disulfates (also referred to as bis-sulfates) were described as a constituent of human urine
205 (Pasqualini and Jayle 1962). Falany and coworkers established for 24-hydroxycholesterol-3,24-
206 disulfate that double sulfation leads to a terminal product that is resistant to re-activation by STS
207 (Cook, et al. 2009). This fueled the idea that a second sulfation step represented a further regulatory
208 step or an irreversible step towards inactivation (Mueller et al. 2015). Double sulfation also changes
209 affinity for organic anion transporters. While estradiol-3-sulfate and estradiol-17-sulfate both were
210 substrates for the sodium-dependent organic anion transporter SOAT (SLC10A6), estradiol-3,17-
211 disulfate no longer was cargo for this transporter (Grosser, et al. 2017); depending on where the
212 second sulfation step may occur within the cell, a steroid disulfate may be confined to that cellular
213 compartment.

214 11-oxo androgenic steroids

215 The C19 steroid 11 β -hydroxy-androstenedione is produced by the adrenal in significant amounts; it
216 has however long been regarded as a dead-end product of adrenal steroidogenesis (Pretorius, et al.
217 2017). In recent years, evidence has accumulated that this steroid could be converted to potent
218 androgenic 11-oxygenated steroids, 11-keto-testosterone and 11-keto-dihydrotestosterone, that
219 have similar potency to testosterone and dihydrotestosterone to activate the human androgen
220 receptor (Storbeck, et al. 2013). Sulfated 11-oxo-steroids have not been reported until now,
221 analogous to other androgens (Schiffer, et al. 2018). Interestingly, 11-oxo-steroids seem to be
222 resistant to glucuronidation in various cancer cell lines (du Toit and Swart 2018) and 11-keto-
223 testosterone and 11-keto-dihydrotestosterone are metabolized at a slower rate than testosterone
224 and dihydrotestosterone (Pretorius, et al. 2016). It seems that the 11-oxo modification prevents
225 conjugation, making these steroids to exert prolonged androgenic effects.

226 Steroid sulfatase action and regulation

227 Steroid sulfatase is a membrane-bound protein with its active site located in the lumen of the
228 endoplasmic reticulum (Thomas and Potter 2013). It catalyzes the hydrolysis of sulfate ester bonds
229 from many chemical structures, and it is heavily involved in the desulfation of steroids. STS's main
230 hormone substrates are estrone sulfate, dehydroepiandrosterone sulfate (DHEAS), pregnenolone
231 sulfate, and cholesterol sulfate. Thus, STS action represents a major intracrine route in regenerating
232 biologically active steroids. The crystal structure of STS has been determined (Hernandez-Guzman, et
233 al. 2003) showing a domain consisting of two antiparallel α -helices that protrude from the roughly

234 spherical structure; this gives it a “mushroom-like” shape. Despite this, very little is known on what
235 factors regulate STS activity. STS undergoes post-translational modifications, the key one being the
236 generation of C-alpha formylglycine (FGly), the catalytic residue in the active site of STS, from a
237 cysteine by sulfatase-modifying factors 1 and 2 (SUMF1 & SUMF2). Furthermore, STS contains four
238 potential N-glycosylation sites, however only two (Asn47 and Asn259) are used (Stein, et al. 1989;
239 von Figura, et al. 1998) and only mutations at these sites decrease activity (Stengel, et al. 2008).

240 Most recent studies have focused on directly measuring STS activity in a range of diseases and
241 conditions in order to shed some light on how this enzyme is molecularly controlled (see **Figure 2**).
242 Evidence from chronic liver disease and pre-osteoblastic cells suggests inflammatory mediators, in
243 particularly TNF α (Newman, et al. 2000), can regulate STS expression and activity most likely through
244 NF-kB signaling (Dias and Selcer 2016; Jiang, et al. 2016); with activity depressed by glucocorticoid
245 treatment (Dias and Selcer 2016). Interestingly, estrogens have also been shown to influence STS
246 activity in leukocytes taken from pregnant patients where STS activity is increased in the 3rd
247 trimester (Miyakawa, et al. 1994). In support of this, Gilligan et al. have shown estradiol (E₂)
248 treatment can increase STS activity in colorectal cancer cells via G-protein coupled estrogen receptor
249 (GPER) action (Gilligan, et al. 2017a). These studies suggest a potential positive feedback mechanism
250 by which elevated local estrogen synthesis can further drive estrogen desulfation and activity. How
251 this system is controlled by down-stream GPER mediators remains unknown. However, it is of
252 interest that many steroids, including estrogens, are anti-inflammatory and thus local
253 sulfation/desulfation regulation may represent a mechanism by which steroids control the local
254 influence of an inflammatory insult.

255 Mutations in the STS gene and X-linked Ichthyosis

256 Mutations or deletions of the *STS* gene result in X-linked ichthyosis (XLI), a condition associated with
257 hyperkeratosis (Ballabio, et al. 1989). XLI is also termed STS deficiency and represents a common
258 inherited metabolic disorder, with 1:6000 live births and no geographical or ethnical variation
259 (Fernandes, et al. 2010). Patients with XLI have no sulfatase activity and thus cholesterol sulfate
260 breakdown is impaired. The subsequent cholesterol sulfate accumulation physiologically stabilizes
261 cell membranes (Williams 1992) and builds-up in the stratum corneum causing partial retention
262 hyperkeratosis with visible scaling (Elias, et al. 1984; Williams and Elias 1981). With this loss of
263 desulfation, it is reasonable to assume XLI patients would also exhibit depleted circulating desulfated
264 steroid concentrations, which would subsequently effect their hormone-related development.
265 However, in healthy adult men STS has no significant impact on systemic androgen reactivation from
266 DHEAS (Hammer, et al. 2005), thus suggesting *STS* loss has less physiological effects than anticipated.
267 Indeed, in XLI patients, a compensatory mechanism has been identified through the upregulation of
268 5 α -reductase which, the authors suggest, maintains peripheral androgen activation despite reduced
269 androgen availability (Idkowiak, et al. 2016). Along with changes in androgen metabolism, XLI
270 patients also have elevated plasma concentrations of 27-hydroxycholesterol-3-sulfate compared to
271 healthy males (Sanchez-Guijo, et al. 2015a). The effects of this increased oxysterol sulfate remains
272 unknown.

273 Greater than 90% of XLI patients harbor complete deletions of the *STS* gene. However, there have
274 been 14 point mutations within the *STS* gene previously reported; 3 nonsense mutations and 11
275 missense mutations (Mueller et al. 2015). More recently, a mutation in exon 3 of the *STS* gene was
276 shown to cause a complete loss of STS activity in the affected patient (del Refugio Rivera Vega, et al.
277 2015). Furthermore, two unrelated Japanese patients with ichthyosis are known to have two
278 different point mutations in exon 7 (Oyama, et al. 2016). A novel indel mutation in exon 5 of the *STS*

279 gene has also been reported leading to a frameshift causing a premature stop codon 81 codons
280 downstream from the substitution site (Takeichi, et al. 2015). Intriguingly, this frameshift did not
281 affect the reported active site of STS thus the encoded transcript may be spared if a truncated
282 mutant protein was synthesized.

283 Steroid Sulfatase and Cancer

284 Breast Cancer

285 The most exciting advancements in steroid desulfation research have come through two recently
286 completed clinical trials of the STS inhibitor Irosustat (STX64, 667Coumate). The IPET trial examined
287 Irosustat in treatment of naive ER+ early breast cancer patients (Palmieri, et al. 2017b) and the
288 Phase II IRIS trial examining the clinical benefit rate of Irosustat combined with aromatase inhibition
289 in advance and metastatic ER+ breast cancer (Palmieri, et al. 2017a). Although patient recruitment
290 numbers were relatively low (IPET n = 13; IRIS n = 27) both trials demonstrated some clinical benefit
291 for STS inhibition. In the IPET trial breast tumors were assessed for the effects of Irosustat on tumor
292 growth as measured by 3'-deoxy-3'-[18F]-fluorothymidine uptake measured by PET scanning (FLT-
293 PET) and Ki67 immunohistochemistry. STS inhibition significantly reduced Ki67 scores and the tumor
294 uptake of FLT as measured by PET. Furthermore, Irosustat also decreased tumor STS expression,
295 with this effect also observed in other estrogen metabolizing enzymes and ER α expression. This
296 suggests STS inhibition may have beneficial effects with regards to dampening down tumor estrogen
297 synthesis.

298 Previous pre-clinical studies have shown combining aromatase inhibitors with STS inhibition was a
299 viable strategy to treat MCF-7 xenografts in mice (Foster, et al. 2008a). Thus, the IRIS trial testing this
300 strategy in breast cancer patients who had lapsed whilst on aromatase therapy. Clinical benefit rate
301 was seen in 18.5% (95% CI 6.3-38.1%) of patients with a median progression-free survival of 2.7
302 months (95% CI 2.5-4.6). Considering the difficulty of treating advanced and metastatic breast
303 cancer, these results are encouraging for the future of STS inhibition in breast cancer treatment.
304 Furthermore, MCF-7 cells resistant to letrozole treatment have been shown to have higher STS
305 mRNA expression and greater expression of organic anion-transporting polypeptides, which mediate
306 estrone sulfate transport into the cell (Higuchi, et al. 2016). This provides some molecular insight
307 into aromatase resistance and how STS inhibition may be beneficial to patients who relapse on
308 aromatase inhibitors. However, more clinical data is still required to examine whether Irosustat, or
309 indeed other STS inhibitors, would be beneficial for ER+ aromatase resistant breast cancer patients.

310 Gynecological Cancers

311 Along with new evidence suggesting the importance of *STS* and *SULT1E1* expression in endometriosis
312 (Piccinato, et al. 2016), there are new insights into how desulfation impacts endometrial (Sinreih, et
313 al. 2017) and ovarian (Mungenast, et al. 2017; Ren, et al. 2015) cancers. This work represents a
314 growing interest in local estrogen metabolism and action in gynecological conditions (Rizner 2016;
315 Rizner, et al. 2017). Indeed, these studies show a lack of aromatase activity and expression in these
316 cancers, implicating STS activity as the most likely pathway through which local estrogen synthesis
317 occurs (Ren et al. 2015; Sinreih et al. 2017). Indeed, high SULT1E1 protein expression is positively
318 associated with better-differentiated epithelial ovarian cancers compared to grade 3 epithelial
319 ovarian cancers (Mungenast et al. 2017). This suggests estrogen sulfation, and thus inactivation,
320 limits estrogen tissue availability reducing the potential mitogenic effects of non-sulfated estrogens.
321 Thus, targeting desulfation (i.e. via STS inhibition) may be an important strategy in treating ovarian
322 and endometrial cancer. Pre-clinical mouse xenograft studies have previously demonstrated that STS

323 inhibition blocks estrone sulfate stimulated growth of endometrial tumors (Foster, et al. 2008b),
324 although this theory remains to be tested clinically. Furthermore, and if the STS pathway dominates
325 estrogen synthesis, then these studies may go some way to explain the clinical failure of aromatase
326 inhibitors to treat endometrial cancer (Bogliolo, et al. 2016).

327 Gastrointestinal Cancers

328 A growing body of evidence on gastrointestinal cancers now implicates sex steroids and their
329 desulfation as important drivers of proliferation (Barzi, et al. 2013; Foster 2013; Ur Rahman and Cao
330 2016). Most research has focused on colorectal cancer (CRC) as previous work has shown a potential
331 prognostic role for STS and SULT1E1 protein expression in CRC (Sato, et al. 2009), implicating a high
332 STS and low SULT1E1 expression as indicative of a poor outcome. More recently, over-expression of
333 STS in the CRC cell line HCT116 increases proliferation in vitro and in vivo xenograft mouse models,
334 with these effects blocked by STS inhibition by STX64 (Gilligan, et al. 2017b). These actions were
335 shown to be through increased estrogen desulfation and activation of the G-protein coupled
336 estrogen receptor (GPER), a finding further supported by evidence these effects may be modulated
337 by a hypoxic environment (Bustos, et al. 2017). Indeed, it is of interest to note STS activity can
338 increase hypoxia inducible factor Hif1 α expression in cervical and prostate cancer cells, suggesting
339 STS action may be further regulated by hypoxic conditions (Shin, et al. 2017). Furthermore, estradiol
340 (E₂) treatment increases both STS activity (Gilligan et al. 2017a) and GPER expression in CRC (Bustos
341 et al. 2017), suggesting a novel positive feedback loop through which E₂ can drive CRC proliferation.

342 Steroid sulfation pathways, the brain and behavior

343 XLI patients have an association with behavioral disorders, which include attention deficit-
344 hyperactivity disorder (ADHD), autism, and social communication deficits (Davies, et al. 2009;
345 Stergiakouli, et al. 2011). A study examining 384 patients with ADHD identified two SNPs in the *STS*
346 gene significantly associated with this condition (Brookes, et al. 2008). Indeed, the polymorphism
347 rs17268988 within the *STS* gene is associated with inattentive behavior in males with ADHD (Humby,
348 et al. 2017). More recently, XLI patients have been shown to be at a significantly increased risk of
349 developmental conditions and psychiatric illness (Chatterjee, et al. 2016). The hormonal implications
350 in these conditions remains ill-defined, although researchers have hypothesized disturbed neuronal
351 DHEA-DHEAS metabolism might result in altered neurotransmitter function contributing to the
352 observed abnormalities. There is some support for this theory, albeit in a different disease context.
353 Evidence suggests declining concentrations of neurosteroids, such as DHEA and DHEAS, are closely
354 associated with increased risk of Alzheimer's disease (AD) (El Bitar, et al. 2014; Wojtal, et al. 2006).
355 STS inhibition attenuated cognitive deficits in spatial learning and memory and in hippocampal
356 synaptic plasticity in rats with amyloid β protein induced AD (Yue, et al. 2016). The authors suggest
357 STS inhibition elevated brain DHEAS concentrations with this accounting for the neuroprotective
358 effects, although neuronal DHEAS levels were not measured. Thus, definitive proof that DHEAS is the
359 key neurosteroid linked to STS action within the brain remains to be seen.

360 Another sulfated steroid, pregnenolone sulfate, is known to inhibit GABA neurotransmission in the
361 brain. Two new studies shed light on the effect of this and other neurosteroids on GABA(A) receptor
362 function. The stimulating neurosteroids tetra-hydrodeoxycorticosteron (THDOC) and pregnanolone
363 bind to the very same site within the transmembrane domain (Lavery, et al. 2017; Miller, et al.
364 2017). Inhibitory pregnenolone-sulfate on the other hand binds to another site within the
365 transmembrane domain and fosters pore opening, which corresponds to the desensitized state
366 (Lavery et al. 2017).

367

368 Conclusion

369 Despite this review only covering the past few years of steroid sulfation and desulfation research, it
 370 highlights the now strong evidence supporting the importance on sulfation and desulfation
 371 pathways in controlling steroid action. Most importantly, early clinical trials in hormone-dependent
 372 breast cancer of the STS inhibitor Irosustat are encouraging and suggest inhibiting desulfation as a
 373 viable strategy. Thus, targeting steroid desulfation in other cancers and conditions remains of
 374 significant interest. Furthermore, improvements in measuring both sulfated and non-sulfated
 375 steroids via mass spectrometry should allow for more sensitive quantification and thus a greater
 376 ability to tease-out how the balance between sulfation and desulfation is regulated.

377 However, there is still much we do not know. Defining which PAPS synthase interacts with which
 378 SULT would lead to a greater understanding on steroid sulfation pathways and may lend itself to
 379 specific inhibitory strategies. Most researchers in this area focus on sulfated estrogens and androgen
 380 precursors (e.g. DHEAS), however we have little grasp of whether other sulfated steroids, such as
 381 vitamin D, represent biologically relevant reservoirs for local desulfation and subsequent action.
 382 Furthermore, we are only beginning to understand about disulfated steroids, and at present, we do
 383 not know how these are formed and whether they possess biological function. Finally, we still do not
 384 clearly understand what factors regulate STS activity, although inflammation seems most likely to
 385 play a role.

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392 Declaration of interest

393 The authors declare that there is no conflict of interest that could be perceived as prejudicing the
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- 742

1 Figure legends

2

3 **Figure 1: Different modes of regulation of sulfotransferase enzymes.** **A**, human SULT1A3 contains a
4 unique glutamate (E) in the substrate binding site, specifically binding catecholamines. **B**, substrates
5 can bind in non-productive conformations, causing substrate inhibition. **C**, dissociation of PAP from
6 the sulfotransferase may be rate-limiting, causing product inhibition. **D**, allosteric protein-protein
7 contacts may regulate SULT function. **E**, non-substrate molecules may allosterically activate
8 sulfotransferases. Please refer to the main text for further explanation.

9

10 **Figure 2: The regulation of STS activity.** Many factors are known to either increase or decrease STS
11 activity. To increase STS activity, sulfatase-modifying factors 1 and 2 (SUMF1 and SUMF2) generate
12 C-alpha formylglycine (FGly), the catalytic residue in the active site of STS, from a cysteine.
13 Estrogens, in particular estradiol, have been shown to increase STS activity in leukocytes in the third
14 trimester of pregnancy and in colorectal cancer cells, with this effect potentially regulated by G-
15 protein coupled estrogen receptors (GPER). Inflammation, mediated by TNF α through NF- κ B
16 signalling, also increases local STS activity. Many cancers, in particular breast, prostate, and
17 colorectal cancer, have all been shown to have higher STS activity compared to non-malignant
18 tissue. Factors that decrease STS activity include mutations in the SUMF1 gene leading to failure of
19 the formation of FGly and thus reduced catalytic activity. Drugs, such as Irosustat, that target STS
20 activity have been developed. Interestingly, glucocorticoids, including dexamethasone, can reduce
21 STS activity in various cell lines. Inherited STS deficient (X-linked ichthyosis) patients have loss of STS
22 activity.

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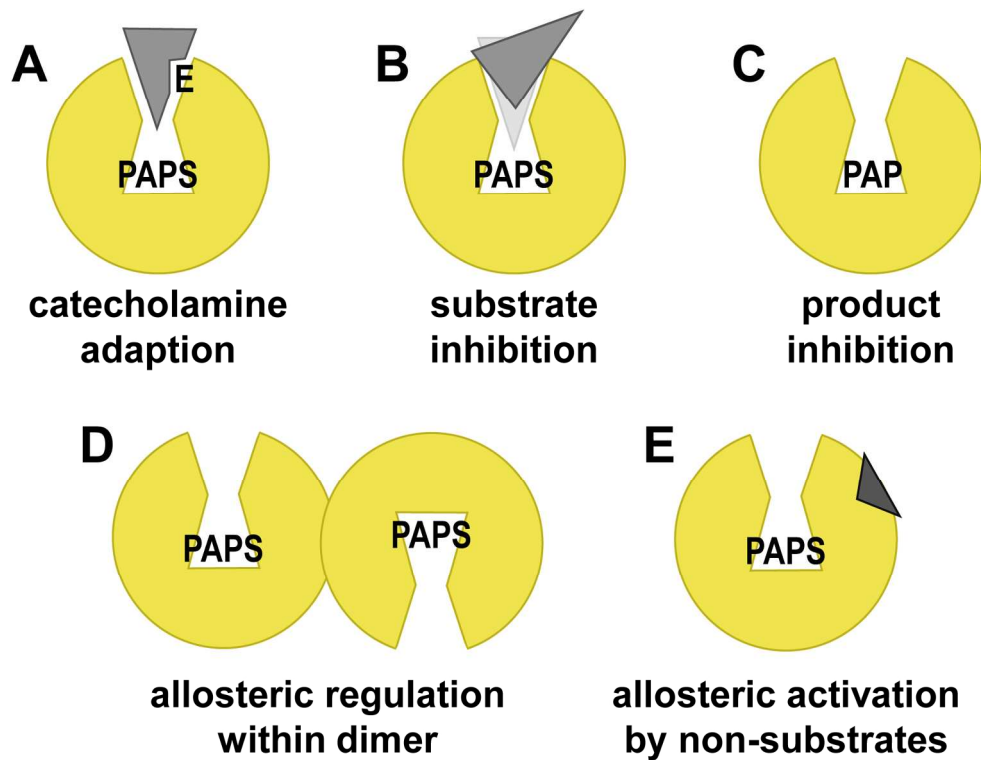


Figure 1: Different modes of regulation of sulfotransferase enzymes. A, human SULT1A3 contains a unique glutamate (E) in the substrate binding site, specifically binding catecholamines. B, substrates can bind in non-productive conformations, causing substrate inhibition. C, dissociation of PAP from the sulfotransferase may be rate-limiting, causing product inhibition. D, allosteric protein-protein contacts may regulate SULT function. E, non-substrate molecules may allosterically activate sulfotransferases. Please refer to the main text for further explanation.

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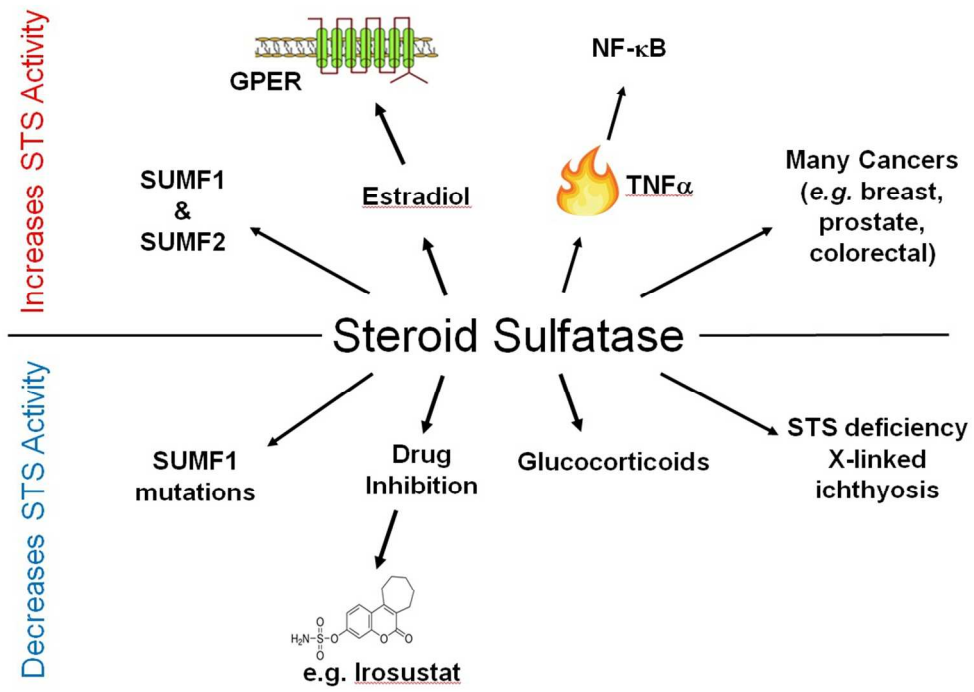


Figure 2

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