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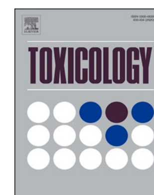

In vitro binding analysis of legacy-linear and new generation-cyclic perfluoro-alkyl substances on sex hormone binding globulin and albumin, suggests low impact on serum hormone kinetics of testosterone

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A R T I C L E I N F O

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A B S T R A C T

In humans, serum testosterone (T) is largely bound to the sex hormone binding globulin (SHBG) and human serum albumin (hSA), resulting in a 2–3 % of unbound or "free" active quote (FT). Endocrine-disrupting chemicals, including perfluoro-alkyl substances (PFAS), are recognized to interfere with the hormonal axes, but the possible impact on the FT quote has not been addressed so far. Here we investigated the possible competition of two acknowledged PFAS molecules on T binding to SHBG and hSA. In particular, perfluoro-octanoic acid (PFOA) and acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)-1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1) (C6O4) were used as, respectively, legacy-linear and new-generation-cyclic PFASs. Human recombinant SHBG 30–234 domain ($SHBG_{30–234}$), produced in HEK293-F cells, and delipidated recombinant hSA were used as in vitro protein models. Isothermal Titration Calorimetry (ITC) and tryptophan fluorescence quencing (TFQ) were used to evaluate the binding modes of T and PFAS to $SHBG_{30–234}$ and hSA. ITC revealed the binding of T to $SHBG_{30–234}$ with a K_d of 44 ± 2 nM whilst both PFOA and C6O4 showed no binding activity. Results were confirmed by TFQ, since only T modified the fluorescence profile of $SHBG_{30–234}$. In hSA, TFQ confirmed the binding of T on FA6 site of the protein. A similar binding mode was observed for PFOA but not for C6O4, as further verified by displacement experiments with T. Although both PFASs were previously shown to bind hSA, only PFOA is predicted to possibly compete with T for the binding to hSA. However, on the base of the binding stoichiometry and affinity of PFOA for hSA, this appears unlikely at the blood concentrations of the chemical documented to date.

1. Introduction

The hypothalamus-pituitary-gonadal axis (HPG) is a major endocrine circuit involed in the regulation of sex steroid production in both males and females (Sze and Brunton, 2020). In males, the late-end effect of the hypotalamic gonadotropin luteinizing hormone (LH) is the stimulation of Leydig cells in testis to produce and release testosterone (T), the main androgenic steroid whose multi-organ anabolic role is widely recognized (Linhares et al., 2022). In humans, peculiar roles in T physiology are played by, respectively, the sex hormone binding globulin (SHBG), the primary serum binding protein of T, and human serum albumin (hSA, Narinx et al., 2022). SHBG is recognized to bind several sex steroids, however T is the one for which it shows the highest affinity with an estimated K_d of ~10^{-9} mol/L. Differently, T shows for hSA a lower affinity, with an estimated K_d of approximately 10^{-5} mol/L. The resulting multiple-equilibrium pattern shows the 44–65 % of T bound to SHBG, the 33–54 % bound to hSA and a remaining 2–3 % which is unbound or "free"(Griffin, J.D.W., 1998). Considering the relatively long time of transit of hSA through the capillary beds, the proportion of T weakly bound to albumin together with FT are thought to be readily

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bioavailable to tissues for the interaction with the classical nuclear androgen receptor.(Manni et al., 1985). An increasing amount of evidence show that FT levels are more reliable than total T in identifying androgen deficiency-like symptoms (Antonio et al., 2016a; Boeri et al., 2017; Facondo et al., 2022). Accordingly, any disrupting agent of this multiple-binding equilibrium is expcted to result in androgen-related disorders, wheather sexual, reproductive or metabolic (Antonio et al., 2016a). Among the known inhibitors of this axis, exogenous chemicals with endocrine-disrupting properties are an emerging threat to male health by mostly acting as anti-androgens or xenoestrogens. In the wide spectrum of endocrine disruptors, perfluoro-alkyl substances (PFASs) are highly persistent pollutants receiving great deal of concerns for their possible and recognized effects on human health (Sunderland et al., 2019). One of the most claimed effects associated with the exposure to PFASs is the interference with the hormonal axes. Environmental exposure to perfluoro-octanoic acid (PFOA), one of the most diffused and studied legacy-linear PFAS, has been associated with the disruption of several hormonal pathawys. As an example, exposure to PFOA has been associated with the disruption of thyroid hormones (TH), resulting in the interference with iodine uptake and TH synthesis (Coperchini et al., 2015; Xin et al., 2018). Moreover, female sex hormones are recognized targets of disruption by PFASs, whose exposure has been variability associated with altered pubertal timing, irregular cycles, reduced fecundity and miscarriage (Fei et al., 2009; Governini et al., 2011; Kristensen et al., 2013; Lopez-Espinosa et al., 2011; Velez et al., 2015; Zhou et al., 2017; Christensen et al., 2011; Darrow et al., 2014; Fei et al., 2009; Governini et al., 2011; Jensen et al., 2015; Louis et al., 2016; Velez et al., 2015; Vestergaard et al., 2012; Whitworth et al., 2012). In males, PFASs have been suggested to interfere at different levels with androgens. Cui et al. in 2020, showed that serum and semen PFOA levels were associated with significant reduction in FT levels (Cui et al., 2020a). Recent data from Xie et al. described a positive association between serum PFASs concentrations and serum T levels in males (Xie et al., 2021a). Previosus findings from our group showed that in male subjects residing in areas at high environmental impact of PFASs, and particularly of PFOA, serum and semen levels of these compounds were positively correlated with serum T, despite showing clear signs of androgen deficiency such as: reduction of either semen quality, testicular volume, penile length, and anogenital distance (Di Nisio et al., 2019a). Taken together, these evidence suggest a possible role of PFAS exposure on the serum FT quote through the imbalance of the binding system of serum T. However, this hypothesis is currently under-investigation. In addition, a possible differential pattern of new-generation PFAS such as the acetic acid, 2,2-difluoro-2-((2,2,4, 5-tetrafluoro-5(trifluoromethoxy)−1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1), (C6O4) is completely missing. The choice of C6O4 as a new-generation PFAS has several orders of reasons. First and foremost, C6O4 was developed and patented in order to cope with the need to replace the perfluorooctanoic acid PFOA. To this regard, C6O4 is structurally unrelated to legacy PFOA. While the first is cyclic, the second is linear. Nevertheless all compounds gave in vitro evidence of some binding affinity to serum albumin (Maso et al., 2021a; Moro et al., 2022). On the other hand, although C6O4 is considered an emerging contaminant, in Europe it falls within the registration range of ≥ 10 to <100 tonnes per annum according to the REACH Regulation. In particular, the only site of cC6O4 manufacture and use is the Piemonte region of Italy and, accordingly, in the last few years, cC6O4 has been included in the list of PFAS monitored in Italy, in water as well as in wastewaters (ARPAV, 2022, 2019, 2021). There is therefore a strong interest in characterizing this molecule in both eco- and bio-toxicological terms (Bizzotto et al., 2023).

Aiming to clarify the possible interference of legacy and new-generation PFASs on the serum T kinetics, in this study we investigated the differential binding pattern and affinity of two structurally different PFASs, PFOA and C6O4, on the two major serum T binding proteins: SHBG and hSA.

2. Materials and methods

2.1. Proteins, chemicals and NMR analysis

Perfluoro-octanoic acid (PFOA) and acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)−1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1) (C6O4) were purchased from Wellington laboratories (Ontario, Canada). Testosterone was purchased from Merck (Milan, Italy). Recombinant human serum albumin (hSA) protein was purchased from Albumin Bioscience (Huntsville, AL, USA). hSA was delipidated according to Maso et al., (Maso et al., 2021b) and stored in PBS buffer (100 mM NaCl e 50 mM Na_3PO_4 at pH 7,4) at -80 °C until use.

^{1}H NMR, ^{13}C{^{1}H}, ^{19}F{^{1}H} NMR, and 2D spectra were recorded with Bruker Ascend 400 spectrometer (Bruker, Milano, Italy), operating at 400 and 100 and 376 MHz (Bruker, Milano, Italy), respectively. Resonance frequencies for ^{1}H NMR, ^{13}C{^{1}H} are referred to tetramethylsilane and for ^{19}F{^{1}H} are referred to $CFCl_3$. Chemical shifts are expressed in ppm with reference to the deuterated solvent residual peak. Multiplicity was addressed as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), and so on. Samples were prepared using 99.8 % deuterated solvents.

2.2. Design of SHBG expression construct

Human SHBG (sex hormone binding protein; Uniprot P04278) was amplified by PCR from a synthetic gene sequence spanning the residues 30–234, encompassing the steroid binding domain (Hildebrand et al., 1995). The construct involved the primers 5'-ttccaggttccactggtgacct-gaggcccgtgctgcc and 5'-tgagagcctccgccaccctcagcctgtgttccaggtgg and was inserted into the expression vector gWiz using the Gibson Assembly method (Thermofisher, Milan, Italy). The integrity of the construct was confirmed by gene sequencing. The SHBG expression vector was designed to allow the secretion of the mature protein by a heterologous signal (IgK signal sequence) and facilitate its enrichment by a C-terminal hexa-histidine tag.

2.3. Expression and purification of SHBG

SHBG 30–234 domain ($SHBG_{30–234}$) was produced in HEK293 F cells (Human Embryonic Kidney cells) grown in suspension in FreeStyle™ 293 Expression Medium (Gibco, ThermoFisher) in shaking flasks at 130 rpm, 37 °C and 8 % CO_2. Cells were sub-cultured every four days to 0.3 $\times 10^6$ cells/mL density and diluted to 1 L at 1×10^6 cells/mL for transfection by polyethylenimine (PEI 40,000, Polyscience Eu GmbH). Briefly, 3 μg of PEI and 1 μg of DNA were resuspended in 20 mL of Opti-MEM (Gibco, ThermoFisher), incubated for 15 min at room temperature (RT) and mixed. After further 15 min, the final solution was added dropwise to the cell culture. Cells cultures were incubated at 37 °C, 8 % CO_2, under constant shaking at 130 rpm for three days. Cells were removed by centrifugation (6000*g* for 20 min at 4 °C in a Backman Coulter® Avanti® 25, rotor JLA 9.1000) and the was supernatant collected and filtered (0.45 μm). The supernatant was loaded into a Ni-NTA resin (CV = 10 mL), pre-equilibrated in buffer A (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, pH 8). After extensive washing with buffer A, protein elution was achieved by treating with 10 mL of 60 % Buffer B (20 mM Tris, 100 mM NaCl, 1 mM CaCl2, 500 mM Imidazole, pH 8) and 20 mL of 100 % Buffer B. The purity grade of SHBG protein was assessed by SDS-PAGE gel and Coomassie stain. Fractions containing isolated SHBG were pooled and desalted by Hitrap Desalting column (Ge Healthcare) in buffer A. Protein oligomeric state and folding were verified by, respectively, analytical size exclusion chromatography, using a Superdex 200 Increase 10/300 GL column (Cytiva) equilibrated in buffer A, and by Circular Dichroism spectroscopy (CD). CD spectra were registered in the range 190–260 nm (Jasco J-810 Spectropolarimeter) at 0.2 nm bandwidth, 0.5 nm data pitch, 0.5 w digital

integration time, 50 nm/min scanning speed with blank subtraction.

2.4. Western blot analysis

Protein samples were treated with Laemmli buffer and incubated for 5 min at 95 °C. Molecular weights of proteins was estimated using the Unstained Protein MW Marker (Thermo Scientific, Waltham, Massachusetts, US). Electrophoresis was performed in SurePAGE Bis-Tris (4–12 %) precast gels (GenScript Biotech, Rijswijk, Netherlands) and Tris-MOPS buffer (pH 7.5). Protein transfer was carried out in wet conditions onto a 0.22 μm nitrocellulose membrane at 90 V for 90 min. Transferred proteins were detected by HRP conjugated anti-His antibody (Abcam, Cambridge, UK 1:4000). Images were processed with Fiji software (https://imagej.net/).

2.5. Fluorescence quenching

The fluorescence spectra were recorded using a Varian Cary Eclipse Spectrofluorometer (Santa Clara, CA, USA). The excitation wavelength was 280 nm and fluorescence spectra were recorded in the wavelength range from 300 nm to 500 nm. Fluorescence quenching measurements were performed using a 0.5 mL quartz cuvette. SHBG (1 μM) and hSA (1 μM) were titrated with increasing concentrations of the ligands (testosterone, PFOA and C6O4). All the measurements were performed at room temperature in buffer A (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, pH 8). Testosterone and PFOA ligands titrations implied the presence of DMSO, never exceeding the 10 % of concentration in the titration buffer. C6O4 stock solutions were prepared in water and diluted in buffer A. The measurement of protein fluorescence intensity for each compound involved three independent experimental replicates. Fluorescence values without protein were used for background corrections for each compound concentration. A titration with DMSO was performed in order to exclude any quenching effects on the protein signal. Fluorescence values plotted against ligand concentration have been obtained by mediating the fluorescence values between 335 and 350 nm, and normalized versus initial fluorescence F0 (F/F0).

2.6. Isothermal titration calorimetry

ITC experiments were performed using a Microcal PEAQ-ITC instrument (Malvern Panalytical, Malvern, UK). SHBG (10 μM) and T (100 μM) were diluted in 20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 % v/v DMSO, pH 8. SHBG (10 μM) and C604 (3 mM) were diluted in 20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, pH 8. In the case of PFOA as ligand (3 mM) and SHBG protein (20 μM), both were diluted in 20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 4 % v/v DMSO, pH 8. Titrations of SHBG with ligands were carried out at 37 °C. In each experiment, an initial 0.4 μL injection (excluded from subsequent data analysis) was followed by 25 independent injections of 1.5 μL with a stirring rate of 750 rpm to ensure rapid mixing. 120 s interval between injections was applied to guarantee the equilibrium at each titration point. Blank experiments (ligands solutions against buffer) were carried out for each ligand and subtracted to corresponding titrations to screen the heat contribution of dilution. Data were analyzed using the MicroCal PEAQ-ITC Evaluation software (Malvern Panalytical, Malvern, UK). Integrated heat signals were fitted by "one identical set of sites" model, since other models gave poor fitting quality. According to this fitting model, were defined:

- K_d as the binding constant;
- n as number of sites;
- $[M]$ as the free concentration of the macromolecule in the active volume;
- M_t as the nominal concentration of the macromolecule
- $[X]$ as the free concentration of the ligand in the active volume;
- X_t as the nominal concentration of the ligand;
- Θ as the fraction of sites occupied by the ligand X
- ΔH as enthalpy change;

given$K_d = \frac{\theta}{(1-\theta)[X]}$.
and $X_t = [X] + n\theta M_t$.
the equation$0 = \theta^2 - \theta\left(1 + \frac{X_t}{nM_t} + \frac{1}{nK_dM_t} + \frac{X_t}{nM_t}\right)$, was used for curve fitting.

Values for K_d and ΔH, together with the stoichiometry of each ligand-SHBG reaction, were obtained from curve fitting. Free energy and the entropy change (ΔS) were calculated from the Gibbs free energy (ΔG) equation: $\Delta G = \Delta H - T\Delta S = -RT\ ln(K_d)$.

3. Results

3.1. Characterization of recombinant $SHBG_{30–234}$ domain and binding profile to testosterone, PFOA and C6O4

In order to address the possible role of SHBG on tissue distribution of legacy and new generation perfluoroalkyl compounds, the N-terminal hormone binding domain (residues from 30 to 234) of SHBG was produced and purified to homogeneity. Analogously to what has been reported by previous structural studies (Grishkovskaya et al., 2000), such domain is composed by a tandem repeat of laminin G-like (LG) folds, behaves as dimers in solution and displays an O-glycosylation profile that slightly increases its final molecular weight by roughly 310 kDa in both electrophoresis and size exclusion chromatography (Supplemental Figure S1A). The circular dichroism spectrum was also compatible with that already reported by HILPERT et al. (2001), Supplemental Figure S1B).

Since it has been suggested that new-generation perfluoro-alkyl ethers display some instability in common organic solvents such as dimethyl-sulfoxide (Liberatore et al., 2020), we performed preliminary experiments to address the possible degradation of C6O4 in DMSO (Supplemental Figure S2). Accordingly, the ^{19}F NMR spectrum of the compound in deuterated DMSO (DMSO-d_6) was performed in a freshly prepared solution and repeated at increasing time intervals, up to 96 h, to monitor the possible molecule degradation. The gradual decrease of the resonances of the compound and the appearance of a peak at − 167 ppm clearly subtended that the compound was not stable for days in DMSO, resulting in an overall 35 % decomposition after 96 h (Supplemental Figure S2A-B). ^{13}C NMR spectrum of C6O4 in DMSO-d_6 acquired after 4 days showed two intense resonances at 124.5 ppm and at 162.7 ppm that are attributed to CO2, and oxalate $(OOC\text{-}COO)_2$- anion, respectively (Supplemental Figure S2C). Differently, C6O4 was completely stable in deuterated water (D_2O), as observed monitoring over time by the ^{19}F NMR spectrum of 5 mM sample in D_2O. After 30 h at room temperature no significant presence of new peaks due to decomposition were observed (Supplemental Figure S2D). Accordingly, the high solubility and stability of C604 in pure water allowed us to avoid the use of DMSO in all the subsequent binding assays.

The affinity of recombinant $SHBG_{30–234}$ dimers toward T was firstly determined by calorimetric measurements at 37 °C in Tris buffer saline, pH 8 (Fig. 1, panel Testosterone). Binding curves fitting by one set of equivalent sites model allowed the estimation of a dissociation constant of 44 ± 2 nM, a ΔH value of − 56 ± 2 kcal/mol and a TΔS value of 13 ± 2 kcal/mol. The estimated number of binding sites roughly corresponded to 0.7, suggesting a possible negative cooperativity. Such values are largely in agreement with the affinities measured in previous studies (DUNN et al., 1981; Hong et al., 2015; Zakharov et al., 2015). Previous studies applied multiple sites models to better describe the dimeric full length SHBG ensemble behavior and the negative cooperativity observed. Despite fitting with a simplified model, our data are in line with the affinity ranges observed elsewhere. Multiple sites model of analysis requires accurate investigations, beyond the purposes of the present study, thus they were not further implemented here.

Subsequently, $SHBG_{30–234}$ was titrated with PFOA and C6O4,

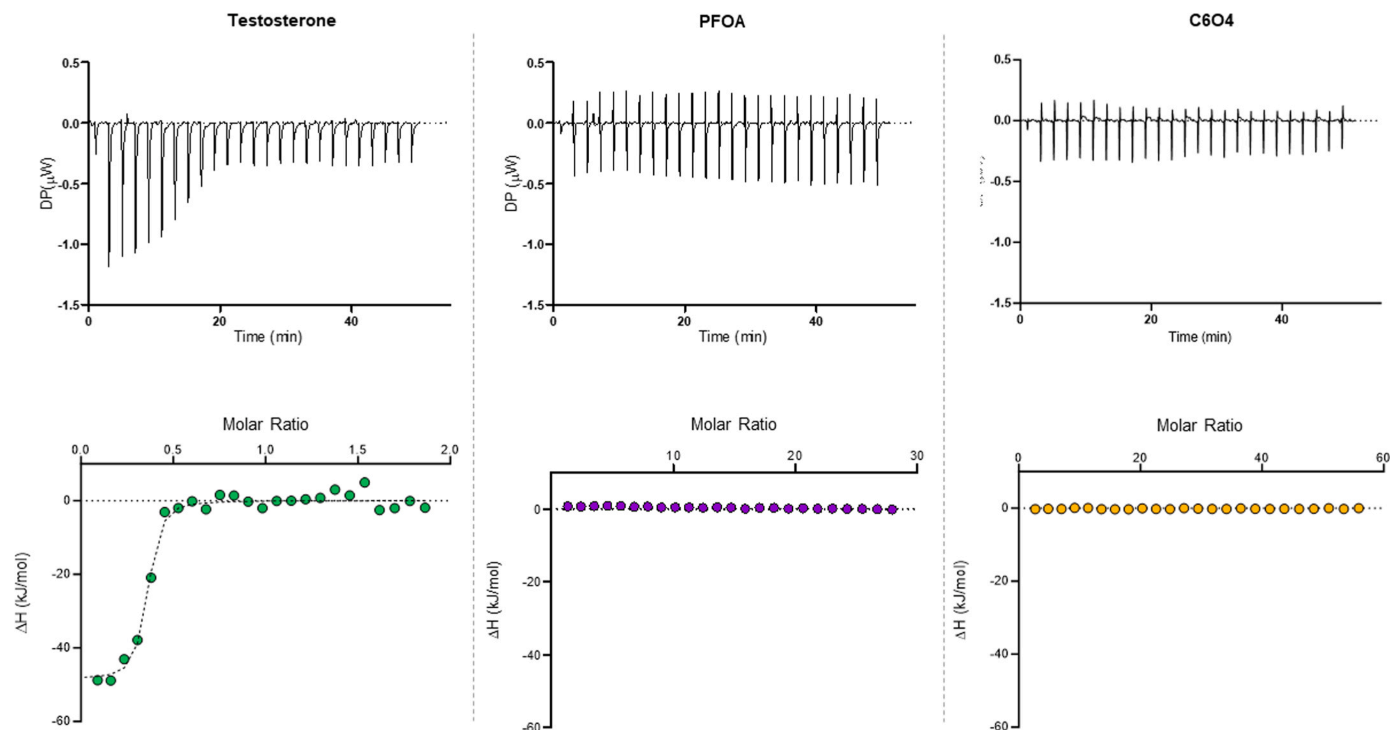

Fig. 1. Isothermal titration calorimetry analysis of human recombinant SHBG 30–234 domain ($SHBG_{30-234}$), binding to perfluoro-surfactants (PFAS) performed at 37 °C. Representative raw trace (top) and integrated binding isotherm (bottom) of the calorimetric titration of testosterone, perfluoro-octanoic acid (PFOA) and acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)−1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1) (C6O4). Data are representatives of three independent experiments.

exploring multiple concentrations ranges up to a maximum ligand:protein molar ratio of approximately 30 (Fig. 1, panels PFOA and C6O4). Both PFOA and C6O4 showed no evident affinity toward SHBG in the tested conditions. It should be noted here that C6O4 was used as a mixture of isomers, as it is supplied in commercial formulations used for industrial applications (Supplemental Figure S3). To confirm these results, the assessment of tryptophan fluorescence quenching (TFQ) was applied to SHBG titration experiments with testosterone, PFOA and C6O4 (Fig. 2). Indeed, of the 11 tryptophan residues in the full-length SHBG, the LG domain possesses five tryptophan residues, two of which, respectively Trp 66 and Trp 170, are close enough to the binding pocket and useful to probe ligands affinity to the ligand binding domain of SHBG [Fig. 2A; PDB: 1D2S (Grishkovskaya et al., 2000)]. On this base, estradiol affinity to SHBG has been previously addressed by TQF, taking advantage of the perturbations in the fluorescence emission from the tryptophan residues in order to monitor the estradiol's binding to SHBG at increasing concentrations of the steroid (Grishkovskaya et al., 2000). TFQ results confirmed both the expected T binding to $SHBG_{30-234}$ and the absence of any detectable fluorescence quenching of the protein due to the interaction with C6O4 or PFOA, even at the highest concentration tested of 200 μM (Fig. 2B).

Taken together, these findings suggest that both legacy perfluoroalkyl compounds, such as PFOA, and new-generation molecules, like C6O4, have no direct impact in the T binding equilibrium to SHBG.

3.2. Binding profile of testosterone, PFOA and C6O4 to human serum albumin

The potential competition of PFOA or C604 for the binding of T to hSA was then assessed. It is recognized that hSA binds and transports roughly 50 % of the total amount of serum T (Griffin, J.D.W., 1998). Previous data on equine serum albumin showed the binding of at least one molecule of T with micromolar affinity between helix h2 of subdomain IIA and helices h2 and h3 of domain IIB, otherwise known as fatty acid binding site 6 (FA6) (Fig. 3A-B; PDB: 6MDQ; Czub et al., 2019a). This main site has been consistently confirmed in hSA by several studies (Kragh-Hansen et al., 1990; Pearlman~ et al., 1967; Zheng et al., 2015). According to the equine SA structure in complex with the androgen, a second molecule of T might be weakly bound to a peripheral pocket between subdomain IA and IB, also known as fatty acid binding site 2 (FA2, Fig. 3C). Such an hypothesis is supported by the conserved pattern of residues in the corresponding hSA region (PDB:6MDQ; Czub et al., 2019b). Other studies suggested at least two binding sites of serum albumin for T, despite no clear evidence at crystal structure analysis have been obtained for hSA so far. In this frame, we have recently investigated the binding of PFOA to hSA, showing the occupation up to four binding sites: one with high affinity in the Sudlow site II (K_d = 0.36 μM) and three additional sites in the mid- micromolar range (cumulative Kd ~ 30 μM). According to our previous structural characterizations of hSA in complex with PFOA, this evidence suggested that the site occupied by T within subdomains IIA and B might also be the target of the perfluoroalkyl substance (Fig. 3D, Maso et al., 2021b) thus potentially competing with the binding of T (Fig. 2 B). Also the possible binding of C604 to hSA has been previously characterized by our group through ITC measurements and competition experiments (Moro et al., 2022), suggesting the occupation of a single binding pocket, distinct from that of T, and corresponding to hSA Sudlow's site II in subdomain IIIA. To verify and compare the affinities of T, PFOA and C604 to hSA, we took advantage of fluorescence quenching of a local Trp probe (hSA Trp 214), positioned roughly 12 Å apart from the main recognized T binding pocket (PDB:7AAE).

The analysis of Trp 124 fluorescence quenching versus ligand concentration showed that the TFQ profiles of T and PFOA were similar, with estimated K_d values falling in the mid-μM range (respectively 118.5 μM and 49.35 μM, Fig. 3E). Differently, no significant binding was detected for C604, as expected. The affinity of the T binding to SHBG measured in our conditions resembled that obtained in previous studies focused on the hormone binding and transport by hSA (Czub et al.,

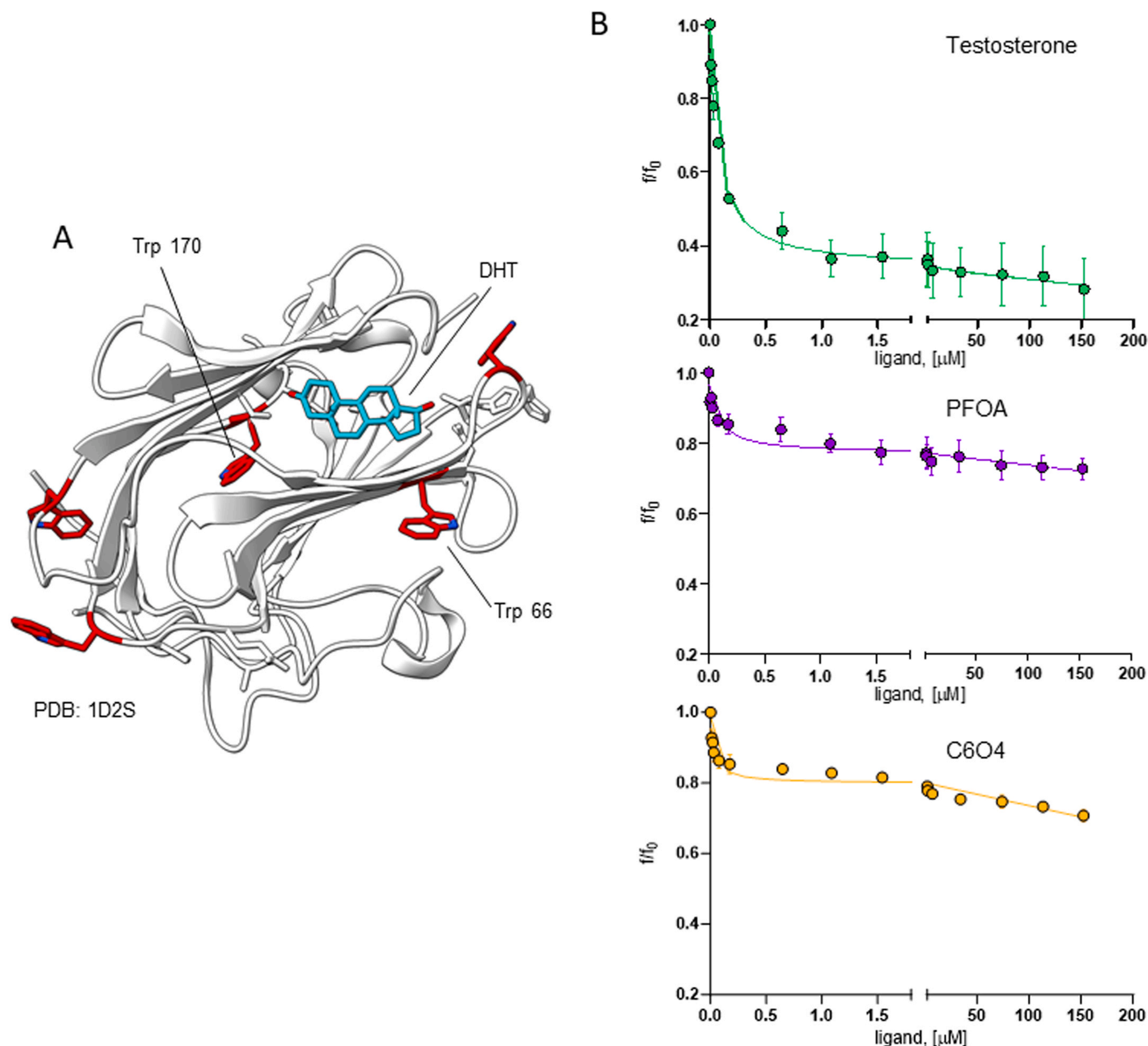

Fig. 2. Tryptophan fluorescence quenching (TFQ) analysis of titration experiments with testosterone, perfluoro-octanoic acid (PFOA) and acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)−1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1) (C6O4) on human recombinant sex hormone binding globulin 30–234 domain ($SHBG_{30–234}$). A) Qualitative evaluation of tryptophan residues potentially diagnostic in TFQ by proximity to the ligand binding domain, based on deposited crystallographic structures (PDB code: 1D2S). B) TFQ profile, obtained by mediating the fluorescence values between 335 and 350 nm and normalized versus initial fluorescence F0 (F/F0), for $SHBG_{30–234}$ (1 μM) titrated with testosterone, PFOA or C6O4. Plots show the mean value of three independent experiments.

2019b). In addition, the TFQ profile of PFOA is in agreement with the affinities detected by ITC within the pull of weakly interacting sites (Maso et al., 2021a). Finally, since our data support the absence of a direct competition of C604 to testosterone, the possible binding of C604 to the Sudlow's site II was evaluated by a competition assay by TFQ method (Fig. 4). The Trp fluorescence spectrum of hSA in the presence of C604, added at concentrations two times higher of its K_d, showed a discrete and progressive quencing following the addition of T at an increasing concentration from 0 to 74 μM (Fig. 4A). On the other hand, the quenched Trp fluorescence spectrum of hSA in the presence of T 99 μM showed to be essentially unaffected by the addition of C6O4 at increasing concentration from 0 to 60 μM (Fig. 4B). Taken together, these data support the existence of two different binding sites for T and C6O4 on hSA and that C6O4 does not interfere with the binding of T to hSA either by direct competition or indirectly.

4. Discussion

In the present study we investigated the possible endocrine disruption by perfluoroalkyl compounds through the competition with testosterone on the two major serum proteins involved in serum transport of the steroid: the sex hormone binding globulin and human serum albumin. PFOA and C6O4, respectively a legacy-linear and new generation-cyclic PFAS, showed negligible or no binding activity to a representative model of human recombinant SHBG, ruling out the possible competition or displacement of T by these compounds. On the other hand, only PFOA showed a binding ability to hSA on the major site recognized for T. However, in a realistic scenario, the concentration of

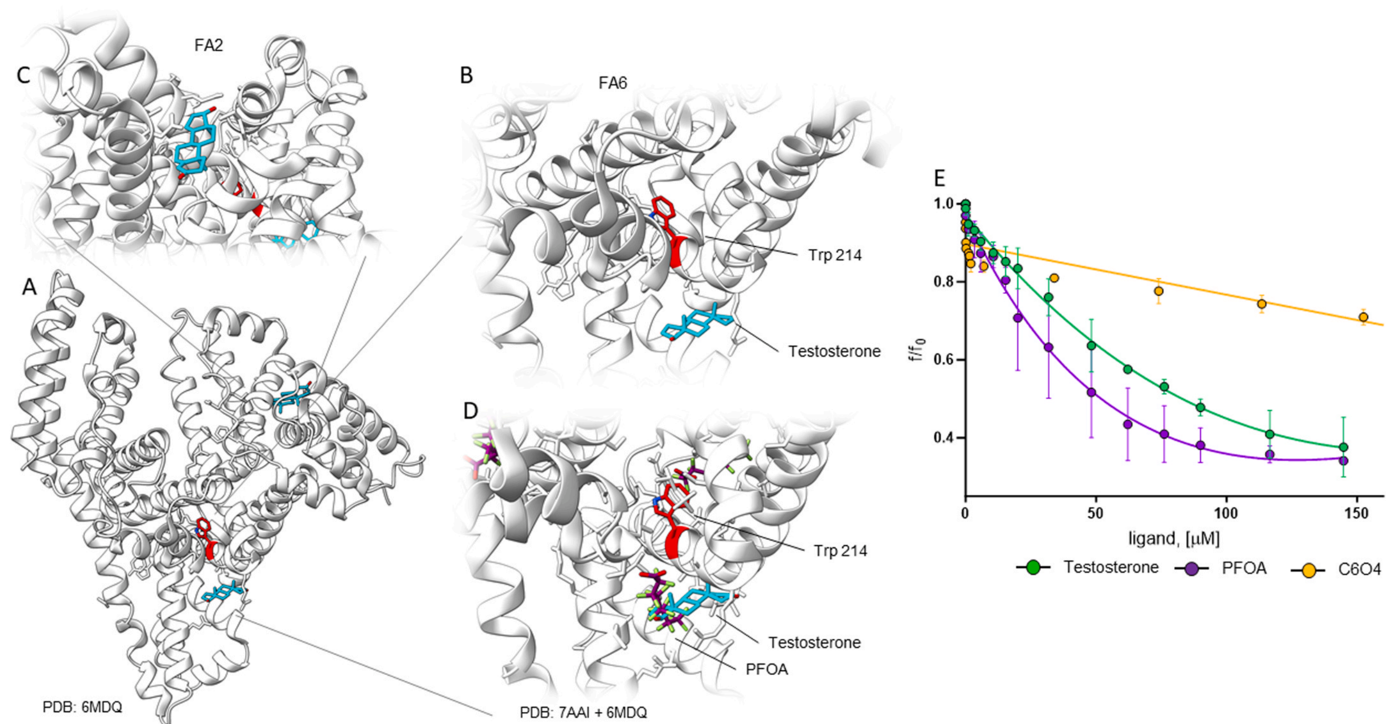

Fig. 3. Tryptophan fluorescence quenching (TFQ) analysis of titration experiments with testosterone, perfluoro-octanoic acid (PFOA) and acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)−1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1) (C6O4) on human serum albumin (hSA). A-D) Qualitative evaluation of tryptophan residues potentially diagnostic in TFQ by proximity to the recognized binding sites for testosterone (cyan), respectively FA2 (C) or FA6 (B) sites, based on deposited crystallographic structures (PDB code: 6MDQ). D) Hypothetical binding competition between testosterone and PFOA (violet), based on deposited crystallographic structures (PDB code: 7AAI). E) TFQ profile, obtained by mediating the fluorescence values between 335 and 350 nm and normalized versus initial fluorescence F0 (F/F0), for hSA (1 μM) titrated with testosterone, PFOA or C6O4. Plots show the mean value of three independent experiments.

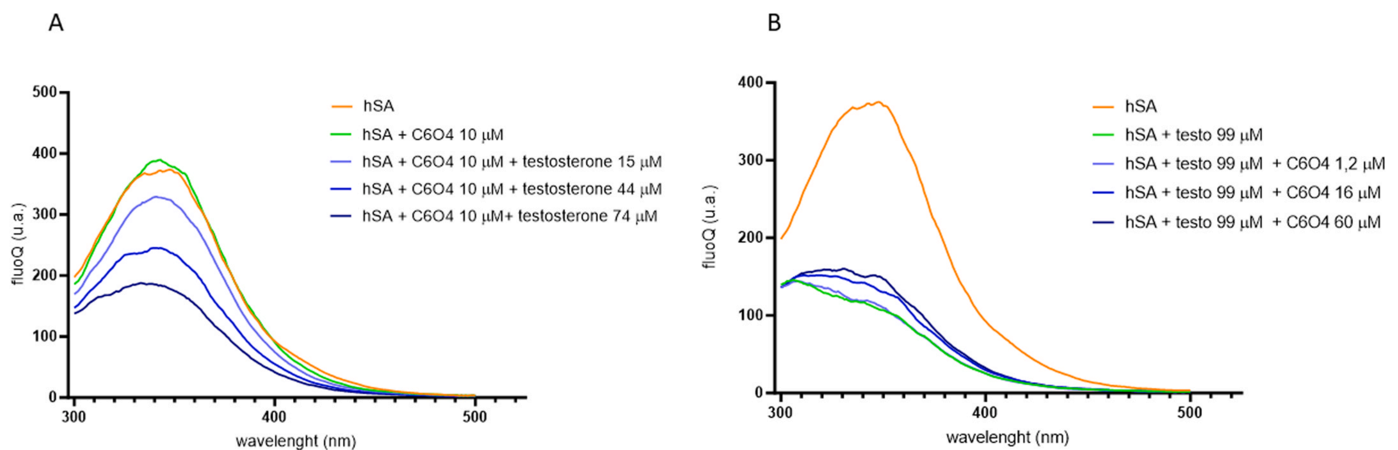

Fig. 4. Competition analysis of testosterone (testo) and acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)− 1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1) (C6O4) on human serum albumin (hSA) by tryptophan fluorescence quenching (TFQ). Representative fluorescence spectra of A) hSA, hSA + 10 μM C6O4 and hSA + 10 μM C6O4 + testosterone at concentrations ranging form 15–74 μM; hSA, B) hSA + 99 μM testosterone and hSA + 99 μM testo + C6O4 at concentrations ranging form 1.2–60 μM.

hSA is so high that a possible competition of PFOA with T is predicted to occur only at plasma concentrations of the pollutant much higher than those documented so far, making this eventuality remote, at least in theory.

The serum hormone kinetics of T are recognized as a major determinant of endocrine derangements. The best describing example of this model is the late onset hypogonadism observed in the aging male (Huhtaniemi and Wu, 2022). According to available epidemiological studies, in spite of a general consensus towards a progressive age-related decline of total serum T levels, the largest part of the ageing men display serum T levels within the reference range adopted for young adults (Sartorius et al., 2012; Travison et al., 2017). Differently, sexual symptoms, recognized as the most specific for the diagnosis of androgen deficiency, are 5-fold more common in the older eugonadal population compared to the background reference (Pye et al., 2014; Sartorius et al., 2012; Veldhuis et al., 2005). In this context, it is interesting to note that patients with normal free T, despite low levels of both total T and SHBG, generally do not show signs of androgen deficiency. Differently, in presence of low free T but normal total T levels, sexual, physical and biological symptoms of hypogonadism are typically observed (Antonio

et al., 2016b). On this bases, any endocrine interference resulting in the variation of free T levels is expected to affect androgen signaling and participate of hormonal derangements.

Health consequences associated with the environmental exposure to PFASs are a current matter of concern worldwide (Sunderland et al., 2019). Available studies, investigating the possible correlation between serum levels of PFASs and semen parameters as outcomes of male hypogonadism, showed a rather consistent association between increased levels of PFOA levels and reduced semen quality. (Di Nisio et al., 2019a). In addition, the exposure to PFOA and PFOS have been associated with impaired sperm motility and morphology (Cui et al., 2020b; Louis et al., 2015; Pan et al., 2019). On the other hand, the possible alteration of semen parameters through the imbalance of sex hormones levels has been addressed less clearly, being the association between PFAS levels and serum sex steroids poorly consistent. To this regard, we showed a positive association between serum PFOA and both total T and LH in 212 exposed Italian males (Di Nisio et al., 2022). Differently, Cui et al. showed a reversed situation, with reduced levels of both total and free T, through the evaluation of serum parameters in 664 adult Chinese men (Cui et al., 2020b). Similar findings were also obtained by Xie et al. evaluating a panel of ten perfluoroalkyl and polyfluoroalkyl substances in 964 males retrieved from the NHANES database (Xie et al., 2021b). Here we show that neither PFOA nor C6O4 displayed an appreciable binding to our SHBG model, evaluated by isothermal titration calorimetry and validated by tryptophan fluorescence quenching. It should be noted that our model of human recombinant SHBG, although oriented towards obtaining the ligand binding domain, was highly representative of the in vivo system, showing a conserved folding pattern at circular dichroism assessment, and displaying spontaneous dimerization at experimental conditions, in agreement with previous data on the minimal sequence requirements for steroid binding and dimerization of human SHBG by Hildebrand et al. (1995). Serum albumin was then evaluated as the possible site of competition between T and PFOA, or C6O4. The binding dynamic of T to hSA is currently a matter of debate. The most recent data on this regard suggest a non-strict 1:1 binding ratio, considering the existence of two or more allosterically coupled binding sites in agreement with previous data on the equine orthologous (Czub et al., 2019b; Jayaraj et al., 2021). However, based on the concurrent binding with serum free-fatty acids, the low affinity binding site with an estimated K_d of ~12 μM, and corresponding to the FA6 site, is the only realistically accessible to T (Jayaraj et al., 2021).

The nature and extent of the interaction between PFOA and albumin is the subject of numerous available studies (Crisalli et al., 2023; Jackson et al., 2021; Li et al., 2021; Salvalaglio et al., 2010). To this regard, previous data from our group showed a differential binding pattern to hSA of PFOA and C6O4 (Maso et al., 2021a; Moro et al., 2022). Indeed, PFOA showed a 4:1 binding ratio to hSA, being respectively 1 high affinity and 3 low affinity sites, corresponding to the FA4, FA6 and FA7 binding sites by displacement experiments with recognized competing drugs (Maso et al., 2021a). Differently, C6O4 showed 2:1 binding ratio to hSA with a low affinity-one set of sites binding mode and encompassing essentially the FA4 binding site (Moro et al., 2022). These data were here confirmed by TFQ experiments, showing that T and PFOA, but not C6O4, displayed a fluorescence quencing on Trp 124 closed to the FA6 site. Most importantly, T and PFOA showed a similar quencing profile, subtending a comparable affinity of these two molecules to hSA. Taken together, these findings can be transposed to a realistic scenario, in which C6O4 is expected to show a low, if any, competition with T for the binding to either SHBG or hSA. On the other hand, PFOA is predicted to possibly displace T from the binding to the FA6-low affinity site of hSA. However, this would only occur after the saturation of both the high affinity sites of hSA and the additional three low affinity sites. Considering the binding stoichiometry of PFOA to hSA, this condition would likely occur at concentrations much greater than 250 ng/mL. According to the available data, such a level of exposure to PFOA is is almost unprecedented for the general population and observed in a minority of subjects occupationally exposed as employed in production plants (Brosset and Ngueta, 2023; Fustinoni and Consonni, 2023; Kim et al., 2023; Řiháčková et al., 2023). On these bases, the most likely endocrine disruption on the androgen signalling, rather than by hormone-kinetic interference with the serum transport protein system of T, would essentially occur at toxicodynamic level by interaction with the androgen receptor. Although non evenly consistent, this has been documented for PFOA in several cell models (Behr et al., 2018; Di Nisio et al., 2019b; Kang et al., 2016; Kjeldsen and Bonefeld-Jørgensen, 2013; Rosenmai et al., 2013). Differently, this possible disrupting mode for C6O4 as a PFOA substitute has not been addressed so far.

We acknowledge the investigation on only PFOA and C6O4 as the main drawback of the present study and, accordingly, no generalized conclusion can be drawn for the overall PFAS family. However, it should be noted that PFOA is the most represented compound in the mixture of PFAS to which the residents were exposed to in the high polluted are of the Veneto Region (Gallo et al., 2022). On the hand, the PFOA substitute C6O4 accounts for an estimated range of production of 10–100 tonnes per annum and is included in the list of PFAS monitored in Italy, in water as well as in wastewaters (ARPAV, 2022, 2019, 2021). Thus, although only two compounds have been studied, these are particularly important PFAS in the current national context.

In conclusion, in this study we showed that PFOA and C6O4 are likely excluded from any form of competition with T on SHBG. On the other hand, even though PFOA, as a legacy-linear PFAS, might compete with the binding of T to hSA, this would occur at very high blood concentrations of the pollutant, rendering this event unlikely in a current context.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2023.153664.

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