Interakcije flavonoida i odabranih ksenobiotika pri vezanju na humani serumski albumin

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FARMACEUTSKO-BIOKEMIJSKI FAKULTET

Hrvoje Rimac

INTERAKCIJE FLAVONOIDA I ODABRANIH KSENOBIOTIKA PRI VEZANJU NA HUMANI SERUMSKI ALBUMIN

DOKTORSKI RAD

Zagreb, 2017.

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DOKTORSKI RAD

Mentori:
Prof. dr. sc. Branka Zorc
Doc. dr. sc. Mirza Bojić

Zagreb, 2017.

University of Zagreb

FACULTY OF PHARMACY AND BIOCHEMISTRY

Hrvoje Rimac

COMPETITION BETWEEN FLAVONOIDS AND SELECTED XENOBIOTICS FOR BINDING SITES ON HUMAN SERUM ALBUMIN

DOCTORAL THESIS

Supervisors:
Prof. Branka Zorc, PhD
Assis. Prof. Mirza Bojić, PhD

Zagreb, 2017.

Rad je predan na ocjenu Fakultetskom vijeću Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta radi stjecanja akademskog stupnja doktora znanosti iz područja biomedicine i zdravstva, polje farmacija, grana farmacija.

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„I have not failed. I've just found 10000 ways that won't work.“

Thomas A. Edison

SAŽETAK

Flavonoidi, kao i veliki broj ksenobiotika, nakon ulaska u cirkulaciju vežu se za humani serumski albumin (HSA) u poddomeni IIA. To vezno mjesto se naziva i Sudlowljevo mjesto I, odnosno vezno mjesto I. S obzirom na veliki broj ksenobiotika koji se ovdje veže, postavlja se pitanje važnosti interakcija istiskivanja pojedinih spojeva s tog veznog mjesta. Iz tog je razloga potrebno bolje opisati svojstva vezanja flavonoida za vezno mjesto I te kliničkih interakcija s lijekovima do kojih bi potencijalno moglo doći.

U prvom dijelu istraživanja ispitivana su vezna svojstva i glavne odrednice vezanja aglikona flavonoida za vezno mjesto u poddomeni IIA HSA. Za određivanje konstanti vezanja koristila se metoda fluorescencijske spektrofotometrije, a za izračunavanje kvantno-kemijskih molekulskih deskriptora računalni program Gaussian 09. U drugom dijelu istraživanja ispitivane su interakcije između aglikona i glikozida flavonoida s odabranim lijekovima te je u tu svrhu korištena metoda fluorescencijske spektrofotometrije zajedno s metodama sidrenja pomoću računalnog programa AutoDock 4.2.6.

Eksperimentalno su utvrđene konstante vezanja 20 flavonoida, koje su iznosile između $5{,}25 \times 10^3$ i $1{,}95 \times 10^5$ M^{-1}. QSAR analizom pronašlo se više strukturnih odlika aglikona flavonoida koje su odgovorne za njihovo vezanje za vezno mjesto I: nukleofilnost i parcijalni naboj atoma C3, parcijalni naboj atoma O4, koplanarnost A i C prstena te koplanarnost AC i B prstena i HOMO i LUMO energije, od čega su najjači utjecaj imala proton-donorska i proton-akceptorska svojstva te koplanarnost prstena. Također je pronađeno da ispitivani lijekovi i flavonoidi mogu ulaziti u interakcije pri vezanju za HSA, ali je vjerojatnost njihova međusobnog istiskivanja pri fiziološkim uvjetima niska jer se ne vežu za isto vezno područje.

Utvrđena su sterička i elektronska svojstva flavonoida koja utječu na jačinu njihova vezanja na HSA. Vjerojatnost istiskivanja ispitivanih lijekova s HSA pomoću flavonoida s klinički važnim posljedicama je niska. Ispitivani lijekovi mogu i pojačavati i smanjivati intenzitet fluorescencije flavonoida vezanih za HSA, tako da je primjena fluorescencije u rutinskom ispitivanju vezanja lijekova na HSA ograničena.

Ključne riječi: humani serumski albumin, flavonoidi, lijekovi, interakcije, fluorescencija, QSAR

SUMMARY

Flavonoids, as well as a large number of xenobiotics, after entering circulation, bind to human serum albumin (HSA) at the IIA subdomain. This binding site is also called Sudlow site I or binding site I. Considering the large number of xenobiotics which bind to this site, it is necessary to evaluate the importance of their displacement interactions from the binding site I. Also, it is important to better describe the characteristics of flavonoid binding to the binding site I and clinical interactions with drugs which could possibly occur.

In the first part of the study, characteristics of flavonoid aglycons' binding to the binding site I, as well as their most important determinants were studied. Fluorescence spectrophotometry was used to determine the binding constants and computer program Gaussian 09 was used to calculate quantum-chemical molecular descriptors. In the second part of the study, interactions between flavonoid aglycons and glycosides with selected drugs were studied by using fluorescence spectrophotometry combined with docking studies carried with AutoDock 4.2.6. computer program.

Binding constants of 20 flavonoids were experimentally determined and they ranged from $5{,}25 \times 10^3$ to $1{,}95 \times 10^5$ M^{-1}. QSAR analysis found several flavonoid features which are key determinants responsible for their binding to the binding site I: nucleophilicity and partial charge of the C3 atom, partial charge of the O4 atom, coplanarity of the A and C rings, as well as coplanarity of the AC and B rings, and HOMO and LUMO energies, with proton-donor and proton-acceptor properties and coplanarity being the most prominent ones. It was also found that the selected drugs and flavonoids can interact in binding to the binding site I, but the possibility of their mutual displacement interactions is not high as they bind to different binding regions.

Flavonoid sterical and electronic characteristics which influence their binding to HSA were determined. A small possibility of displacement of selected drugs by flavonoids with clinically important consequences was found. Examined drugs can both increase and decrease fluorescence intensity of flavonoids bound to HSA, thus the application of fluorescence in routine determination of drug binding to HSA is limited.

Keywords: human serum albumin, flavonoids, drugs, interactions, fluorescence, QSAR

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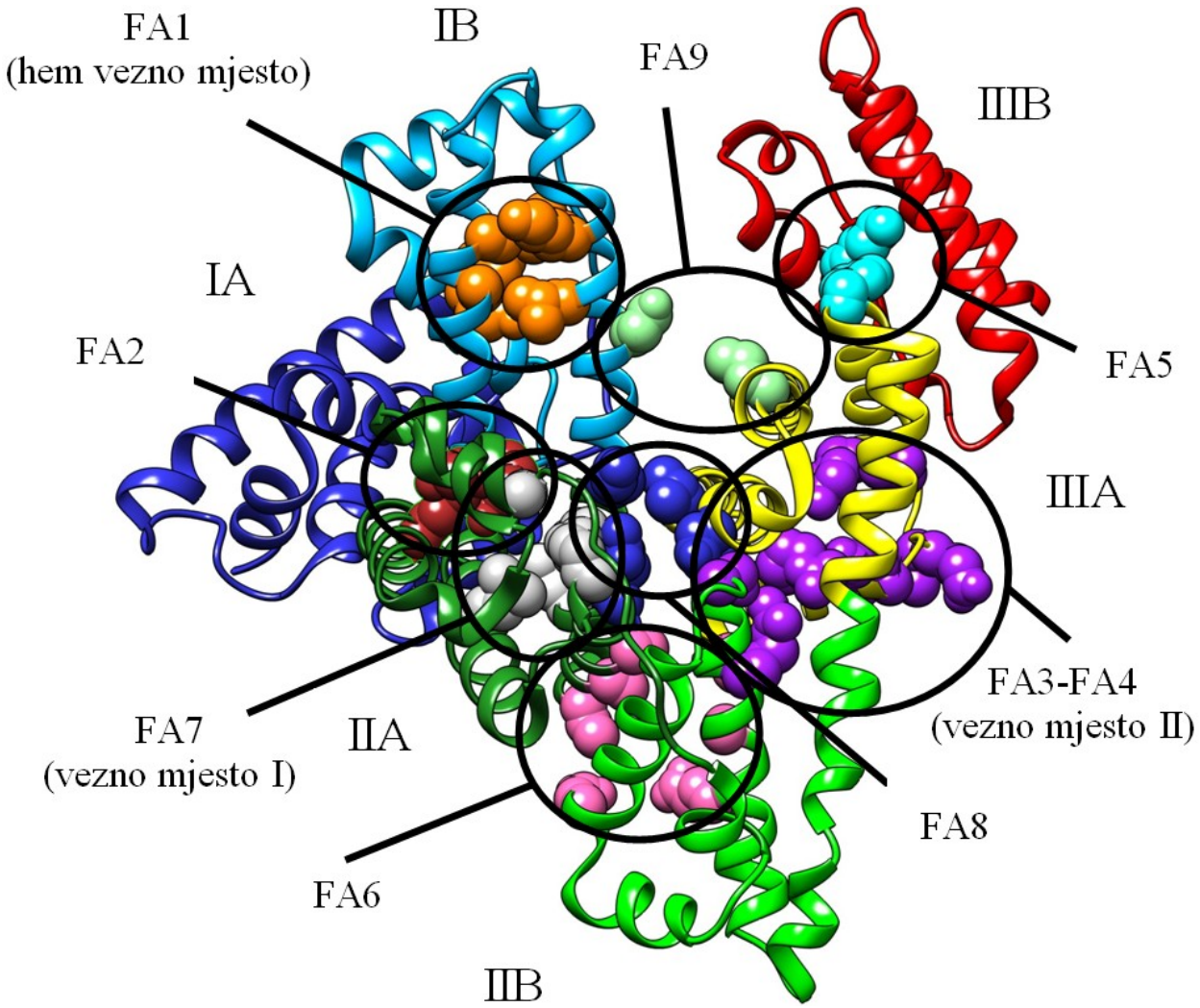
1. UVOD

1.1. Humani serumski albumin

Humani serumski albumin (HSA) je najzastupljeniji protein u ljudskoj plazmi s koncentracijom u zdravih ljudi 500–750 μM (35–50 g/L) te predstavlja oko 60% mase svih proteina u plazmi [1]. Sintetizira se u hepatocitima kao jednolančani neglikozilirani protein relativne molekulske mase 66500 Da, a sastoji se od 585 aminokiselinskih ostataka koji su organizirani u tri strukturno slične domene oblika α uzvojnice (I-III), od koji se svaka sastoji od dvije poddomene (A i B) [2] (slika 1). Trodimenzijski, HSA je srcolikog oblika s približnim dimenzijama 80 × 80 × 30 Å s 35 cisteinskih aminokiselinskih ostataka, od kojih svi osim jednog (Cys 34 u domeni I) tvore disulfidne mostove i time stabiliziraju molekulu. Zbog svog visokog neto naboja od –15 pri pH 7,0 HSA je iznimno topljiv u krvnoj plazmi [1,2]. U tijelu ima mnogobrojne uloge, kao što su regulacija onkotskog tlaka i pH krvne plazme, posjeduje i (pseudo)enzimska i antioksidativna svojstva, a služi i za skladištenje i prijenos endobiotika i ksenobiotika [3], posebice hidrofobnih organskih aniona srednje veličine (100–600 Da), kao što su bilirubin, dugolančane masne kiseline, hematin, tiroksin i mnogi drugi, na taj im način produžujući poluživot u cirkulaciji i regulirajući koncentraciju u krvi [4,5]. Mnogi se lijekovi vežu za jedno od dva glavna vezna mjesta, smještena u poddomenama IIA i IIIA (Sudlowljeva mjesta I i II), s time da se više spojeva veže u poddomeni IIA [6,7]. Vezno mjesto I, u poddomeni IIA, je prostrano i sastoji se od nekoliko zasebnih veznih područja u koja se mogu smjestiti ligandi vrlo različitih kemijskih struktura. Vezno mjesto II, u poddomeni IIIA, je manjeg volumena te je manje fleksibilno i stoga se tu mogu vezati samo strukturno slični ligandi [8].



Slika 1. Helikalna struktura HSA s označenim poddomenama i mjestima vezanja masnih kiselina (FA1-FA9) (PDB unos 2BXD) [8].

1.1.1. Vezanje fizioloških liganada

HSA je glavni nosač neesterificiranih masnih kiselina (engl. *Fatty Acid*) u plazmi, što predstavlja njegovu osnovnu fiziološku funkciju. Dosad je otkriveno 9 veznih mjesta za masne kiseline (FA1-FA9), koja se nalaze u raznim poddomenama, pri čemu se neka od njih preklapaju s veznim mjestima za druge spojeve, uključujući i ksenobiotike (lijekove) (slika 1). Najvažnija od njih su FA1 koje se preklapa s veznim mjestom za hem, FA3-FA4 koje se preklapa s veznim mjestom II u poddomeni IIIA te FA7 koje se preklapa s veznim mjestom I u poddomeni IIA. Sadržaj masnih kiselina u plazmi nije stalan te na taj način može utjecati na istovremeno vezanje drugih liganada [3,9–11].

Drugi fiziološki ligand HSA je bilirubin, završni produkt katabolizma hema. Vezanjem na HSA (99,99%), toksičnost bilirubina je značajno smanjena. Prijašnja su istraživanja pokazivala da se vezno mjesto visokog afiniteta (engl. *High Affinity Site*, HAS) nalazi u poddomeni IIA, kao i u blizini veznog mjesta II, ali novije studije su pokazale da se primarno vezno mjesto bilirubina nalazi u poddomeni IB, u blizini veznog mjesta za hem [12].

Tiroksin se veže za HSA koji na sebi nema vezanih masnih kiselina na četiri mjesta, koja se označavaju od Tr-1 do Tr-4, a djelomično se preklapaju s FA7, FA3-FA4 i FA5. Masne se kiseline natječu s tiroksinom za vezanje na sva četiri vezna mjesta, sprječavajući njegovo vezanje. Pri visokim koncentracijama, masne kiseline također mogu inducirati promjene u 3D strukturi HSA, što za posljedicu ima formiranje petog tiroksinskog veznog mjesta, Tr-5 na području FA9 [3,13]. Određene HSA genetske varijante pokazuju viši afinitet za tiroksin, što dovodi do manjeg postotka slobodnog tiroksina u krvi i posljedično do povećane sinteze tiroksina [3]. Pomoću ovog mehanizma koncentracija slobodnog tiroksina ostaje unutar normalnog raspona, ali je njegova ukupna koncentracija viša, što može dovesti do pogrešne dijagnoze hipertireodizma te do nepotrebnog liječenja ovog simptoma, poznatog kao obiteljska disalbuminemična hipertiroksinemija (engl. *Familial Dysalbuminemic Hyperthyroxinemia*, FDH) [14].

1.1.2. Povezanost strukture i afiniteta (SAR) lijekova pri vezanju za HSA

Tipični lijekovi koji se vežu za HSA su lipofilni lijekovi i većina ih se veže ili u poddomeni IIA ili u poddomeni IIIA. Tipični lijek poddomene IIA je dikarboksilna kiselina ili velika heterociklična molekula s negativnim nabojem u sredini molekule, a tipični lijek poddomene IIIA je karboksilna kiselina s negativno nabijenom kiselinskom skupinom na jednom kraju molekule s hidrofobnim središnjim dijelom molekule. Nažalost, osim ovih općenitih pravila, ne može se puno više reći da bi se objasnilo ili predvidjelo vezanje liganada na HSA [11]. Kragh-Hansen je u svom preglednom radu usporedio nekoliko strukturno sličnih lijekova koji se značajno razlikuju u vezanju za HSA [10]. Primjerice, jopanoat se razlikuje od jofenoksata u tome što sadrži amino skupinu umjesto hidroksilne skupine. Unatoč tome, ta dva lijeka imaju različite HAS-ove na HSA. Nadalje, ampicilin, koji se razlikuje od penicilina G u jednoj amino skupini, može istisnuti bilirubin, za razliku od penicilina G. Još jedan primjer je benzoat koji također istiskuje bilirubin, dok vezanje parahidroksibenzoata rezultira stvaranjem

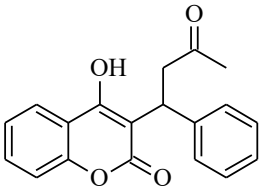
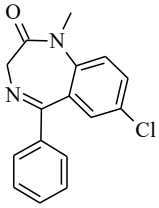
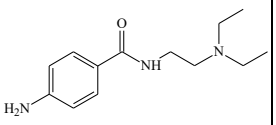
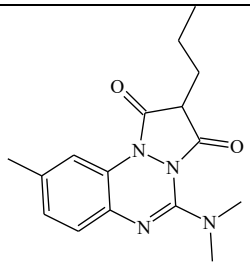
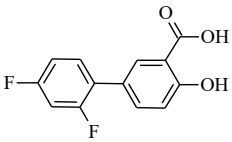
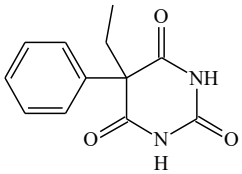
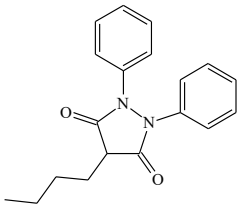
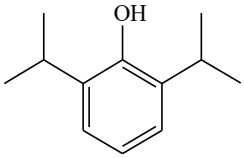
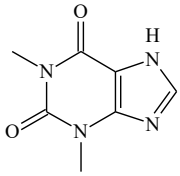
stabilnog ternarnog kompleksa. Također, *L*-triptofan se veže otprilike 100 puta jače od *D*-triptofana, dok je razlika u jačini vezanja između *R*- i *S*-oksazepama 30–40 puta. S druge strane, afiniteti za *R*- i *S*-varfarin su otprilike jednaki, kao i za *R*- i *S*-progesteron i aldosteron, iako se konstante vezanja steroida razlikuju u slučaju α- ili β-vezanih supstituenata [10].

Lijekove koji imaju visoki afinitet za HSA treba davati u višim dozama da bi se postigla učinkovita koncentracija; ti lijekovi posljedično sporo dolaze do mjesta djelovanja i sporo se eliminiraju [15]. Kao što je već rečeno, lijekovi koji se vežu za HSA su uglavnom lipofilni i vezanjam za HSA im se povećava metabolička stabilnost i poluvrijeme eliminacije ($t_{1/2}$). U takvim slučajevima, učestalost doziranja se može smanjiti, a lijek vezan za HSA može služiti kao skladište. S druge strane, povećavanje nevezanog udijela lijeka čini ga manje podložnim iznenadnim povišenjima aktivne koncentracije, ali također i snizuje $t_{1/2}$ [16,17].

Iako je visok postotak vezanja za HSA svojstvo lipofilnih lijekova, korelacija između lipofilnosti i vezanja za HSA zasad se utvrdila samo za kongeneričke serije spojeva, dok su za strukturno različite skupine molekula lipofilnost i vezanja na HSA slabo korelirani [18], što se objašnjava vezanjem strukturno različitih spojeva na različita vezna mjesta [15]. Zanimljiv slučaj je vezanje fenilbutazona i oksifenbutazona. Iako se obje molekule vežu u središtu veznog mjesta u poddomeni IIA, zbog prisutnosti hidroksilne skupine, oksifenbutazon je zarotiran 180° s obzirom na fenilbutazon kad su vezani na HSA bez masnih kiselina, dok im je u prisutnosti masnih kiselina orijentacija slična. To nam govori kako čak i neznatne strukturne promjene u molekulskoj strukturi mogu imati nepredvidljive učinke na vezanje lijekova [8].

Primjeri nekoliko lijekova koji su u velikom postotku vezani za vezna mjesta u poddomenama IIA i IIIA, kao i lijekova koji nisu značajno vezani za HSA prikazani su u tablici 1. Lijekovi vezani u poddomenama IIA i IIIA imaju središnje i krajnje smještenu elektronegativnu skupinu. S druge strane, prokainamid je pri pH 7,4 pozitivno nabijen i veže se samo 10–15%, dok su fenobarbiton i teofilin 40% vezani. To se može objasniti činjenicom da, iako fenobarbiton i teofilin imaju parcijalno negativne keto skupine, one su jednoliko raspoređene po cijeloj molekuli što rezultira smanjenom lokalizacijom negativnog naboja i slabijim vezanjem. Drugi primjeri lijekova s niskim postotkom vezanja za HSA su etosuksimid, primidon, različiti cefalosporini, aminoglikozidi, digitoksin i dr. [15,19].

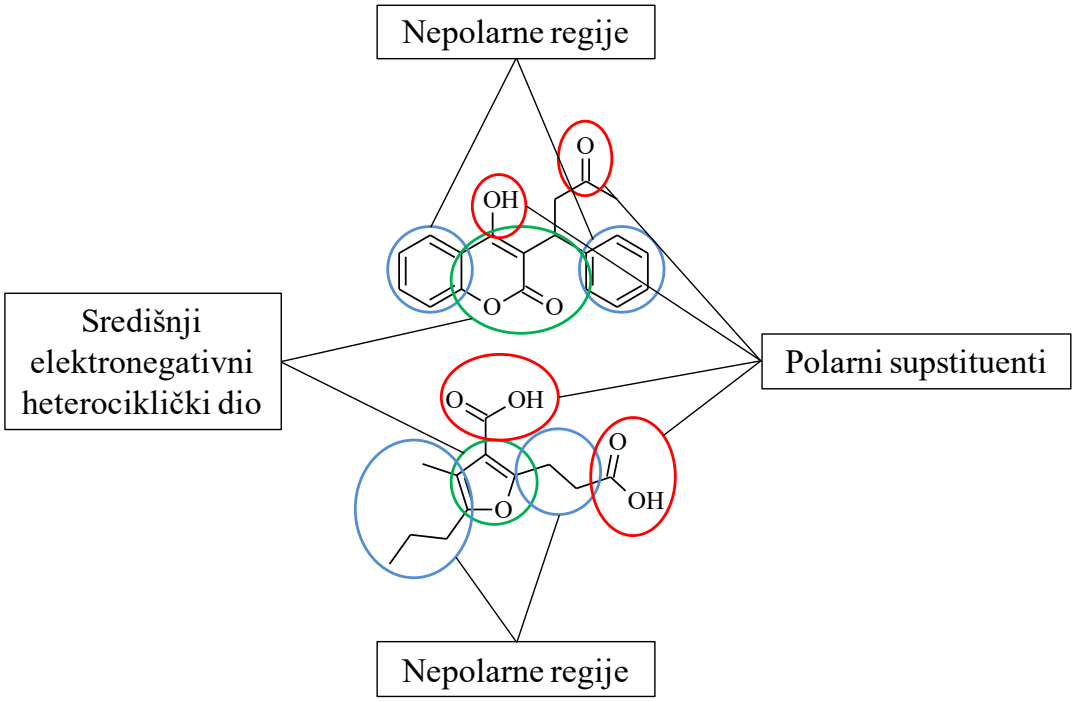
Tablica 1. Primjeri lijekova koji se snažno vežu u poddomenama IIA i IIIA i lijekova koji nisu značajno vezani za HSA.

Lijekovi koji se vežu u poddomeni IIA	Postotak vezanja u poddomeni IIA	Lijekovi koji se vežu u poddomeni IIIA	Postotak vezanja u poddomeni IIIA	Lijekovi koji nisu značajno vezani za HSA	Postotak vezanja za HSA
 varfarin	99% [15]	 diazepam	99% [15]	 prokainamid	10–15% [19]
 azapropazon	99% [15]	 diflunisal	99% [15]	 fenobarbiton	40% [19]
 fenilbutazon	97,8% [15]	 propofol	95–99% [20]	 teofilin	40% [19]

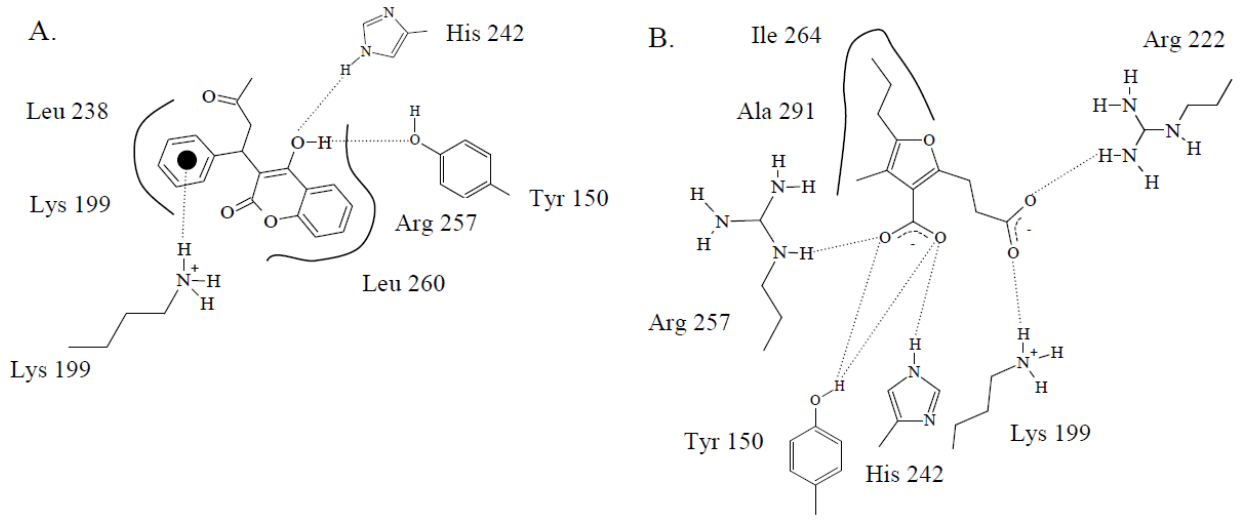
1.1.3. Vezno mjesto u poddomeni IIA

Vezno mjesto u poddomeni IIA još se naziva i Sudlowljevo mjesto I (ili jednostavnije, vezno mjesto I) odnosno varfarin-azapropazonsko mjesto. Na to se vezno mjesto veže najveći broj lijekova i ono se nalazi u blizini triptofanskog aminokiselinskog ostatka Trp 214. Fehske i sur. [21,22] su utvrdili da se to vezno mjesto sastoji od dva preklapajuća vezna područja za varfarin i azapropazon. Kasnije su Yamasaki i sur. [23] predložili tri vezna područja, podmjesta Ia, Ib i Ic, koja se nalaze unutar veznog mjesta I, što su kasnije kristalografski potvrdili Zhu i sur. [24].

Kao što je već rečeno, ligandi koji se snažno vežu za to vezno mjesto općenito su dikarboksilne kiseline ili velike heterocikličke molekule s negativnim nabojem u sredini molekule (slike 2 i 3) te se istovremeno mogu vezati dva ili više liganda [11]. Cijelo se vezno mjesto sastoji od dva nepolarna vezna džepa s nekoliko središnje smještenih polarnih aminokiselina. Vezno mjesto I pokazuje preferenciju prema koplanarnim aromatskim spojevima koji mogu ući u pukotinu i tvoriti vodikove veze, kao što je opisano u poglavlju 4.2. „DFT proračuni SAR-a aglikona flavonoida pri vezanju na HSA“. SAR studija vezanja cefalosporina na HSA je pokazala da se anionski cefalosporini vežu najjače, dok se kationski cefalosporini vežu najslabije, a vezanje neioniziranih cefalosporina raste porastom njihove lipofilnosti [25].



Slika 2. Strukturna svojstva varfarina i 3-karboksi-4-metil-5-propil-2-furanpropionata (CMPF).

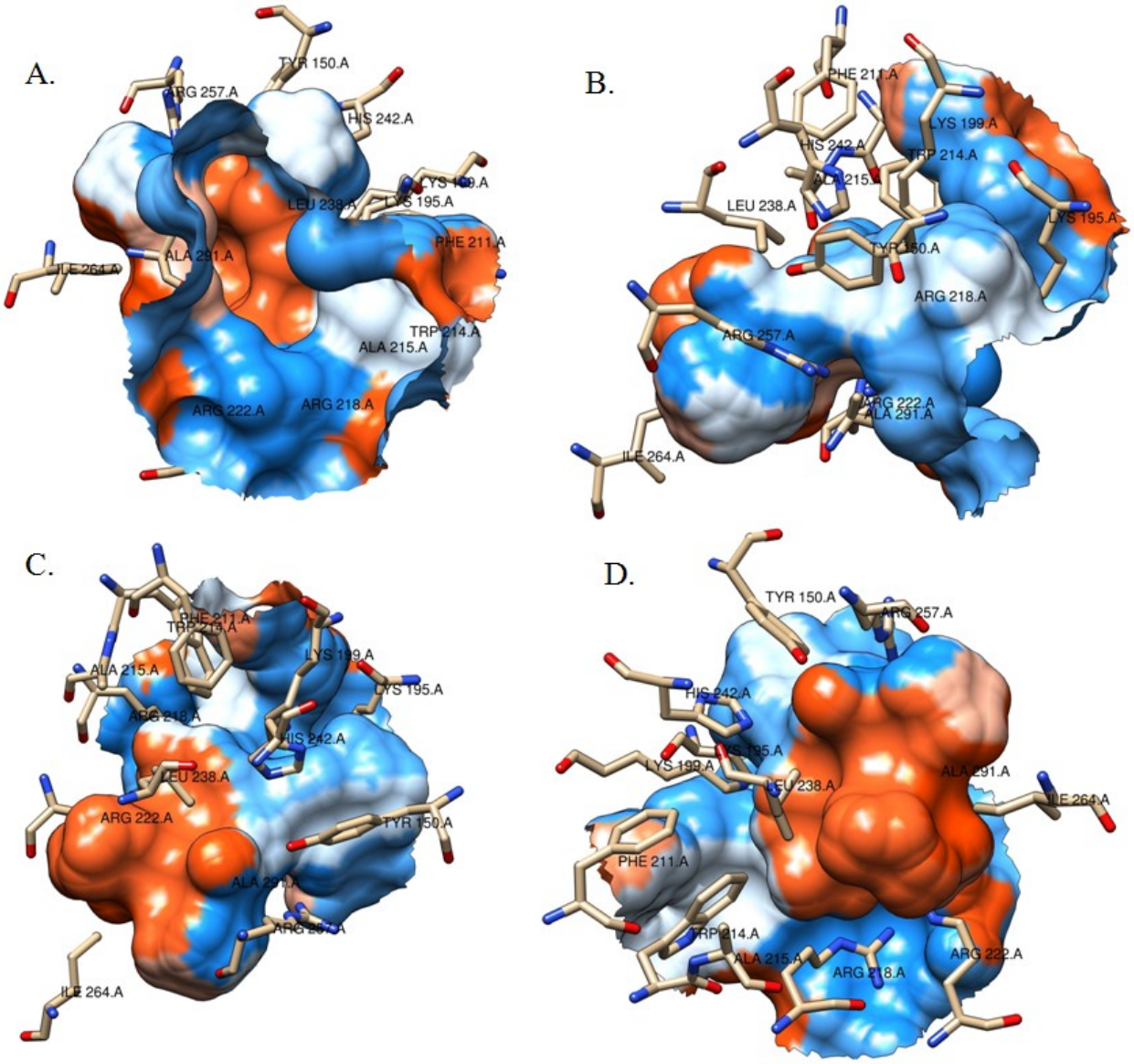


Slika 3. Interakcije između A. varfarina (PDB unos 2BXD) i B. 3-karboksi-4-metil-5-propil-2-furanpropionata (CMPF) (PDB unos 2BXA) s veznim mjestom I [8].

1.1.3.1. Svojstva veznog mjesta u poddomeni IIA

Vezno mjesto u poddomeni IIA se sastoji od tri vezna područja koja se djelomično preklapaju što predstavlja problem u definiranju osnovnih strukturnih svojstava njegovih liganada [23]. Skupina bazičnih aminokiselinskih ostataka (Lys 195, Lys 199, Arg 218 i Arg 222) nalazi se na ulazu u vezno mjesto (slika 4A), dok se druga skupina (Tyr 150, His 242 i Arg 257) nalazi na kraju dubljeg (lijevog) džepa (slika 4B). Središnja regija dijeli vezno mjesto u dva džepa. Dublji džep je još jednom podijeljen u dva dijela s Ile 264 (slika 4C), dok je drugi (desni) džep omeđen s Phe 211, Trp 214, Ala 215, Leu 238 i alifatskim dijelovima Lys 199 i Arg 218 (slika 4D) [8]. Ulaz veznog mjesta je širok i sastoji se od fleksibilnih postraničnih lanaca, što mu omogućuje vezanje različitih supstituentskih skupina. S druge strane, ligandi poput 3-karboksi-4-metil-5-propil-2-furanpropionata (CMPF), oksifenbutazona, fenilbutazona, varfarina, pa čak i glukoze vežu se u središtu džepa sa svojom planarnom grupom umetnutom između nepolarnih postraničnih lanaca Leu 238 i Ala 291 i tvore vodikovu vezu s hidroksilnom skupinom Tyr 150 (slika 4B), koja ima središnju ulogu u vezanju lijekova [8,26]. Zbog prostranosti ovog veznog mjesta, ligandi nisu sterički ograničeni i različiti se ligandi mogu istovremeno vezati, što omogućuje vezanje i molekula vode. Molekule vode mogu pomoći pri stvaranju interakcija između liganada i HSA [8], ali isto tako mogu objasniti nedostatak stereoselektivnosti za varfarin [27]. S obzirom da se u veznom mjestu I nalaze dvije skupine polarnih aminokiselinskih ostataka, „savršeni“ ligand bi bila molekula s dva anionska ili elektronegativna dijela, udaljena

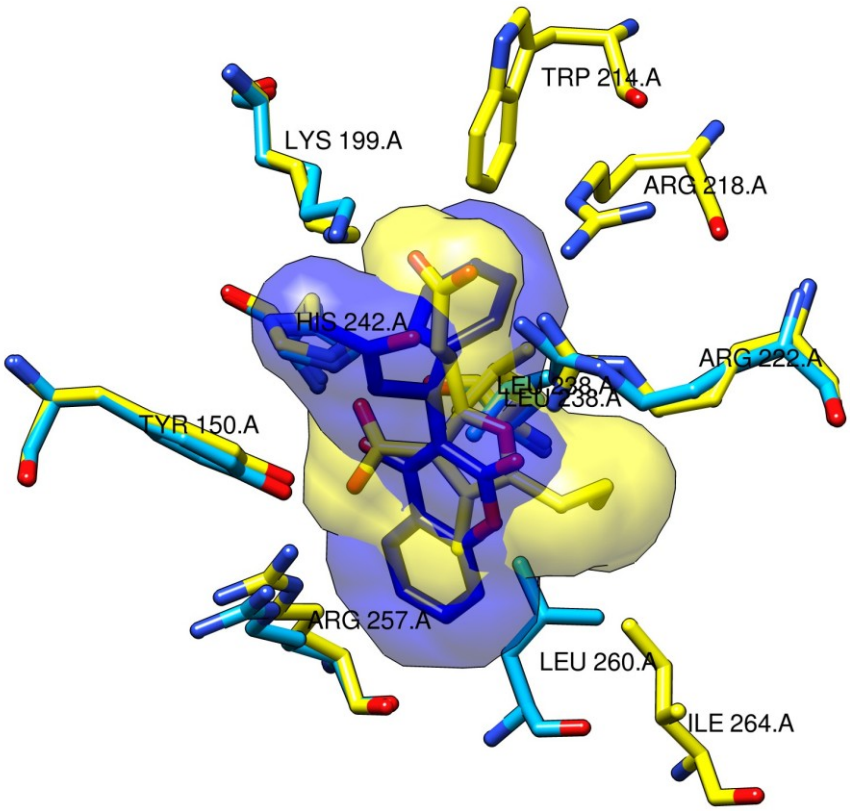
5–6 veza jedna od druge. Jedna takva molekula je i CMPF (slike 2 i 3B), koji se veže za HSA s visokim afinitetom iako je hidrofilna molekula [8,15].



Slika 4. Vezno mjesto u poddomeni IIA. Hidrofilne aminokiseline su označene plavo, hidrofobne narančasto, a neutralne bijelo. A. Hidrofilni ulaz veznog mjesta omeđenog polarnim aminokiselinama Lys 195, Lys 199, Arg 218 i Arg 222, B. Druga skupina polarnih aminokiselina Tyr 150, His 242 i Arg 257, C. Dno dubljeg veznog džepa podijeljenog u dva dijela s Ile 264, D. Manji hidrofobni džep omeđen s Phe 211, Trp 214, Ala 215, Leu 238 i alifatskim dijelovima Lys 199 i Arg 218, gledano odostraga.

Prostranost i vezna svojstva ovog veznog mjesta najbolje se mogu vidjeti kad se preklope kristalografske strukture varfarina i CMPF-a (slika 5). Može se primijetiti da se konformacija obližnjih aminokiselina ne razlikuje previše, dok se strukture i orijentacije varfarina i CMPF-a

značajno razlikuju. Oba spoja se prostiru u različite dijelove veznog mjesta, dok je središnja planarna skupina u oba slučaja smještena u blizini Leu 238. To pokazuje kako se dva strukturno i prostorno vrlo različita liganda mogu vezati za isto vezno mjesto zahvaljujući njegovoj veličini.



Slika 5. Preklopljene strukture varfarina (plavo) i CMPF-a (žuto) vezanih za vezno mjesto u poddomeni IIA i aminokiseline s kojima dolaze u kontakt. Iako su ligandi prostorno vrlo različiti, vezno mjesto je dovoljno veliko da veže oba liganda bez značajnih promjena u konformaciji aminokiselinskih ostataka.

Vezanje masnih kiselina također utječe na tercijarnu strukturu HSA [8] i vezanje liganada za to vezno mjesto [10,28,29]. Kad se masna kiselina veže za FA2, postranični lanac Tyr 150 se premješta i ulazi u interakciju s karboksilnom skupinom masne kiseline u FA2. Dolazi do konformacijskih promjena HSA i značajnog pregrupiranja ostalih aminokiselina, čime se povećava veličina i smanjuje polarnost unutarnje polarne skupine aminokiselina. Iako Tyr 150 ima središnju ulogu u vezanju lijekova kod HSA na koji nisu vezane masne kiseline, kad HSA uđe u interakcije s masnom kiselinom u FA2 mjestu, ulogu Tyr 150 nadomještaju drugi aminokiselinski ostaci (Lys 199, Arg 222, His 242, i u manjoj mjeri Arg 218 i Arg 257), zbog čega je vezno mjesto hidrofobnije, što može čak i povećati konstantu vezanja, npr. varfarina

[8,27]. Kao što je već rečeno, prisutnost masnih kiselina utječe na orijentaciju oksifenbutazona, ali isto tako i na raspored aminokiselina u blizini indometacina [8]. Pri višim koncentracijama masnih kiselina, vezanje liganada se smanjuje zbog izravne kompeticije s masnim kiselinama za vezno mjesto malog afiniteta (engl. *Low Affinity Site*, LAS) FA7 [27].

1.2. Istiskivanje lijekova s proteina plazme

Kad su u pitanju interakcije lijekova, vrlo se malo pažnje posvećuje interakcijama uzrokovanim istiskivanjem lijekova s proteina plazme, te se u najvećem broju slučajeva smatraju klinički neznačajnima [16,17]. Ovakva situacija je u kontrastu sa standardnom praksom prepisivanja lijekova, gdje je polifarmakoterapija jako zastupljena, posebice kod starijih pacijenata s dodatnim medicinskim problemima, kao što su ograničena funkcija jetre ili bubrega [30–33]. Istovremeno uzimanje više lijekova, određene hrane (posebice masne hrane) i interakcije s različitim endogenim spojevima poput hormona mogu dovesti do istiskivanja pojedinih lijekova s proteina plazme i na taj način povećati njihovu toksičnost i/ili ubrzati njihovu eliminaciju [17]. Dodatni problem predstavlja činjenica da se koncentracija proteina plazme razlikuje među osobama ili je do nje došlo zbog različitih medicinskih stanja. Koncentracija proteina plazme može biti značajno niža kod npr. pacijenata s oštećenjem jetre ili bubrega, što dovodi do veće vjerojatnosti istiskivanja lijekova [30,32,34]. S druge strane, u situacijama gdje se HSA koristi u liječenju opeklina, fetalne eritroblastoze, hemoragijskog šoka, hipoproteinemije i ascitesa [3], iznenadno povećanje ukupnog broja veznih mjesta može uzrokovati prolaznu fluktuaciju u koncentraciji slobodnog lijeka, što je važno kod npr. antimikrobnih lijekova opisanih minimalnom učinkovitom koncentracijom [17].

Lijekovi se za proteine plazme vežu s različitim afinitetima. Neki lijekovi ne pokazuju afinitet za HSA (npr. litij), dok su neki vezani više od 99% (npr. varfarin) [19]. Smatra se da samo slobodni, nevezani lijek može proći kroz različita tkiva i stanične membrane i tako doći do receptora odgovornog za farmakološku aktivnost lijeka [17]. Kad se molekule nevezanog lijeka uklone iz cirkulacije, dodatna količina lijeka disocira s proteina plazme prema zakonu o djelovanju masa, tako da s konstantnom koncentracijom proteina [P], omjer koncentracija vezanog liganda [LP] i nevezanog liganda ostaje konstantan (K_A)

$$[L] + [P] \rightleftarrows [LP]$$

$$K_A = [LP] / [L][P]$$

U slučaju da se neki drugi spoj (spoj B) veže na isto vezno mjesto kao i spoj koji je već vezan (spoj A), moguće je da spoj B istisne spoj A i poveća mu slobodnu koncentraciju, a na taj način i toksičnost. Klinička značajnost istiskivanja lijekova s proteina plazme je kontroverzna tema. Na početku se smatralo da je važno znati točna vezna mjesta i postotak vezanog lijeka kako bi se

izbjeglo potencijalno istiskivanje lijeka te posljedično povećanje koncentracije slobodnog lijeka uzrokovano primjenom nekog drugog lijeka. Općenito, važnost istiskivanja lijekova je bila precijenjivana jer je većina istraživanja bila provođena u *in vitro* uvjetima i mnoge su studije bile usredotočene na postotak lijeka koji je nevezan, umjesto na koncentraciju nevezanog lijeka, što ne mora nužno biti korelirano [16,35,36]. Iako je ova tema još uvijek kontroverzna, novija istraživanja govore da je istiskivanje lijekova s proteina plazme od male kliničke važnosti, osim u određenim slučajevima kao što su narušene funkcije jetre ili bubrega [3,16,36,37]. Kad je istiskivanje od kliničke značajnosti, ono može dovesti do prijelaznog povećanja farmakološke aktivnosti primijenjenog lijeka ili njegovog bržeg izlučivanja, s obzirom da je veća količina lijeka dostupna za vezanje na metu ili za ekskreciju. Potonji slučaj je od naročite važnosti kad se koncentracija lijeka mora održavati iznad određene koncentracije da bi lijek bio učinkovit, kao kod npr. antiinfektivnih lijekova [17].

Većina se lijekova u određenom postotku veže za proteine plazme, najviše na HSA. Kao što je već rečeno, samo nevezana, slobodna frakcija lijeka može prouzročiti željena, ali i neželjena djelovanja lijeka, bilo prije ili nakon biotransformacije nakon koje slijedi izlučivanje. U ravnotežnom stanju i u odsutnosti aktivnog transporta, koncentracija lijeka je jednaka s obje strane plazmatske membrane tako da se može smatrati da je koncentracija lijeka u plazmi jednaka koncentraciji lijeka na mjestu djelovanja. Istiskivanje lijekova, kao i endogenih spojeva, s njihovih veznih mjesta na proteinima plazme, može utjecati na njihov slobodni udio, kao i na njihovu slobodnu koncentraciju. To može biti uzrokovano bilo kompetitivnim istiskivanjem (gdje se dva spoja natječu za isto vezno mjesto) ili alosteričkim istiskivanjem (gdje vezanje spoja B inducira konformacijske promjene u proteinu koje dovode do disocijacije spoja A s proteina). Ove interakcije mogu utjecati na farmakokinetiku (raspodjelu, metabolizam i izlučivanje), kao i na farmakodinamiku lijeka, iako u većini slučajeva nisu klinički značajne.

1.2.1. Nužni uvjeti za klinički važno istiskivanje lijekova s proteina plazme

Osnovni uvjet za klinički važnu interakciju istiskivanja lijekova je da se lijek koji se istiskuje u visokom postotku veže za proteine plazme (>90%) i da ima usku terapijsku širinu. U takvom slučaju, malo smanjenje količine vezanog lijeka značajno povisuje njegovu slobodnu koncentraciju, odgovornu za njegove farmakološke učinke. Spoj koji istiskuje lijek također mora biti vezan u visokom postotku i mora biti u dovoljno visokoj koncentraciji da bi bio u

mogućnosti natjecati se s lijekom od interesa. Kapacitet HSA za oba spoja također se mora uzeti u obzir. Još jedan uvjet koji se mora uzeti u obzir jest da je lijek koji se istiskuje visokog postotka ekstrakcije, odnosno da protok krvi kroz organe koji eliminiraju lijek mora biti manji od umnoška postotka nevezanog lijeka i klirensa organa. To znači da je protok krvi kroz organ koji eliminira lijek ograničavajući faktor za eliminaciju lijeka i da povišenje koncentracije lijeka neće biti adekvatno kompenzirano ubrzanjem eliminacije lijeka. U slučaju lijekova niskog postotka ekstrakcije, istisnuti lijek će biti dostupan za eliminaciju i u tom slučaju će mu se smanjiti $t_{1/2}$. Također, s obzirom da postoji vremenski odmak između promjene u slobodnoj koncentraciji lijeka i nastupa farmakoloških posljedica, spoj koji istiskuje mora biti primijenjen ili intravenski ili oralno uz izostanak hepatičkog klirensa. U suprotnome, kompenzacijski mehanizmi će biti u stanju smanjiti potencijal istiskivanja prije nego drugi spoj dođe u cirkulaciju. Taj vremenski odmak je svojstvo svakog lijeka posebno te s kraćim vremenskim odmakom potrebnim za ponovno uspostavljanje ravnoteže, farmakološki učinci će biti izraženiji [16,17,37,38].

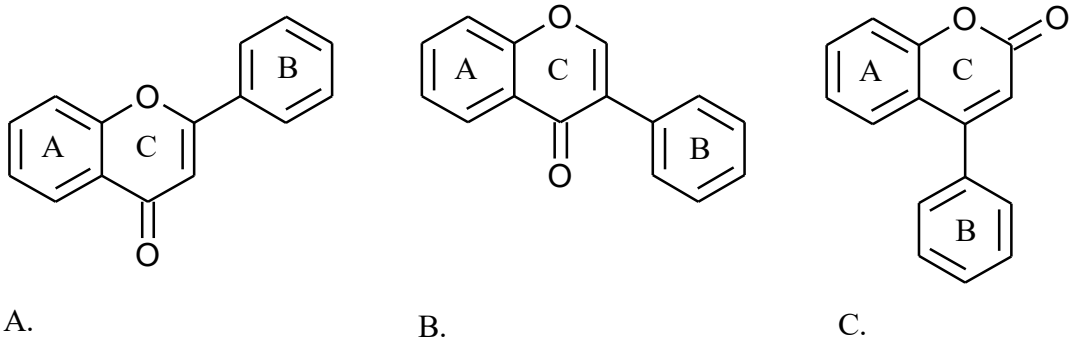
1.2.2. Terapijsko praćenje lijeka

Terapijsko praćenje lijeka (engl. *Therapeutic Drug Monitoring*, TDM) predstavlja upravljanje režimom primjene lijekova na temelju njihove koncentracije u krvi. TDM obično nije potreban za lijekove čije su terapijske koncentracije puno niže od njihovih toksičnih koncentracija. Međutim, za lijekove s uskim rasponom terapijskih koncentracija, kod kojih se toksičnost postiže pri koncentracijama koje su malo iznad njihove najviše terapijske koncentracije, TDM može biti od velike koristi [19]. S obzirom da je samo slobodna koncentracija lijeka farmakološki aktivna, promjene u postotku vezanja lijeka za proteine plazme mogu utjecati na njegovu aktivnu koncentraciju bez puno utjecaja na njegovu ukupnu koncentraciju. To može dovesti do poddoziranja, toksičnih učinaka ili čak do pogrešne dijagnoze [14,32]. Za lijek s uskom terapijskom širinom, ako je vezanje za proteine plazme veće od uobičajenog, može se zaključiti da je režim primjene lijeka dobar, iako slobodna koncentracija lijeka nije dovoljna da dovode do farmakološkog učinka. S druge strane, ako je vezanje za proteine plazme niže od uobičajenog, visoki klirens će kompenzirati početnu višu koncentraciju lijeka. Konačna slobodna koncentracija lijeka će se vratiti na očekivanu koncentraciju, ali će ukupna koncentracija biti niža te će lijek trebati češće uzimati [16,17].

1.3. Spojevi korišteni u istraživanju

1.3.1. Flavonoidi

Flavonoidi su skupina sekundarnih metabolita viših biljaka. Ime im potječe od latinske riječi *flavus*, što znači žut, a opisuje njihovu boju. Kemijski, osnovna struktura im je 15-eročlani kostur koji se sastoji od dva benzenska (A i B) i jednog heterocikličnog prstena (C) u obliku C6-C3-C6. Dijele se na flavonoide (derivati 2-fenil-1,4-benzopirona (flavona)), izoflavonoide (derivati 3-fenil-1,4-benzopirona) i neoflavonoide (derivati 4-fenil-1,2 benzopirona) (slika 6), koji se dalje dijele u dodatne podskupine ovisno o prisutnosti C2-C3 dvostruke veze (u slučaju odsutstva dvostruke veze spojevi se nazivaju „flava**n**oidi“) i 3-OH skupine (u tom se slučaju na kraju imena stavlja nastavak -ol) [39,40].



Slika 6. Podskupine flavonoida. A. flavonoidi, B. izoflavonoidi, C. neoflavonoidi.

U prirodi postoji više od 5000 vrsta flavonoida, a u biljkama služe kao pigmenti, sudjeluju u filtraciji UV zraka, fiksaciji dušika te djeluju kao prijenosnici signala, fiziološki regulatori i inhibitori staničnog ciklusa [39,40]. Također su zastupljeni u ljudskoj prehrani putem voća i povrća [41] te imaju brojne blagotvorne učinke na zdravlje, od kojih su najistraživaniji antioksidativni učinak [42], odnosno sprječavanje lipidne peroksidacije [43] i prevencija krvožilnih bolesti [44–46], a postoje snažne indikacije da djeluju i antikancerogeno [47].

Jednom kad uđu u cirkulaciju, većina se flavonoida ekstenzivno konjugira, pretežito glukuronidacijom [48], te se i nemetabolizirani i metabolizirani oblici vežu u poddomeni IIA HSA [49–52], s iznimkom flavanona [53]. Njihove koncentracije mogu dosegnuti i

mikromolarne razine, npr. nakon konzumacije velikih količina luka, zelenog čaja, soka od naranče i sl. [46,54,55]. Visoke koncentracije flavonoida i njihov visok postotak vezanja na vezno mjesto I potencijalno bi mogli dovesti do istiskivanja lijekova te njihovih toksičnih učinaka [56,57].

1.3.2. Varfarin

Varfarin je tipičan lijek od kojeg se može očekivati da ulazi u interakcije s drugim lijekovima jer, između ostaloga, ima niski postotak ekstrakcije, niski volumen distribucije (V_d), uskog je raspona terapijskih koncentracija, s gornjom granicom od približno 9 μM (3 mg/mL) [58,59], s time da je više od 99% vezan za HSA [16,17]. Interakcije varfarina s više različitih lijekova su proglašene klinički značajnima, iako s blagim nuspojavama [3,60]. Većina ih je kombinacija istiskivanja i inhibicije metabolizma, npr. s fenilbutazonom [61] i amiodaronom [62], ali za interakcije s klofibratom i kloralhidratom se pretpostavlja da su uzrokovane isključivo istiskivanjem [3,63]. Također, postoje indikacije da FDH varijacije na položajima R218H i R218P smanjuju afinitet vezanja varfarina za HSA pet puta [64], što nema utjecaja na povećanje toksičnosti varfarina, ali može dovesti do promjena u njegovom $t_{1/2}$ [17]. Potvrđeno je i da je pri visokim molarnim koncentracijama masnih kiselina ([masne kiseline]:[HSA] > 3), afinitet vezanja varfarina za HSA smanjen [10], dok je u slučaju kovalentnog vezanja glukoze za HSA (npr. kod *diabetes mellitusa*), afinitet vezanja varfarina povećan [3]. Dostupne su i kristalografske strukture kompleksa varfarina vezanog u poddomeni IIA HSA [8,27].

1.3.3. Indometacin i piroksikam

Indometacin je nesteroidni protuupalni lijek (engl. *NonSteroidal Anti-Inflammatory Drug*, NSAID) iz skupine arilalkanskih kiselina (derivat metiliranog indola) i inhibira sintezu prostaglandina, prostaciklina i tromboksana iz arahidonske kiseline [65], tako da inhibira i COX-1 i COX-2 enzime, s većim afinitetom za COX-1 enzim [66]. Veže se za HSA >99%. Kao i kod varfarina, dostupna je kristalografska struktura indometacina vezanog za HAS (u poddomeni IIA) i LAS na HSA, koji su različiti od veznog područja varfarina [8,67].

Piroksikam, kao i indometacin, pripada NSAID skupini lijekova, u podskupinu oksikama te imaju isti mehanizam djelovanja. Kao i indometacin, i piroksikam se u vrlo visokom postotku veže za HSA (>99%).

S obzirom na njihovu široku upotrebu, potencijalno opasne nuspojave i vrlo visoki postotak vezanja na HSA, istraživanje kliničke značajnosti njihova istiskivanja s HSA je opravdano.

1.3.4. Furosemid

Furosemid je diuretik Henleove petlje koji se veže za HSA >99%. Njegova koncentracija u krvi iznosi do 10 μg/mL [68–70]. Koristi se kod edema, ciroze jetre, oštećenja bubrega i visokog krvnog tlaka. Zbog učestalosti njegove primjene, pogotovo kod starijih pacijenata, ali i kliničke značajnosti interakcija u koje ulazi [71,72], također je dobar kandidat za ispitivanje istiskivanja s HSA.

1.3.5. Mikofenolat mofetil

Mikofenolat mofetil je morfolinoetilni ester mikofenolne kiseline, imunosupresiva koji se koristi u svrhu prevencije odbacivanja organa pri njihovoj transplantaciji [19,73,74]. Mikofenolna kiselina je reverzibilni inhibitor inozin monofosfat dehidrogenaze te na taj način inhibira *de novo* sintezu purina i DNA te proliferaciju T i B limfocita [74]. Mikofenolna kiselina se veže za HSA >90%, ali može dosegnuti i 98% kod pacijenata s transplantiranom jetrom [75], dok klinički podaci za mikofenolat mofetil nisu dostupni. Mikofenolat mofetil u cirkulaciji brzo prelazi u mikofenolnu kiselinu koja postiže najvišu koncentraciju nakon jednog sata [76]. Neovisno o tome, mikofenolat mofetil je uključen u ovo istaživanje jer je, za razliku od ostalih ispitivanih lijekova, dosta veće i fleksibilnije strukture te je kao takav zanimljiv za usporedbu dobivenih rezultata.

2. CILJ I HIPOTEZE ISTRAŽIVANJA

Nakon što uđu u cirkulaciju, većina flavonoida se veže za vezno mjesto IIA HSA (vezno mjesto I), najzastupljenijeg proteina u ljudskoj plazmi. Na to se vezno mjesto također veže i veliki broj endobiotika, kao i ksenobiotika. S obzirom na visoku zastupljenost flavonoida u svakodnevnoj prehrani, postavlja se pitanje važnosti interakcija flavonoida i njihovih metabolita s lijekovima koji se u visokom postotku vežu za isto vezno mjesto. Iz tog je razloga nužno bolje opisati vezno mjesto I humanog serumskog albumina i interakcije do kojih na njemu dolazi. Time bi se mogao bolje procijeniti rizik neželjenih učinaka lijekova koji su posljedica njihovog istiskivanja s veznog mjesta.

Hipoteza istraživanja je da strukturna svojstva, tj. supstituenti na flavonoidima značajno utječu na sposobnost i jačinu njihovog vezanja na vezno mjesto I, a posljedično i na mogućnost njihovih interakcija s lijekovima. Ciljevi istraživanja su utvrditi strukturne značajke flavonoida odgovorne za njihovo vezanje na HSA i mogućnost interakcija s lijekovima, kao i ispitati mogućnost primjene flavonoida u svrhu procjene zauzetosti veznog mjesta IIA HSA u optimizaciji doziranja lijekova uskog raspona terapijskih koncentracija.

Specifični ciljevi ovog doktorskog rada su:

1. Bolje razumijeti svojstva veznog mjesta I
    a. Utvrđivanje strukturnih svojstava flavonoida odgovornih za jačinu vezanja na navedeno vezno mjesto. Važna strukturna svojstva će se utvrditi fluorescencijskim određivanjem konstanti vezanja 20 flavonoida na HSA te povezivanjem dobivenih konstanti s molekulskim deskriptorima dobivenih upotrebom računalnog programa Gaussian 09.
    b. Utvrđivanje interakcija između odabranih flavonoida i ksenobiotika na razini vezanja za HSA. Interakcije između flavonoida i ksenobiotika će se utvrditi na način da će se različiti postotci veznog mjesta I zasititi određenim lijekom te će se mjeriti utjecaj zasićenja veznog mjesta na fluorescenciju flavonoida. Rezultati eksperimenata će se interpretirati zajedno s rezultatima dobivenih sidrenjem ispitivanih lijekova i flavonoida.
2. Utvrđivanje rizika klinički značajnih interakcija odabranih flavonoida i lijekova pri vezanju za HSA. Iz rezultata dobivenih pod točkom 1. će se procijeniti mogućnost kliničke značajnosti interakcija između ispitivanih lijekova i flavonoida.

3. Ispitati mogućnost razvijanja metode za određivanje postotka zasićenosti veznog mjesta I pomoću flavonoida. Iz rezultata dobivenih pod točkom 1. će se utvrditi mogućnost razvijanja općenite metode za ispitivanje postotka zasićenosti veznog mjesta I HSA.

3. MATERIJALI I METODE

3.1. Materijali

HSA bez vezanih masnih kiselina čistoće ≥ 96% te varfarin natrij i piroksikam su kupljeni od Sigma-Aldrich, SAD. Indometacin, furosemid i mikofenolat mofetil su nabavljeni od Acros Organics, Belgija.

Flavonoidi su nabavljeni od Extrasynthèse, Francuska:

- krizin dimetileter
- diosmetin
- fisetin
- formononetin
- genistein
- kvercetin-3-*O*-glukuronid
- luteolin
- pinocembrin-7-metileter
- prunetin
- sakuranetin
- tamariksetin,

ChromaDex, SAD:

- 3,6-dihidroksiflavon
- 7-hidroksiflavon
- flavanon,

Sigma-Aldrich, SAD

- apigenin
- krizin
- flavon
- galangin
- kvercetin,

TransMIT GmbH, Njemačka

- izokvercitrin
- rutin
- hiperozid
- cinarozid
- izoorientin i

BioChemika, Švicarska

- ramnetin

Svi standardni flavonoida su imali deklariranu čistoću ≥ 98% i bili su korišteni bez dodatnog pročišćavanja.

3.2. *In vitro* fluorescencijska spektroskopija ravnotežnog stanja

Vezanje liganada na vezno mjesto u poddomeni IIA vrlo se jednostavno može pratiti metodom fluorescencijske spektroskopije. Dobro poznato svojstvo tog veznog mjesta je prisutnost triptofanskog aminokiselinskog ostatka, Trp 214, jedinog u cijeloj molekuli HSA, a koji ima sposobnost fluorescencije u vodenim otopinama. Kad se neki ligand veže u njegovoj blizini, intenzitet fluorescencije triptofana se smanjuje. To svojstvo omogućuje određivanje konstante vezanja ispitivanog liganda [77,78].

3.2.1. Mjerenje konstanti vezanja aglikona flavonoida

Svježa otopina HSA pripremana je svaki dan prije mjerenja u Dulbecco fosfatnom puferu (137 mM natrijev klorid, 2,7 mM kalijev klorid, 8,1 mM dinatrijev hidrogenfosfat, 1,47 mM kalijev dihidrogenfosfat) [79]. Flavonoidi su otopljeni u DMSO-u i niz otopina je bio priređen za svaku kombinaciju HSA-flavonoid: koncentracija HSA je bila konstantna, 1 μM, a koncentracija flavonoida je bila u rasponu 0,03–10 μM. U svim je pokusima koncentracija DMSO-a bila ispod 4% *V*/*V*. Utjecaj DMSO-a kao suotapala je bio anuliran dodatkom alikvota DMSO-a otopini HSA u kojoj nije bilo flavonoida.

Fluorescencijska mjerenja u ravnotežnom stanju su izvršena pomoću OLIS RSM 1000F spektrofluorimetra (Olis Inc., SAD) opremljenog termostatiranim držačem kivete pri 25±1°C. Korištena je kiveta za fluorescencijska mjerenja Helma Analytics 105.253-QS s putem svjetlosti od 10 × 2 mm (ekscitacija × emisija). Valna duljina ekscitacije je bila 280 nm (maksimum apsorpcije HSA), a emisijski spektar je sniman u rasponu 310–370 nm, gdje samo HSA ima fluorescencijska svojstva, s maksimumom pri 340 nm. Sva mjerenja su provedena u duplikatu koristeći širine proreza pri ekscitaciji i emisiji od 1,24 mm. Sve otopine su bile mjerene nakon inkubacijskog razdoblja od 2 sata.

Pri navedenim valnim duljinama i flavonoidi apsorbiraju elektromagnetsko zračenje. Na temelju njihovih molarnih apsorpcijskih koeficijenata pri 280 i 340 nm (ε_{280} i ε_{340}) dobivenih pomoću UV-Vis spektrofotometra Varian Carry 50 Bio, kod izmjerenih vrijednosti fluorescencija učinak unutarnjeg filtra je uzet u obzir [80]. Izmjerena vrijednost intenziteta fluorescencije, F_{obs}, je ispravljena u F_{corr} prema jednadžbi (1), gdje je $A_{ex} = \varepsilon_{280} \times c \times l$ apsorbancija flavonoida pri valnoj duljini ekscitacije (c predstavlja koncentraciju flavonoida, a l

duljinu puta svjetla kroz kivetu i iznosi 1 cm), a $A_{em} = \varepsilon_{340} \times c \times l$ (l = 0,2 cm) apsorbancija flavonoida pri valnoj duljini emisije:

$$F_{\text{corr}} = F_{\text{obs}} \times e^{\left(\frac{A_{\text{ex}}+A_{\text{em}}}{2}\right)} \tag{1}$$

3.2.2. Računanje konstanti vezanja aglikona flavonoida

Svaki spektar dobiven tijekom titracije je prosjek 10000 fluorescencijskih spektara izmjerenih kroz 10 sekundi. Konstanta vezanja izračunata je na globalnoj razini pri svim mjerenim valnim duljinama pomoću SPECFIT računalnog programa (Zavod za anorgansku kemiju, Sveučilište u Baselu, Švicarska) [81–84]. Metodom rastava singularnih vrijednosti (engl. *Singular Value Decomposition,* SVD) pronađena je jedna spektralno značajna specija, koja je pripisana poznatom spektru HSA. Ova je analiza također predložila stvaranje kompleksa u omjeru 1:1 bez indikacija stvaranja komleksa većeg reda. Stoga, model vezanja je dan jednadžbama (2) i (3), gdje K_A predstavlja konstantu vezanja kompleksa:

$$\text{HSA + flavonoid} \underset{}{\overset{K_A}{\rightleftharpoons}} \text{HSA-flavonoid} \tag{2}$$

$$K_A = \frac{[HSA-flavonoid]}{[HSA][flavonoid]} \tag{3}$$

Konstante vezanja izračunate za pojedine komplekse HSA-flavonoid su dane u $mol^{-1}dm^3$. Više vrijednosti konstanti vezanja ukazuju na jače vezanje i veću stabilnost kompleksa. U svim slučajevima se izračunavao log K_A.

3.2.3. Mjerenje interakcija varfarina s kvercetinom i luteolinom te njihovim derivatima

Otopina HSA pripremana je kao i kod mjerenja konstanti vezanja aglikona flavonoida. Varfarin je otopljen u metanolu, luteolin u smjesi metanol:DMSO = 2:1, a ostali flavonoidi u smjesi metanol:DMSO = 4:1. U svim je pokusima koncentracija suotapala bila manja od 5%.

Fluorescencijska su mjerenja provedena na Safas Xenius termostatiranom fluorimetru s ugrađenim mješačem. Svi eksperimenti su provedeni pri 25±1°C koristeći širine proreza pri ekscitaciji i emisiji od 10 mm. Otopine varfarina i flavonoida su dodane u 2 mL 6 μM (0,4 g/L) otopine HSA koja se nalazila u kvarcnoj kiveti. Svi su pokusi izvedeni u duplikatu.

3.2.3.1. Određivanje konstanti vezanja kvercetina, luteolina i njihovih derivata te varfarina

Otopine pojedinih liganada su postepeno dodavane u 2 mL 6 μM otopine HSA smještene u kvarcnoj kiveti. Mjerenja su provedena pri valnoj duljini ekscitacije od 295 nm, a emisijski spektar je sniman u području 310–400 nm. S obzirom da i flavonoidi i varfarin apsorbiraju elektromagnetsko zračenje pri navedenim valnim duljinama, njihovi molarni apsorpcijski koeficijenti (ε) su izračunati pomoću termostatiranog UV-Vis spektrofotometra HP 8543 pri 25±0,1°C kako bi se uzeo u obzir učinak unutarnjeg filtra. Konstante vezanja su određene prema Dufour i Dangles [49]. Provedena je Scatchardova analiza s pretpostavljenim *n* identičnih veznih mjesta na HSA. Vrijednosti konstante vezanja (K_A), intenzitet molarne fluorescencije kompleksa (*f*) i stehiometrijski koeficijent (*n*) su dobiveni iz ovisnosti intenziteta fluorescencije (*F*) o ukupnoj koncentraciji liganda [L_t] prema jednadžbama (4) i (5) koristeći računalni program za regresiju pomoću metode najmanjih kvadrata (Scientist, MicroMath, SAD). [*L*] predstavlja koncentraciju slobodnog liganda, a *c* ukupnu koncentraciju HSA. S obzirom da su sve vrijednosti *n* blizu 1, korišten je jednostavni 1:1 model vezanja. U svim slučajevima je uzet u obzir i učinak unutarnjeg filtra korištenjem apsorpcije liganada pri valnim duljinama ekscitacije i emisije (ε_{ex} i ε_{em}) (jednadžba (6)), pri čemu je F_{corr} ispravljena, a F_{obs} izmjerena vrijednost intenziteta fluorescencije, ε je zbroj ε_{ex} i ε_{em}, *l* je duljina puta svjetlosti kroz kivetu, koji iznosi 0,65 cm, a *c* koncentracija liganda.

$$F_{\mathrm{corr}} = fc\frac{nK[L]}{1+K[L]} \tag{4}$$

$$[L_t] = [L]\left(1 + \frac{ncK}{1+K[L]}\right) \tag{5}$$

$$F_{\mathrm{obs}} = F_{\mathrm{corr}} \times 10^{-\varepsilon lc} \tag{6}$$

3.2.3.2. Ispitivanja istiskivanja varfarina s HSA

Ispitivanje istiskivanja varfarina s HSA pomoću flavonoida se temeljilo na činjenici da određeni flavonoidi i varfarin, kad su slobodni u otopini, ne fluoresciraju ili slabo fluoresciraju. Međutim, kad su vezani za HSA, intenzitet njihove fluorescencije značajno raste [49,85], što omogućuje praćenje njihovog vezanja i istiskivanja s HSA. U svim je eksperimentima koncentracija HSA bila 6 μM, dok je udio HSA zasićen varfarinom iznosio 0, 22, 38, 63 i 92%. Zasićeni udio HSA je izračunat pomoću jednadžbi (7) i (8):

$$K_{\mathrm{A}} = \frac{[cL]}{[c_{\mathrm{f}}][L]} \tag{7}$$

$$p = \frac{[cL]}{[c]} \tag{8}$$

pri čemu je K_{A} konstanta vezanja, $[cL]$ koncentracija HSA-ligand kompleksa, $[cf]$ koncentracija nevezanog HSA, $[L]$ koncentracija slobodnog liganda, $[c]$ ukupna koncentracija HSA i p udio zasićenog HSA. Mjerenja vezanja flavonoida su provedena na valnoj duljini ekscitacije od 450 nm, a emisijski spektri su snimani u rasponu 466–580 nm. Interakcije između varfarina i flavonoida pri vezanju na HSA su određene na temelju maksimalnog intenziteta fluorescencije (F_{max}) flavonoida i koncentracije flavonoida potrebne da se taj intenzitet postigne (c_{max}). Dodatna mjerenja su provedena na valnim duljinama eksitacije i emisije varfarina, 317 i 379 nm, s ciljem potvrde rezultata dobivenih mjerenjem intenziteta fluorescencije flavonoida.

3.2.4. Ispitivanja interakcija indometacina, piroksikama, furosemida i mikofenolat mofetila s kvercetinom i kvercetin-3-*O*-glukuronidom

Priprema otopina liganada i mjerenje njihovih konstanti vezanja su provedeni na način analogan onome opisanom u poglavlju 3.2.1. („Mjerenje konstanti vezanja aglikona flavonoida“), s time da je konačna koncentracija HSA iznosila 0,4 μM, koncentracija liganada je bila u rasponu 0,03–36 μM, a valna duljina ekscitacije je iznosila 295 nm. Sve ostale postavke eksperimenata su bile istovjetne.

Ispitivanja istiskivanja pojedinih lijekova s HSA pomoću kvercetina i kvercetin-3-*O*-glukuronida su provedena na način istovjetan onome opisanom u poglavlju 3.2.3.2. („Ispitivanja istiskivanja varfarina s HSA“).

3.3. DFT proračuni aglikona flavonoida

Kvantno-kemijski proračuni na molekulama aglikona flavonoida su provedeni korištenjem računalnog programa Gaussian 09 [86]. Sve strukture (neutralni i anionski oblici, gdje je bilo moguće) su bili optimirani pomoću B3LYP funkcionala [87,88] koristeći Popleove 6-31G(d) i 6-311++G(d,p) bazne skupove [89,90]. B3LYP funkcional je bio izabran kao jedan od najčešće korištenih funkcionala u početnom probiru velikih molekula zbog svog povoljnog omjera preciznosti i kvalitete proračuna. Dodatnu potvrdu za opravdanost korištenja ovog funkcionala u istraživanju struktura flavonoida daje njegova uspješna primjena i od drugih istraživačkih grupa [91,92]. Analitička vibracijska analiza je provedena pri odgovarajućim razinama kako bi se opisala svaka stacionarna točka na površini potencijalne energije kao minimum (Nimag = 0).

Strukture flavona, flavonola i izoflavona su jednostavne u smislu konformacijske stabilnosti. Glavni parametar koji je odgovoran za strukturnu različitost je diedarski kut C2-C10 (u slučaju flavona i flavonola), odnosno C3-10 (u slučaju izoflavona). Diedralna pretraživanja su provedena kroz relaksirano skeniranje površine potencijalne energije kod 300 intervala. U postupku pretraživanja, za svaku promjenu odgovarajućeg torzijskog kuta, struktura je bila potpuno optimirana za sve stupnjeve slobode, čime je omogućeno pronalaženje minimuma slobodne energije.

U slučaju flavanona korištena je identična procedura pronalaženja konformacija, međutim posebna je pozornost posvećena piranonskom C prstenu koji poprima djelomično modificiranu konformaciju stolca u svim strukturama globalnih minimuma.

Boltzmannova razdioba je bila korištena kako bi se identificirali konformeri s više od 5% udjela u ukupnoj populaciji (temeljeno na Gibbsovoj slobodnoj energiji i sobnoj temperaturi) i te su strukture bile uključene u daljnju analizu.

Kako bi se odgovarajuće modelirao utjecaj otapala u vodenom mediju (dielektrična konstanta ε = 78,4), tri različite metode su bile primijenjene: (a) implicitna solvatacija korištenjem solvatacijskog modela temeljenog na gustoći (engl. *Solvation Model based on Density*, SMD) [93], gdje je voda tretirana kao kontinuum, (b) eksplicitna solvatacija koja uključuje jednu molekulu vode postavljenu u blizini najelektronegativnijeg atoma i (c) supramolekularni pristup gdje su uključene i implicitna i eksplicitna solvatacija [94]. Zbog sličnosti rezultata u *in vacuo* i

različitim solvatacijskim modelima, u opisu rezultata će se komentirati samo *in vacuo* model i model eksplicitne solvatacije.

Analiza prirodnih veznih orbitala (engl. *Natural Bond Orbital* (NBO) *analysis*) je provedena primjenom računalnog programa NBO 3.1 [95] koji je uključen u Gaussian 09 paket. NBO analiza (NPA vrijednosti, engl. *Natural Population Analysis*, analiza prirodne populacije) i populacijska analiza (Mulliken i APT vrijednosti (engl. *Atomic Polar Tensor*, atomski polarni tenzor)) supstituentskih funkcionalnih skupina su dobivene zbrajanjem pojedinačnih naboja/populacija na svakom atomu u funkcionalnoj skupini. Fukui parametri (elektrofilnost i nukleofilnost) su izračunati korištenjem NBO i populacijske analize jedne točke na odgovarajućim $N - 1$ i $N + 1$ sustavima, gdje N predstavlja broj elektrona.

Kako bi se olakšala kvantitativna usporedba različitih konformacija, kondenzirana Fukui funkcija [96–98] temeljena na atomskim nabojima je bila izračunata. NPA naboji su temeljeni na NBO analizi. Naboji (q) su izračunati za sve flavonoide u njihovim N, $N + 1$ i $N - 1$ elektronskim stanjima kako bi se dobili kondenzirani f^- i f^+ deskriptori prema jednadžbama za nukleofilnost (9) i elektrofilnost (10):

$$f_{\mathrm{A}}^{-} = q_{\mathrm{A}}(N) - q_{\mathrm{A}}(N-1) \tag{9}$$

$$f_{\mathrm{A}}^{+} = q_{\mathrm{A}}(N+1) - q_{\mathrm{A}}(N) \tag{10}$$

gdje je $q_{\mathrm{A}}(N)$ izračunati naboj na atomu A za ukupni broj elektrona N. $N - 1$ odgovara broju elektrona u neutralnoj molekuli, s jednim elektronom uklonjenim iz najviše popunjene molekulske orbitale (engl. *Highest Occupied Molecular Orbital*, HOMO) aniona, dok $N + 1$ odgovara broju elektrona u analognom kationskom sustavu.

Svi statistički proračuni bili su provedeni pomoću računalnog programa Statistica 7.0 (Statsoft, SAD). p je mjera statističke značajnosti: vrijednosti p niže od 0,05 su smatrane značajne u cijelom tekstu.

3.4. Sidrenje i vizualizacija interakcija liganada i HSA

Računalni program AutoDock 4.2.6. (The Scripps Research Institute, SAD) [99] koristi disperziju, vodikove veze, elektrostatske i desolvatacijske komponente kako bi odredio najvjerojatniju konformaciju kompleksa. Pomoću ovog programa pronađena su vjerojatna vezna mjesta kvercetina i kvercetin-3-*O*-glukuronida, kao i sekundarno vezno mjesto (*R*)- i (*S*)-varfarina na molekuli HSA. U ovim je pokusima kvercetin bio odabran kao predstavnik aglikona flavonoida, a kvercetin-3-*O*-glukuronid kao predstavnik metabolita kvercetina. 3D koordinate molekule HSA ko-kristalizirane s (*R*)-varfarinom i 3D koordinate molekule HSA ko-kristalizirane s miristinskom kiselinom i indometacinom dobivene su iz RCSB Protein Data Bank (unosi 2BXD i 2BXM) [8]. Kristalografska struktura pod unosom 2BXD je jedina dostupna kristalografska struktura gdje je HSA kristaliziran samo s varfarinom (bez miristinske kiseline), što najbolje odgovara našim eksperimentalnim uvjetima. S druge strane, kristalografska struktura HSA s vezanom molekulom indometacina sadrži vezanu i miristinsku kiselinu, dok kristalografske strukture drugih ispitivanih lijekova nisu dostupne.

Za proces sidrenja je bio izabran monomer A PDB unosa 2BXD kojemu su nadodani bočni lanci koji su nedostajali. Molekule varfarina i vode su bile uklonjene iz datoteke koja je bila korištena za sidrenje. Molekuli HSA su također bili dodani atomi vodika gdje je bilo potrebno, a svi lizinski, argininski i cisteinski bočni lanci su bili protonirani, dok su svi aspartatni i glutamatni bočni lanci bili deprotonirani. Amino i karboksilni krajevi lanaca su bili nabijeni, histidin na položaju 242 je bio protoniran na položaju N_{ε} položaju, dok su svi ostali histidinski bočni lanci bili nabijeni na položaju N_{δ}, temeljeno na vizualnoj provjeri, što je rezultiralo svukupnim nabojem molekule HSA od –14.

Zbog svoje strukturne sličnosti flavonoidima, sidrenje (*R*)- i (*S*)-varfarina je također bilo provedeno i utvrđeno je da su njihovi položaji međusobno jednaki i odgovaraju položajima kristaliziranog (*R*)-varfarina. To je u skladu s prethodnim istraživanjima koja pokazuju da se (*R*)- i (*S*)-varfarin vežu za isto područje veznog mjesta I [27] te je služilo kao potvrda da su postavke sidrenja optimalno postavljene za provedbu sidrenja ostalih liganada.

Dodatno, sidrenje (*R*)- i (*S*)-varfarina je također bilo provedeno u prisutnosti kristaliziranog (*R*)-varfarina, kako bi se utvrdilo njihovo sekundarno vezno područje unutar veznog mjesta I. 3D oblici liganada su nacrtani i njihova inicijalna geometrija je bila određena u računalnom

programu HyperChem 8.0 (Hypercube, Inc., SAD), a njihov je naboj postavljen da predstavlja njihove najzastupljenije specije pri pH 7,4, što je objašnjeno u poglavlju 3.3. („DFT proračuni aglikona flavonoida“) te potvrđeno na internetskoj stranici chemicalize.com. U slučaju (*R*)- i (*S*)-varfarina, sidrene su bile anionske specije, dok su u slučaju kvercetina i kvercetin-3-*O*-glukuronida bile sidrene dvije najzastupljenije anionske i fluorescentne specije [100]. Nakon toga je slijedilo učitavanje liganada u AutoDock program u odgovarajućem obliku datoteke i parcijalni naboji u molekuli su bili određeni prema Ionescu i sur. [101].

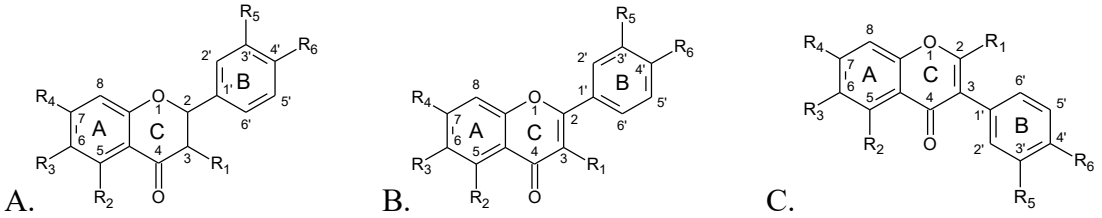
Nakon što je globalnim sidrenjem bilo potvrđeno da se vezanje flavonoida događa u veznom mjestu I, mreža veličine 80 × 80 × 80 Å s razmakom između pojedinih točaka od 0,375 Å i središtem na dušikovom atomu u bočnom lancu Trp 214 (koordinate 3,466, –10,477, –4,189) je bila generirana pomoću AutoGrid programa [99]. U postupku sidrenja bio je primijenjen lamarkijanski genetski algoritam (engl. *Lamarckian Genetic Algorithm*, LGA) [102]. Molekula HSA se tretirala kao rigidna dok je svim jednostrukim vezama liganada bila dozvoljena rotacija tijekom postupka Monte Carlo simuliranog traženja globalnog minimum (engl. *Simulated Annealing*, SA). Sidrenje svih liganda se provelo 100 puta, s populacijom veličine 150, maksimalnim brojem procjena energije od 25000000, 27000 generacija, učestalošću mutacije od 0,02 te učestalošću krosovera od 0,080. Korijen srednje kvadratne devijacije (engl. *Root-Mean-Square-Deviation*, RMSD) od 2,0 Å je uzet kao kriterij za analizu grozdova rezultata sidrenja (s ciljem utvrđivanja jesu li dvije usidrene konformacije dovoljno slične da bi bile smatrane jednakima) i nekoliko takvih grozdova s najnižim energijama vezanja svakog liganda je korišteno za daljnje usporedbe.

Provjera i modifikacija kristalografskih struktura, kao i vizualizacija i interpretacija rezultata sidrenja je provedena pomoću računalnog programa UCSF Chimera 1.10.1. (Sveučilište u Kaliforniji, SAD).

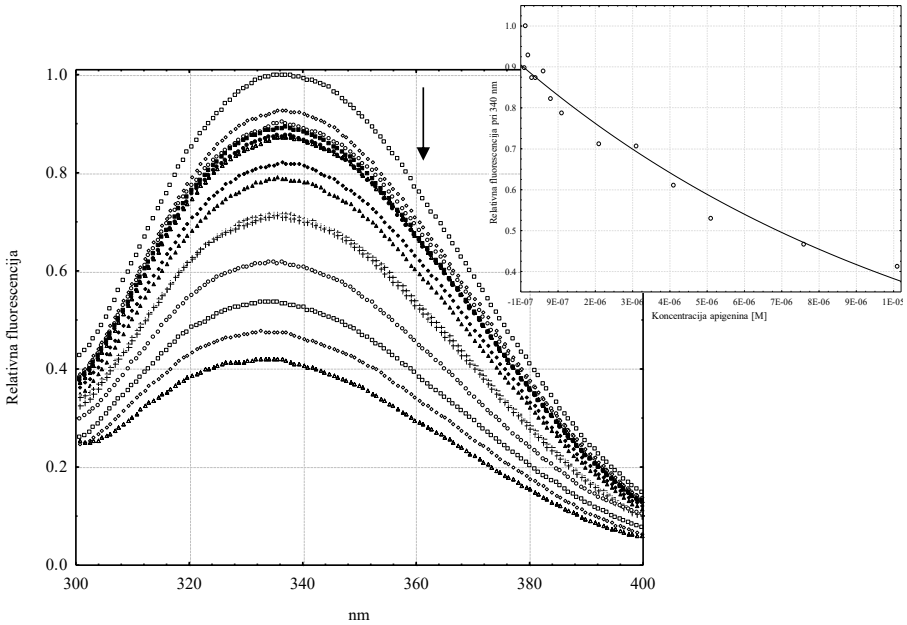
4. REZULTATI I RASPRAVA

4.1. Spektrofluorimetrijsko određivanje konstanti vezanja aglikona flavonoida za HSA

Slika 7 prikazuje osnovne strukturne formule ispitivanih podskupina flavonoida, dok tablica 2 prikazuje izmjerene konstante vezanja flavonoida i njihovu usporedbu s dostupnim literaturnim podacima. Općenito, flavanoni su pokazali najniže konstante vezanja za HSA, nakon čega slijede izoflavoni, dok su flavoni i flavonoli okarakterizirani najvišim konstantama vezanja. Slika 8 prikazuje izgled spektrofluorimetrijske titracije na primjeru apigenina.



Slika 7. Osnovna strukturna formula ispitivanih podskupina flavonoida: A. flavanoni, B. flavoni i flavonoli, C. izoflavoni.



Slika 8. Spektrofluorimetrijska titracija HSA apigeninom pri pH 7,4 i 25°C. Ukupna koncentracija HSA je bila konstantna i iznosila je 1 µM, dok je koncentracija apigenina varirala od 0 (najviši spektar) do 10 µM (najniži spektar). Umetak: izgled titracijske krivulje apigenina pri 340 nm.

Tablica 2. Ispitivani flavonoidi, njihovi supstituenti i konstante vezanja

#	Flavonoid	R_1	R_2	R_3	R_4	R_5	R_6	K_A [M^{-1}]	K_A [M^{-1}] iz literature	Referenca
	Flavanoni									
1	Flavanon	H	H	H	H	H	H	$(5{,}25 \pm 0{,}79) \times 10^3$	N/A	N/A
2	Pinocembrin-7-metileter	H	OH	H	OCH_3	H	H	$(2{,}19 \pm 0{,}09) \times 10^4$	N/A	N/A
3	Sakuranetin	H	OH	H	OCH_3	H	OH	$(2{,}19 \pm 0{,}04) \times 10^4$	N/A	N/A
	Flavoni									
4	6-Hidroksiflavon	H	H	OH	H	H	H	$(1{,}58 \pm 0{,}60) \times 10^4$	N/A	N/A
5	7-Hidroksiflavon	H	H	H	OH	H	H	$(1{,}95 \pm 0{,}10) \times 10^5$	$9{,}44 \times 10^4 – 3{,}82 \times 10^5$	[103,104]
6	Apigenin	H	OH	H	OH	H	OH	$(1{,}32 \pm 0{,}05) \times 10^5$	$9{,}85 \times 10^4 – 1{,}95 \times 10^6$	[57[a],105]
7	Krizin	H	OH	H	OH	H	H	$(1{,}95 \pm 0{,}08) \times 10^5$	$1{,}82 \times 10^5 – 3{,}09 \times 10^6$	[57[a],104,106]
8	Krizin dimetileter	H	OCH_3	H	OCH_3	H	H	$(2{,}95 \pm 0{,}15) \times 10^4$	N/A	N/A
9	Diosmetin	H	OH	H	OH	OH	OCH_3	$(8{,}91 \pm 0{,}18) \times 10^4$	$5{,}28 \times 10^4 – 1{,}18 \times 10^5$	[49[b],107]
10	Flavon	H	H	H	H	H	H	$(6{,}17 \pm 0{,}56) \times 10^4$	$4{,}96 \times 10^4$	[104]
	Flavonoli									
11	3,6-Dihidroksiflavon	OH	H	OH	H	H	H	$(7{,}41 \pm 0{,}15) \times 10^4$	$5{,}28 \times 10^4$	[108[c]]
12	3,7-Dihidroksiflavon	OH	H	H	OH	H	H	$(1{,}66 \pm 0{,}05) \times 10^5$	$(1{,}51 \pm 0{,}23) \times 10^5$	[109]
13	Fisetin	OH	H	H	OH	OH	OH	$(1{,}20 \pm 0{,}05) \times 10^5$	$(1{,}38 \pm 0{,}02) \times 10^5$	[110]
14	Galangin	OH	OH	H	OH	H	H	$(2{,}34 \pm 0{,}09) \times 10^4$	$(3{,}80 \pm 0{,}91) \times 10^6$	[57[a]]
15	Kvercetin	OH	OH	H	OH	OH	OH	$(1{,}70 \pm 0{,}03) \times 10^5$	$3{,}1 \times 10^4 – 3{,}31 \times 10^5$	[49[b],57[a],106,111–114]
16	Ramnetin	OH	OH	H	OCH_3	OH	OH	$(1{,}29 \pm 0{,}03) \times 10^5$	N/A	N/A
17	Tamariksetin	OH	OH	H	OH	OH	OCH_3	$(2{,}34 \pm 0{,}07) \times 10^4$	$(7{,}46 \pm 0{,}30) \times 10^4$	[49[b]]
	Izoflavoni									
18	Formononetin	H	H	H	OH	H	OCH_3	$(2{,}14 \pm 0{,}11) \times 10^4$	$1{,}60 \times 10^5$	[115]
19	Genistein	H	OH	H	OH	H	OH	$(4{,}90 \pm 0{,}10) \times 10^4$	$1{,}14 \times 10^4 – 1{,}5 \times 10^5$	[49[b],113,116,117]
20	Prunetin	H	OH	H	OCH_3	H	OH	$(3{,}80 \pm 0{,}14) \times 10^4$	N/A	N/A

[a] Proračuni su temeljeni na kompetitivnom vezanju s varfarinom korištenjem metode fluorescentne anizotropije.
[b] Izračunato za goveđi serumski albumin (BSA) korištenjem metode gašenja fluorescencije.
[c] Proračuni su temeljeni na kompetitivnom vezanju s kvercetinom korištenjem metode gašenja fluorescencije.
N/A podaci nisu dostupni.

Konstante vezanja ostalih flavonoida izračunate su na jednaki način. Većina dobivenih rezultata je u skladu s objavljenim podacima prijašnjih studija, s nekoliko iznimaka. Za neke flavonoide literaturni podaci nisu mogli biti nađeni. U slučaju 3,6-dihidroksiflavona i tamariksetina postoji mala razlika s obzirom na literaturne podatke, što se može objasniti različitom metodologijom izračunavanja konstanti vezanja. Literaturna konstanta vezanja 3,6-dihidroksiflavona je niža od konstante vezanja dobivene u ovoj studiji, a može se objasniti činjenicom da i 3,6-dihidroksiflavon ima fluorescentna svojstva pri uvjetima mjerenja fluorescencije kvercetina, odnosno gašenje fluorescencije kvercetina i njegovo istiskivanje mogu dovesti do lažno sniženih vrijednosti konstante vezanja 3,6-dihidroksiflavona. U slučaju tamariksetina, razlike mogu biti objašnjene činjenicom da je umjesto HSA korišten BSA, koji, iako je strukturno sličan, ne mora pokazivati jednaki afinitet za tamariksetin [49]. Značajnije su razlike uočene u slučajevima galangina i formononetina. Literaturni podaci konstante vezanja galangina dolaze iz eksperimenata istiskivanja varfarina, gdje se smanjenje u anizotropiji varfarina, odnosno povećanje rotacijske slobode varfarina smatra potvrdom istiskivanja varfarina. Yamasaki i sur. [118] su dokazali da promjene u rotacijskoj slobodi nisu nužno dokaz istiskivanja ili vezanja liganda. Slično razmišljanje se može primijeniti i u slučaju formononetina. Također, u nekim slučajevima su studije gašenja fluorescencije HSA provedene pri 280 nm (pri čemu i triptofanski i tirozinski ostaci apsorbiraju svjetlost), dok su druge studije provedene pri 295 nm (pri čemu samo triptofanski aminokiselinski ostatak apsorbira), što može dovesti do razlika u izračunatim konstantama vezanja. Iako ta razlika nije pretjerano značajna, za kvalitetno i pouzdano određivanje SAR-a, eksperimentalni uvjeti moraju biti jednaki za sve ligande, pogotovo ako su ti ligandi strukturno slični i ako se njihove konstante vezanja značajnije ne razlikuju.

4.2. DFT proračuni SAR-a aglikona flavonoida pri vezanju na HSA

U svim globalnim minimumima, odnosno u konformacijama s najmanjom slobodnom energijom za sve flavonoide diedralni kut C2-C10 (u slučaju flavona i flavonola) ili C3-10 (u slučaju izoflavona) iznosi oko 0°. Dodatni doprinos koplanarnosti između AC i B prstena flavona, flavonola i izoflavona dolazi od hidroksilnih, odnosno metoksi grupa koje su zarotirane kako bi tvorile unutarmolekulske vodikove veze. Svi globalni minimumi konformacija pronađeni na B3LYP/6-311++g(d,p) razini su u skladu s prethodno objavljenim literaturnim podacima [119,120]. U slučaju flavanona bila je korištena ista procedura, ali s posebnom pozornošću na piranonski prsten C koji se nalazi u djelomično modificiranom konformaciji stolca u svim globalnim minimumima. Ti rezultati također odgovaraju ranije objavljenim konformacijskim istraživanjima flavanona [121,122].

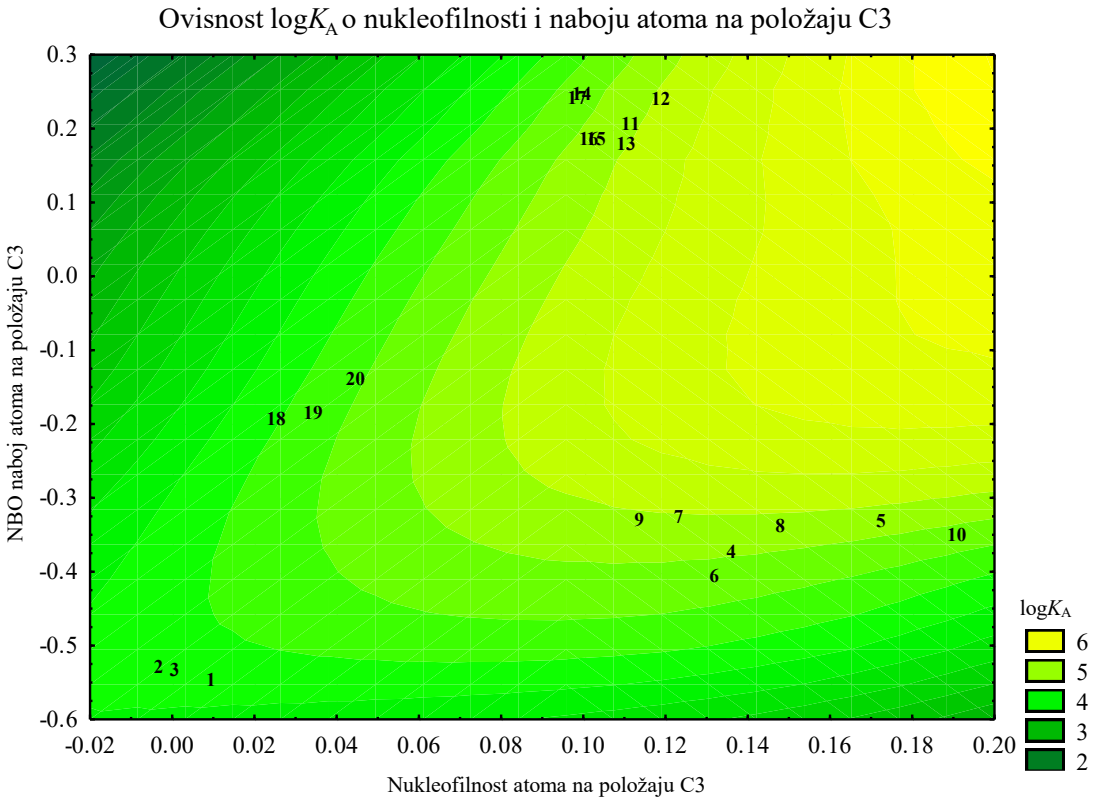
Pri fiziološkom pH, analizirani flavonoidi se vežu za vezno mjesto I u obliku aniona. Pronađeno je više svojstava flavonoida koje su povezane s njihovim afinitetom vezanja za navedeno vezno mjesto: (1) nukleofilnost atoma C3 i parcijalni naboj povezan s (2) parcijalnim nabojem atoma O4, (3) elektrofilnost supstituenta vezanog na atom C8, (4) visoke HOMO i LUMO energije i (5) koplanarnost između AC i B, kao i koplanarnost između A i C prstena. Kao što je već bilo rečeno, vrlo slični rezultati su dobiveni i za *in vacuo* i modele s otapalom, te će se komentirati samo podaci za *in vacuo* model i model s eksplicitnim otapalom ($\varepsilon = 78{,}4$), pri čemu se eksplicitna solvatacija može smarati i kompleksacijom te, s obzirom da je vezno mjesto I vrlo hidrofobno [2], predstavlja najvjerojatniji utjecaj vode na sustav HSA-flavonoid. Ako nije izričito rečeno, podaci se odnose na *in vacuo* proračune.

4.2.1. Supstitucija na atomu u položaju C3

Glavne strukturne razlike između četiri podvrste proučavanih aglikona flavonoida se nalaze na ili u blizini C3 atoma. Za očekivati je da će se različiti supstituenti i veze na položaju C3 odraziti i na distribuciju elektrona u blizini atoma C3.

Prema slici 9, nukleofilnost i parcijalni naboj atoma C3 omogućavaju odijeljivanje ispitivanih podvrsta flavonoida što korelira s afinitetom prema HSA. Nažalost, ta svojstva ne omogućavaju razlikovanje pojedinih flavonoida s visokim ili niskim afinitetom prema HSA unutar pojedine podvrste flavonoida. Također se može primijetiti da visoka nukleofilnost C3

atoma inducirana prisutstvom hidroksilne skupine povećava afinitet prema HSA. Flavonoli i flavoni posjeduju najveći afinitet prema HSA: promjena steričkih svojstava uzrokovana supstitucijom na položajima C2 i C3 značajno smanjuje njihov afinitet prema HSA, kao što je pokazano u slučajevima izoflavona [123].



Slika 9. Prikaz ispitivanih aglikona flavonoida s obzirom na nukleofilnost i parcijalni naboj atoma C3, izračunato na B3LYP razini (brojevi odgovaraju rednim brojevima flavonoida u tablici 2).

4.2.2. Parcijalni naboj atoma O4

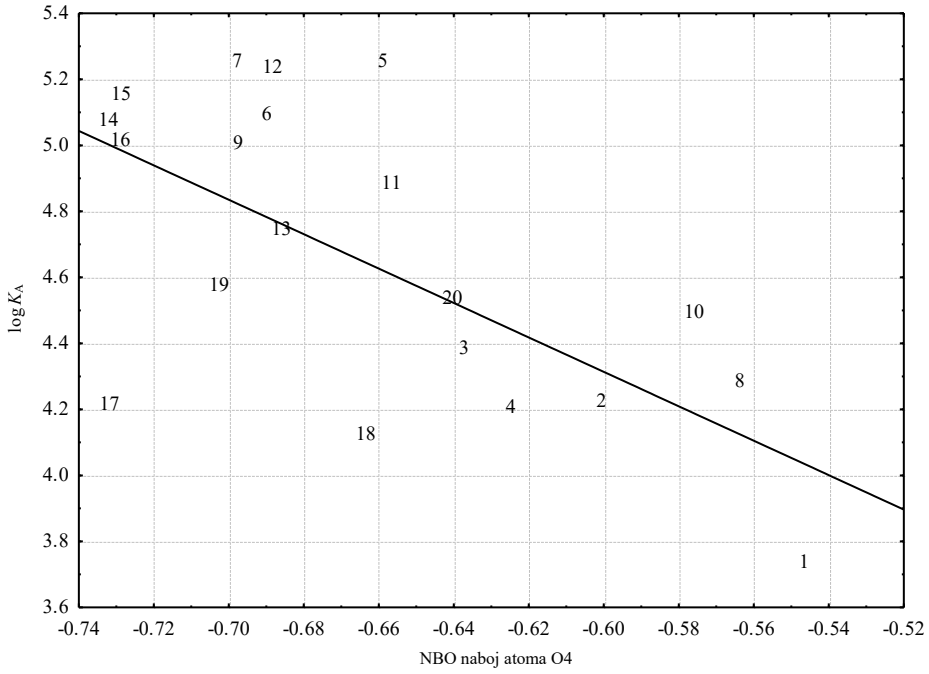
Elektronska svojstva atoma C3 su velikim dijelom određena supstitucijom susjednih atoma, posebice elektron-odvlačećim atomom kisika vezanog na atom C4. Slika 10 pokazuje jaku ovisnost log K_A o parcijalnom naboju atoma O4: afinitet flavonoida za HSA se smanjuje smanjivanjem negativnog naboja. Ta povezanost potvrđuje značajnost polarnih interakcija između atoma O4 i HSA koja je prethodno bila opisana za luteolin [124] i moguću interakciju s Lys 195 koji se nalazi u blizini [51]. Od ostalih vrsta polarnih interakcija, vodikove veze su

najvjerojatniji način interakcija: atom kisika vezan na atom C4 predstavlja dobrog akceptora vodikove veze, što može povećati afinitet flavonoida za HSA.

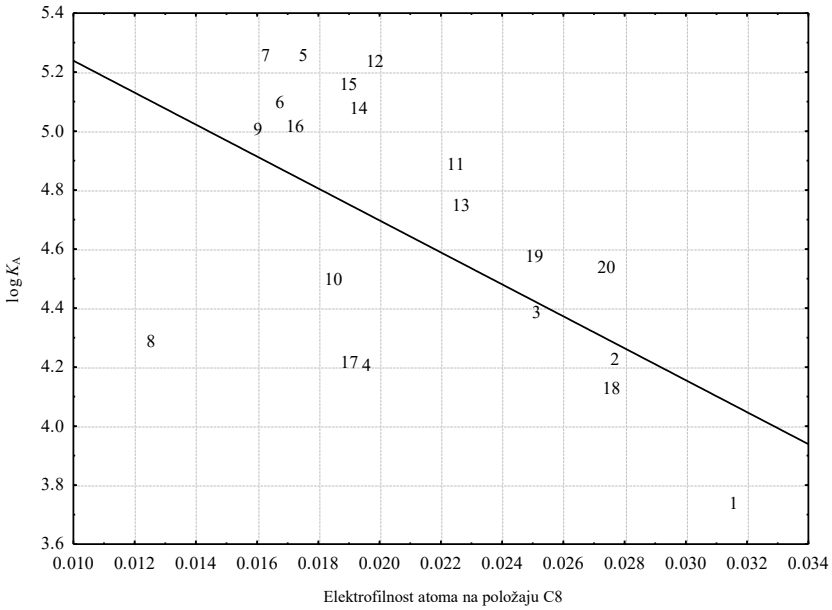
4.2.3. Ostale supstitucije

Neke studije su utvrdile značajni učinak polarnih interakcija između supstituenata na prstenu B i HSA [51,52,112,123,125]. Prema literaturi, negativni naboj 3'- ili 4'-OH skupine povisuje konstantu vezanja flavonoida za HSA. Međutim, naša studija nije uspjela utvrditi povezanost između naboja prstena B i afiniteta za vezanja, ni u *in vacuo* modelu (r = –0,3742; p = 0,1041), ni u modelu s eksplicitnom solvatacijom (r = –0,4181; p = 0,0666). Umjesto analiziranja svojstava pojedinih atoma, u našoj se studiji gledao samo zbroj doprinosa svih pojedinih atoma u prstenu B. Eksperimentima sidrenja utvrđeno je da se prsten B djelomično nalazi izvan veznog mjesta I te da je usmjeren prema mjestu dodira poddomena IIB i IIIA [51,124]. Iako u našem slučaju veza između naboja prstena B i konstante vezanja nije statistički značajna, ukazuje na povoljne učinke negativnog naboja lokaliziranog na prstenu B.

Većina prijašnjih studija također naglašava važnost nepolarnih interakcija prstena A i njegovih supstituenata s HSA [116,124,125]. Iz tog su se razloga ispitala i elektronska svojstva prstena A i njegovih supstituenata. Jedina statistički značajna korelacija između elektronskih svojstava supstituenata prstena A i HSA prikazana je na slici 11; vrlo niska elektrofilnost supstituenta na položaju C8 povezana je s višim afinitetom za HSA. Iz toga se može zaključiti da niska elektrofilnost supstituenta na položaju C8 ima utjecaja, ali nije presudna za učinkovito vezanje flavonoida za HSA.



Slika 10. Ovisnost log K_A o parcijalnom naboju atoma O4 temeljeno na NBO analizi (r = –0,6514; p = 0,0019), izračunato na B3LYP razini (brojevi odgovaraju rednim brojevima flavonoida u tablici 2).



Slika 11. Odnos između log K_A i elektrofilnosti supstituenta na položaju C8 izračunata na B3LYP razini (r = –0,5862; p = 0,0066) (brojevi odgovaraju rednim brojevima flavonoida u tablici 2).

4.2.4. Granične molekulske orbitale

Sklonost stvaranju unutarmolekulskih vodikovih veza dobro je dokumentirano svojstvo flavonoida [119]. Također se može očekivati da će snažne proton-donorske i proton-akceptorske skupine vezane za kostur flavonoida utjecati i na formiranje međumolekulskih vodikovih veza između flavonoida i HSA.

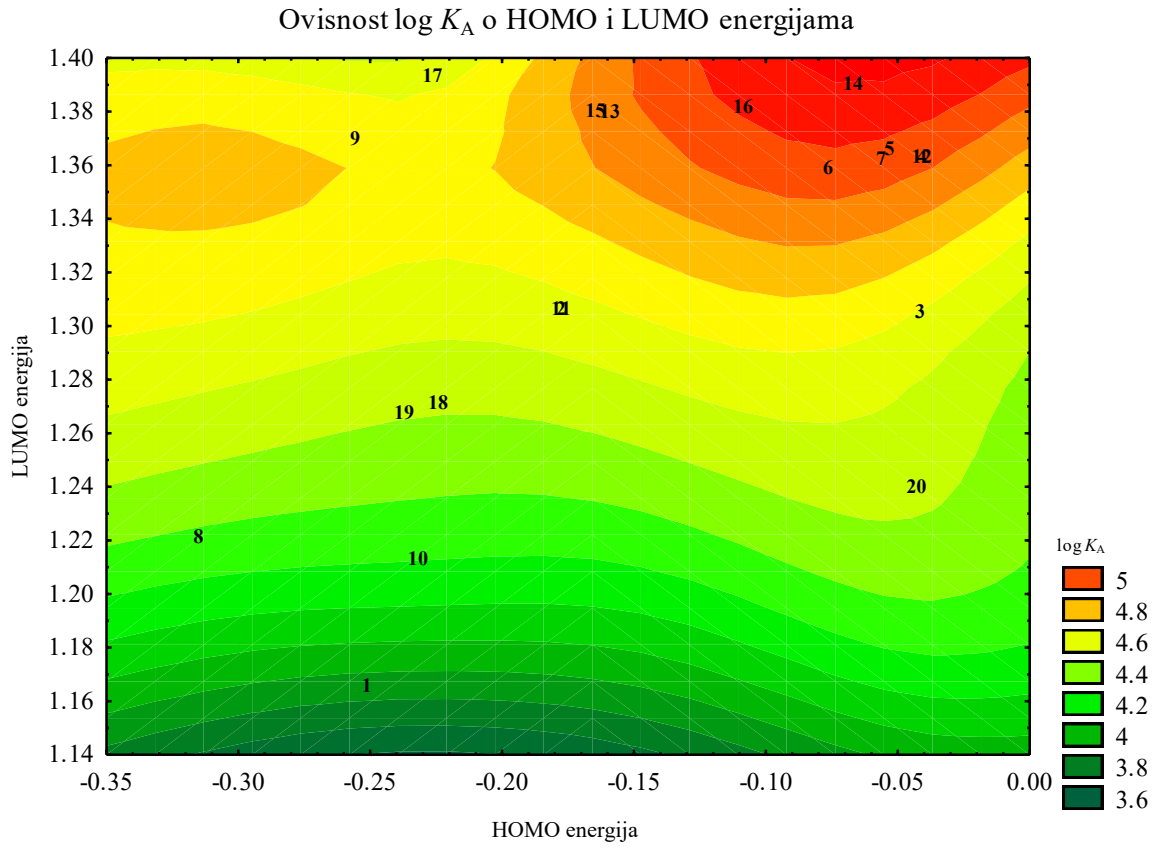
Slika 12 pokazuje da su snažno vezani flavonoidi okarakterizirani visokim HOMO i LUMO energijama. To je u skladu s njihovom sklonošću stvaranja višestrukih vodikovih veza u kojima flavonoidi mogu igrati ulogu i proton-donora i proton-akceptora.

4.2.5. Planarnost

Potpuni nedostatak korelacije između razlike u vrijednostima energija HOMO i LUMO i log K_A u *in vacuo* modelu (r = 0,0206; p = 0,9313), kao i u modelu s ekplicitnom solvatacijom (r = 0,1654; p = 0,4858) pokazuje da konjugacija nema nikakvog utjecaja na vezanje aglikona flavonoida na HSA. Taj se rezultat ne slaže sa zaključcima prijašnjih studija [123,125], kao ni s rezultatima utjecaja elektrofilnosti C8 atoma. Iako ova studija obuhvaća relativno veliki broj flavonoida, razlike u energijama između HOMO i LUMO se značajno ne razlikuju. Ta činjenica ograničava značajnost ovog rezultata samo na izabrane četiri podskupine aglikona flavonoida.

Važno je napomenuti i da su geometrije flavonoida izračunate bez uzimanja u obzir HSA. Dobiveni rezultati su u skladu s rezultatima koje je objavio Aparicio [119], koji je pokazao da aglikoni flavonoida mogu tvoriti unutarmolekulske vodikove veze koje stabiliziraju koplanarne geometrije. Zanimljivo je primjetiti da je koplanarnost AC i B prstena (slika 7) u obliku diedralnog kuta (kuta između atoma 3-2-1'-2' za flavanone, flavone i flavonole i atoma 2-3-1'-2' za izoflavone) povezana s afinitetom za vezanja na HSA *in vacuo* (r = 0,6832; p = 0,0009), ali ta povezanost nije prisutna u solvatacijskom modelu (r = 0,0349; p = 0,8838). S obzirom da geometrije flavonoida vezanih za HSA izračunate u studijama sidrenja pokazuju nedostatak koplanarnosti AC i B prstena [51,116,126,127], smatramo da je koplanarnost dobivena u *in vacuo* uvjetima samo pokazatelj sklonosti flavonoida stvaranju vodikovih veza s HSA. Također, utvrđeno je da je koplanarnost prstena AC (diedralni kut između ravnina definiranih atomima 4-4a-5 i 4a-5-6) nužna, ali ne i dovoljna za učinkovito vezanje za HSA (r = 0,5097; p = 0,0217 za *in vacuo* i r = 0,5093; p = 0,0218 za eksplicitnu solvataciju): čak i malo odstupanje od

koplanarnosti je povezano s gubitkom afiniteta za HSA, ali planarne molekule se ne vežu za HSA jednakim afinitetom. S obzirom da se planarnost često povezuje s konjugacijom, neki su autori smatrali da flavonoidi s visokim konstantama vezanja uspostavljaju veze s HSA kroz konjugaciju [123,125]. Prema predstavljenim rezultatima, planarnost flavonoida ima veći utjecaj na vezanje za HSA nego konjugacija: flavanoni koji su okarakterizirani značajno nižom konstantom vezanja od ostalih flavonoida ukazuju na točnost te tvrdnje. Nadalje, primarno vezno mjesto flavanona na HSA nije, za razliku od ostalih flavonoida, u poddomeni IIA, već se nalazi bliže veznom mjestu u poddomeni IIIA [53]. Mogući razlog te pojave je prisutnost neplanarnog prstena C, koji onemogućuje vezanje u blizini Trp 214 aminokiselinskog ostatka u poddomeni IIA.



Slika 12. Ovisnost log K_A o HOMO i LUMO energijama (brojevi odgovaraju rednim brojevima flavonoida u tablici 2).

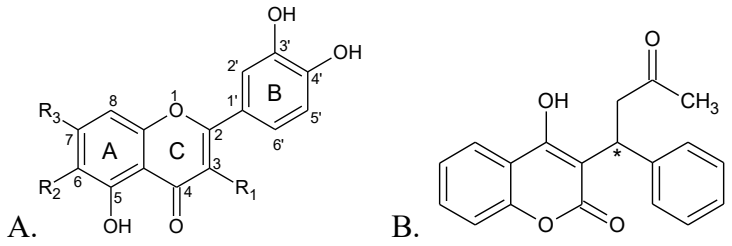
4.2.6. Strukturna svojstva flavonoida koje utječu na jačinu njihova vezanja na HSA

U ovom je istraživanju pronađeno više strukturnih odlika aglikona flavonoida koje su zaslužne za njihovo vezanje na vezno mjesto I HSA. 1) Nukleofilnost i parcijalni naboj atoma C3 omogućavaju klasifikaciju flavonoida u podskupine: flavanoni (jako niska nukleofilnost i visoki negativni parcijalni naboj), izoflavoni (niska nukleofilnost i niski negativni parcijalni naboj), flavoni (srednje visoka do visoka nukleofilnost i srednje visoki negativni parcijalni naboj) i flavonoli (srednje visoka nukleofilnost i pozitivni parcijalni naboj). 2) Povišeni negativni parcijalni naboj atoma O4 je snažno povezan s konstantom vezanja za HSA te oslikava dobra proton-donorska i proton-akceptorska svojstva flavonoida. 3) Koplanarnost A i C prstena je povezana s višim konstantama vezanja i to je preduvjet za vezanje flavonoida u hidrofobnoj šupljini. Nadalje, 4) koplanarnost AC i B prstena oslikava sklonost flavonoida stvaranju vodikovih veza, što je u skladu s 5) visokim HOMO i LUMO energijama. S druge strane, konjugacija ni AC ni B prstena nije bila povezana s jačinom vezanja flavonoida s HSA, ali negativni naboj B prstena ima utjecaja na konstantu vezanja.

4.3. Spektrofluorimetrijsko određivanje interakcija između kvercetina, luteolina i njihovih derivata s varfarinom pri vezanju na HSA

4.3.1. Određivanje konstanti vezanja kvercetina, luteolina i njihovih derivata te varfarina

S obzirom na strukturnu sličnost varfarina s flavonoidima (derivati benzopiranona, slika 13), može se pretpostaviti da se varfarin i flavonoidi vežu za isto područje veznog mjesta I. Među ispitivanih ligandima, kvercetin je pokazao najvišu konstantu vezanja, nakon čega slijedi luteolin te varfarin (tablica 3). Iz toga se može zaključiti da bi kvercetin i luteolin trebali istiskivati varfarin s njegovog veznog mjesta. Niže konstante vezanja glikozida kvercetina i luteolina se mogu objasniti njihovom veličinom, što se može potvrditi primjerom rutina, koji kao prostorno najveća molekula ima najnižu konstantu vezanja. Negativni naboj kiselinskog glukuronskog dijela kvercetin-3-*O*-glukuronida blago povisuje njegovu konstantu vezanja s obzirom na ostale glikozide, što je u skladu s općom premisom o jačem vezanju aniona za vezno mjesto IIA [2,4].



Slika 13. Strukturna formula A. flavonoida derivata kvercetina i luteolina, B. varfarina (vidjeti tablicu 3 za pojedine flavonoide).

Tablica 3. Ispitivani ligandi i njihove konstante vezanja na HSA (izračunato prema jednadžbama (4)-(6) i pretpostavljenom vezanju 1:1)

Ime	R_1	R_2	R_3	K_A±S.D. (× 10^4 M^{-1})
Varfarin	N/A	N/A	N/A	10,56 ± 0,68
Luteolin	-H	-H	-OH	12,20 ± 0,53
Izoorientin	-H	-Glc	-OH	4,57 ± 0,30
Cinarozid	-H	-H	-O-Glc	4,20 ± 0,25
Kvercetin	-OH	-H	-OH	14,40 ± 0,52
Izokvercitrin	-O-Glc	-H	-OH	4,47 ± 0,21
Hiperozid	-O-Gal	-H	-OH	3,24 ± 0,19
Kvercetin-3-*O*-glukuronid	-O-Gluc	-H	-OH	5,36 ± 0,13
Rutin	-O-Glc-Rha	-H	-OH	2,92 ± 0,13

N/A – podaci nisu dostupni, Glc – glukoza, Gal – galaktoza, Gluc – glukuronska kiselina, Rha – ramnoza

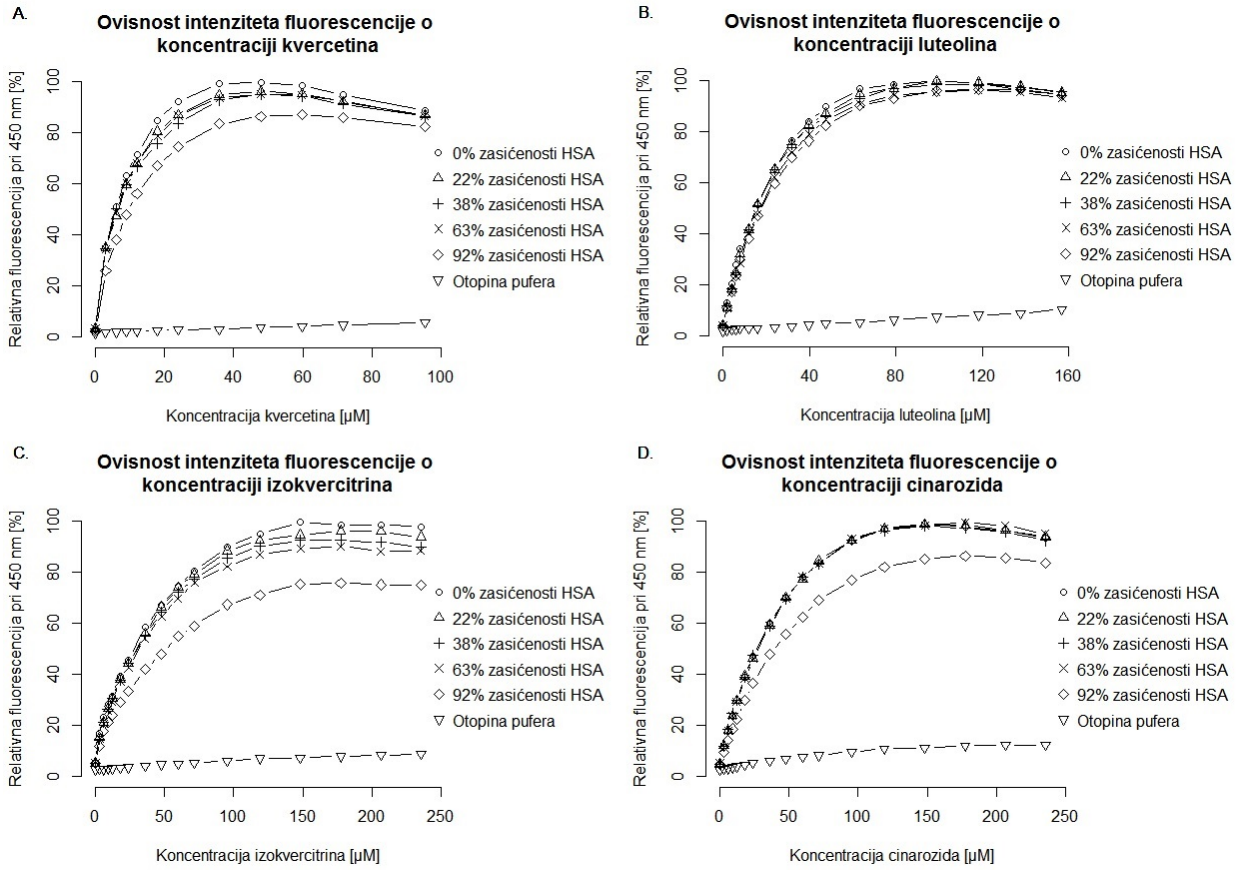
4.3.2. Studije istiskivanja varfarina s veznog mjesta I HSA flavonoidima

Kako bi se utvrdila vjerojatnost istiskivanja lijekova sa HSA, uobičajeno se koriste *in vitro* metode koje uključuju niske koncentracije HSA [128–130]. Rezultati dobiveni na ovakav način ne mogu izravno biti ekstrapolirani na fiziološke uvjete, ali mogu pružiti važan uvid u potencijalne probleme [36]. Dosadašnja istraživanja istiskivanja varfarina s veznog mjesta IIA flavonoidima raznim metodama su dala kontroverzne rezultate [49,51,56,57,131], od onih koji tvrde da nema nikakvog rizika za istiskivanje [49] do onih koji tvrde da je rizik istiskivanja varfarina i njegovih nuspojava vrlo visok [57]. U ovoj se studiji za ispitivanje istiskivanja varfarina s njegovog veznog mjesta koristila metoda fluorescencije flavonoida. Svi flavonoidi korišteni u studiji posjeduju 5-OH skupinu za koju se zna da gasi njihovu intrinzičnu fluorescenciju [85]. Međutim, kada su vezani za HSA, flavonoidi fluoresciraju na valnoj duljini ekscitacije od 450 nm i emisije 500–540 nm. U slučaju da flavonoid nije u mogućnosti vezati se za HSA ili se veže s nižom ili višom konstantom vezanja zbog nekog vanjskog faktora, to se može primijetiti pomoću promjena u njihovim F_{max} i c_{max}. S konstantom vezanja varfarina za HSA od 10,56 ± 0,68 × 10^4 M^{-1}, korištenjem jednadžbi (7) i (8), moguće je odrediti koncentraciju, odnosno količinu varfarina potrebnu da se zasiti određeni udio veznog mjesta I (0, 22, 38, 63 i 92%). Također je potrebno naglasiti da je varfarin natrij korišten u ispitivanjima kao racemična smjesa te da konstante vezanja (*R*)- i (*S*)-varfarina nisu jednake; (*S*)-enantiomer ima

neznatno višu konstantu vezanja [132], međutim oba enantiomera se vežu na isto područje poddomene IIA [27].

4.3.2.1. Istovremeno vezanje varfarina i aglikona flavonoida

Nakon inkubacije s varfarinom, koncentracija flavonoida se postepeno povećavala sve dok nije bio postignut F_{max}. Istiskivanje varfarina s HSA zbog vezanja kvercetina, luteolina, izokvercitrina ili cinarozida je prikazan na slici 14. Činjenica da se neki flavonoidi jače vežu za HSA od varfarina može dovesti do zaključka da će posljedično doći do istiskivanja varfarina. U slučaju da se varfarin jače veže, zauzeće određenog postotka veznog mjesta od strane varfarina će smanjiti fluorescenciju zbog smanjenog nastajanja kompleksa HSA-flavonoid.

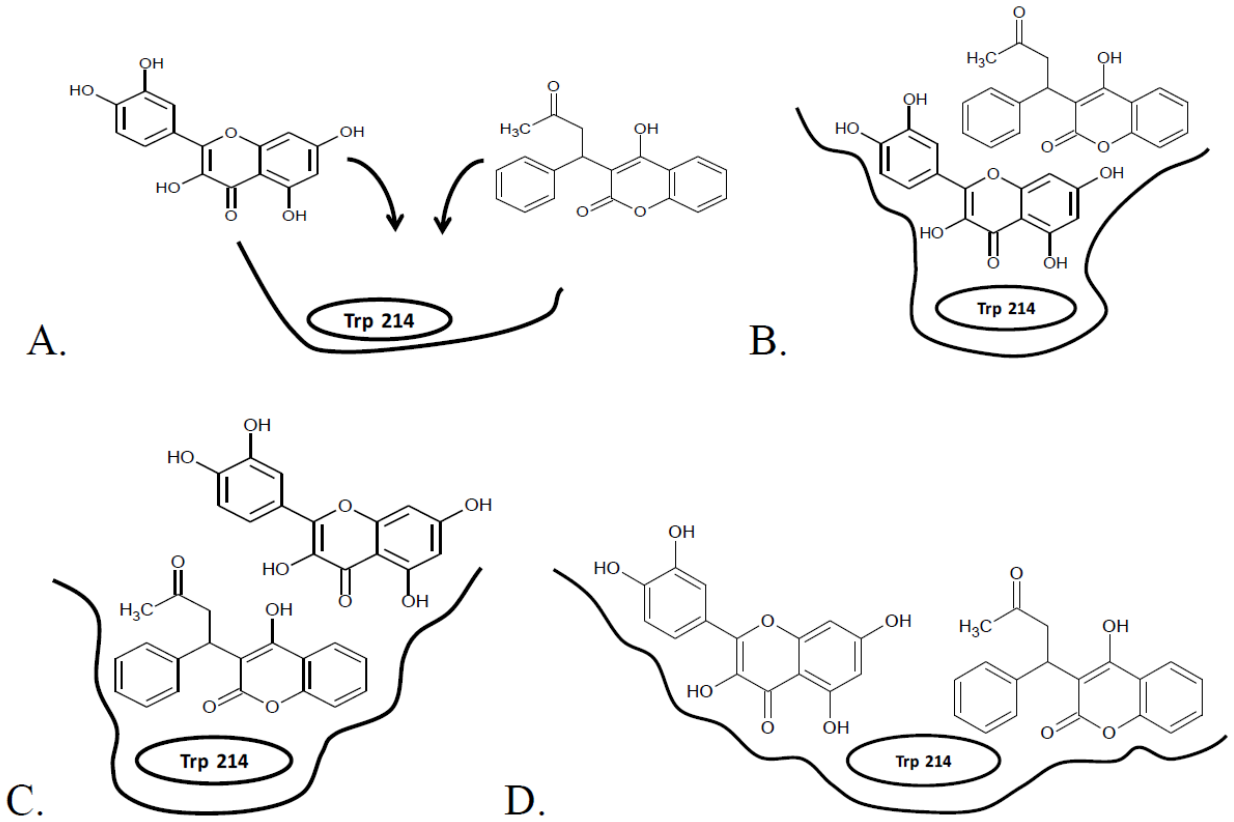


Slika 14. Utjecaj različitih koncentracija varfarina na fluorescenciju kompleksa HSA-flavonoid.

Dodatak varfarina neznatno smanjuje F_{max} kvercetina s malim povišenjem c_{max} (slika 14A). Smanjenje F_{max} kvercetina povišenjem koncentracije varfarina je već bilo utvrđeno za

kompleks BSA-kvercetin [133]. Ovo smanjenje je najvidljivije pri zasićenju veznog mjesta I od 92%. U tom slučaju koncentracija varfarina iznosi 120 μM, dok je koncentracija kvercetina niža od 60 μM, što je mnogo više od fiziološki mogućih koncentracija. Za niže vrijednosti zasićenosti HSA nema značajnih razlika bilo u F_{max}, bilo u c_{max}. Iz toga se može zaključiti da varfarin i kvercetin ne dijele isto vezno područje iako se oboje vežu u IIA poddomeni (slika 15A). Ako bi se vezno mjesto kvercetina nalazilo dublje unutar veznog džepa nego vezno mjesto varfarina (slika 15B.), varfarin bi ometao vezanje kvercetina te bi pri visokim koncentracijama varfarina fluorescencija pri 526 nm bila potpuno ugašena. Međutim, rezultati dobiveni spektrofluorimetrijskom analizom, a potvrđeni i eksperimentima sidrenja, to ne potvrđuju. Dodatna ispitivanja su također provedena u kojima je HSA bio zasićen kvercetinom, a koncentracija varfarina se postupno povećavala kako bi se otkrilo nalazi li se vezno područje varfarina dublje unutar veznog džepa u odnosu na vezno područje kvercetina (slika 15C). Ovi rezultati također potvrđuju hipotezu da nema izravne interakcije između kvercetina i varfarina. U oba slučaja, čak i pri najvišim koncentracijama ometajućeg liganda, mali dodatak ispitivanog liganda uzrokuje značajan porast intenziteta fluorescencije. Iz toga se može zaključiti da niti varfarin i kvercetin dijele isto vezno područje unutar veznog mjesta I, niti se vezno područje jednog liganda nalazi ispred veznog područja drugog liganda, nego se oba liganda mogu istovremeno vezati za svoja vezna područja unutar poddomene IIA (slika 15D), tvoreći manje fluorescentni ternarni HSA-kvercetin-varfarin kompleks, bez da značajno utječu na afinitet vezanja drugog liganda. Ovo je nadalje potvrđeno i nedostatkom karakteristične sigmoidalne krivulje u slučaju kooperativnog [134,135], odnosno Langmuirove krivulje u slučaju antikooperativnog vezanja [23,136] (slika 14A).

Slični rezultati su dobiveni i za luteolin (slika 14B); jedina je razlika što u slučaju luteolina nije bilo primjetne razlike u F_{max} i c_{max} između različitih postotaka zasićenosti HSA, čak ni u slučaju 92%-tne zasićenosti HSA. Može se zaključiti da je 3-OH supstituent od velike važnosti za intenzitet fluorescencije flavonoida i njegov položaj u prostoru značajno utječe na F_{max}. Drugo objašnjenje je moguće prisustvo drugog varfarinskog veznog mjesta, niskog afiniteta (LAS), na koje se varfarin veže tek nakon što je primarno vezno mjesto, HAS, popunjeno. Ovo bi potencijalno moglo objasniti veći utjecaj varfarina na intenzitet fluorescencije kvercetina nego luteolina, s obzirom da kvercetin ima 3-OH skupinu koja bi mogla ući u interakcije s varfarinom vezanim za LAS pri višim koncentracijama, što je potvrđeno i studijom sidrenja.



Slika 15. Predloženi položaji molekula varfarina i kvercetina u poddomeni IIA HSA (temeljeno na Petitpas i sur. [27] i Ghuman i sur. [8]). A. Varfarin i kvercetin se vežu za isto vezno područje, B. Područje vezanja varfarina se nalazi ispred područja vezanja kvercetina, C. Područje vezanja varfarina se nalazi iza područja vezanja kvercetina i D. Područja vezanja varfarina i kvercetina su neovisna jedno o drugome.

Dobiveni rezultati su u skladu s rezultatima koje su objavili Zsila i sur. [51] te Dufour i Dangles [49], a slažu se i s rezultatima kristalografskih struktura koje su objavili Petitpas i sur. [27] te Yamasaki i sur. [23,118] koji tvrde da se za vezno mjesto u poddomeni IIA mogu vezati dodatni ligandi u blizini varfarina. Razlika u fluorescenciji flavonoida može biti pripisana konformacijskim promjenama i potencijalnim promjenama veličine i oblika veznog mjesta I uzrokovanih varfarinom. To uzrokuje promjene u veznom području flavonoida i F_{max} kompleksa HSA-flavonoid bez značajnog utjecaja na njihovu konstantu vezanja. Razlika u intenzitetu fluorescencije pri 92%-tnom zasićenju HSA može biti objašnjena izraženijom konformacijskom promjenom veznog mjesta, ali mogućnost druge molekule varfarina vezane za LAS pri višim koncentracijama ne može biti odbačena [131]. Ta druga molekula varfarina bi mogla i izravno utjecati na intenzitet fluorescencije flavonoida.

S druge strane, rezultati koje su objavili Di Bari i sur. [56] i Poór i sur. [57] govore da kvercetin, kao i neki drugi flavonoidi, može istisnuti varfarin s njegovog veznog mjesta čak i pri nižim koncentracijama. Di Bari i sur. su utvrdili da smanjenje intenziteta vrpci kvercetina dobivenih metodom induciranog cirkularnog dikroizma (ICD) ukazuje na njegovo istiskivanje s HSA. Nadalje, Poór i sur. tvrde da smanjenje vrijednosti polarizacije mjerene fluorescencijskom anizotropijom odgovara većoj rotacijskoj slobodi slobodnog lijeka, odnosno istiskivanju varfarina iz njegovog kompleksa s HSA. Međutim, Yamasaki i sur. [23,118] su utvrdili da vezanje drugog liganda može utjecati na mobilnost prvog liganda i imati učinak na rezultate dobivene metodama ICD-a i fluorescencijske anizotropije bez nužnog istiskivanja liganda. Drugim riječima, promjene u signalima fluorescencijske anizotropije i ICD-a ne ukazuju jednoznačno na istiskivanje liganda. Nadalje, tehnika nuklearne magnetske rezonance razlike prijenosa zasićenja (engl. *Saturation Transfer Difference Nuclear Magnetic Resonance*, STD-NMR) koju su koristili Di Bari i sur. je okarakterizirna ovisnošću intenziteta signala o udaljenosti vodikovih atoma liganda od vodikovih atoma HSA. Intenzitet signala ovisi o recipročnoj vrijednosti šeste potencije udaljenosti ligand-HSA. U njihovom slučaju, relativni intenzitet signala kvercetina je iznosio ~40% njegovog intenziteta bez varfarina. Dakle, vezanje varfarina utječe na udaljenost između kvercetina i HSA, ali rezultati eksplicitno ne pokazuju istiskivanje, nego mogu ukazivati i na konformacijske promjene.

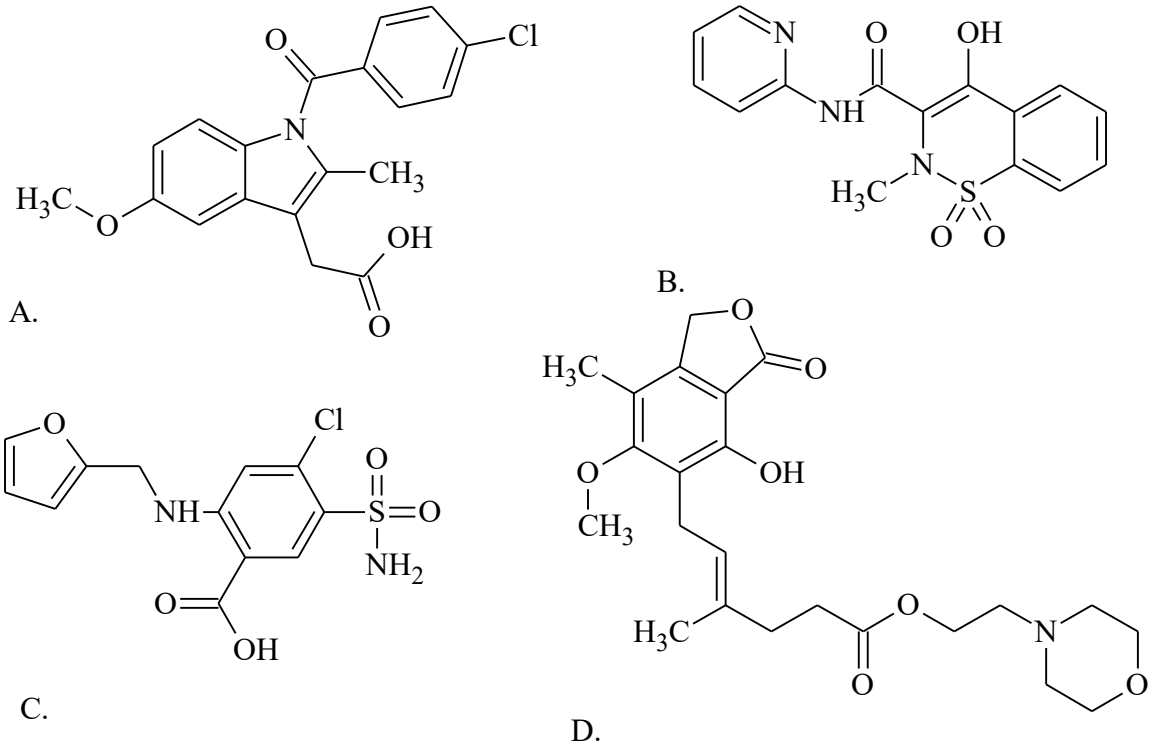
4.3.2.2. Istovremeno vezanje varfarina i glikozida flavonoida

Dodatna mjerenja su provedena pod pretpostavkom da bi varfarin mogao ometati vezanje glikozida flavonoida. Glikozidi flavonoida imaju niže konstante vezanja i prostorno su veće molekule od svojih aglikona, tako da se očekivalo da će lakše ulaziti u interakcije s varfarinom. Kao i kod aglikona, dvije različite vrste ponašanja su primjećene. Kod derivata kvercetina (svi su supstituirani na položaju 3, slika 13A) dolazi do blagog smanjenja F_{max} povećanjem zasićenosti veznog mjesta s neprimjetnim utjecajem na c_{max}, osim u slučaju kada su vezna mjesta u poddomeni IIA zasićena 92% (slika 14C, podaci za ostale derivate kvercetina nisu prikazani zbog sličnosti). S druge strane, kod derivata luteolina (supstituirani na položajima 6 i 7, slika 13A) nije uočeno nikakvo smanjenje ni u F_{max} ni u c_{max}, s iznimkom slučaja kada su vezna mjesta u poddomeni IIA zasićena 92% (slika 14D, podaci za izoorientin nisu prikazani zbog sličnosti). Supstituenti na položaju 3 utječu na intenzitet fluorescencije s obzirom da su dio

velikog konjugiranog sustava koji spaja prsten B s kisikovim atomom na položaju 4, ali nemaju skoro nikakvog učinka na interakcije s varfarinom. Supstitucija na položaju 6 ili 7 nema utjecaja ni na fluorescenciju flavonoida ni na interakcije s varfarinom. Kao i kod kvercetina, jedini učinak je primjećen pri 92%-tnom zasićenju HSA. To se može objasniti vezanjem varfarina za LAS [8,27,133], što uzrokuje alosteričke konformacijske promjene i formiranje slabije fluorescentnog ternarnog kompleksa ili sudjeluje u izravnoj kompetitivnoj interakciji. Također, kao i kod kvercetina i luteolina, može se primijetiti da varfarin ima veći utjecaj na glikozide kvercetina nego luteolina, naglašavajući utjecaj supstituenta na položaju 3 na intenzitet fluorescencije.

4.4. Spektrofluorimetrijsko određivanje interakcija kvercetina i kvercetin-3-*O*-glukuronida s indometacinom, piroksikamom, furosemidom te mikofenolat mofetilom pri vezanju na HSA

Nakon ispitivanja istiskivanja varfarina s njegovog veznog mjesta i zaključka da se flavonoidi ne vežu na isto vezno područje kao i varfarin, ispitale su se interakcije kvercetina i kvercetin-3-*O*-glukuronida s indometacinom, piroksikamom, furosemidom i mikofenolat mofetilom (slika 16). Korištenjem metode gašenja fluorescencije, najjače vezanje za vezno mjesto u poddomeni IIA je pokazao piroksikam (K_A = (24,13 ± 3,08) × 10^4 M^{-1}), nakon kojeg su slijedili indometacin (K_A = (17,29 ± 6,39) × 10^4 M^{-1}), furosemid (K_A = (7,53 ± 0,01) × 10^4 M^{-1}) i mikofenolat mofetil (K_A = (1,27 ± 0,34) × 10^4 M^{-1}).



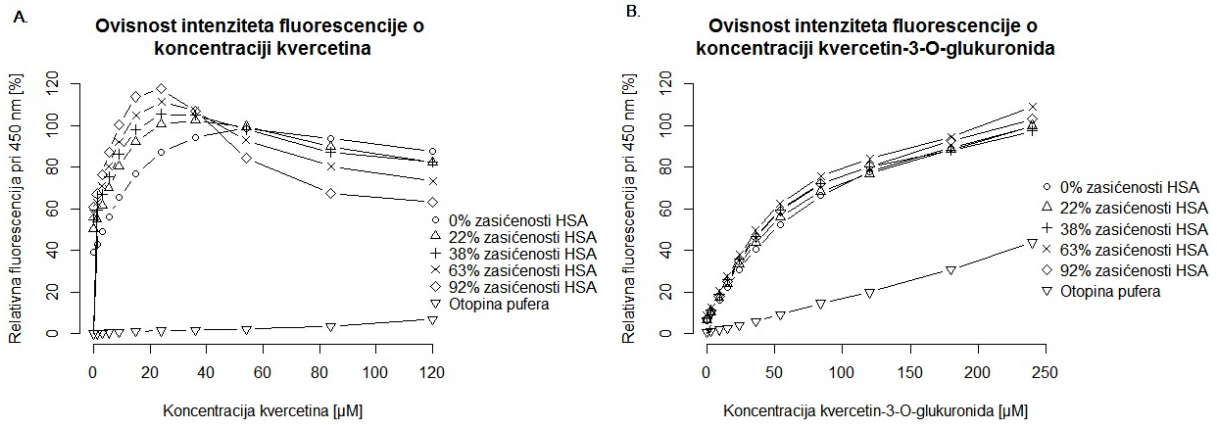
Slika 16. Strukturne formule: A. indometacina, B. piroksikama, C. furosemida i D. mikofenolat mofetila.

Nakon određivanja konstanti vezanja, korištenjem jednadžbi (7) i (8), analogno kao i kod varfarina, određene su koncentracije ispitivanih lijekova potrebne za zasićenje 0, 22, 38, 63 i 92% veznog mjesta u poddomeni IIA HSA, s time da zbog niske topljivosti mikofenolat

mofetila, nije bilo moguće postići njegovu koncentraciju koja bi zasitila 92% navedenog veznog mjesta. Zatim je uslijedilo mjerenje istiskivanja lijekova jednim aglikonom flavonoida, kvercetinom, i jednim glikozidom flavonoida, kvercetin-3-*O*-glukuronidom.

4.4.1. Interakcije kvercetina i kvercetin-3-*O*-glukuronida s indometacinom

Intenzitet fluorescencije kvercetina (A) i kvercetin-3-*O*-glukuronida (B) pri različitim postocima zasićenosti veznog mjesta u poddomeni IIA HSA indometacinom se mogu vidjeti na slici 17. Pri koncentracijama kvercetina nižim od 30 μM (slika 17A), povišenjem koncentracije indometacina, odnosno što je viši postotak zasićenosti veznog mjesta I, to je i intenzitet fluorescencije kvercetina pri određenoj koncentraciji veći, suprotno od rezultata dobivenih zasićivanjem veznog mjesta varfarinom. Pri koncentracijama kvercetina višim od 30 μM, intenzitet fluorescencije kvercetina počinje opadati te pri koncentracijama kvercetina višim od 40 μM, intenzitet fluorescencije kvercetina pri određenoj koncentraciji je niži što je postotak zasićenosti veznog mjesta viši. Ta činjenica se može objasniti na način da prisutnost indometacina u blizini veznog mjesta kvercetina stabilizira molekulu kvercetina, odnosno smanjuje joj rotacijsku i translacijsku slobodu. Na taj se način povećava udio kvercetina koji se nalazi u konformaciji koja može apsorbirati zračenje te posljedično dolazi i do veće emisije fluorescentnog zračenja. Drugo objašnjenje može biti da dolazi do prijenosa energije između molekula indometacina i kvercetina što doprinosi većem intenzitetu fluorescencije. Pri koncentracijama kvercetina višima od 30 μM intenzitet fluorescencije opada zbog međusobne kompeticije molekula kvercetina za vezna mjesta. Pri tome se ne može postići stabilizirajući ili učinak prijenosa energije indometacina te zbog visoke koncentracije kvercetina dolazi do promjene konformacije veznog mjesta, što anulira učinak indometacina. Za razliku od varfarina, u kristalografskoj strukturi kompleksa HSA-indometacin je utvrđen i LAS indometacina [8] koji se ne nalazi blizu njegovog HAS-a te se pretpostavlja da vezanje indometacina na LAS alosterički ne utječe na vezanje kvercetina, za što dodatnu potvrdu daje i vezanje kvercetin-3-*O*-glukuronida.



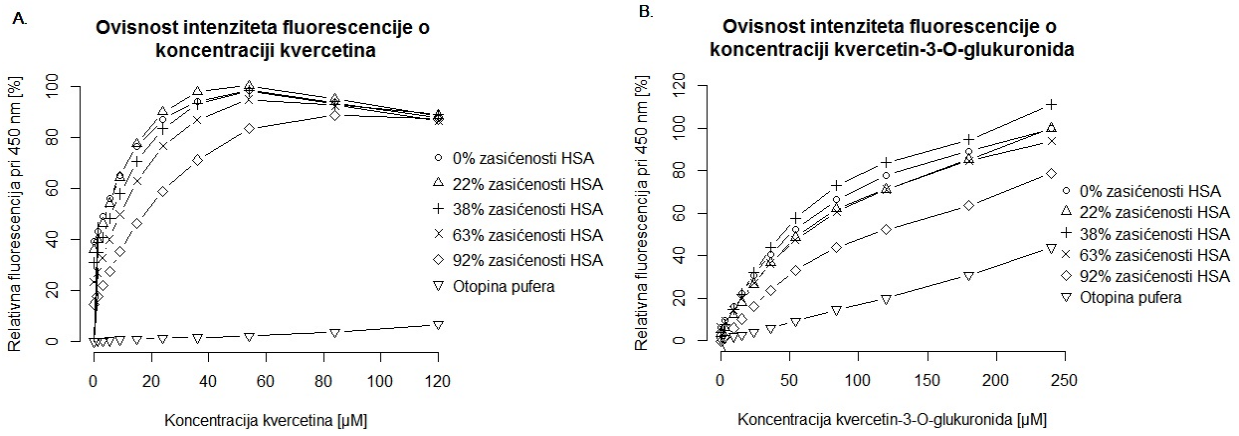
Slika 17. Ovisnost intenziteta fluorescencije A. kvercetina i B. kvercetin-3-*O*-glukuronida o postotku zasićenosti veznog mjesta u poddomeni IIA HSA indometacinom.

U slučaju vezanja kvercetin-3-*O*-glukuronida (slika 17B) iz rezultata se ne može zaključiti o značajnijoj povezanosti F_{max} o postotku zasićenosti veznih mjesta. Bitno je naglasiti kako je zbog različite tehnike ispitivanja interakcija, greška u mjerenjima veća nego kod eksperimenata s varfarinom te nije moguće odrediti manje razlike u intenzitetu fluorescencije između pojedinih postotaka zasićenost veznih mjesta. Unatoč tome, može se primijetiti kako je F_{max} kvercetin-3-*O*-glukuronida u slučaju nezasićenog HSA niži nego pri jednakim koncentracijama u slučajevima djelomično zasićenog HSA. Moguće je da dolazi do malog učinka stabilizacije ili prijenosa energije na molekulu flavonoida, ali s obzirom da je molekula kvercetin-3-*O*-glukuronida značajno veća od molekule kvercetina, kvercetin-3-*O*-glukuronid i indometacin ne dolaze u toliko bliski kontakt tako da je i učinak indometacina značajno manji. Očekivalo bi se da, ako bi vezanje indometacina za LAS alosterički modificiralo strukturu HSA, bi to imalo veći učinak na vezanje kvercetin-3-*O*-glukuronida nego kvercetina zbog svoje veličine i niže konstante vezanja. Međutim, takav učinak iz dobivenih rezultata nije vidljiv te se može zaključiti da vezanje indometacina za LAS alosterički ne mijenja (ili barem ne u dovoljnoj mjeri da bi imao učinka) konformaciju veznog mjesta u poddomeni IIA.

4.4.2. Interakcije kvercetina i kvercetin-3-*O*-glukuronida s piroksikamom

Podaci dobiveni za interakciju kvercetina i kvercetin-3-*O*-glukuronida s piroksikamom su najsličniji podacima dobivenima u slučaju varfarina (slike 18A i B). U slučaju interakcija s kvercetinom (slika 18A), uočljiv je trend smanjivanja F_{max} kvercetina s povišenjem postotka zasićenosti veznog mjesta, s najvećim padom između 63 i 92%, kao i kod varfarina. Činjenicu da

je intenzitet fluorescencije 22% zasićenog HSA neznatno veći od nezasićenog HSA bi se moglo pripisati greški u mjerenju zbog primijenjene tehnike. U slučaju 92% zasićenog veznog mjesta uočava se značajni pad u F_{max}, ali isto kao i kod varfarina, taj pad je vjerojatnije posljedica vezanja piroksikama za LAS i izravne kompeticije s kvercetinom (uočljivo po povišenju u c_{max} kvercetina) ili alosteričkoj promjeni konformacije veznog mjesta koja posljedično utječe na konformaciju kvercetina i na intenzitet fluorescencije. S druge strane, kod nižih postotaka zasićenosti veznog mjesta, F_{max} se ostvaruje kod jednakog c_{max} kvercetina od 55 μM, iz čega se može zaključiti da nema značajnijih interakcija između piroksikama i kvercetina pri vezanju na njihove HAS-ove.

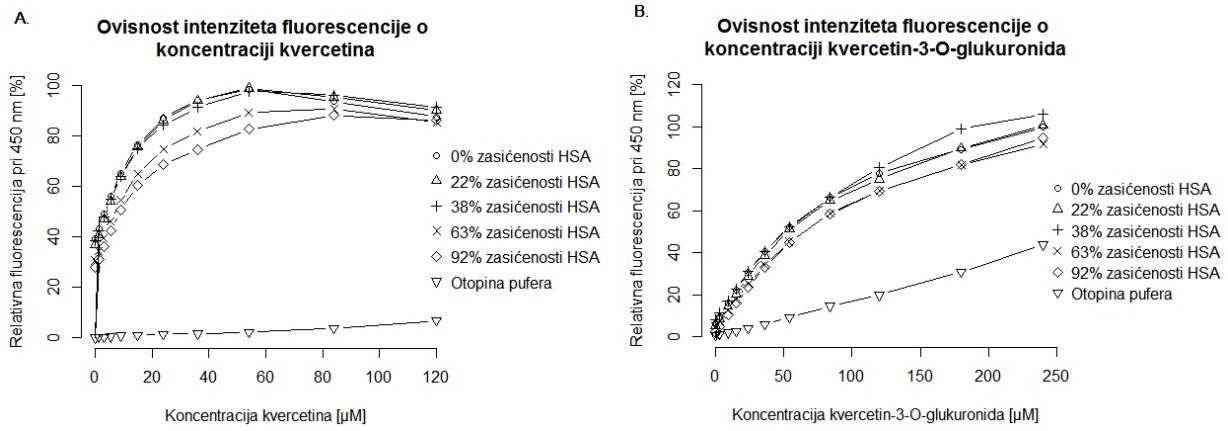


Slika 18. Ovisnost intenziteta fluorescencije A. kvercetina i B. kvercetin-3-*O*-glukuronida o postotku zasićenosti veznog mjesta u poddomeni IIA HSA piroksikamom.

Pri vezanju kvercetin-3-*O*-glukuronida (slika 18B), kao i u slučaju vezanja kvercetina, uočljiv je znatan pad F_{max} pri 92% zasićenom HSA. To daje dodatnu težinu zaključku o interakciji piroksikama vezanog na LAS ili alosteričkoj promjeni konformacije HSA koja se uočava kroz smanjeni intenzitet fluorescencije kvercetin-3-*O*-glukuronida. Kao što se može vidjeti iz grafa, F_{max} 38% zasićenog HSA je veći i od F_{max} nezasićenog i 22% zasićenog HSA, dok su F_{max} 22 i 63% zasićenog HSA jednaki, ali niži od F_{max} nezasićenog HSA. Ovo bi se isto moglo pripisati greškom korištene metode, iako se ne može odbaciti mogućnost da bi dodatno istraživanje drugom metodom moglo ukazati na neke druge fenomene.

4.4.3. Interakcije kvercetina i kvercetin-3-*O*-glukuronida s furosemidom

Rezultati interakcija kvercetina i kvercetin-3-*O*-glukuronida s furosemidom (slike 19A i B) pokazuju slične značajke onima od varfarina i piroksikama, ali s malim razlikama. U slučaju interakcija s kvercetinom (slika 19A), značajna razlika u F_{max} se uočava i za 63 i za 92% zasićenosti HSA, a također se uočava da je F_{max} za niže postotke zasićenosti praktički identičan. Također se može primjetiti i pomak c_{max} s 55 µM kod 0, 22 i 38% zasićenog HSA na 80 µM kod 63 i 92% zasićenog HSA. I ovdje je vjerojatni uzrok pada F_{max} postojanje LAS-a za furosemid u blizini veznog područja kvercetina, s time da je razlika u konstanti vezanja furosemida za HAS i LAS niža nego ona kod piroksikama.



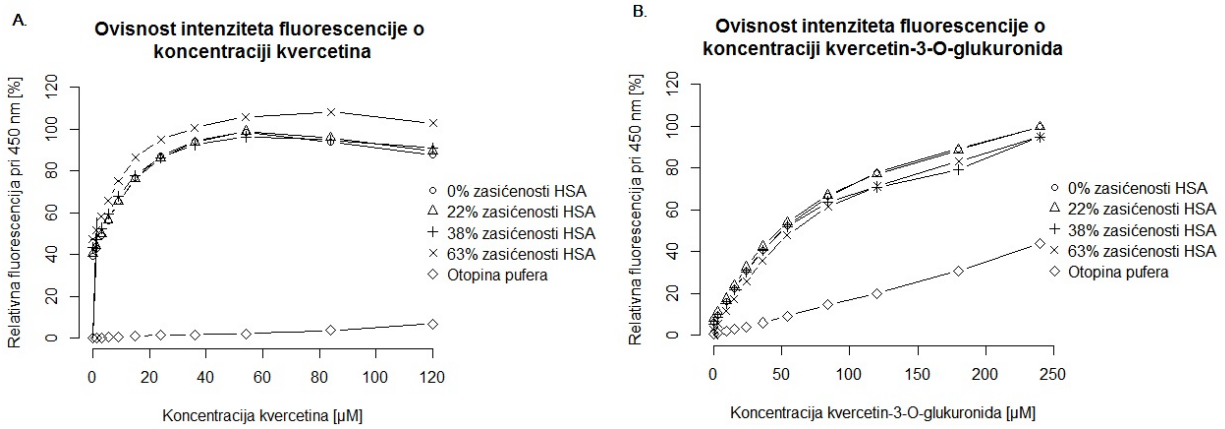
Slika 19. Ovisnost intenziteta fluorescencije A. kvercetina i B. kvercetin-3-*O*-glukuronida o postotku zasićenosti veznog mjesta u poddomeni IIA HSA furosemidom

Rezultati interakcija furosemida s kvercetin-3-*O*-glukuronidom (slika 19B) pokazuje neznatno manji F_{max} u slučaju 63 i 92% zasićenog HSA s obzirom na manje zasićeni HSA, što je u skladu s vezanjem furosemida za LAS, kao i u slučaju interakcija s kvercetinom, ali razlika nije toliko izražena da bi mogli tvrditi da je i statistički značajna. I u ovom slučaju, kao i kod interakcija piroksikama s kvercetin-3-*O*-glukuronidom (slika 18B), uočava se povišeni F_{max} pri 38% zasićenom HSA.

4.4.4. Interakcije kvercetina i kvercetin-3-*O*-glukuronida s mikofenolat mofetilom

Rezultati interakcija mikofenolat mofetila s kvercetinom i kvercetin-3-*O*-glukuronidom se mogu vidjeti na slikama 20A i B. Ovdje je važno napomenuti da su, s obzirom na puno nižu

konstantu vezanja mikofenolat mofetila za vezno mjesto u poddomeni IIA, koncentracije mikofenolat mofetila potrebne da bi se zasitio jednaki postotak veznih mjesta bile 5–12 puta veće od ostalih lijekova. Isto tako, moguće je da vezno mjesto u poddomeni IIA nije HAS mikofenolat mofetila. Na slici 20A je prikazan intenzitet fluorescencije kvercetina pri različitim postocima zasićenosti veznog mjesta I mikofenolat mofetilom. Može se uočiti da prisutnost mikofenolat mofetila ne utječe niti na vezanje kvercetina, niti na njegov F_{max}, osim pri najvišoj, 63% zauzetosti veznih mjesta, gdje je vidljiv porast F_{max}, ali i c_{max} s oko 55 μM na oko 80 μM (kao što je već rečeno, zauzetost veznog mjesta od 92% nije mogla biti postignuta zbog nedovoljne topljivosti mikofenolat mofetila). Za razliku od mjerenja s indometacinom, u ovom je slučaju povišenje F_{max} najvjerojatnije posljedica alosteričkih modifikacija HSA nastalih vezanjem mikofenolat mofetila na više veznih područja zbog visoke koncentracije (138,15 μM u odnosu na koncentraciju HSA od 6 μM) ili posljedica vezanja mikofenolat mofetila na svoj LAS koji se nalazi u blizini kvercetina. Isto tako, u slučaju da bi povišenje F_{max} bilo posljedica izravnog utjecaja vezanja mikofenolat mofetila na njegov HAS u blizini kvercetina, povišenje F_{max} bi bilo vidljivo i pri nižim postocima zasićenja.

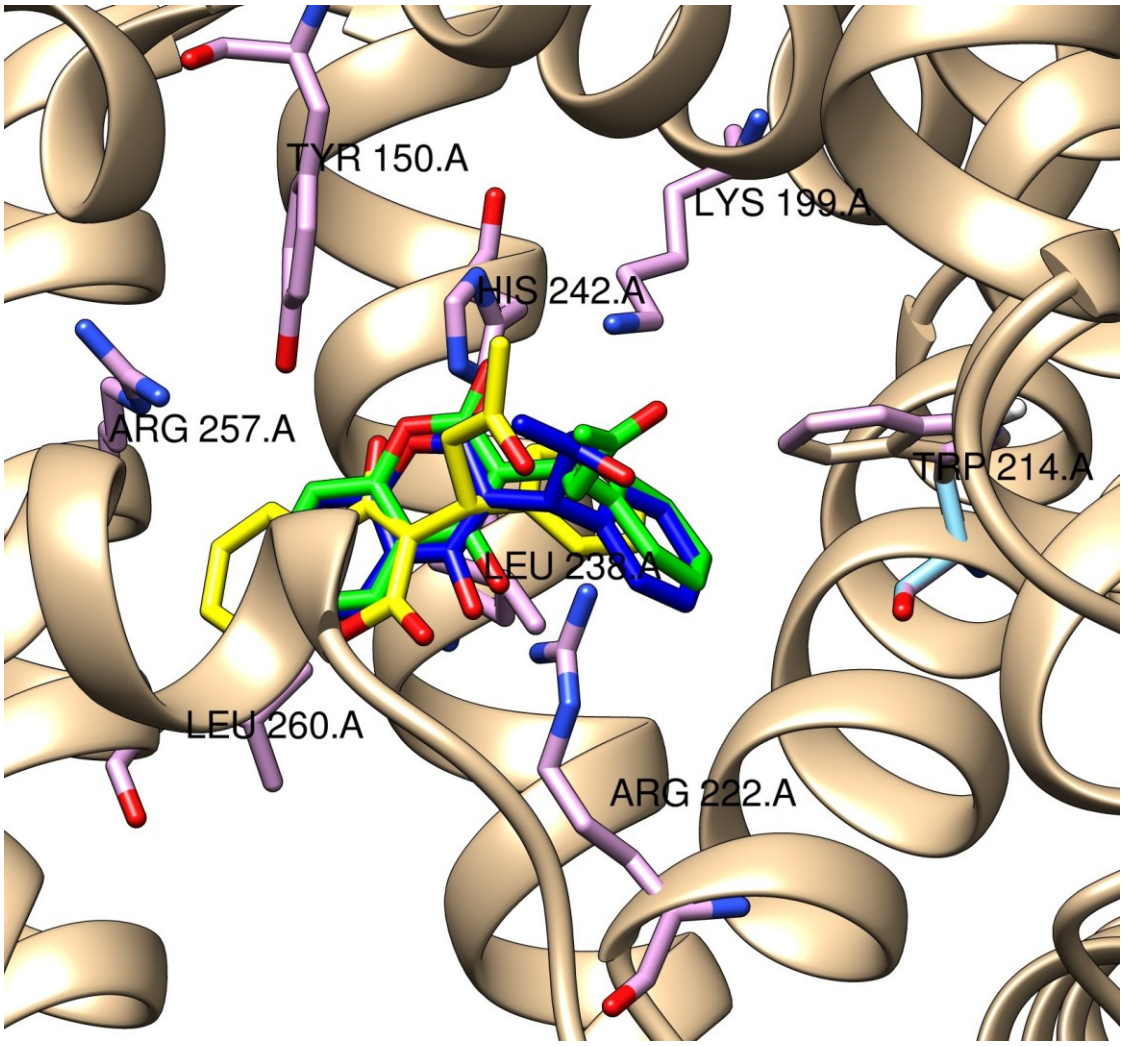


Slika 20. Ovisnost intenziteta fluorescencije A. kvercetina i B. kvercetin-3-*O*-glukuronida o postotku zasićenosti veznog mjesta u poddomeni IIA HSA mikofenolat mofetilom.

U slučaju interakcija mikofenolat mofetila s kvercetin-3-*O*-glukuronidom (slika 20B), može se uočiti malo smanjenjenje F_{max} pri zasićenjima od 38 i 63%, što također govori u prilog alosteričkih modifikacija HSA uslijed vezanja mikofenolat mofetila, ali u ovom slučaju, jer je kvercetin-3-*O*-glukuronid veća molekula, dolazi do snižavanja vrijednosti F_{max}.

4.5. Molekulsko modeliranje kompleksa HSA-kvercetin i HSA-kvercetin-3-*O*-glukuronid s i bez varfarina

Usporedba usidrenih molekula (*R*)- i (*S*)-varfarina s kristalografskom strukturom (*R*)-varfarina vezanog za HSA (PDB unos 2BXD) je pokazala visoki stupanj sličnosti položaja liganada (slika 21) i na taj način validirala prikladnost ovog pristupa. U blizini molekule varfarina nalaze se četiri pozitivno nabijena aminokiselinska ostatka, Lys 199, Arg 222, His 242 i Arg 257 i tri nenabijena aminokiselinska ostatka, Tyr 150, Leu 238 i Leu 260. Varfarin također tvori i tri vodikove veze, i to s Tyr 150, Arg 222 i His 242.



Slika 21. Prikaz kristalografske strukture (*R*)-varfarina (žuto) i obližnjim aminokiselinskim ostacima te preklapanje s usidrenim (*R*)-varfarinom (plavo) i (*S*)-varfarinom (zeleno).

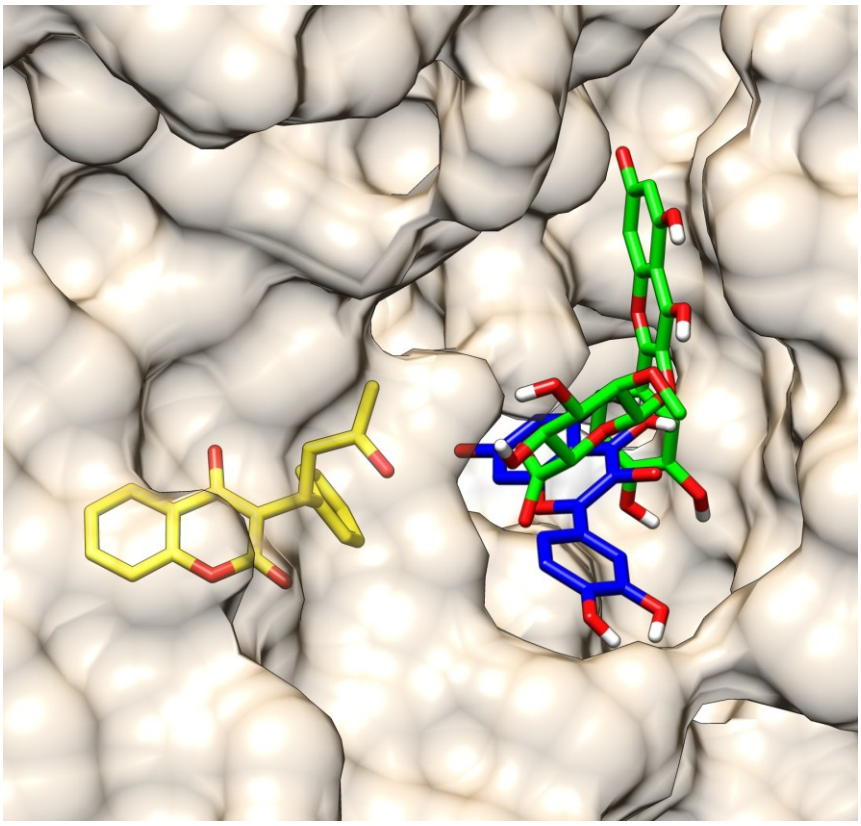
Sažeti podaci sidrenja specija kvercetina se mogu vidjeti u tablici 4. Od svih navedenih grozdova, samo grozdovi pod rednim brojevima 1. i 3. aniona na položaju 7 i 4' te grozd 4 fluorescentnog aniona na položaju 3,4' pokazuju preklapanje u veznom mjestu s varfarinom, ali mora se uzeti u obzir da su ti grozdovi formirani od malog broja sidrenja (3 i 2 za anion kvercetina u položaju 7, 1 i 1 za anion u položaju 4' te 3 za fluorescentni anion u položajima 3 i 4') te je stoga mala vjerojatnost da dolazi do vezanja na tim područjima u stvarnim uvjetima. Za sva ostala sidrenja, i za nefluorescentne i za fluorescentne specije, vezno mjesto kvercetina se ne preklapa s veznim mjestom varfarina i nalazi se na hidrofilnom ulazu u IIA poddomenu (slika 22).

Tablica 4. Sažetak podataka sidrenja specija kvercetina

Anion kvercetina na položaju 7			Anion kvercetina na položaju 4’		
Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)	Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)
1.	3	–4,79	1.	1	–4,68
2.	1	–4,42	2.	19	–4,38
3.	2	–4,22	3.	1	–4,30
4.	4	–4,20	4.	2	–4,16
5.	12	–4,12	5.	9	–4,11
6.	23	–4,07	6.	9	–4,08
7.	3	–3,94	7.	2	–3,69
8.	14	–3,94	8.	8	–3,68

Fluorescentni anion kvercetina na položajima 3 i 7			Fluorescentni anion kvercetina na položajima 3 i 4’		
Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)	Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)
1.	43	–4,66	1.	5	–4,42
2.	1	–4,61	2.	18	–4,04
3.	4	–4,60	3.	13	–3,87
4.	7	–4,24	4.	3	–3,82
5.	4	–4,20	5.	1	–3,81

S druge strane, vezno mjesto kvercetin-3-*O*-glukuronida se ne preklapa s veznim mjestom varfarina ni u jednom od sidrenja, ali se preklapa s veznim mjestom kvercetina. Također broj sidrenja po grozdu za kvercetin-3-*O*-glukuronid je niži nego kod kvercetina (tablica 5), što se može objasniti većom fleksibilnošću glukuronidnog liganda, kao i prostranošću ulaza u IIA poddomenu, koja je u mogućnosti vezati kvercetin-3-*O*-glukuronid u različitim konformacijama. U ovom slučaju su vezna mjesta svih grozdova smještena na ulazu u IIA poddomenu (slika 22), s time da su aglikonski dio anionske i fluorescentne anionske specije međusobno zarotirani za otprilike 180° (slike 23A i B). Glukuronski dio specija se nalazi u istoj ravnini, ali je u slučaju fluorescentne anionske specije zarotiran za 90° i usmjeren je prema površini proteina (slike 23A i B), što može biti uzrok slabijeg vezanja kvercetin-3-*O*-glukuronida (tablica 5).

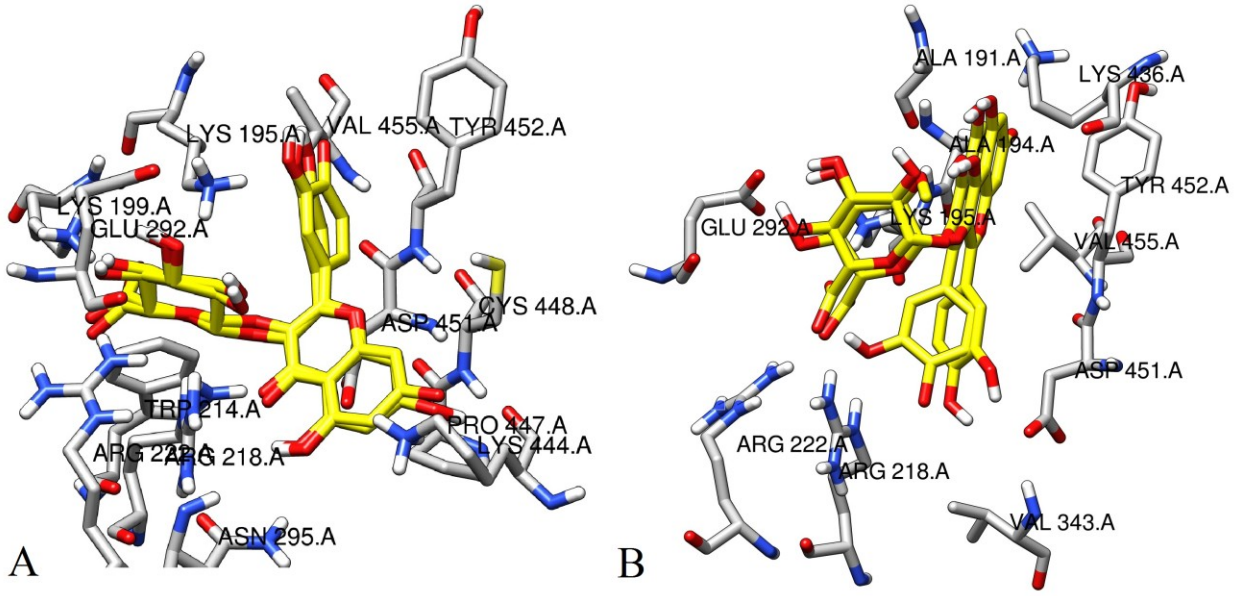


Slika 22. Preklapanje kristalografske strukture (*R*)-varfarina (žuto) s usidrenim fluorescentnim anionima kvercetina (plavo) i kvercetin-3-*O*-glukuronida (zeleno).

Tablica 5. Sažetak podataka sidrenja specija kvercetin-3-*O*-glukuronida.

Anion kvercetin-3-*O*-glukuronida na položaju 7			Anion kvercetin-3-*O*-glukuronida na položaju 4’		
Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)	Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)
1.	4	–5,02	1.	5	–6,09
2.	5	–4,76	2.	3	–4,89
3.	1	–4,62	3.	2	–4,78
4.	4	–4,49	4.	4	–4,74
5.	2	–4,49	5.	6	–4,52
6.	9	–4,45	6.	1	–4,16
7.	5	–4,45	7.	4	–4,02
8.	1	–4,35	8.	4	–3,99

Fluorescentni anion kvercetin-3-*O*-glukuronida na položaju 7			Fluorescentni anion kvercetin-3-*O*-glukuronida na položaju 4’		
Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)	Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)
1.	13	–4,21	1.	3	–4,18
2.	2	–4,07	2.	4	–4,16
3.	11	–4,06	3.	6	–3,29
4.	1	–4,05	4.	6	–3,20
5.	3	–3,95	5.	1	–3,19

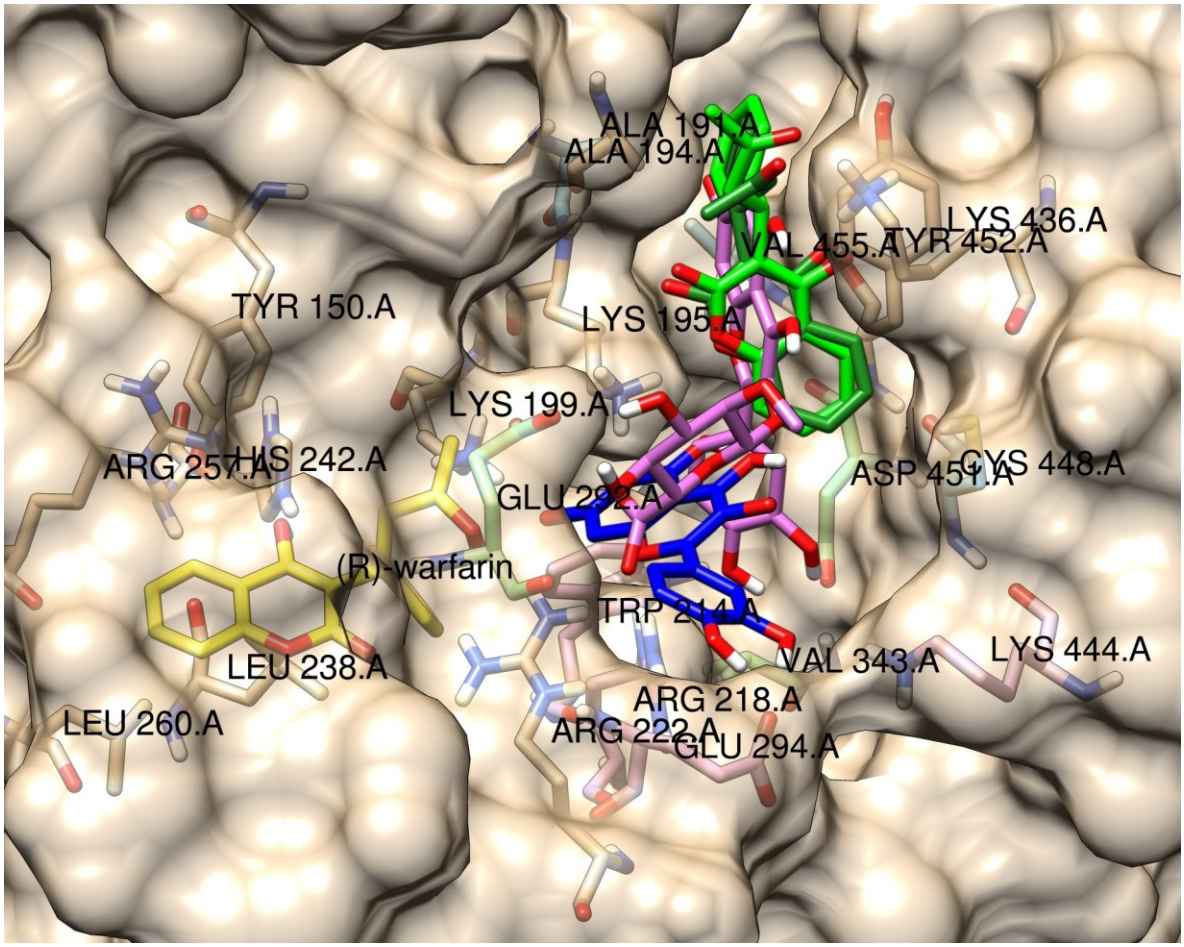


Slika 23. Usporedba usidrenih položaja specija kvercetin-3-*O*-glukuronida s obližnjim aminokiselinskim ostacima. A. Anionske specije kvercetin-3-*O*-glukuronida, B. Fluorescentne anionske specije kvercetin-3-*O*-glukuronida.

Rezultati sidrenja i (*R*)- i (*S*)-varfarina s ciljem pronalaženja potencijalnog LAS-a varfarina (tablica 6) su pokazali (praktički jednoglasno za sva sidrenja za oba enantiomera) visoku sklonost za područje u blizini Ala 191, Lys 195 i Tyr 452, aminokiselinskih ostataka koji također ulaze u interakciju s kvercetin-3-*O*-glukuronidom pri njegovom vezanju za HSA, ali nemaju veliku značajnost u vezanju kvercetina (slika 24). Može se zaključiti da varfarin vezanjem za svoj LAS može ući u kompeticiju s glikozidima flavonoida tijekom vezanja za HSA, s obzirom da se položaji kumarinskog i flavonoidnog AC prestena preklapaju. To može objasniti niži intenzitet fluorescencije glikozida flavonoida pri visokim koncentracijama varfarina. S druge strane, varfarin vezan za svoj LAS ne ulazi u interakcije s aglikonima flavonoida. Međutim, blizina konjugiranog AC prstena i varfarinskog kumarinskog prstena može objasniti niži intenzitet fluorescencije kvercetina pri visokim koncentracijama varfarina pomoću prijenosa energije. Luteolin je manje pod utjecajem ovog prijenosa energije u smislu relativne promjene intenziteta fluorescencije (slika 14), ali je njegov apsolutni intenzitet fluorescencije općenito znatno niži od intenziteta fluorescencije kvercetina zbog nedostatka 3-OH skupine.

Tablica 6. Sažetak podataka sidrenja (*R*)- i (*S*)-varfarina za LAS.

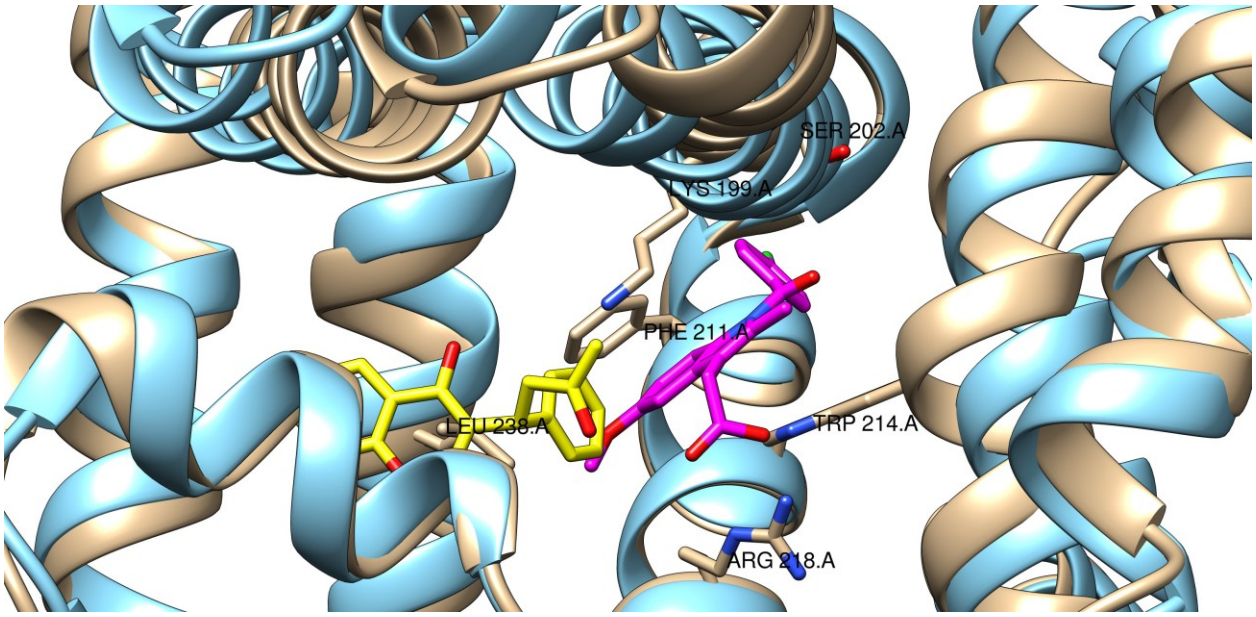
(*R*)-varfarin			(*S*)-varfarin		
Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)	Rang grozda	Broj sidrenja	Energija vezanja (kcal/mol)
1.	9	–6,70	1.	27	–6,70
2.	6	–6,52	2.	6	–6,56
3.	4	–6,49	3.	5	–6,51
4.	35	–6,28	4.	15	–6,42
5.	4	–6,27	5.	2	–6,31



Slika 24. Usporedba kristalografskih podataka (*R*)-varfarina (žuto) s usidrenim položajem (*R*)- i (*S*)-varfarina na potencijalnom LAS-u (oboje zeleno), fluorescentnim anionom kvercetina u položajima 3 i 7 (plavo) i fluorescentnim anionom kvercetin-3-*O*-glukuronida na položaju 7 (ružičasto) s obližnjim aminokiselinskim ostacima.

4.6. Molekulsko modeliranje ternarnih kompleksa HSA-indometacin-kvercetin i HSA-indometacin-kvercetin-3-*O*-glukuronid

Uz simulacije sidrenja kompleksa kvercetina i kvercetin-3-*O*-glukuronida s i bez varfarina, provedena je usporedba kristalografskih struktura 2BXD (svijetlo plavo) i 2BXM (sivo) (slika 25). S obzirom na razliku u tercijarnoj strukturi, koja je posljedica prisustva molekula miristinske kiseline, uzete su samo koordinate molekula indometacina te nadodane u rezultate sidrenja kvercetina i kvercetin-*O*-glukuronida na temelju strukture 2BXD.

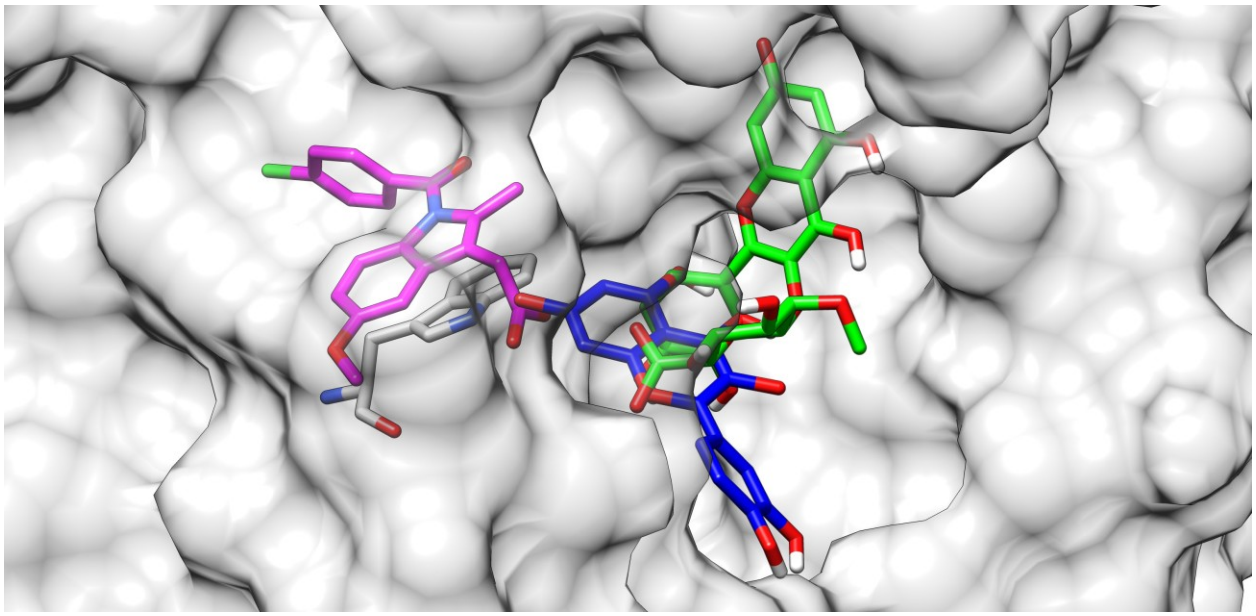


Slika 25. Preklapanje kristalografskih struktura 2BXD i 2BXM s vezanim molekulama (*R*)-varfarina (žuto) i indometacina (ljubičasto) te aminokiselinama s kojima indometacin dolazi u kontakt.

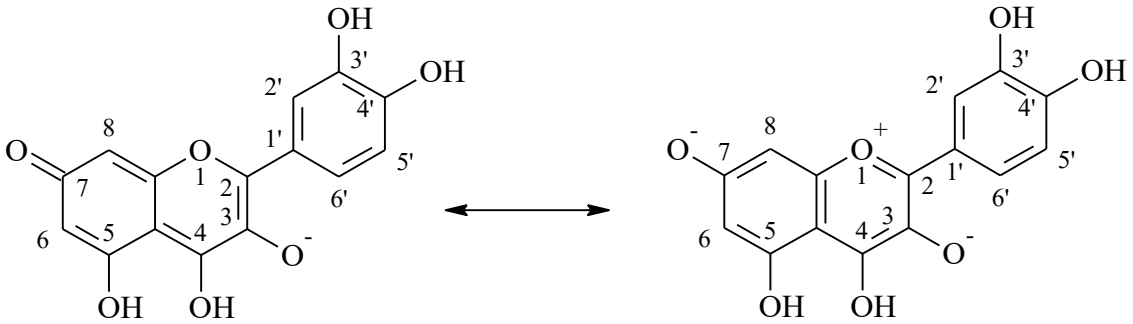
Kao što se može vidjeti na slici 25, vezno područje indometacina je znatno bliže Trp 214 te vezno mjesto I može istovremeno za sebe vezati i molekulu varfarina i molekulu indometacina. Molekula indometacina ostvaruje kontakte s aminokiselinskim ostacima Lys 199, Ser 202, Phe 211, Trp 214, Arg 218, Leu 238, od kojih varfarin također dolazi u kontakt s Lys 199 i Leu 238 (slika 21).

Analogno slici 22, na slici 26 se može uočiti preklapanje kristalografske strukture indometacina s usidrenim fluorescentnim anionima kvercetina i kvercetin-3-*O*-glukuronida. Može se uočiti kako se indolski prsten indometacina nalazi antiparalelno s obzirom na indolski prsten Trp 214 te između njih dolazi do π-π interakcija. Također, karboksilna se skupina

indometacina nalazi u blizini hidroksilne, odnosno okso skupine kvercetina u položaju 7 (slika 27). S obzirom na položaj indometacina u ovakvom ternarnom kompleksu, može doći do interakcija između indometacina i kvercetina pri čemu se kvercetin konformacijski stabilizira te dolazi do povišenja njegovog F_{max}.



Slika 26. Preklapanje kristalografske strukture indometacina (ljubičasto) s usidrenim fluorescentnim anionima kvercetina (plavo) i kvercetin-3-*O*-glukuronida (zeleno) i prikazanim Trp 214.



Slika 27. Prikaz tautomernih oblika fluorescentnog aniona kvercetina [100].

S druge strane, iz slike 26 je također vidljivo da indometacin ne ulazi ni u kakve interakcije s kvercetin-3-*O*-glukuronidom, što objašnjava činjenicu da prisutnost indometacina ne utječe na fluorescenciju kvercetin-3-*O*-glukuronida (slika 17B).

4.7. Procjena rizika interakcija između flavonoida i odabranih ksenobiotika pri vezanju na HSA

S obzirom na utvrđenu ovisnost F_{max} i c_{max} različitih flavonoida o zasićenosti veznog mjesta u poddomeni IIA odabranim ksenobioticima, može se reći kako je to vezno mjesto izrazito veliko i prilagodljivo te je u mogućnosti istovremeno na sebe vezati i više liganada. U svim slučajevima u kojima je došlo do potencijalnih interakcija (određenih na temelju promjena u F_{max} i c_{max}), koncentracije i lijekova i flavonoida su bile puno više od fiziološki ostvarivih. S obzirom da koncentracije flavonoida u krvi dosežu koncentracije od nekoliko μM [46,54,55], a koncentracija HSA kod zdravih ljudi iznosi 500–750 μM [1], to također ide u prilog tom zaključku. Neovisno o tome, kod nekih lijekova (indometacin) je utvrđena interakcija s kvercetinom i pri nižim koncentracijama te je potrebno bolje objasniti prirodu njihove interakcije, ali se čini da ni u ovom slučaju ne dolazi do istiskivanja.

S druge strane, na temelju dobivenih rezultata reakcije istiskivanja se ne smiju olako odbaciti kao klinički neznačajne. Postoji veliki broj slučajeva gdje i pri nižim koncentracijama dolazi do međusobnih reakcija istiskivanja lijek-lijek, iako su rijetke. Takve reakcije su, kao što je već spomenuto, između varfarina i klofibrata [3] te varfarina i kloral hidrata [63], ali i ceftriaksona i probenecida [137], valproata i fenitoina s acetilsalicilnom kiselinom [138], fenitoina s antibioticima [139] i dr. Češće su interakcije lijekova s fiziološkim ligandima HSA, naročito s masnim kiselinama [68,140–144], ali i s hemom, bilirubinom [3,32,34,145–147] te s patofiziološkim ligandima, kao što su indoksil sulfat, indol-3-acetat, hipurna kiselina i CMPF, koji se u tijelu također mogu nalaziti u značajnim koncentracijama [11,32,34,148,149]. U tim će slučajevima doći do smanjenja sveukupnog kapaciteta HSA za vezanje lijekova te će se na taj način povećati njihova slobodna koncentracija, što će posljedično najčešće dovesti do njihovog bržeg izlučivanja, ali moguće i do neželjenih nuspojava.

4.8. Procjena mogućnosti razvijanja rutinske metode za određivanje postotka zasićenosti veznog mjesta IIA HSA pomoću flavonoida

Iako je kod nekih lijekova pokazana ovisnost F_{max} i c_{max} flavonoida o postotku zasićenosti veznih mjesta u poddomeni IIA, ta metoda nije primjenjiva kao opći test procjene postotka slobodnih veznih mjesta iz dva razloga: 1) Vezno mjesto u poddomeni IIA je preveliko i previše prilagodljivo da bi se, bilo aglikonima, bilo glikozidima flavonoida uspjelo ispitati vezanje drugih spojeva u cijelom veznom mjestu. 2) Interakcije između lijekova i flavonoida ne moraju nužno smanjivati F_{max} i povisivati c_{max} flavonoida, što komplicira, ako ne i onemogućuje određivanje postotka slobodnih veznih mjesta s obzirom da jedan lijek može smanjivati, a drugi istovremeno povisivati F_{max}.

Iz svega navedenog, fluorescencija flavonoida bi se mogla koristiti kao indikator zasićenosti veznog mjesta I za pojedine lijekove kod kojih je ovisnost F_{max} flavonoida o koncentraciji lijeka dobro utvrđena, ali za primjenu ove metode za procjenjivanje postotka zasićenosti veznih mjesta u uvjetima gdje je prisutna polifarmakoterapija, potrebno je pronaći molekulu koja će pokrivati veće područje veznog mjesta I i koja će biti osjetljivija na vezanje drugih spojeva u svojoj blizini. Također, takva indikatorska molekula ne bi smjela apsorbirati ni emitirati svjetlost pri valnim duljinama apsorpcije ili emisije drugih fluorescentnih spojeva u plazmi, primjerice bilirubina. Isto tako, razrijeđivanje plazme bi se trebalo izbjeći ako je moguće jer se uz istu količinu liganda postotak zasićenosti veznog mjesta smanjuje povećanjem razrijeđenja plazme.

5. ZAKLJUČCI

Flavonoidi se, kao i većina lijekova, vežu za vezno mjesto u poddomeni IIA HSA (vezno mjesto I). U ovom su doktorskom radu korištene metode *in vitro* fluorescencijske spektroskopije ravnotežnog stanja i molekulskog modeliranja (DFT proračuni i sidrenje) kako bi se bolje razumjela svojstva vezanja veznog mjesta I, utvrdila strukturna svojstva flavonoida odgovorna za njihovo vezanja za vezno mjesto, objasnile njihove interakcije s različitim ksenobioticima, vjerojatnost istiskivanja lijekova s HSA pomoću flavonoida te ispitala mogućnost korištenja flavonoida za određivanje postotka zasićenosti veznog mjesta I.

Zbog vezanja velikog broja ksenobiotika, ali i endobiotika te posljedično mogućih interakcija istiskivanja, potrebno je bolje opisati vezno mjesto I. Na taj bi se način moglo bolje utvrditi koje strukturna svojstva molekula utječu na njihovo vezanje za ovo vezno mjesto. Na modelu aglikona flavonoida je pronađeno više strukturnih odlika koje su zaslužne za njihovo vezanje na vezno mjesto I. 1) Nukleofilnost i parcijalni naboj atoma C3 omogućava klasifikaciju flavonoida u podskupine: flavanoni (jako niska nukleofilnost i visoki negativni parcijalni naboj), izoflavoni (niska nukleofilnost i niski negativni parcijalni naboj), flavoni (srednje visoka do visoka nukleofilnost i srednje visoki negativni parcijalni naboj) i flavonoli (srednje visoka nukleofilnost i pozitivni parcijalni naboj). 2) Povišeni negativni parcijalni naboj atoma O4 je snažno povezan s konstantom vezanja za HSA te oslikava dobra proton-donorska i proton-akceptorska svojstva flavonoida. 3) Koplanarnost A i C prstena je povezana s višim konstantama vezanja i to je preduvjet za vezanje flavonoida u hidrofobnoj šupljini. Nadalje, 4) koplanarnost AC i B prstena oslikava sklonost flavonoida stvaranju vodikovih veza, što je u skladu s 5) visokim HOMO i LUMO energijama. S druge strane, konjugacija ni AC ni B prstena nije bila povezana s jačinom vezanja flavonoida s HSA, ali negativni naboj B prstena ima učinak na konstantu vezanja.

Također su ispitane i interakcije odabranih flavonoida i ksenobiotika (varfarina, indometacina, piroksikama, furosemida i mikofenolat mofetila) pri vezanju za vezno mjesto I. Utvrđeno je kako varfarin i flavonoidi nemaju isti HAS unutar veznog mjesta I te da dolazi do neznatnog smanjenja F_{max} flavonoida vjerojatno zbog alosteričke promjene konformacije HSA uslijed vezanja varfarina. Pri većim koncentracijama varfarina, koje nisu ostvarive u uvjetima *in vivo*, dolazi do značajnijeg pada F_{max}, moguće zbog vezanja varfarina za LAS. Slične rezultate interakcija s kvercetinom i kvercetin-3-*O*-glukuronidom pokazuju i piroksikam i furosemid, uz postojanje razlika u koncentracijama pri kojima se navedeni lijekovi vežu za svoj LAS i ulaze u

značajnije interakcije s flavonoidima. U slučaju interakcija s mikofenolat mofetilom, uočava se povišenje F_{max} kvercetina pri visokim koncentracijama mikofenolat mofetila, što je vjerojatno posljedica vezanja mikofenolat mofetila na LAS u blizini veznog područja kvercetina. Pri tome se kvercetin stabilizira u konformaciji koja ima fluorescentna svojstva. U slučaju interakcija kvercetina s indometacinom, uočeno je značajno povišenje F_{max} kvercetina koje je bilo proporcionalno postotku zasićenja veznog mjesta I indometacinom. Ova činjenica se objašnjava interakcijom indometacina i kvercetina pri vezanju na njihove HAS-ove. Do porasta F_{max} kvercetina dolazi zbog bliskog položaja indometacina i kvercetina u veznom mjestu I, što je potvrđeno i metodama sidrenja, a posljedica je ili stabiliziranja kvercetina u fluorescentnoj konformaciji ili u prijenosu energije s indometacina na kvercetin. Slični fenomeni kod kvercetin-3-*O*-glukuronida nisu uočeni zbog različitog veznog područja aglikona i glikozida u veznom mjestu I, pri čemu se aglikoni vežu dublje u hidrofobnom džepu.

Što se tiče mogućnosti klinički značajnih interakcija istiskivanja između lijekova i flavonoida, utvrđeno je da je između ispitivanih flavonoida i lijekova ta vjerojatnost niska, odnosno da do interakcija dolazi pri vrlo visokim koncentracijama, koji nisu ostvarive u fiziološkim uvjetima. S druge strane, potrebno je naglasiti da nije ispitivana mogućnost interakcija na razini inhibicije i indukcije enzima, isto kao i da postoje primjeri klinički značajnog istiskivanja lijekova od strane (pato)fizioloških liganda.

S obzirom da je kod nekih lijekova utvrđena ovisnost F_{max} flavonoida o postotku zasićenosti veznog mjesta I određenim lijekom, upotreba flavonoida kao indikatora zasićenosti veznog mjesta je moguća. Međutim, upotreba flavonoida kao općeg indikatora zasićenosti veznog mjesta I u optimizaciji polifarmakoterapije nije moguća jer se neki lijekovi vežu za ovo vezno mjesto bez utjecaja na fluorescenciju flavonoida, dok pri interakcijama drugih lijekova s flavonoidima dolazi do snižavanja F_{max}, a u nekim slučajevima i do povišenja F_{max} flavonoida, što onemogućuje procjenu zasićenosti veznog mjesta. U ovom je slučaju potrebno pronaći molekulu koja pokriva više različitih veznih područja od flavonoida i čiji se rezultati mogu jednoznačno objasniti.

6. POPIS KRATICA I SIMBOLA

2D – dvodimenzijski

3D – trodimenzijski

ε – molarni apsorpcijski koeficijent

APT – engl. *Atomic Polar Tensor*, atomski polarni tenzor

c_{max} – koncentracija potrebna da se postigne maksimalni intenzitet fluorescencije flavonoida

CMPF – 3-karboksi-4-metil-5-propil-2-furanpropionat

DFT – engl. *Density Functional Theory*, teorija funkcionala gustoće

DMSO – dimetilsulfoksid

FA – engl. *Fatty acid*, masna kiselina

FDH – engl. *Familial Dysalbuminemic Hyperthyroxinemia*, obiteljska disalbuminemična hipertiroksinemija

F_{max} – maksimalni intenzitet fluorescencije

Gal – galaktoza

Glc – glukoza

Gluc – glukuronska kiselina

HOMO – engl. *Highest Occupied Molecular Orbital*, najviša popunjena molekulska orbitala

HAS – engl. *High Affinity Site*, mjesto vezanja visokog afiniteta

HSA – humani serumski albumin

ICD – inducirani cirkularni dikroizam

K_A – konstanta vezanja

LAS – engl. *Low Affinity Site*, mjesto vezanja niskog afiniteta

LGA – engl. *Lamarckian Genetic Algorithm*, lamarkijanski genetski algoritam

LUMO – engl. *Lowest Unoccupied Molecular Orbital*, najniža nepopunjena molekulska orbitala

N/A – podaci nisu dostupni

NBO – engl. *Natural Bond Orbital*, prirodna vezna orbitala

NPA – engl. *Natural Population Analysis*, analiza prirodne populacije

NSAID – engl. *NonSteroidal Anti-Inflammatory Drug*, nesteroidni protuupalni lijek

PDB – engl. *Protein Data Base*, baza podataka proteina

Rha – ramnoza

RMSD – engl. *Root-Mean-Square-Deviation*, korijen srednje kvadratne devijacije

SA – engl. *Simulated Annealing*, simuliranje traženje globalnog minimuma funkcije

SAR – engl. *Structure-Activity/Affinity Relationship*, povezanost strukture i aktivnosti/afiniteta

SMD – engl. *Solvation Model based on Density*, solvatacijski model temeljen na gustoći

STD-NMR – engl. *Saturation Transfer Difference Nuclear Magnetic Resonance*, nuklearna magnetska rezonanca razlike prijenosa zasićenja

SVD – engl. *Singular Value Decomposition*, rastav singularnih vrijednosti

$\boldsymbol{t_{1/2}}$ – poluvrijeme eliminacije

TDM - engl. *Therapeutic Drug Monitoring*, terapijsko praćenje lijeka

$\boldsymbol{V_d}$ – volumen distribucije

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8. PRILOZI

Ovaj dodatak sadrži tri znanstvena rada objavljena u časopisima zastupljenim u bazama Current Contents koji obrađuju problematiku iznesenu u ovom doktorskom radu:

1. H. Rimac, Ž. Debeljak, D. Šakić, T. Weitner, M. Gabričević, V. Vrček, B. Zorc, M. Bojić. Structural and electronic determinants of flavonoid binding to human serum albumin: An extensive ligand-based study, RSC Adv. 6 (2016) 75014–75022.
2. H. Rimac, Ž. Debeljak, M. Bojić, L. Millerd. Displacement of drugs from human serum albumin: from molecular interactions to clinical significance, Curr. Med. Chem. 24 (2017) 1–18.
3. H. Rimac, C. Dufour, Ž. Debeljak, B. Zorc, M. Bojić. Warfarin and flavonoids do not share the same rinding region in binding to the IIA subdomain of human serum albumin, Molecules 22 (2017) 1153.

PRILOG I

H. Rimac, Ž. Debeljak, D. Šakić, T. Weitner, M. Gabričević, V. Vrček, B. Zorc, M. Bojić. Structural and electronic determinants of flavonoid binding to human serum albumin: An extensive ligand-based study, RSC Adv. 6 (2016) 75014–75022.

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Structural and electronic determinants of flavonoid binding to human serum albumin: an extensive ligand-based study

Hrvoje Rimac,[a] Željko Debeljak,[bc] Davor Šakić,[a] Tin Weitner,[a] Mario Gabričević,[a] Valerije Vrček,[a] Branka Zorc[a] and Mirza Bojić*[a]

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Flavonoids are ubiquitous plant metabolites that interfere with different biological processes in the human organism. After absorption they bind to human serum albumin (HSA), the most abundant carrier protein in the blood which also binds various hormones and drugs. Binding of flavonoids to HSA may impact their distribution, influencing the active concentration in the blood. To determine the most prominent features responsible for binding of 20 different flavonoid aglycones to the IIA region of HSA, *in vitro* fluorescence measurements and density functional theory (DFT) calculations were conducted. These results were then integrated to elucidate structure–affinity relationships. The presented results reveal that flavones and flavonoles bind most strongly to the IIA region of HSA. There are several electronic and structural determinants associated with flavonoid binding to this HSA region: high C3 nucleophilicity and partial charge of O4, high HOMO and LUMO energies, and coplanarity of AC and B rings. Both steric and electronic characteristics of flavonoids have a great impact on their binding to HSA, with hydrogen donor and acceptor properties and coplanarity being the most prominent.

1. Introduction

Human serum albumin (HSA) is the most abundant protein in human plasma (60%, w/w),[1] which structure has been determined crystallographically. It is a 585-residue monomeric protein comprised of three homologous domains (I–III), each of which is composed of two subdomains (A and B) (Fig. 1).[2] These domains play a central role in binding of various endo- and exogenous compounds, particularly hydrophobic organic anions of medium size (100 to 600 Da), *e.g.* bilirubin, long-chain fatty acids, hematin, thyroxin.[3] Many drugs bind to one of the two primary binding sites, located in subdomains IIA and IIIA (Sudlow sites I and II, respectively), with IIA being the most prominent one.[4,5] The IIA subdomain appears to be spacious and is comprised of several individual binding sites which can accommodate ligands characterized by very different chemical structures. The IIIA site is smaller and less flexible and thus can accommodate only structurally similar ligands.[6]

A large number of drugs characterized by narrow therapeutic windows, such as warfarin,[7] amlodipine,[8] various antiepileptic drugs[9,10] *etc.* bind to HSA. It has been demonstrated that their binding significantly influences their distribution, free blood concentration and metabolism,[9,11] primarily in kidney and liver patients.[12] Apart from drugs, various other exogenous compounds bind to HSA as well, such as flavonoids. Flavonoids are a group of phenolic compounds, ubiquitous in fruits and vegetables.[13] They have many salutary properties, among which antioxidative properties are best described.[14] They also help to prevent lipid peroxidation[15] and cardiovascular diseases.[16–18] Once they reach circulation, most of these compounds bind in subdomain IIA of HSA,[19–22] with flavanones being an

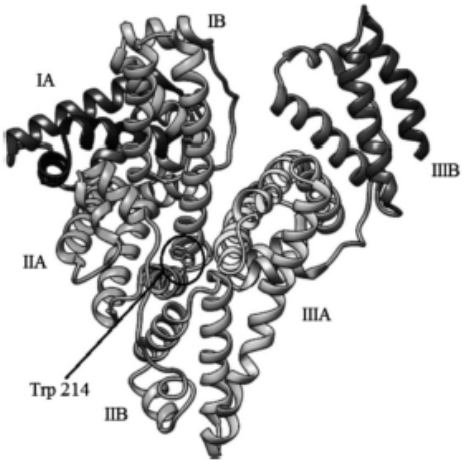

Fig. 1 Structure of HSA (PDB entry 1AO6).

[a]*University of Zagreb, Faculty of Pharmacy and Biochemistry, Department of Medicinal Chemistry, A. Kovačića 1, HR-10000 Zagreb, Croatia. E-mail: mbojic@pharma.hr*
[b]*Clinical Hospital Center Osijek, Osijek, Croatia*
[c]*J.J. Strossmayer University of Osijek, Faculty of Medicine, Osijek, Croatia*

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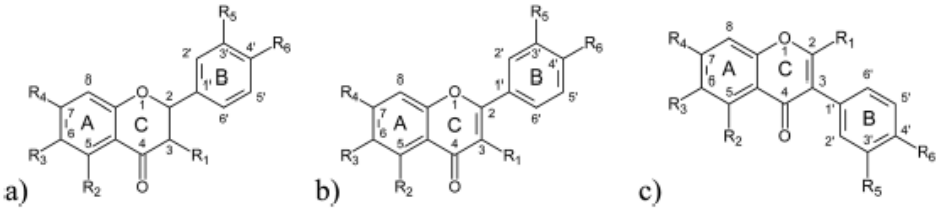

Fig. 2 Structural formulae of investigated classes of flavonoids: (a) flavanones, (b) flavones and flavonols, (c) isoflavones.

exception.[23] When flavonoid binds to the same site as a hormone, drug or even a toxin;[24] the displaced compound's free concentration in blood may increase. This can lead to changes in pharmacodynamics and pharmacokinetics of the compound in question, such as enhanced pharmacological or adverse effects and its faster elimination.

Binding of flavonoids to HSA has been extensively studied using different fluorescence spectroscopy techniques,[19,20,23,25–30] circular dichroism,[21,25,27,29,30] and molecular modeling.[21,25–30] Binding of ligands in the IIA subdomain of HSA can be monitored by measuring the fluorescence intensity of the Trp 214 residue. Change in tryptophan fluorescence intensity is observed when a ligand binds to HSA in its vicinity. The observed change in fluorescence enables the calculation of HSA–ligand complexes stability constants and also the distance between the ligand and Trp 214.[31]

The IIA subdomain of HSA is comprised of a neutral hydrophobic cavity and a positively charged hydrophilic part.[2,32] At physiological pH, the B ring of flavonoids (Fig. 2) possess a partial negative charge, while chromene part (rings A and C) is hydrophobic, creating ideal conditions for flavonoid binding.[33] This has been modeled by docking studies of flavone luteolin,[27] flavonol quercetin,[21] and isoflavone daidzein.[25,26,34] These studies only included few structurally similar flavonoids, so general structure–affinity relationship of HSA–flavonoid binding have not yet been determined.

To determine a common structure–affinity relationship of HSA–flavonoid binding 20 flavonoid aglycones were chosen in this study. *In vitro* binding constants of these compounds were determined using fluorescence spectrophotometry and their geometries and electronic properties were calculated at the density functional theory (DFT) level of theory. B3LYP model was used to determine electronic and structural features of flavonoids that may influence their binding to the IIA subdomain and effects of solvation have been evaluated. To the best of our knowledge, this is the most extensive study of HSA–flavonoid binding. Significance of obtained results is more accentuated by the fact that in the recent years flavonoid scaffolds have been recognized as privileged scaffolds in drug discovery.[35,36]

2. Materials and methods

2.1. Materials

Fatty acid free HSA was purchased from Sigma-Aldrich, USA (purity ≥ 96%). Flavonoids were obtained from Extrasynthèse, France (chrysin dimethylether, diosmetin, fisetin, formononetin, genistein, pinocembrin-7-methylether, prunetin, sakuranetin, and tamarixetin), ChromaDex, USA (3,6-dihydroxyflavone, 3,7-dihydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, and flavanone), Sigma-Aldrich, USA (apigenin, chrysin, flavone, galangin, and quercetin), and BioChemika, Switzerland (rhamnetin). All flavonoid standards had a specified purity of ≥98%.

2.2. *In vitro* fluorescence measurements

Albumin was dissolved daily before measurements in Dulbecco's phosphate-buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, 1.47 mM potassium dihydrogen phosphate).[37] Flavonoids were dissolved in DMSO. A series of solutions were made for each HSA–flavonoid combination: the concentration of HSA was held constant at 1 μM and the flavonoid concentration ranged 0.03–10 μM. In all experiments the maximal DMSO concentration was 4% v/v. The effect of DMSO as a co-solvent was annulated by adding a small aliquot to a pure HSA solution.

Steady-state fluorescence spectra were recorded on the OLIS RSM 1000F spectrofluorimeter (Olis Inc., Bogart, GA, USA) equipped with a thermostated cell holder at 25 °C. Hellma Analytics 105.253-QS fluorescence cells with a light path of 10 × 2 mm (excitation × emission) were used. Excitation wavelength was 280 nm (albumin absorption maximum) and the emission spectra were recorded in the range 310–370 nm, where only HSA has fluorescent properties, with the observed maximum at 340 nm. All studies were performed in duplicate at 25 °C using 1.24 mm excitation and emission slit widths. All solutions were analyzed after 2 hours incubation period.

In the studied wavelength range flavonoids absorb light as well. Based on the molar absorbance coefficients at 280 and 340 nm, the inner filter effect cannot be neglected.[38] The observed fluorescence, F_{obs}, has been corrected to F_{corr} according to eqn (1), where $A_{ex} = \varepsilon_{280} \times c \times l$ is absorbance at the excitation wavelength (c is flavonoid concentration and $l = 1$ cm) and $A_{em} = \varepsilon_{340} \times c \times l$ is absorbance at the emission wavelength ($l = 0.2$ cm):

$$F_{corr} = F_{obs} e^{\left(\frac{A_{ex}+A_{em}}{2}\right)} \quad (1)$$

2.3. Calculation of binding constants

Each spectrum obtained during titration is an average of 10 000 fluorescence spectra measured in 10 seconds. Equilibrium constants were calculated by a global fit at all wavelengths with SPECFIT[39–42] software. A single significant spectrally active

species was suggested by singular value decomposition analysis and attributed to the known spectrum of HSA. This analysis also suggested 1-to-1 complex formation and did not indicate any higher order complexes. Therefore, the proposed binding model is given by eqn (2) and (3), where K_A is the association constant of the complex:

$$\text{HSA} + \text{flavonoid} \xrightleftharpoons{K_A} \text{HSA} - \text{flavonoid} \quad (2)$$

$$K_A = \frac{[\text{HSA} - \text{flavonoid}]}{[\text{HSA}][\text{flavonoid}]} \quad (3)$$

The association constant values calculated from fluorescence titrations for the suggested HSA–flavonoid complexes are given in $\text{mol}^{-1}\ \text{dm}^3$. Higher values of K_A indicate stronger binding and higher complex stability. In all cases, $\log K_A$ was calculated.

2.4. DFT calculations

Quantum chemical calculations on flavonoids were performed using the Gaussian09 program.[43] All structures (neutral and anionic forms, where available) were optimized with B3LYP functional,[44,45] using Pople's 6-31G(d) and 6-311++G(d,p) basis sets.[46,47] B3LYP functional was selected as one of the most used functional for initial screening of large molecules, due to good precision/performance ratio. Flavonoid structures were recently explored using this method.[48,49] Analytical vibrational analysis was performed at the corresponding levels to characterize each stationary point on the potential energy surface as a minimum (NImag = 0).

Structures of flavones, flavonols, and isoflavones are simple in terms of conformational flexibility. The main parameter which governs the structural diversity is a dihedral angle along C2–C1′ (flavones and flavonols) or C3–C1′ (isoflavones). Dihedral scans were calculated through relaxed scanning of potential energy surfaces at 30° intervals. In this scanning procedure, for each change of the corresponding torsional angle, the structure is fully optimized for all degrees of freedom, affording unconstrained minima to be located. In all global minima, aka lowest lying energy minimum for each flavonoid structure, corresponding dihedral angles are around 0°. An additional contribution to the coplanarity between AC and B rings of flavones, flavonols, and isoflavones comes from hydroxyl/methoxy groups which are rotated to form intramolecular hydrogen bonds, in corresponding global minima. All global minima located at the B3LYP/6-311++G(d,p) level are in agreement with calculated data reported earlier.[50,51]

In case of flavanones the same conformational procedure was followed, with special attention to the pyranone ring C which adopts a slightly modified sofa conformation in all global minimum structures. These results match the earlier conformational searches on flavanones.[52,53]

Boltzmann distribution was used to identify the conformers that have more than 5% ratio in total population (based on Gibbs free energy and room temperature), and those structures have been included in further analysis.

To properly model solvent effects in aqueous medium (dielectric constant of $\varepsilon = 78.4$), three different methods were included: (a) implicit solvation using Solvation Model based on Density (SMD),[54] where water is treated as a continuum, (b) explicit solvation that includes one water molecule placed in the vicinity of the most electronegative atom, and (c) supramolecular approach where both implicit and explicit solvation is included.[55] Due to high similarity between *in vacuo* and various solvation models, in the remainder of the text only the *in vacuo* and the explicit solvation are commented. Explicit solvation can also be considered as complexation, and since the IIA binding site is highly hydrophobic,[2] it represents the most likely influence of water on the HSA–flavonoid system. If not mentioned otherwise, results refer to *in vacuo* data.

Natural bond orbital (NBO) analysis was performed using NBO 3.1 program[56] included in the Gaussian09 package. NBO analysis (NPA values) and population analysis (Mulliken and APT values) of substituent functional groups were obtained by summarizing individual charge/population on each atom in the functional group. Fukui parameters (electrophilicity and nucleophilicity) were calculated using single-point NBO and population analysis calculations on corresponding $N - 1$ and $N + 1$ (N equals number of electrons) systems.

To facilitate quantitative comparison between different sites, the condensed Fukui function[57–59] based on atomic charges was calculated. NPA charges from NBO analysis were used. Charges (q) were calculated for all flavonoids in their N, $N + 1$, and $N - 1$ electrons states to obtain the condensed f^- and f^+ descriptors according to equations for the nucleophilicity (4) and electrophilicity (5):

$$f_A^- = q_A(N) - q_A(N - 1) \quad (4)$$

$$f_A^+ = q_A(N + 1) - q_A(N) \quad (5)$$

where $q_A(N)$ is the calculated charge on atom A for N total electrons. The $N - 1$ corresponds to the number of electrons in the neutral molecule, with an electron removed from the HOMO of the anion, and the $N + 1$ corresponds to the number of electrons in the respective cation system.

All statistical calculations have been done by Statistica 7.0 (Statsoft, USA). p is a measure of statistical significance: p values below 0.05 were considered as significant throughout the text. Univariate linear regression and 2D contour plots have been used in some instances to describe relationship between HSA binding affinity and different structural and electronic features of flavonoids.

3. Results and discussion

3.1. Determination of binding constants

General structural formulas (Fig. 2) and binding constants of the investigated flavonoids are shown below (Table 1). In general, flavanones are characterized by the lowest binding constants, followed by isoflavones. Flavones and flavonols have the highest values of binding constants. Determination of apigenin binding constant (Table 1, entry 6) is shown in Fig. 3.

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Table 1 Binding constants of investigated flavonoids

#	Flavonoid	R_1	R_2	R_3	R_4	R_5	R_6	K_A [M^{-1}]	K_A [M^{-1}] from literature	Reference
Flavanones										
1	Flavanone	H	H	H	H	H	H	$(5.25 \pm 0.79) \times 10^3$	N/A	N/A
2	Pinocembrin-7-methylether	H	OH	H	OCH_3	H	H	$(2.19 \pm 0.09) \times 10^4$	N/A	N/A
3	Sakuranetin	H	OH	H	OCH_3	H	OH	$(2.19 \pm 0.04) \times 10^4$	N/A	N/A
Flavones										
4	6-Hydroxyflavone	H	H	OH	H	H	H	$(1.58 \pm 0.60) \times 10^4$	N/A	N/A
5	7-Hydroxyflavone	H	H	H	OH	H	H	$(1.95 \pm 0.10) \times 10^5$	9.44×10^4 to 3.82×10^5	61 and 62
6	Apigenin	H	OH	H	OH	H	OH	$(1.32 \pm 0.05) \times 10^5$	9.85×10^4 to 1.95×10^6	63 and 64[a]
7	Chrysin	H	OH	H	OH	H	H	$(1.95 \pm 0.08) \times 10^5$	1.82×10^5 to 3.09×10^6	62, 64[a] and 65
8	Chrysin dimethylether	H	OCH_3	H	OCH_3	H	H	$(2.95 \pm 0.15) \times 10^4$	N/A	N/A
9	Diosmetin	H	OH	H	OH	OH	OCH_3	$(8.91 \pm 0.18) \times 10^4$	5.28×10^4 to 1.18×10^5	19[b] and 66
10	Flavone	H	H	H	H	H	H	$(6.17 \pm 0.56) \times 10^4$	4.96×10^4	62
Flavonoles										
11	3,6-Dihydroxyflavone	OH	H	OH	H	H	H	$(7.41 \pm 0.15) \times 10^4$	5.28×10^4	67[c]
12	3,7-Dihydroxyflavone	OH	H	H	OH	H	H	$(1.66 \pm 0.05) \times 10^5$	$(1.51 \pm 0.23) \times 10^5$	68
13	Fisetin	OH	H	H	OH	OH	OH	$(1.20 \pm 0.05) \times 10^5$	$(1.38 \pm 0.02) \times 10^5$	69
14	Galangin	OH	OH	H	OH	H	H	$(2.34 \pm 0.09) \times 10^4$	$(3.80 \pm 0.91) \times 10^6$	64[a]
15	Quercetin	OH	OH	H	OH	OH	OH	$(1.70 \pm 0.03) \times 10^5$	3.1×10^4 to 3.31×10^5	19,[b] 34, 64,[a] 65 and 70–72
16	Rhamnetin	OH	OH	H	OCH_3	OH	OH	$(1.29 \pm 0.03) \times 10^5$	N/A	N/A
17	Tamarixetin	OH	OH	H	OH	OH	OCH_3	$(2.34 \pm 0.07) \times 10^4$	$(7.46 \pm 0.30) \times 10^4$	19[b]
Isoflavones										
18	Formononetin	H	H	H	OH	H	OCH_3	$(2.14 \pm 0.11) \times 10^4$	1.60×10^5	73
19	Genistein	H	OH	H	OH	H	OH	$(4.90 \pm 0.10) \times 10^4$	1.14×10^4 to 1.5×10^5	19,[b] 25, 71 and 74
20	Prunetin	H	OH	H	OCH_3	H	OH	$(3.80 \pm 0.14) \times 10^4$	N/A	N/A

[a] Calculations are based on competitive binding with warfarin using fluorescence anisotropy approach. [b] Calculated for bovine serum albumin (BSA) using fluorescence quenching approach. [c] Calculations are based on competitive binding with quercetin using fluorescence quenching approach. N/A not available.

Binding constants for other flavonoids were calculated in the same manner. Most of the binding constants are in accordance with data from previously published studies, with a few exceptions: for some flavonoids literature data could not be found. For 3,6-dihydroxyflavone and tamarixetin, there is a slight discrepancy with literature data, which could be explained by different techniques used to obtain their binding constants. The literature binding constant of 3,6-dihydroxyflavone is lower than the one obtained in this study, which can be explained by fact that 3,6-dihydroxyflavone is also slightly fluorescent under the same conditions, *i.e.* fluorescence quenching and displacement of quercetin could produce falsely decreased values. In the case of tamarixetin, discrepancies can be explained by the fact that albumins from two different species were used.[19] Greater differences are detected for galangin and formononetin. Published galangin binding constant comes from warfarin displacement experiments, where decrease in warfarin anisotropy, *i.e.* higher rotational freedom of warfarin was taken as a proof of warfarin displacement. As shown by Yamasaki *et al.*,[60] changes in rotational freedom are not necessarily equal to ligand displacement or binding. Similar reasoning can be applied in the case of formononetin. In some cases fluorescence quenching studies are performed at excitation wavelength of 280 nm (where both tryptophan and tyrosine residues absorb) and in another at 295 nm (where only the warfarin residue absorbs), which can also lead to discrepancies. These differences are not so pronounced, but even small discrepancies may compromise relative comparison of ligands, especially if their binding constants are not very different. Therefore, a study of binding constants conducted under the uniform conditions is a necessary prerequisite for a reliable determination of the structure–affinity relationship.

3.2. DFT calculations for structure–affinity relationship of flavonoid binding to HSA

At physiological pH analyzed flavonoids have a tendency to bind to the IIA binding site of HSA in the form of an anion. There are several key flavonoid properties that are associated with respective binding affinity: (1) C3 nucleophilicity and the partial charge linked to (2) the O4 partial charge, (3) electrophilicity of C8 substituent, (4) high HOMO and LUMO energies, and (5) coplanarity of both AC and B rings and A and C rings. Very similar results for structures optimized *in vacuo* and in the model solvent ($\varepsilon = 78.4$) were obtained.

3.2.1. C3 substitution. Major structural differences between four flavonoid aglycone classes included in the study are located at or near the C3 atom. It is expected that different

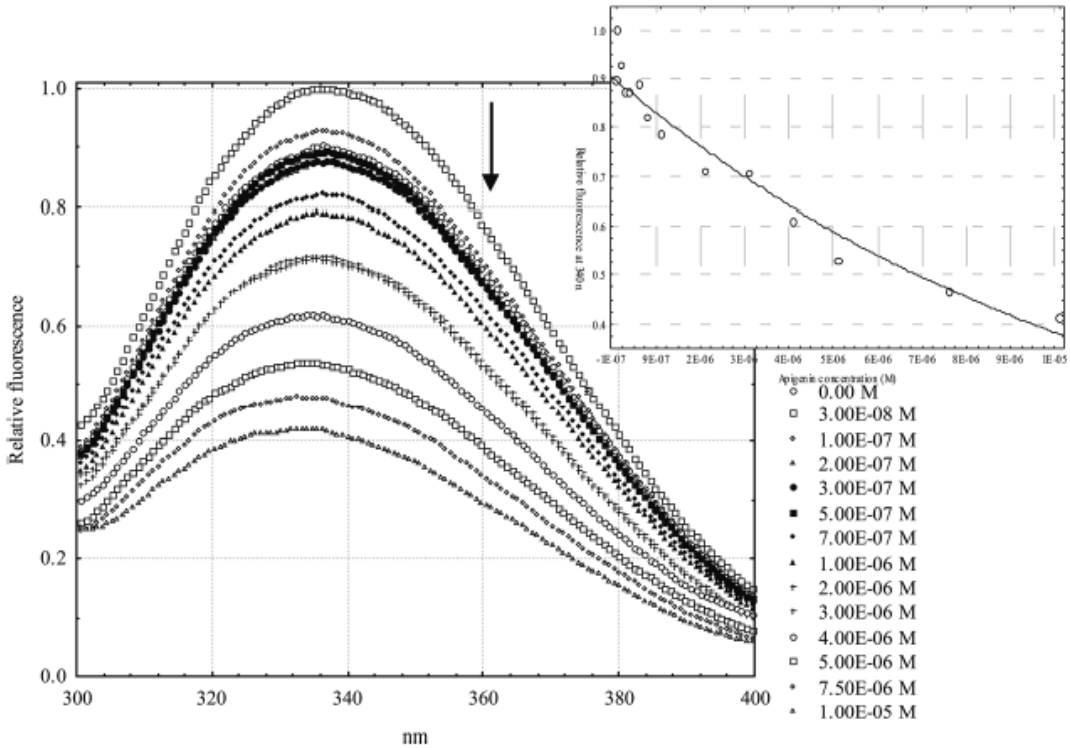

Fig. 3 Spectrofluorimetric titration of HSA with apigenin. Total concentration of HSA was held constant at 1 μM and total concentrations of apigenin varied from 0 (top spectra) to 10 μM (bottom spectra) incubated in pH 7.4 buffer at 25 °C. Inset: fitting curve at 340 nm.

C3 substitutions and bonds are reflected in the electronic distribution around the C3 atom.

According to Fig. 4, nucleophilicity and the partial charge of C3 atom enable separation of analyzed flavonoid classes which correlates with the affinity for HSA. Unfortunately these properties of the C3 atom do not enable within-group differentiation between molecules with high and low HSA affinity. It may be noticed that high C3 nucleophilicity induced by the presence of a hydroxyl group increases flavonoid affinity for HSA. Flavonols and flavones possess the highest affinity for HSA: alteration of steric properties due to substitutions at C2 and C3 atoms significantly reduces their affinity for HSA, as it is shown for isoflavones.[24]

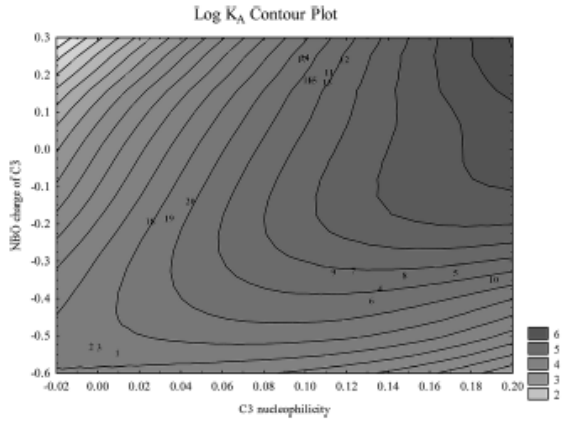

Fig. 4 Flavonoid aglycone classes depicted in space nucleophilicity and partial charge of C3 atom calculated at the B3LYP level (numbers correspond to flavonoids in Table 1).

3.2.2. Partial charge of the O4 atom. Electronic properties of the C3 atom are largely determined by substitutions of neighboring atoms, particularly by electron-withdrawing oxo group at C4 atom.

Fig. 5 reveals strong dependence of log K_A on partial charge of the O4: flavonoid affinity for HSA decreases with decreasing negative charge. This association underlines the general significance of polar interactions between O4 and HSA previously described for luteolin[27] and also possible interaction with the Lys 195 residue located nearby.[21] Among other types of polar interactions hydrogen bonding is as the most probable type of interaction: oxo-group attached to the C4 atom represents a good hydrogen bond acceptor that may increase the affinity for HSA.

3.2.3. Other substitutions. Some studies assigned a significant impact of polar interactions between the B ring substituents and HSA.[21,22,24,70,75] According to the literature, a negative charge of the 3′- or 4′-OH groups increases the binding constant. However, our study failed to reveal any association

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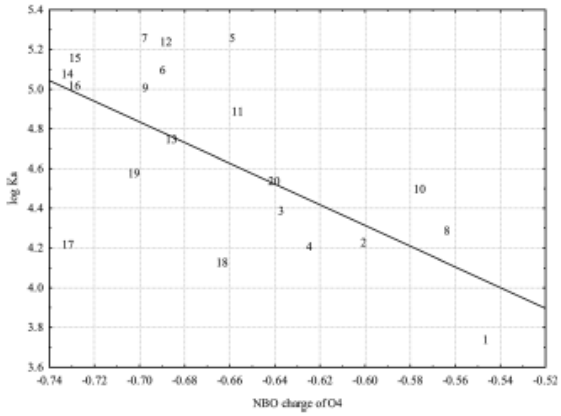

Fig. 5 Dependence of log K_A on O4 partial charge based on NBO analysis ($r = -0.6514$; $p = 0.0019$) calculated at the B3LYP level (numbers correspond to flavonoids in Table 1).

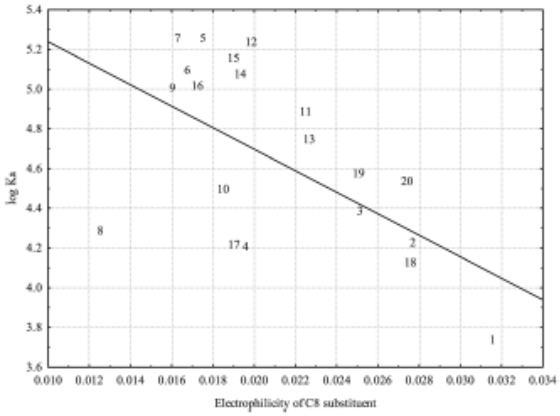

Fig. 6 Relationship between log K_A and electrophilicity of C8 substituent calculated at the B3LYP level ($r = -0.5862$; $p = 0.0066$) (numbers correspond to flavonoids in Table 1).

between the charge of the B ring and binding affinity, neither *in vacuo* ($r = -0.3742$; $p = 0.1041$) nor in the explicit solvation ($r = -0.4181$; $p = 0.0666$). Instead of analyzing properties of individual atoms, only the sums of atomic contributions aggregated over the B ring have been studied here. It was shown by docking experiments that the B ring protrudes outside of the binding pocket towards the interface of IIB and IIIA subdomains.[21,27] In some cases substituents on the B ring may form polar bonds with HSA. The relationship between the B ring charge and the binding constant is not statistically significant, but it shows favorable effects of the negative charge localized on the B ring.

Most of the previous studies emphasized the significance of nonpolar interactions of the ring A and its substituents with the HSA.[25,27,75] Electronic properties of A ring atoms and substituents have been analyzed in that respect.

The only significant association between electronic properties of A ring substituents and HSA binding established is depicted in Fig. 6: very low electrophilicity of C8 substituent is associated with higher affinity for HSA. Fig. 6 suggests that low electrophilicity of the C8 substituent is relevant, but not sufficient for effective binding to HSA.

3.2.4. Frontier molecular orbitals. Propensity towards intramolecular hydrogen bonds formation is a well-documented property of flavonoids.[51] Strong hydrogen bond donor and acceptor groups attached to the flavonoid scaffold are also expected to promote formation of intermolecular hydrogen bonds between a flavonoid and HSA.

Fig. 7 shows that tightly bound flavonoid ions are characterized by high HOMO and LUMO energies. This is consistent with their tendency towards multiple hydrogen bond formations in which flavonoid ions may play both hydrogen bond acceptor and hydrogen bond donor roles.

3.2.5. Planarity. A complete lack of correlation between HOMO–LUMO gap and log K_A in both *in vacuo* ($r = 0.0206$; $p = 0.9313$) and the explicit solvation ($r = 0.1654$; $p = 0.4858$) shows that conjugation has no effect on HSA binding. This is in contradiction with some earlier studies,[24,75] as well as with the

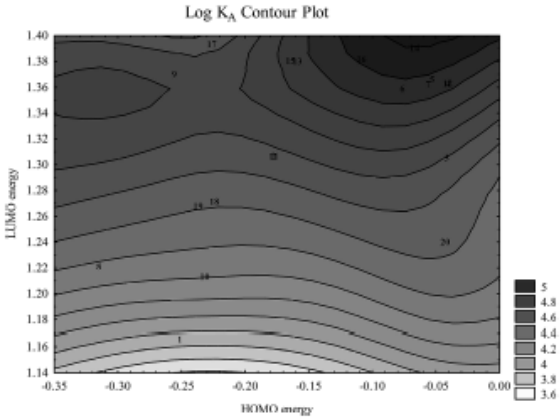

Fig. 7 Dependence of log K_A on HOMO and LUMO energies (presented in a.u.) (numbers correspond to flavonoids in Table 1).

impact of the C8 electrophilicity. Although the present study includes a large number of flavonoids, there is still a relatively low variation in energy gaps. This limits the significance of this notion strictly to the four selected classes of flavonoid aglycones.

Flavonoid aglycone geometries presented here were calculated without taking HSA into account. Obtained results are consistent with the results published by Aparicio,[51] who has revealed that flavonoid aglycones can form intramolecular hydrogen bonds that stabilize coplanar geometries. It is interesting to notice that coplanarity of AC and B rings (Fig. 2) in terms of dihedral angle (angle between atoms 3-2-1′-2′ for flavanones, flavones and flavonols, and 2-3-1′-2′ for isoflavones), is associated with HSA binding affinity *in vacuo* ($r = -0.6832$; $p = 0.0009$), but this association is not present in the solvation water model ($r = -0.0349$; $p = 0.8838$). Considering that flavonoid geometries attached to HSA calculated in docking studies show lack of coplanarity of AC and B rings,[21,25,26,28] we

incline to the conclusion that coplanarity obtained *in vacuo* only reflects the propensity of flavonoids towards hydrogen bonding to HSA.

Also, it has been determined that AC ring planarity is necessary (dihedral angle between planes defined by atoms 4-4a-5 and 4a-5-6), but not sufficient for effective binding to HSA ($r = -0.5097$; $p = 0.0217$ and $r = -0.5093$; $p = 0.0218$ for *in vacuo* and explicit solvation, respectively): even a small deviation from planarity is associated with the loss of affinity for HSA, but all planar molecules do not bind to HSA with equal affinity. Since planarity is frequently associated with conjugation some authors expected that flavonoids tightly bound to HSA establish respective interaction through conjugation.[24,75] According to our results, it seems that the planarity of flavonoids itself makes greater impact on HSA binding than conjugation: flavanones which are characterized by significantly lower binding constants than other flavonoids speak in favor of this notion. Moreover, according to the literature, it has been established that the primary binding site for flavanones is not, as opposed to other flavonoids, in subdomain IIA, but is located closer to the binding site in the IIIA subdomain.[23] Possible reason for this effect could be the presence of a non-planar C ring, which disables binding in the vicinity of Trp 214 residue of the IIA subdomain.

4. Conclusions

In this study spectroscopically determined binding affinities of flavonoid aglycones for HSA were associated with their steric and electronic features. Nucleophilicity and partial charge of the C3 atom enabled classification of flavonoids into subgroups: flavanones (very low nucleophilicity and high negative partial charge), isoflavones (low nucleophilicity and low negative partial charge), flavones (medium to high nucleophilicity and medium negative partial charge) and flavonols (medium nucleophilicity and positive partial charge), with flavones and flavonols being most tightly bound. Increased negative partial charge of the O4 atom has shown strong association with the HSA binding affinity, reflecting its good hydrogen acceptor properties. Also, it has been shown that planarity is of great importance for the flavonoid binding. Coplanarity of A and C rings is associated with higher binding constants. According to our calculations it is a prerequisite for binding in the hydrophobic cavity. Additionally, coplanarity of AC and B rings reflects the propensity of flavonoids towards hydrogen bonding to HSA, consistent with corresponding high HOMO and LUMO energies. Contrary to previously published results, this study shows that neither conjugation of B and AC rings nor hydrogen acceptor and donor properties of the B ring are common determinants of flavonoid binding to HSA; however a negative charge located on the B ring shows a favorable effect on the binding constant.

This study represents the most extensive study of flavonoids binding to HSA, complementing an experimental technique of fluorescence spectrophotometry with results of quantum chemical approach to provide explanations of flavonoid binding properties. However, it is important to emphasize that all of the laboratory measurements were done *in vitro*. Further studies are needed to evaluate biological implications of the described phenomenon like flavonoid–drug and flavonoid–hormone interactions.

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PRILOG II

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REVIEW ARTICLE

Displacement of Drugs from Human Serum Albumin: From Molecular Interactions to Clinical Significance

Hrvoje Rimac[a], Željko Debeljak[*,b,c], Mirza Bojić[a] and Larisa Miller[d]

[a]*Department of Medicinal Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia;* [b]*Institute of Clinical Laboratory Diagnostics, Osijek University Hospital, Osijek, Croatia;* [c]*Department of Pharmacology, Faculty of Medicine, University of Osijek, Osijek, Croatia;* [d]*Sanofi Genzyme, Boston, MA, USA*

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Abstract: ***Background:*** Human serum albumin (HSA) is the most abundant protein in human serum. It has numerous functions, one of which is transport of small hydrophobic molecules, including drugs, toxins, nutrients, hormones and metabolites. HSA has the ability to interact with a wide variety of structurally different compounds. This promiscuous, nonspecific affinity can lead to sudden changes in concentrations caused by displacement, when two or more compounds compete for binding to the same molecular site.

Objective: It is important to consider drug combinations and their binding to HSA when defining dosing regimens, as this can directly influence drug's free, active concentration in blood.

Conclusion: In present paper we review drug interactions with potential for displacement from HSA, situations in which they are likely to occur and their clinical significance. We also offer guidelines in designing drugs with decreased binding to HSA.

Keywords: Human serum albumin, drug displacement, pharmacokinetic interactions, free concentration, drug design, Sudlow's site I, Sudlow's site II.

1. INTRODUCTION

Drug interactions caused by altered drug binding to plasma proteins have received relatively small proportion of attention when it comes to drug-drug interactions; in the majority of cases these interactions are not considered to be clinically significant [1,2]. Displacement of drugs from serum proteins is typically studied *in vitro* or *in vivo*, in pair-wise fashion. This situation is in contrast with the real prescribing practices, where polypharmacotherapy is highly prevalent. This is especially the case in elderly patients with underlying medical conditions such as limited hepatic or renal function [3-6]. In such cases, co-administration of several drugs, certain foods (especially fatty food), and interactions with different endogenous compounds like hormones, can cause displacement of individual drugs and therefore can increase their toxicity and/or decrease their elimination [2]. Additional problems are caused due to varying concentration of plasma proteins between individuals or due to certain medical conditions. Concentration of plasma proteins can be significantly lower, *e.g.* in patients with renal and hepatic impairments, leading to a higher probability of drug displacement [3,5,7]. On the contrary, in situations where HSA is used in the treatment of burn injuries, fetal erythroblastosis, hemorrhagic shock, hypoproteinemia, and ascites [8], sudden increase in the total number of binding sites can cause transient fluctuations in free drug concentrations which is important in case of antimicrobial agents characterized by a minimum effective concentration [2]. Drugs bind to plasma proteins with different affinities. Some drugs show no affinity for HSA (*e.g.* lithium), while other are more than 99% bound (*e.g.* warfarin) [9]. It is considered that only the free, unbound drug can cross different tissue and cellu-

*Address correspondence to this author at the Institute of Clinical Laboratory Diagnostics, Osijek University Hospital, 31000 Osijek, Croatia, Tel/Fax: +385-(0)31-511-650;
E-mail: zeljko.debeljak@gmail.com

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lar membranes, thus reaching the receptor responsible for pharmacological activity of the drug [2]. When the unbound drug molecules are removed from the circulation, additional amount of the drug dissociates from plasma proteins according to the law of mass action, so that with a given protein concentration [*P*], the ratio of the bound ligand concentration [*LP*] and unbound ligand concentration [*L*] stays constant (*K*).

$$[L] + [P] \rightleftarrows [LP]$$

$$K = [LP] / [L][P]$$

If a compound B (either endogenous or exogenous) binds to the same location as the compound A, it is possible that the compound B could displace compound A and increase its free concentration, leading to the increased toxicity of the compound A. Clinical significance of such drug displacement from plasma proteins is a controversial subject. At first it was considered important to know the exact binding sites and the percentage of the bound drug in order to avoid potential displacement followed by the elevation of free drug concentration caused by administering another drug. In general, importance of drug displacement was overestimated due to the fact that most of the research has been done *in vitro* and many of the studies were focused on the free drug fraction given in percentage of the total drug instead of the free drug concentration, which are not necessary tightly correlated [1,10,11]. Even though this subject is still controversial, recent studies point in the direction that displacement interactions are of little clinical value, except in certain cases, such as in liver or kidney impairment [1,8,11,12]. When displacement interactions are clinically important, they can lead to a transient increase in the pharmacological activity of the administered drug or its faster elimination, since a larger amount of the drug is readily available for target binding or excretion. The latter case is especially important when a concentration of the drug has to be maintained above a certain level to be effective, as in cases of anti-infective drugs [2].

The two most important carrier proteins in human blood are HSA and α_1-acid glycoprotein (AGP). AGP is a 40 kDa protein with a concentration of 0.5-1 g/L (12.5-25 μM) which mostly binds many basic drugs, such as local anesthetics. On the other hand, HSA is a 66.5 kDa protein with a concentration of 35-50 g/L (500-750 μM), representing around 60% of all plasma proteins by mass and binding mostly acidic and neutral compounds [13]. This review will be focused on displacements taking place on HSA, but displacements involving α_1-acid glycoprotein must not be overlooked.

2. HSA

HSA is a major component of blood plasma and has numerous functions such as regulation of oncotic pressure and pH of blood plasma, enzymatic and antioxidative properties and storage and transportation of hydrophobic molecules [8]. HSA is synthesized in hepatocytes as a single-chain, non-glycosilated protein consisting of 585 amino acids comprising three structurally similar α-helical domains (I-III) which are further dived into two subdomains (A and B) (Fig. **1**). Three-dimensionally, HSA is a heart-shaped structure with approximate dimension 80×80×30 Å with 35 cystein residues, of which all except one (C34 in domain I) form disulfide bonds and thus stabilize the molecule. Due to its high net charge of -15 at pH 7.0 it is highly soluble in blood plasma [13,14].

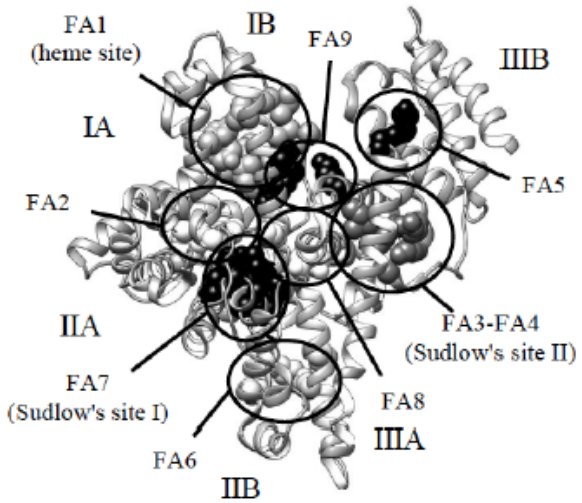


Fig. (1). Helical structure of HSA and locations of fatty acid binding sites (PDB entry 2BXA) [15].

2.1. Binding Sites

2.1.1. Binding of Physiological Ligands

HSA is a principal carrier of non-esterified fatty acids (FA) in human plasma, which represent its primary physiological ligands. Nine FA binding sites (FA1-FA9) on HSA have been determined located in various subdomains and some of them overlap with binding sites for other compounds, including xenobiotics (drugs) (Fig. **1**). Most important of them are FA1 which overlaps with the heme site, FA3-FA4 which overlap with Sudlow's site II and FA7 which overlaps with Sudlow's site I. The content of fatty acids in plasma can vary and thus influence the simultaneous binding of other ligands [8,16-18]. A more detailed overview of drugs binding to HSA subdomains with

their crystallographic data can be found in Ghuman *et al.* [15].

Another physiological ligand for HSA is bilirubin, an end product of heme catabolism. By binding to HSA (99.99%), bilirubin toxicity is greatly diminished. It was previously believed that the high affinity binding site for bilirubin is located in the IIA subdomain, as well as near the Sudlow's site II, but more recent studies have shown that the primary binding site is in the subdomain IB, in close proximity of the heme site [19].

Thyroxin binds to fatty acid-free HSA at four sites, labeled Tr-1 to Tr-4 which partly overlap with FA7, FA3-FA4 and FA5. Fatty acids compete with thyroxin for binding at all four sites, preventing its binding. At high fatty acids concentrations they also induce changes in HSA three-dimensional structure with the formation of the fifth thyroxin site, Tr-5, at the location of FA9 [8,20]. Certain HSA mutants show a higher affinity for thyroxin, leading to smaller percentage of free thyroxin in blood and consequential increase in thyroxin synthesis [8]. Through this mechanism concentration of free thyroxin remains within normal range, but total concentration of thyroxin is higher, which can lead to misdiagnosis as hyperthyroidism with consequent inappropriate treatment for this syndrome, known as familial dysalbuminemic hyperthyroxinemia (FDH) [21].

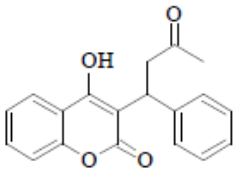
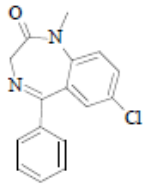
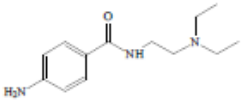
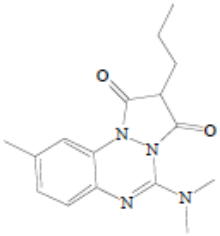
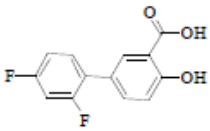
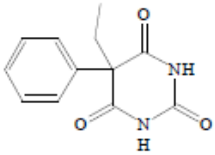
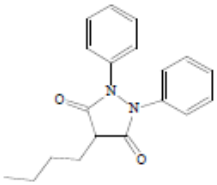
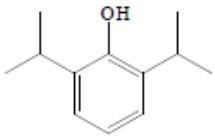
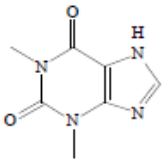
2.2. SAR of Drugs Binding to HSA

Drugs that typically bind to HSA are lipophilic drugs and most of them bind to either Sudlow's site I or Sudlow's site II. A typical site I drug is a dicarboxylic acid or a bulky heterocyclic molecule with a negative charge in the middle of the molecule and a typical site II drug is a carboxylic acid with a negatively charged acidic group at one end of the molecule with a hydrophobic center. Unfortunately, apart from these general rules, little can be said to further explain or predict binding to HSA [18]. Kragh-Hansen in his review article compared several structurally similar drugs which differ significantly in their binding [17]. For example, iopanoate differs from iophenoxate such that one has an amino group vs. the other with a hydroxy group. Yet, these two molecules have different high-affinity sites on HSA. Moreover, ampicillin, which differs from penicillin G by an amino group, can displace bilirubin as opposed to penicillin G. Another example is benzoate which also displaces bilirubin, while binding of parahydroxybenzoate results in a stable ternary complex. Also, *L*-tryptophan binds approximately 100-fold stronger than *D*-tryptophan, while the difference in binding of *R*- and *S*-oxazepam is 30-40 times. On the other hand, affinities for the *R*- and *S*-warfarin are practically the same, as well as *R*- and *S*-progesterone and aldosterone. However, steroid binding constants differ in cases of α- or β-bound substituents [17].

As stated by Smith *et al.* [12], no consensus has been established on how plasma protein binding affects *in vivo* efficacy of drugs in general terms. However, more can be said about interactions and stable and predictable drug concentrations for drugs with narrow therapeutic index. Drugs with a high affinity for HSA require higher doses to achieve their effective concentration; these drugs can be slow to distribute to sites of action and may not be efficiently eliminated [22]. As already mentioned, drugs that bind to HSA are mostly lipophilic and by binding to plasma proteins, their metabolic stability and half-life of elimination ($t_{1/2}$) increase. In such cases, the frequency of dosing can be decreased and drug bound to plasma proteins can serve as a depot. On the other hand, increasing the unbound fraction of a drug makes it less prone to a sudden increase in the active concentration, but also decreases its $t_{1/2}$ [1,2]. For this reason, in specific cases, drug binding to plasma proteins should be explored in greater detail.

Even though a high level of binding to HSA is a characteristic of lipophilic drugs, correlation between lipophilicity and HSA binding has been established only for congeneric series of compounds, while for diverse sets of molecules lipophilicity and HSA binding are poorly correlated [23]. This can be explained by binding of structurally different compounds to different binding sites [22]. An interesting case is binding of phenylbutazone and oxyphenbutazone. While both molecules bind in the center of the Sudlow's site I, the presence of a hydroxyl group in oxyphenbutazone causes the molecule to be rotated 180° with respect to phenylbutazone in defatted HSA, while their orientation are similar in presence of fatty acids. This shows that even minor structural changes in molecular structure can have the unpredictable effects on drug binding [15]. A general way to design drugs with decreased HSA binding is to lower lipophilicity, make the drug more acidic or basic and to increase the polar surface area [24]. Since this will also influence the drug metabolism and elimination [25], a more subtle approach should be used, one that is based on the structural properties of specific drug. Several examples of drugs highly bound to Sudlow's sites I and II, as well as several drugs not significantly bound to plasma proteins are shown in Table **1**. As it can be seen, drugs binding to Sudlow's sites I and II have centrally and terminally

Table 1. Examples of drugs highly bound to Sudlow's sites I and II and drugs not significantly bound to HSA.

Bound to Sudlow's site I	Percentage of binding to site I	Bound to Sudlow's site II	Percentage of binding to site II	Drugs not significantly bound to HSA	Percentage of binding to HSA
 warfarin	99% [22]	 diazepam	99% [22]	 procainamide	10-15% [9]
 azapropazone	99% [22]	 diflunisal	99% [22]	 phenobarbitone	40% [9]
 phenylbutazone	97.8% [22]	 propofol	95-99% [32]	 theophylline	40% [9]

located electronegative group, respectively. On the other hand, procainamide is at pH 7.4 positively charged and binds only 10-15%, while phenobarbitone and theophylline are only 40% bound. This can be explained by the fact that, even though phenobarbitone and theophylline possess partially negative keto groups, they are more evenly distributed in the molecule resulting in lower localization of the negative charge and thus lower binding. Other examples of drugs with low binding to HSA are ethosuximide, primidone, different cephalosporins, aminoglycosides, digitoxin and various other drugs [9,22], all following the same rules.

2.2.1. Sudlow's Site I (Warfarin-Azapropazone Site)

The highest percentage of commonly used drugs bind to one of the two primary binding sites, Sudlow's sites I and II. Site I is located in the IIA subdomain of HSA in the vicinity of the tryptophan residue, W214. Initially, it was reported by Fehske *et al.* [26,27] that this site is comprised of the overlapping binding sites for warfarin and azapropazone. Later Yamasaki *et al.* [28] proposed three binding regions, subsites Ia, Ib and Ic located within the site I, confirmed by Zhu *et al.* crystallographically [29]. An additional site which is located at the entrance of the site I pocket is suggested by our group (unpublished data). More hydrophilic compounds, such as glucuronides, are able to bind at this site. Ligands that are strongly bound to the binding site I are generally dicarboxylic acids or bulky heterocyclic molecules with a negative charge in the middle of the molecule (Figs. **2** and **3**), but site I is still a large and flexible region able to accommodate a wide variety of structurally different ligands, even at the same time [18]. The site is comprised of two non-polar binding pockets with several centrally located polar amino acids. It shows preference for flat aromatic compounds that are able to fit into the crevice and form hydrogen bonds, as described for flavonoid aglycons using a quantitative structure-activity relationship (QSAR) [30]. A SAR study of cephalosporins binding to HSA has shown that anionic cephalosporins have the strong-

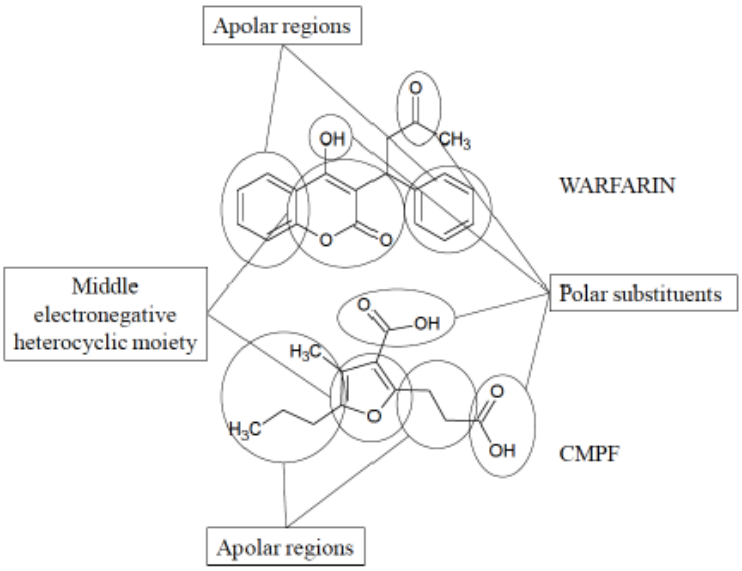


Fig. (2). Binding characteristics of warfarin and 3-carboxy-4-methyl-5-propyl-2-furan propionate (CMPF).

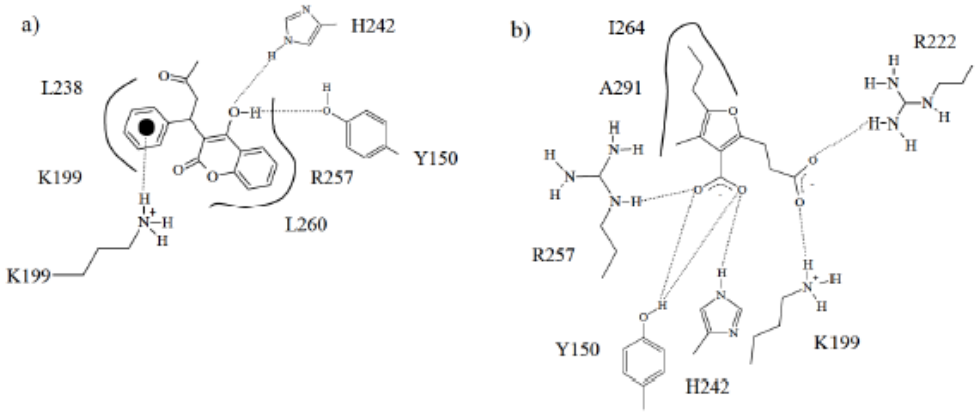


Fig. (3). Interactions between HSA and **a)** warfarin (PDB entry 2BXD) and **b)** 3-carboxy-4-methyl-5-propyl-2-furan propionate (CMPF) (PDB entry 2BXA) [15].

est binding, with cationic cephalosporins being on the opposite side of this binding spectrum [31]. Binding of non-ionized cephalosporins increases with their increasing lipophilicity. Additionally, site I shows poor stereo selectivity, (*e.g.* warfarin) due to the flexibility of this site [8].

2.2.2. Characteristics of Sudlow's Site I

This binding site is located in the IIA subdomain and is pre-formed. It is comprised of three binding regions which partially overlap and this represents a problem in defining structural general characteristics of site I ligands [28]. A cluster of basic polar residues (K195, K199, R218, R222) is located at the entrance of the binding site (Fig. **4a**), as well as another cluster (Y150, H242, R257) at the back of the deeper (left) pocket (Fig. **4b**). Sudlow's site I has a central region which divides this binding site into two pockets. The deeper pocket is yet again divided into two parts by I264 (Fig. **4c**), while the second (right) pocket is delimited by F211, W214, A215, L238 and aliphatic portions of K199 and R218 (Fig. **4d**) [15]. The entrance of the binding site is wide and consists of flexible side chains, which enables it to accommodate different substituent groups. On the other hand, ligands like CMPF, oxyphenbutazone, phenylbutazone, warfarin and even glucose cluster in the center of the site 1 pocket and have a planar group pinned snugly between the apolar side-chains of L238 and A291 and form a hydrogen bond

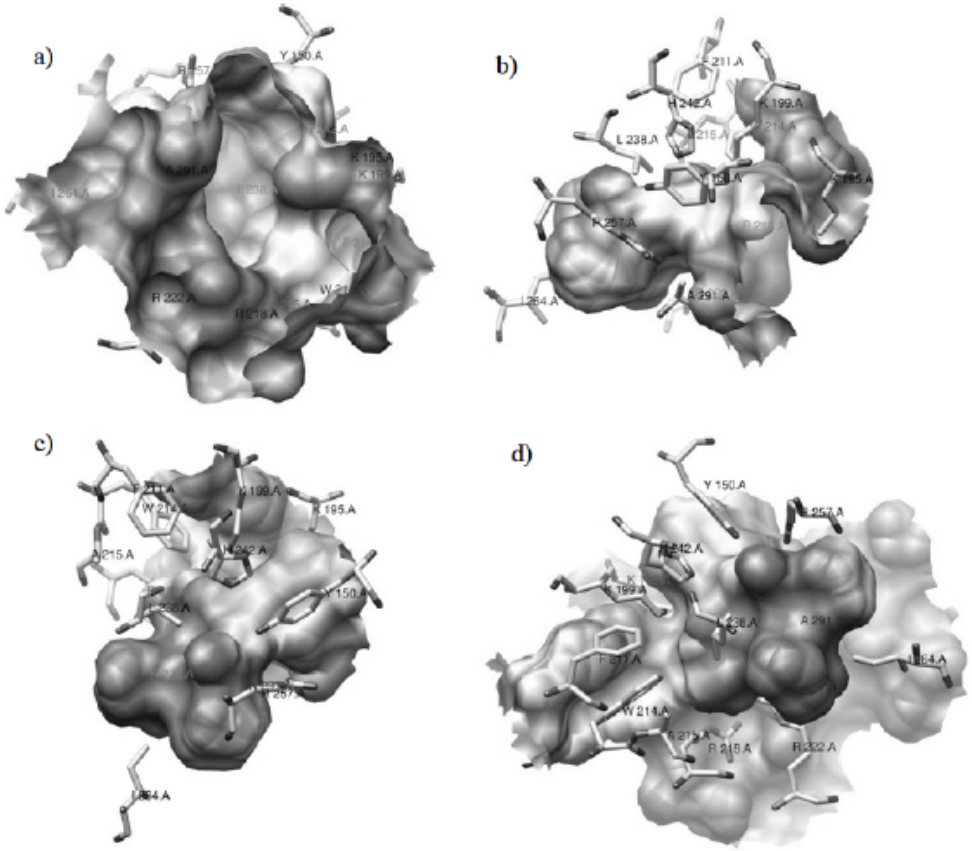


Fig. (4). Sudlow's site I binding pocket: **a)** the hydrophilic entrance of the binding pocket delimited by polar residues K195, K199, R218, and R222, **b)** second polar cluster with residues Y150, H242, and R257, **c)** the bottom of the deeper pocket divided in two parts by I264, **d)** smaller hydrophobic pocket delimited by F211, W214, A215, L238 and aliphatic portions of K199 and R218, viewed from the back.

with the hydroxyl group of Y150 (Fig. **4b**), which has a central role in drug binding interactions [15,33]. Due to the capaciousness of the binding site, ligands are not sterically constrained and different ligands can simultaneously be bound, which allows co-binding of water molecules. These water molecules can mediate interactions between the ligand and HSA [15], and can also explain the lack of stereoselectivity for warfarin [34]. Since there are two polar clusters in the site I, a "perfect" ligand would be a molecule with two anionic or electronegative features on opposites of the molecule, separated by five to six bonds. One such molecule is 3-carboxy-4-methyl-5-propyl-2-furan propionate (CMPF) (Fig. **3b**), which binds with high affinity even though it is a hydrophilic molecule [15,22].

The spaciousness and the characteristics of the Sudlow's site I can be best observed if crystallographic structures of warfarin and CMPF bound to HSA are overlaid (Fig. **5**). It can be seen that the direction of the nearby amino acid residues is not very different, while the conformation and orientation of warfarin and CMPF differs significantly. Both compounds extend to different compartments of the binding site, while the central planar group is in both cases pinned near the L238 residue. This is a perfect example how two structurally and spatially very different compounds fit into the same binding site due to the volume of the binding site.

Binding of fatty acids also influences tertiary structure of HSA [15] and binding of site I ligands [17,35,36]. When a fatty acid is bound to FA2, the Y150 side chain is relocated and interacts with the carboxylic moiety of fatty acid in the FA2 site. Also, a major rearrangement of other amino acids occurs, increasing the volume and decreasing the polarity of the inner polar cluster. Even though the Y150 has a central role in binding of drugs with defatted HSA, when it interacts with a fatty acid at the FA2 binding site, its

influence is compensated by different side residues (K199, R222, H242, and to smaller extent R218 and R257) rendering the environment more hydrophobic and can even increase the binding constant (*e.g.* warfarin) [15,34]. As already mentioned, presence of fatty acids also influences orientation of oxyphenbutazone, as well as the amino acid environment of indomethacin [15]. At higher fatty acids concentration, ligand binding decreases due to direct competition with fatty acids for binding to the low affinity FA7 binding site [34]. Co-binding and its effects on the binding constants between different drugs are described in more detail by Yang *et al.* [37].

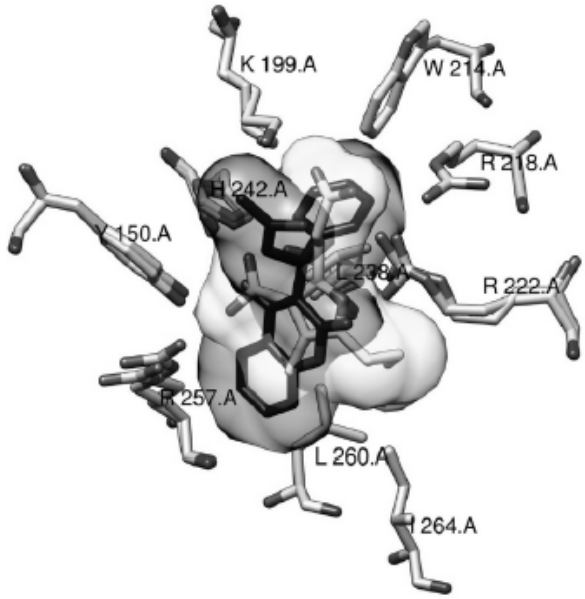


Fig. (5). Overlaid structures of warfarin (darker) and CMPF (lighter) bound in the Sudlow's site I. While the ligands are spatially very different, the binding side is large enough to be able to accommodate both ligands without major rearrangements in amino acid residues.

2.2.3. Sudlow's Site II (Indole-Benzodiazepine Site)

Site II is located in the IIIA subdomain and appears to be smaller and narrower than site I, as well as more stereoselective. Site II is composed of a long hydrophobic region and a polar patch formed by tyrosine, arginine and serine residues. This structure supports selectivity for carboxylic acids with a negatively charged acidic group at one end of the molecule, separated from a hydrophobic center (*e.g.* diflunisal and ibuprofen) [8,15,18]. Through a QSAR study, Wanwimolruk *et al.* [38]. has shown that the site II is comprised of a hydrophobic cleft 16Å deep and 8Å wide with a cationic group located near the surface. A negative charge is not a prerequisite for binding as long as an electronegative center is present, while positively charged ligands do not bind to this site (*e.g.* benzodiazepines) (Figs. **6** and **7**) [38,39]. Due to its smaller size, this site is stereoselective (*e.g.* (*R*)-ibuprofen binds 2.3 times stronger than (*S*)-ibuprofen and *L*-tryptophan binds about 100 times stronger than *D*-tryptophan) and has not yet been divided into sub-binding regions as site I [18]. Since the site II overlaps with the high affinity FA3-FA4 binding sites, displacement interactions involving fatty acids and drugs are more prone to occur at this site than at site I, which overlaps with the low affinity FA7 binding site [35,38].

2.2.4. Characteristics of Sudlow's Site II

Similar to site I, Sudlow's site II is pre-formed hydrophobic cavity containing polar clusters. It is structurally similar to the site I, only smaller: it appears that site II possesses only one hydrophobic pocket; the other pocket is not present due to the location of Y411, which is located at the position which corresponds to L219 in the site I. Additionally, the entrance of the site II is more exposed to the solvent and the polar cluster (R410, K414, and S489) is located close to the one side of the entrance (Fig. **8a**). Ligands interact with the hydroxyl group of Y411, the polar cluster at the entrance, and the hydrophobic pocket (Fig. **8b**), thus this binding site is selective for drugs with a peripherally located electronegative group, such as diflunisal, diazepam, and ibuprofen [15]. However, certain compounds such as propofol, halothane and indoxyl sulfate bind to the carbonyl oxygen of L430 [40]. In the case of propofol, steric effects of the isopropyl groups make the interaction between the single polar hydroxyl group with the main polar patch in Sudlow's site II impossible and instead, propofol adopts a conformation that allows formation of a hydrogen bond to the carbonyl oxygen of L430 [40]. As well as the binding site I, binding site II is also adaptable, able to accommodate a large, branched structure such as diazepam, and it can also bind larger molecules, such as thyroxin and two molecules of fatty acids (FA3-FA4). In binding of these molecules, the adaptability of this binding site comes into play. Significant rotations of side chains L387 and L453 are accompanied by the binding of diazepam and fatty acids, with possible influence of water molecules to further adapt the binding site in case of diazepam [15].

As in the case of Sudlow's site I, different ligands can be overlaid in the site II to show its adaptability

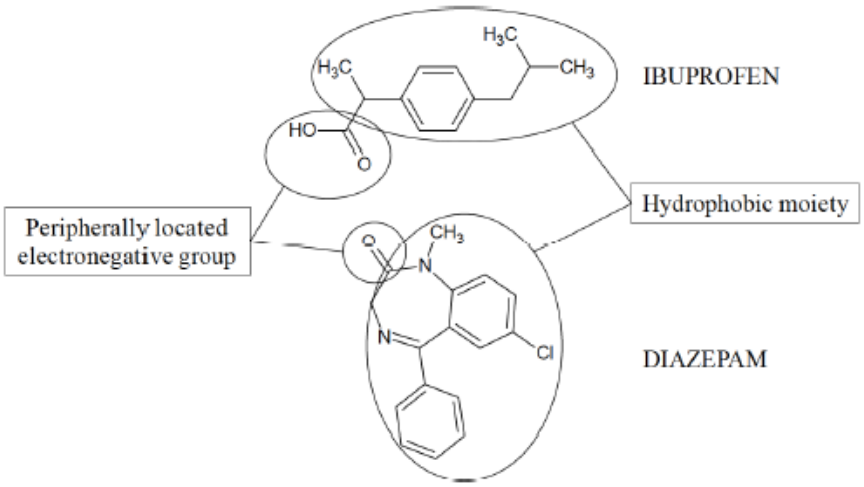


Fig. (6). Binding characteristics of ibuprofen and diazepam.

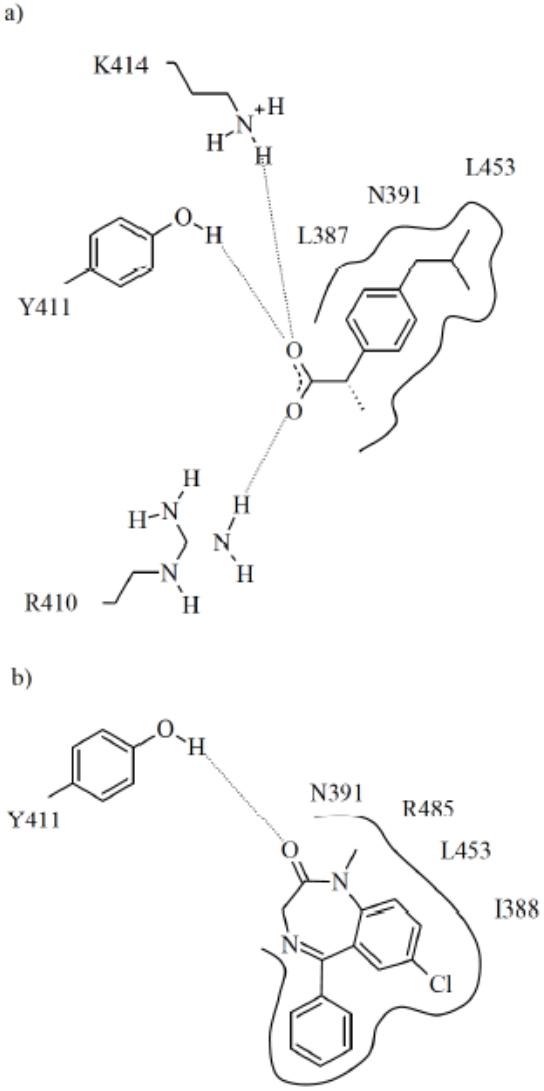


Fig. (7). Interactions between HSA and **a)** ibuprofen (PDB entry 2BXG) and **b)** diazepam (PDB entry 2BXF).

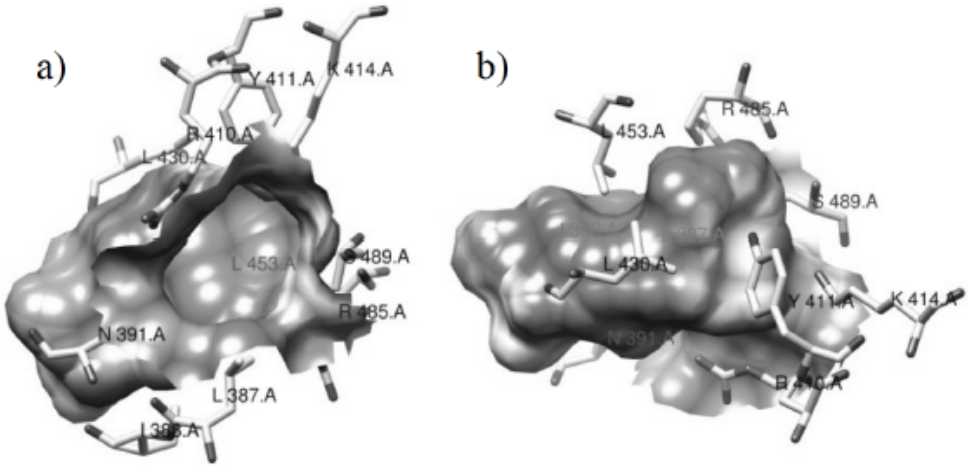


Fig. (8). Sudlow's site II binding pocket: **a)** the hydrophilic mouth of the binding pocket with polar residues R410, Y411, K414, and S489 located more closely to one side of the entrance, **b)** side view of the hydrophobic part of the binding pocket.

and volume (Fig. **9**). In this case, crystallographic structure of ibuprofen and diazepam are overlaid. As can be seen, more volume of the binding site is shared by both ligands. Exceptions are additional volume needed to accommodate the phenyl ring of diazepam which causes significant difference in orientation of L387 and the carboxylic moiety of ibuprofen which causes changes in orientation of Y411. As can be concluded, both Sudlow's sites can accommodate different ligands, but due to its size, Sudlow's site I can bind a much larger variety of ligands, some even at the same time and with less conformational changes of the protein required to do so.

2.2.5. Binding Site in the IB Subdomain

Importance of the IB binding site has been recognized as being primary and secondary binding site for many compounds [41]. Apart from being the primary binding site for endogenous compounds such as bilirubin and hemin [8], this region has been identified as the primary binding site for lidocaine [42] and fusidic acid (an antibiotic with a steroid structure) [19] as well as several other drugs as either primary or secondary binding site [8,29].

2.2.6. Other Binding Sites

C34 located in a crevice of the IA subdomain is the only cystein residue that does not form an intramolecular disulfide bridge. This cystein residue can thus bind certain drugs and form covalent interactions with them (*e.g.* bucillamine derivatives, *D*-penicillamine, captopril, and ethacrynic acid) [8,18], as well as bind and degrade disulfiram into two diethyldithiocarbamate molecules [43]. This binding site also has a high specificity for metal ions (*e.g.* Au(I), Pt(II), Ag(I), Hg(II)), as well as drugs containing these metals (*e.g.* auranofin, cisplatin) [8,18]. Additional metal binding sites also exist; the first one is located at the *N*-terminus and is called *N*-terminal site, NTS. This site binds Cu(II), Co(II) and Ni(II). Another important metal binding site is the multimetal binding site or Cadmium site A (MBS-A) and is the primary binding site for Cd(II) and Zn(II). Apart from the already mentioned, there are several more binding sites able to bind different compounds. Examples of such ligands are oxyphenbutazone, propofol, iodopamide, diflunisal, halothane, ibuprofen *etc* [15]. Binding sites located in all subdomains have been identified at which only a few compounds have been found to bind [8,18]. A list of three-dimensional structures of HSA with various ligands at their binding sites can be found in Fanali *et al.* [8].

2.3. Rebinding of Displaced Drugs

In certain cases, a displaced drug can rebind at another site, thus the increase of the free drug concentration can be lower than expected. Such situations have been best described with certain non-steroidal anti-inflammatory drugs [44,45]. In case of diclofenac and ibuprofen, Yamasaki *et al.* [44]. have shown an *in vitro* site-II-to-site-I displacement of HSA-bound diclofenac by ibuprofen. Diclofenac primarily binds to Sudlow's site II, as well as ibuprofen. In the presence of ibuprofen, diclofenac is displaced from the site II, however it rebinds to the site I, resulting in much lower free concentrations of diclofenac than those expected for purely

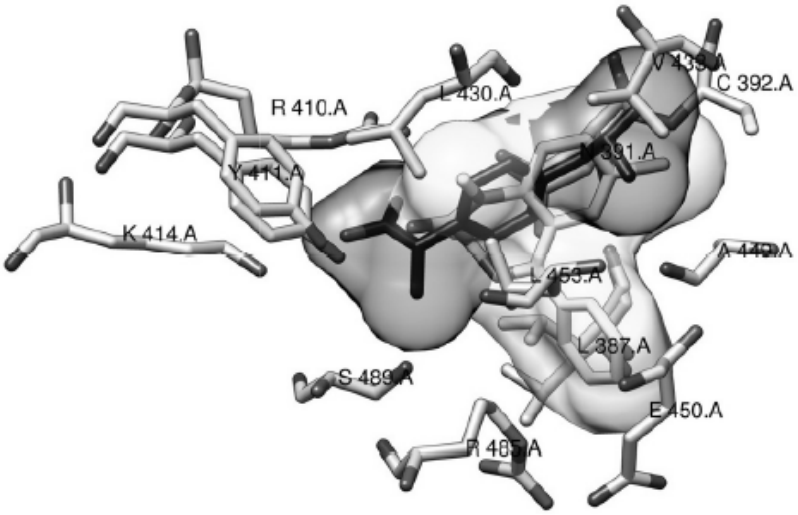


Fig. (9). Overlaid structures of ibuprofen (darker) and diazepam (lighter) bound in the Sudlow's site II. The central parts of the molecules significantly overlap, while to accommodate the phenyl ring of diazepam L387 must be reoriented and in the case of the carboxylic moiety of ibuprofen the location of Y411is also significantly changed.

competitive interaction [44]. This site-II-to-site-I displacement was also shown to be stereoselective, as in case of carprofen, with the most profound interaction between *S*(+)-carprofen and *S*(+)-ibuprofen [45].

3. DRUG-DRUG DISPLACEMENT INTERACTIONS

Majority of drugs bind to some extent to plasma proteins, with HSA being the most prevalent protein carrier in plasma. As already mentioned, only the unbound, free fraction of the drug can cause desired, as well as adverse effects, either prior to or through metabolic conversion which is then followed by elimination processes affecting the overall dynamics of the system. At steady state and in the absence of active transport, the concentration of the drug is the same on both sides of the plasma membrane, so the free concentration of the drug in plasma can be considered the same as the free drug concentration at the target site. Displacement of drugs, as well as endogenous compounds from their binding sites on plasma proteins can influence their free fraction, as well their free concentration. This can be caused by either competitive displacement (where two drugs compete for the same binding site) or allosteric displacement (where binding of the displacer drug induces conformational changes in the protein leading to dissociation of the drug being displaced). This interaction can have effects on pharmacokinetics (distribution, metabolism and excretion), as well as on pharmacodynamics of the drug, but for the most part, displacement interactions are not of clinical significance. However, here are mentioned certain conditions in which they could be highly relevant.

3.1. Necessary Conditions for Clinically Important Drug-Drug Displacement Interactions

The primary condition for a clinically important displacement interaction is that the drug being displaced is highly protein bound (>90%) and has a narrow therapeutic index. In such cases, a small decrease of the bound drug can significantly increase the free drug concentration responsible for the pharmacological effects. The displacer compound also has to be highly protein bound and has to be in a high enough concentration to be able to compete with the drug of interest. The capacity of HSA for both compounds also has to be taken into account. Another condition that has to be met is that the drug being displaced has to be high extraction ratio (ER) drug, meaning that the blood flow through the eliminating organ has to be smaller than the product of the percentage of the unbound drug and intrinsic organ clearance. This means that the blood flow through the eliminating organ is the limiting factor for the drug excretion and that the increase in drug concentration will not be adequately compensated with an increase in elimination. For drugs with low ER, and high unbound fraction, the displaced drug will be more available for elimination and its $t_{1/2}$ will decrease. Lastly, since there is a time delay between the change in the free concentration and the onset of the pharmacological consequences, the displacer drug has to be taken either intravenously or orally with non-hepatic

clearance. Otherwise, compensatory mechanisms will be able to diminish the displacement potential of displacer drug before it reaches circulation. This time delay is an individual characteristic of every drug, and the shorter the time delay needed to achieve this equilibrium, the more enhanced the pharmacological effects will be [1,2,12,46]. A more thorough explanation of these conditions, as well as the list of drugs that meet these demands can be found in Benet & Hoener [1].

Concentration of both the drug being displaced and the displacer drug varies over time. The extreme case is a bolus injection of the displacer drug, which will increase the concentration of the displaced drug, especially of drugs with low volume of distribution (V_d) and low ER. The increased concentration of the displaced drug will return to normal levels with time, but with possible toxicity and faster elimination in the meantime, resulting in more pronounced difference between maximum (c_{max}) and minimum (c_{min}) steady-state concentrations [2,46]. Therapeutic drug monitoring (TDM) represents management of patient's drug regimen based on drug's blood concentration. TDM is usually not necessary for drugs for which therapeutic concentrations are far below their toxic concentrations. However, for drugs with a narrow therapeutic index whose toxicity can occur at concentrations slightly above the therapeutic range, TDM can be very valuable [9]. Since only the free drug concentration is pharmacologically active, changes in the percentage of plasma binding can influence the active concentration without much effect on the total concentration. This can lead to under-dosing, toxic effects or even misdiagnosis [5,21]. For a drug with a narrow therapeutic index, if protein binding is higher than normal, clinician might conclude that the dosage regimen is in order, while the free concentration of the drug is insufficient to produce a pharmacological effect. On the other hand, if protein binding is lower than normal, higher clearance will compensate for initially higher free concentration. The final free concentration will return to normal, while the total concentration will be lower [1]. This could persuade the clinician to increase the dose, leading to adverse effects.

3.2. Consequences of Displacement on drug $t_{1/2}$ and its Metabolites

It can be said that the purpose of drug metabolism is to make them more hydrophilic so they can be more readily eliminated from the body. On the other hand, HSA represents a depot for lipophilic drugs protecting them, among other things, from the aforementioned metabolism and prolonging their $t_{1/2}$ [8]. As already mentioned, in a case of a drug being displaced, its concentration in blood is increased which leads to its increased metabolism (if the enzymes are not already fully saturated) and decreased $t_{1/2}$ of that species. This increase in concentration also leads to the faster elimination of drug from the body [1,2]. If the drug species being displaced from HSA is the active form of drug and is a high extraction drug, this can lead to transient increase in pharmacological effects. In a situation where the drug species being displaced is a prodrug, the situation slightly changes. If the enzymes which are responsible for the metabolic activation of the prodrug are fully saturated, an additional increase in prodrug free concentration does not affect the concentration of the active species. In the case that they are not fully saturated, the concentration of the active species can increase, but with a lesser impact on pharmacological effects due to all the compensatory mechanisms described before, so the largest impact this could have is an increased elimination rate of the prodrug from the body associated with the short lasting pharmacological effects [1,2,12,46]. A question of binding of the newly formed drug metabolites to HSA and their influence on drug displacement might also come to mind. Since the newly formed metabolites are more hydrophilic than the parent drug, their affinity for HSA is usually much lower and they are also in lower concentrations so their contribution to additional displacement may be disregarded [8,13].

3.3. Modifying Factors

3.3.1. Altered Hepatic Function

People with decreased hepatic function can have a substantially lower concentration of HSA, since it is synthesized in the liver. In this case, there will be less albumin in the blood available for binding drugs and therefore the possibility of displacement is greater. In some cases, structural changes of the albumin molecules can occur, which further decreases binding capacity [7]. Patients with hepatic impairment can also experience altered metabolism and decreased hepatic clearance which can lead to accumulation, i.e. increase of the drug concentration [3]. Decreased drug binding can also be due to accumulation of endogenous ligands, *e.g.* bilirubin and fatty acids, as well as in hemolysis where heme concentration can be increased from standard 1×10^{-6} M to 4×10^{-5} M [5,7]. In these situations, HSA binding sites can be extensively occupied by endogenous substrates thus influencing binding of other ligands [8], as mentioned in section 4.

3.3.2. Altered Renal Function

In patients with renal diseases binding of drugs to serum albumin is usually decreased. As in hepatic diseases, albumin concentration can be affected by renal dysfunction and thus significantly lower (as low as 7 g/L). This condition coupled with the accumulation of endogenous inhibitors, such as different uremic toxins (indoxyl sulfate, indole-3-acetate, hippuric acid, and CMPF) can decrease binding of many drugs, such as warfarin, furosemide, diazepam and phenytoin [5,7,18]. Indoxyl sulfate, indole-3-acetate and hippuric acid bind to the site II and CMPF binds to the site I [5,9,18]. In hemodialysis patients, oxidative modification of HSA can lead to alterations in HSA conformation, which decreased binding of drugs to the site II of HSA [8].

3.3.3. Diabetes Mellitus

In hyperglycemic conditions albumin is highly prone to glycation which can alter its tertiary structure and function [8]. Normal level of glycated HSA is below 6%, but can be increased up to 20-30% in hyperglycemic patients [47]. The glycation reaction occurs typically between ε-amino groups of lysine residues and glucose, leading to the formation of a Schiff base, but more complex Amadori products can also form, leading to formation of even more advanced glycation products. As the glycation can occur at Sudlow's sites I and II, changes in drug binding can also occur [8,48]. Wang *et al.* have thoroughly investigated glycation of the Sudlow's site I. Crystallographic data show that two molecules of glucose can be accommodated in this site. The first one forms hydrogen bonds with Y150, R222, H242, and R257 while the pyranose ring is pinched between the side-chains of L238 and A291 [33], as many other drugs that bind to this site [15]. The second glucose was found to be in the linear open form and located at the entrance of Sudlow's site I, covalently linked to K195. This glucose molecule is anchored by the positively charged residues K199, R218, R222, and A291. The glycated K195 forms hydrogen bonds with R218 located at the entrance to the Sudlow's site I and consequently blocks the access of other ligands to this binding site [33]. However, studies with glycated HSA and several drugs, endogenous ligands and probes have been conducted [47,49-51], but clinical significance of HSA glycation is still under debate [8,47].

3.3.4. (Pseudo)-enzymatic Properties of HSA

HSA has been shown to possess enzymatic properties such as an enolase activity toward dihydrotestosterone, thioesterase activity toward disulfiram, dehydration of prostaglandin D2 and dehydration and subsequent isomerization of 15-keto-prostaglandin E2 (reactions involving prostaglandins can be inhibited by phenylbutazone and warfarin) [18]. Also, enzymatic activity of HSA can be stereoselective, [11]. *e.g.* site I can degrade sulbenicillin, with preference for the R-isomer [52] and both site I and site II have an esterase activity [18,53,54] as well as hydrolase activity for drug glucuronides, catalyzing the transformation of acyl glucuronides to parent drugs, but can also lead to covalent binding of drugs to HSA and toxicity [8,18,55,56]. Additionally, this esterase activity can also be used to activate prodrugs [18]. In this way HSA can influence the total and free drug concentrations, as well as their metabolism. The non-classical enzymatic activities of HSA could have an impact on the pharmacokinetics of drugs [53]. However, this is not directly associated with the drug displacement [8,13].

3.3.5. Polymorphism of the ALB Gene

ALB gene shows significant degree of polymorphism and according to Fanali *et al.* [8] 83 different genetic variants of HSA and proalbumin have been characterized at the protein and/or gene level with prevalence 1:1000. These mutations usually show small if any effects on ligand binding, but several exceptions exist [57]. Studies have connected albumin polymorphism with altered binding of fatty acids, metal, steroid hormones, thyroxin, diazepam, salycilate and warfarin, with some of them showing increased and some of them showing decreased binding [8,57,58].

3.3.6. Hypoalbuminemia

Hypoalbuminemia can be present in conditions such as hepatic and renal diseases, but also in rheumatoid arthritis due to high uptake of HSA at the sites of inflammation [59] and in pregnancy where it is coupled with an increase of endogenous ligands, such as fatty acids [60]. Furthermore, HSA levels in the elderly population have been shown to be approximately 19% lower than in young population because of the reduced renal and hepatic function [4,61]. Another form of "temporary" hypoalbuminemia is the decrease of the HSA concentration during inflammatory diseases [62] and different conditions, such as renal failure, burns, surgery, and malnutrition [11], as well as and in neonates [61]. Neonates are in particular risk because in addition to having lower HSA concentration, they also have decreased hepatic function, as well as increased risk of hyperbilirubinemia [18,61].

3.3.7. Analbuminemia

Analbuminemia is a condition characterized by concentration of albumin concentration <1 g/L. Even though this condition does not result in serious phenotype in adults, as other proteins take over the role of albumin, few clinical symptoms do occur, such as mild edema, hypotension, fatigue *etc* [8,13]. Problems with analbuminemia can be regarded as extreme cases of hepatic or renal diseases and can also have significant clinical consequence causing increased free concentration of drugs, higher peak activity, earlier onset of toxicity and faster clearance.

3.3.8. Medical Use of HSA

HSA is widely used in the treatment of following conditions: hypovolemia, shock, burns, surgical blood loss, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, acute liver failure, chronic liver disease, nutrition support, resuscitation, and hypoalbuminemia [8,11,63]. In such cases, the initially low HSA concentration can be increased several times, which can lead to changes, i.e. decrease, in free concentrations of both endogenous and exogenous ligands.

4. CLINICALLY SIGNIFICANT DISPLACEMENT INTERACTIONS

4.1. Interactions with Endogenous Ligands

Increased binding of heme to its binding site (*e.g.* in severe hemolytic anemia or crush syndrome) decreases the binding constant of Sudlow's site I ligands approximately by one order of magnitude. On the other hand, plasma levels of heme-Fe(III) can be increased due to administration of site I ligands [64-66]. Fatty acids represent the most important dietary displacer molecules as they are highly abundant in everyday diet, are found in high concentrations in the body, have multiple binding sites on HSA and can influence binding of other ligands both directly and allosterically [8,16]. Even though other dietary constituents, *e.g.* flavonoids, may influence pharmacokinetics of certain drugs in different ways, they are found in much too low concentrations to have any effect in displacement interactions. Binding of fatty acids generally decreases binding of HSA ligands (*e.g.* thiamylal, diazepam, furosemide, ibuprofen, flurbiprofen, and steroids [5,17,18]), while in cases of heme site ligands and bilirubin, binding is increased [17,35]. Another issue of concern is the displacement of bilirubin by a large number of drugs. As mentioned by Kragh-Hansen [18] and Maruyama *et al.* [67], numerous drugs (*e.g.* valproate, ceftriaxone, sulfonamides, oxyphenbutazone, phenylbutazone, sulfinpyrazone, ketophenylbutazone, glibenclamide, tolbutamide, warfarin, salicylate, and furosemide) have bilirubin-displacing properties. In such cases, the recommendation is that these drugs should not be given to neonates in danger of kernicterus. It has also been suggested that all drugs used for newborns should be tested for bilirubin-displacing properties [18].

4.2. Warfarin

Warfarin is a classic low ER and low V_d drug with narrow therapeutic index which is 99% bound to HSA [1,2]. As such, interactions with several drugs have been reported as clinically significant, but with mild side-effects [8,68]. Most of them are a combination of displacement and metabolism inhibition, *e.g.* phenylbutazone [69] and amiodarone [70], but so far only two have been hypothesized to occur exclusively through displacement, warfarin-clofibrate and warfarin-chloral hydrate interactions [8,71]. There are some indications that FDH mutations at positions R218H and R218P decrease affinity of warfarin binding to HSA five-fold [72]. However, even though this decline should not have any influence on the warfarin toxicity, some changes in warfarin elimination half-time might occur [2] and a case report showing that in patients undergoing chronic warfarin therapy, a loading dose of valproic acid can potentially lead to increased risk of bleeding due to increased free warfarin concentration [73]. Additionally, it has also been confirmed that at higher molar concentrations of fatty acids (fatty acids:HSA >3), warfarin binding to HSA is decreased [17], while if HSA is glycated, binding of warfarin is increased [8].

4.3. Ceftriaxone

Ceftriaxone is an anti-infective agent with a low ER and a low V_d, so similar problems occur as with warfarin. When administered alongside with probenacide, $t_{1/2}$ of ceftriaxone was 30% decreased, which resulted in lower c_{min} [74]. Another example is a decrease of ceftriaxone $t_{1/2}$ in patients with sepsis with normal renal function, but approximately 50% decreased albumin concentration. In this case albumin concentration was the sole cause of decreased c_{min} which fell under minimum effective concentration and ceftriaxon dosage had to be adjusted [75].

4.4. Anticonvulsant Drugs

Anticonvulsant drugs are associated with more drug-drug interactions than any other therapeutic class of drugs and this is primarily attributed to the first-

generation drugs. Most of these interactions are due to the enzyme induction and inhibition, but some of them are also involved in the displacement interactions [76]. Valproic acid is an important anticonvulsant drug with low ER and low V_d. It binds to HSA up to 95%. Within its therapeutic range, its molar concentration can be higher than the concentration of serum albumin [77]. Due to the limited number of binding sites, if this situation occurs, every valproic acid dosage elevation will dangerously increase its free, active concentration. Even more extreme situation can occur if the concentration of albumin is lower than normal: in normal dosage regimen the free drug concentration can increase from regular 5% up to 26% [78]. Interindividual differences in valproic acid free fractions are a regular occurrence, but this is further emphasized in the presence of uremia and liver disease or in case of hypoalbuminemia [9]. Valproic acid can also enter displacement interactions with other drugs, *e.g.* warfarin [73]. and acetylsalicylic acid. In the latter case, it is an example of displacement accompanied by metabolic inhibition. In such cases, the free valproic acid concentration can increase more than eightfold, while the total concentration increase is less than twofold [79].

Phenytoin is another anticonvulsant drug with low ER and low V_d which binds to albumin up to 90%. It was shown that in hypercholesterolemia and in mixed hyperlipidemia, the serum level of free phenytoin was elevated, probably due to displacement by fatty acids [9]. As with valproic acid, binding of phenytoin is altered in patients with hypoalbuminemia, uremia and hepatic diseases [5]. While some of phenytoin displacement interactions are accompanied by metabolism inhibition, such as with valproic acid [79], ibuprofen [9,79], and omeprazole [79], others are a result of purely displacement interactions, *e.g.* acetylsalicylic acid [79]. Some case reports have been published that associate increased phenytoin free concentration with the displacement in the presence of valproic acid without involvement of metabolism inhibition [77,80]. Displacement interactions between phenytoin and different antibiotics [81], as well as interactions between anticonvulsant drugs and parenteral nutrition [82] have also been reported.

Results observed in case of carbamazepine are similar to those of valproic acid and phenytoin, but to a lesser extent. Carbamazepine binds to HSA 70-80%, while the primary and active metabolite 10,11-epoxide is only 50% bound [9]. Since the epoxide metabolite is almost equipotent as the parent drug, it contributes significantly to its pharmacological effects, while at the same time buffers the effects of displacement [83].

Second- and third-generation anticonvulsant drugs are less prone to interactions due to their lower binding to HSA (and non-hepatic metabolism). An example of such drug is eslicarbazepine acetate, a carbamazepine derivative, which is only 30% protein bound. Even though it still interacts with other anticonvulsant drugs, such as phenytoin, lamotrigine, and topiramate through enzyme inhibition and induction, the displacement component of the interaction is not clinically significant. Similar example is oxcarbazepine, which is 60% serum protein bound with its active metabolite 10-hydroxycarbazepine, which is 40% bound. They are both displaced from their binding sites by valproic acid, but the clinical significance of this interaction is still uncertain [76].

Tiagabine, which is 96% protein bound also represents an interesting case. Tiagabine may lower valproic acid serum levels, but the mechanism of its action is still unknown and could be connected to tiagabine metabolism as 40% of its metabolites are yet to be identified. However, valproic acid also displaces tiagabine from its HSA binding sites and increases the free active tiagabine concentration, as do salicylates and naproxen [84].

4.5. Desirable Displacement Interactions

Not all displacement interactions are unwanted. In some cases they are used to increase therapeutic effects. In nephrotic patients, loop diuretics usually have little effect because of their binding to HSA in urine, but when taken with a displacer, such as bucolome, their diuretic effect is increased [18]. The pain relieving properties of diclofenac suppositories have been shown to increase with concurrent use of nabumetone. Since diclofenac and 6-methoxy-2-naphthylacetic acid (6-MNA, the main nabumetone metabolite) both bind to the IIA site and 6-MNA does not inhibit CYP2C9, the rate-limiting enzyme in the metabolic clearance of diclofenac, it was concluded that the increased pharmacological activity of the diclofenac suppository-nabumetone therapy was due to the transient increase in the free concentration of diclofenac [85]. Displacement is also used for faster elimination of radiological contrast agents, *e.g.* the use of technetium-99m-labeled mercaptoacetylglycylglycylglycine (99mTc-MAG3) with bucolome. When they are administered together, the radiopharmaceutical free concentration in blood is higher, leading to enhanced transfer and higher concentrations in target tissues. This results in better diagnostic images with shorter waiting times, faster clearance and lower radiation doses for patients [86].

CONCLUSION

Drug displacement interactions are of limited clinical significance but they should be taken into account in the following scenarios. Firstly, more and more patients are undergoing polypharmacotherapy. Considering the number of drugs taken simultaneously and due to the fact that binding of one drug can directly or indirectly (allosterically) displace another drug, it is hard to predict all of the possible interactions. Therefore, drugs with a narrow therapeutic index which are prone to displacement should be monitored more closely. Secondly, a lot of patients taking drugs also have additional health conditions that can complicate the situation even more. From today's perspective, drugs that are the most prone to displacement by another drug, with or without inhibition of metabolism are antiepileptic drugs. A bigger concern are drug displacement interactions with endogenous compounds, which concentrations can vary significantly, such as bilirubin, heme, CMPF and fatty acids, as well as conditions such as hypovolemia and hypoalbuminemia. At the same time, not all displacement interactions are necessarily undesired. In cases of diuretics and diagnostics, displacement is sometimes beneficial due to the increase of the active concentration or due to faster excretion, respectively.

Drugs mostly bind to one of the two main HSA binding sites, Sudlow's site I and Sudlow's site II. Both sites are hydrophobic pockets able to bind small electronegative compounds. Since site I is more capacious, it binds a larger number of ligands which can structurally be different and can bind simultaneously. Drugs binding to this site are more prone to drug-drug displacement due to the number of different drugs binding there, although displacements involving endogenous ligands also occur. On the other hand, drugs which bind to site II are more prone to interactions with fatty acids due to the high affinity FA3-FA4 binding sites. Our recommendation is that if a drug has a narrow therapeutic range, HSA binding should be avoided. Situations in which HSA is used as a depot in order to decrease the dosing frequency or to stabilize the drug concentration in the blood represent special cases. A simple rule of thumb for designing drugs that are not tightly bound to HSA would be: 1.) Increasing potency. Higher potency translates to lower drug concentrations needed to achieve the same pharmacological effect. Therefore, the drug will occupy less HSA binding sites and the risk of displacement interactions will be lower. 2.) Decreasing lipophilicity. Due to the fact that HSA binds mostly lipophilic compounds, decreasing drug lipophilicity will lower the percentage of the drug bound. 3.) Decreasing the drug molecular volume. Since Sudlow's site I is capacious and can accommodate two different ligands at the same time, lowering the drug volume decreases the chance of the drug being displaced by another compound. 4) Decreasing the electronegative character of substituents. Since drugs binding to both Sudlow's sites I and II are anions or have an electronegative group, decreasing their electronegativity would also decrease their binding to these sites. And 5) Decreasing planarity of rings. Binding to Sudlow's site I is also correlated to planarity of ring systems so replacement of some of the double bonds in the ring systems would also decrease affinity of drugs for HSA. It also must be taken into account that modifying any of these properties will most likely also influence other characteristics of the drug: any attempt to optimize HSA binding should not be done at the expense of drug efficacy.

The risk of sudden toxic effects caused by displacement is valid for a) high ER drugs administered intravenously and b) high ER drugs which are given orally and have non-hepatic clearance. On the other hand, low ER drugs might experience changes in steady state c_{max} and c_{min} with lower $t_{1/2}$, especially in case of a low V_d. Moreover, underlying conditions also have to be considered as they can change not only drug binding capacity of HSA, but also drug metabolism and excretion. The extent of drug binding to HSA influences not only a) the free drug concentration in blood, but also b) the time needed for equilibration of drug concentration between the blood and the effect compartment, and c) the time needed to eliminate the drug from the effect compartment, as well as the blood. In situations where there is a high possibility of drug toxicity, therapeutic drug monitoring should be implemented, with determination of the free drug concentration, measured at the receptor site if possible.

As the computational research gets less expensive, less time-consuming and more reliable, we expect increased interest in application of computational techniques in studies of the protein-ligand and even protein-protein interactions. The main disadvantage of computational statistics studies is that they require a large pool of well studied ligands. On the other hand, docking studies deal with specific ligands, but their main pitfalls are inability to predict the binding constant with high accuracy and the lack of reliable methods for validation of obtained results. These pitfalls are partially associated with the computational restrains. Another computational method, molecular dynamics

simulation, is also a highly used method for studying conformational changes of biomacromolecules upon ligand binding [87]. It is a reliable method for investigating local conformational changes of biomacromolecules and can also serve as a validation tool to verify the rationality of docking results, limited only by computational cost. However, until the time comes when we will be able to simulate conformational changes and adjustments of the whole protein in its natural environment, docking results, and computational results in general will have to be taken with a grain of salt.

CONFLICT OF INTEREST

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HR reviewed the literature and prepared figures. Most of the text was prepared by HR and ŽD who initiated and guided preparation of the review. MB and LM took part in preparation of paragraphs dealing with the medicinal chemistry and clinical aspects of drug displacement from HSA, respectively.

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PRILOG III

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Warfarin and Flavonoids Do Not Share the Same Binding Region in Binding to the IIA Subdomain of Human Serum Albumin

Hrvoje Rimac [1], Claire Dufour [2], Željko Debeljak [3,4], Branka Zorc [1] and Mirza Bojić [1,*]

1 Department of Medicinal Chemistry, University of Zagreb, Faculty of Pharmacy and Biochemistry, Ante Kovačića 1, 10000 Zagreb, Croatia; hrimac@pharma.hr (H.R.); bzorc@pharma.hr (B.Z.)
2 UMR408 SQPOV, Safety and Quality of Plant Products, INRA, Avignon University, 228 Route de l'Aérodrome, 84000 Avignon, France; claire.dufour@inra.fr
3 Institute of Clinical Laboratory Diagnostics, Osijek University Hospital Center, Josipa Huttlera 4, 31000 Osijek, Croatia; debeljak.zeljko@kbo.hr
4 Department of Pharmacology, School of Medicine, University of Osijek, Cara Hadrijana 10/E, 31000 Osijek, Croatia
* Correspondence: mbojic@pharma.hr; Tel.: +385-1-4818-304

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Abstract: Human serum albumin (HSA) binds a variety of xenobiotics, including flavonoids and warfarin. The binding of another ligand to the IIA binding site on HSA can cause warfarin displacement and potentially the elevation of its free concentration in blood. Studies dealing with flavonoid-induced warfarin displacement from HSA provided controversial results: estimated risk of displacement ranged from none to serious. To resolve these controversies, in vitro study of simultaneous binding of warfarin and eight different flavonoid aglycons and glycosides to HSA was carried out by fluorescence spectroscopy as well as molecular docking. Results show that warfarin and flavonoids do not share the same binding region in binding to HSA. Interactions were only observed at high warfarin concentrations not attainable under recommended dosing regimes. Docking experiments show that flavonoid aglycons and glycosides do not bind at warfarin high affinity sites, but rather to different regions within the IIA HSA subdomain. Thus, the risk of clinically significant warfarin–flavonoid interaction in binding to HSA should be regarded as negligible.

Keywords: flavonoids; HSA binding; subdomain IIA; fluorimetry; docking; molecular modeling

1. Introduction

Human serum albumin (HSA) is a plasma protein capable of binding and transporting a wide variety of ligands, both endogenous and exogenous. It serves as a depot for xenobiotics, prolonging their half-life in circulation and regulating their blood concentration [1]. HSA is a protein with a molar mass of 66,500 g/mol and its blood concentration in healthy subjects ranges from 500–750 μM (35–50 g/L) [2]. It consists of three domains (I–III), each of which is comprised of two subdomains (A and B) and the two most important binding sites for drugs are situated in subdomains IIA and IIIA, which are also called warfarin and benzodiazepine site, respectively [3].

A large number of drugs characterized by a narrow therapeutic range, one of which is warfarin, binds to HSA [4]. The upper limit of therapeutic range for warfarin is approximately 9 μM (3 mg/L) [5,6]. Since most of warfarin molecules in blood are bound to HSA (>99%) [7] the presence of another compound which also binds to HSA can theoretically cause displacement of warfarin leading to elevation of free warfarin concentration [8,9]. These interactions could lead to adverse effects in patients suffering from a kidney or liver disease [10–12]. Although some of the aforementioned in vitro data implies

otherwise, displacement of warfarin from HSA is regarded as clinically insignificant under the normal dosing regime [11,13].

Flavonoids are plant products abundant in every day diet [14]. They have many salutary effects, of which their antioxidative [15], cardiovascular [16,17], and anticarcinogenic [18] are the most extensively studied. In some instances, their plasma concentrations may rise to micromolar values, e.g., after consumption of high quantities of onions, green tea, orange juice etc. [17,19,20]. They generally bind to the IIA site of HSA [21,22], suggesting that their binding may cause displacement of drugs from these binding sites, leading to adverse effects [23,24]. Once in circulation, flavonoids are extensively conjugated, mostly by glucuronidation [25] and sulfatation [26]. This is of critical importance for appropriate assessment of possible flavonoid interactions with other endogenous or exogenous ligands.

Recent research about flavonoid-induced displacement of warfarin from HSA produced controversial results [21,23,24,27,28]. Several different methods were used, including fluorescence spectroscopy [21,24], circular dichroism [23,28], nuclear magnetic resonance [23], ultracentrifugation [27], electronic absorption spectroscopy and molecular modeling [28], but the results vary from no risk of displacement [21] to significant risk of adverse effects associated with warfarin [24]. In this research, simultaneous binding of warfarin and different flavonoids (both aglycons and glycosides) (Figure 1) to the IIA HSA binding site was studied. Binding of ligands to the IIA binding site of HSA can easily be monitored by fluorescence spectroscopy [29,30]. This binding site is characterized by a hydrophobic cavity and a hydrophilic, positively charged entrance, ideal conditions for binding of small, negatively charged ligands such as warfarin or flavonoids [31]. A well-known property of this binding site is the presence of a single tryptophan residue (Trp-214), which fluoresces in aqueous solutions. When a ligand binds in the tryptophan vicinity, the fluorescence intensity decreases. This property enables determination of the ligand binding constant as well as calculation of the distance from the tryptophan residue [29,30]. Additionally, when unbound in a solution, warfarin and certain flavonoids are slightly fluorescent, but when they are bound to HSA, the intensity of their fluorescence increases considerably [21,32], making it possible to monitor their binding or displacement.

Table 1. Investigated flavonoids and their binding constants: correlation between theoretical and experimental values.

Name	R_1	R_2	R_3	$K \pm$ S.D. ($\times 10^4$ M^{-1})	Corr [1]
Warfarin	N/A	N/A	N/A	10.56 ± 0.68	0.998
Luteolin	-H	-H	-OH	12.20 ± 0.53	0.999
Isoorientin	-H	-Glc	-OH	4.57 ± 0.30	0.999
Cynaroside	-H	-H	-*O*-Glc	4.20 ± 0.25	0.999
Quercetin	-OH	-H	-OH	14.40 ± 0.52	1.000
Isoquercitrin	-*O*-Glc	-H	-OH	4.47 ± 0.21	1.000
Hyperoside	-*O*-Gal	-H	-OH	3.24 ± 0.19	0.999
Quercetin-3-*O*-glucuronide	-*O*-Gluc	-H	-OH	5.36 ± 0.13	1.000
Rutin	-Glc-Rha	-H	-OH	2.92 ± 0.13	1.000

[1] Correlation between experimental data and hypothesized 1:1 binding ratio calculated according to Equations (1)–(3). N/A: not applicable; S.D.: standard deviation.

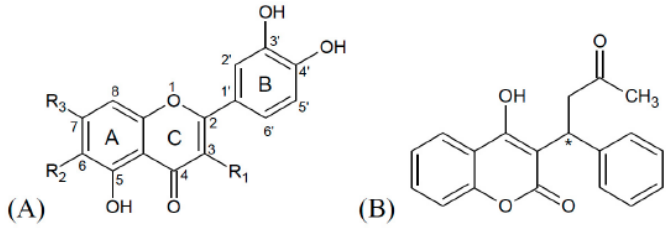

Figure 1. Chemical structures of (A) investigated flavonoids and (B) warfarin. See Table 1 for individual flavonoids.

Displacement studies were conducted at excitation and emission wavelengths of warfarin and flavonoids. Quercetin-3-*O*-glucuronide was included in this study as a representative of flavonoid glucuronides. Docking calculations for the HSA-quercetin and the HSA-quercetin-3-*O*-glucuronide complexes were also performed to improve the interpretation of in vitro observations and to determine their precise binding locations on HSA, as well as their interaction with a putative second, low affinity warfarin binding site.

2. Results and Discussion

2.1. Ligand Binding Constants

Quenching of the tryptophan fluorescence can be used to determine binding constants of ligands which bind in this region of HSA. Results are shown in Table 1.

Warfarin and flavonoids are derivatives of benzopyranone (Figure 1), so it may be hypothesized that they share the same binding site on HSA. Among the investigated ligands, quercetin is characterized by the highest binding constant, followed by luteolin and warfarin (Table 1). Lower values of binding constants for glycosides can be explained by their size. This is shown by rutin which, as the largest tested glycoside, has the lowest binding constant. The negative charge of the glucuronic acid moiety of quercetin-3-*O*-glucuronide slightly increases its binding constant compared to other glycosides, consistent with the premise about the strong binding of anions to IIA binding site [31,33].

2.2. Warfarin Displacement Studies

In order to evaluate the risk of drug displacement from HSA, in vitro techniques involving low HSA concentrations are usually employed [8,34,35]. Results obtained in this way cannot be directly extrapolated to physiological conditions, but can provide valuable insights [36]. For warfarin displacement studies, flavonoid fluorescence was used. All of the tested flavonoids have the 5-OH group, which is known to quench their intrinsic fluorescence [32]. However, when bound to HSA, these flavonoids fluoresce at excitation and emission wavelengths of 450 and 500–540 nm, respectively. If a flavonoid is unable to bind to HSA or binds with a lower or higher constant due to an external factor, this can be observed from the intensity of their maximum fluorescence (F_{max}) and the concentration needed to achieve it (c_{max}). With the binding constant of warfarin for HSA calculated to be $(10.56 \pm 0.68) \times 10^4$ M^{-1} and by application of Equations (4) and (5), it is possible to determine warfarin concentration needed to saturate a certain fraction of HSA binding sites, with presumed 1:1 binding ratio. It has to be pointed out that the warfarin sodium used in this study was racemic mixture: the binding constants for (*R*)- and (*S*)-warfarin are not identical, with the (*S*)-enantiomer having a slightly higher binding constant [37]. However, as already stated, they do bind to the exact same region of the IIA subdomain [38].

2.2.1. Simultaneous Binding of Warfarin and Flavonoid Aglycons

After incubation with warfarin, flavonoid concentration was gradually increased until F_{max} was achieved. Warfarin displacement due to its simultaneous binding to HSA with quercetin, luteolin, isoquercitrin, or cynaroside is represented by changes in fluorescence (Figure 2). The fact that some flavonoid aglycons bind more firmly to HSA in comparison to warfarin can suggest consequential warfarin displacement. The determination of the warfarin binding constant enabled calculation of the concentration needed to saturate a desired fraction of the IIA binding site. In such cases, obstruction of flavonoid binding will also decrease their fluorescence.

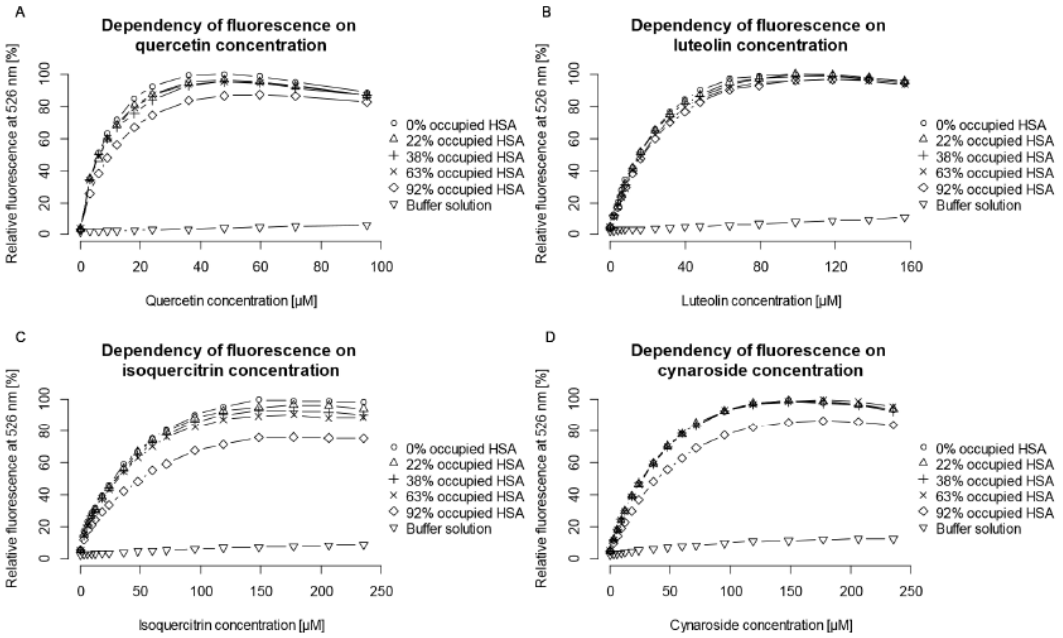

Figure 2. Effect of different warfarin concentrations on fluorescence of human serum albumin (HSA)-flavonoid complexes. (A) Quercetin; (B) luteolin; (C) isoquercitrin; and (D) cymaroside.

Addition of warfarin slightly decreased quercetin F_{max} with a slight increase in c_{max} (Figure 2A). A decrease of quercetin F_{max} with increasing warfarin concentration was already established for a bovine serum albumin (BSA)-quercetin complex [39]. This decrease is most evident when 92% of the IIA binding site is saturated with warfarin. In this case warfarin concentration is 120 μM, as opposed to a quercetin concentration of less than 60 μM, which is much higher than physiologically obtainable concentrations. For lower values of HSA saturation there is no significant difference in either F_{max} or c_{max}. It can be concluded that warfarin and quercetin do not share the same binding region even though they are both located in the IIA subdomain (Figure 3A). If the quercetin binding region would be situated farther inside the binding pocket than that of warfarin (Figure 3B), warfarin would impede quercetin binding and at high warfarin concentrations, the fluorescence at 526 nm would be quenched completely. However, these results, supported by docking experiments, do not confirm this scenario. Additional experiments (see Table S1 and Figure S1) were conducted where HSA was saturated with quercetin and warfarin concentration was gradually increased to determine if warfarin binding region is located farther inside the binding pocket than quercetin (Figure 3C). These results also support the hypothesis that there are no direct interactions between quercetin and warfarin. In both cases, even at the highest concentrations of the obstructing ligand, a small addition of the tested ligand causes significant rise in fluorescence intensity. Thus, warfarin and quercetin neither share the same binding region, nor is the binding region of one ligand situated in front of the other (Figure 3D), but rather a formation of a less fluorescent ternary HSA-quercetin-warfarin complex occurs, where quercetin and warfarin bind in the same, IIA binding domain, but in different regions and do not significantly influence each other's binding affinity. This is further confirmed by a lack of distinctive sigmoidal or Langmuir-like curves in cases of cooperative [40,41] or anti-cooperative binding [42,43], respectively (Figure 2). Similar results were obtained for luteolin (Figure 2B): the only difference is that there was no noticeable change in F_{max} and c_{max} between different percentages of saturated HSA, even in the case of 92% HSA saturation. It can be concluded that the 3-OH substituent is of high significance for fluorescence intensity of flavonoid molecules, and its position highly influences flavonoid F_{max}. Another explanation could be the presence of warfarin low affinity site (LAS), which could potentially

explain its higher impact on quercetin than on luteolin molecule as quercetin has an extra 3-OH group that could interact with warfarin bound to LAS at higher concentrations.

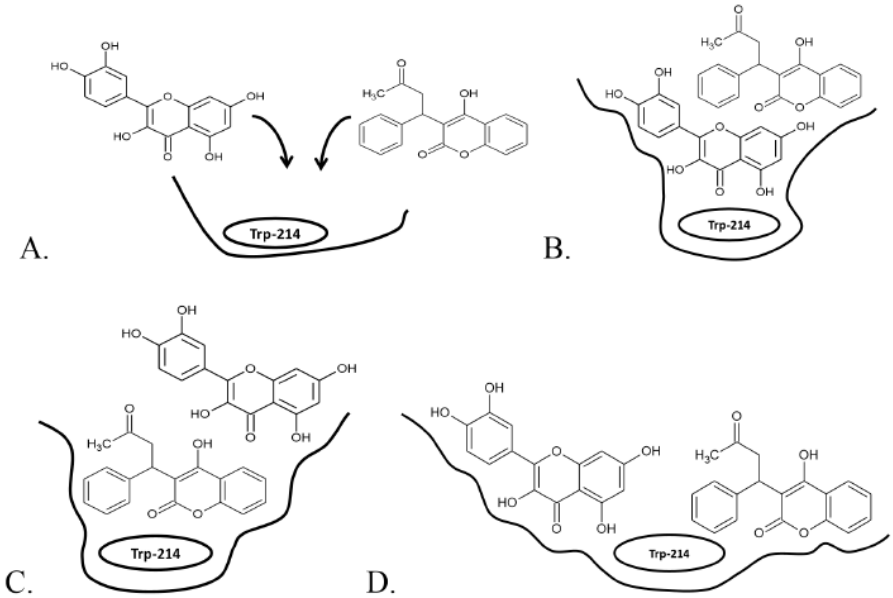

Figure 3. Proposed locations of warfarin and quercetin molecules in the IIA site of HSA. (Based on Petitpas et al. [38] and Ghuman et al. [44]). (**A**) Warfarin and quercetin bind to the same binding region; (**B**) warfarin binding region is situated in front of the quercetin binding region; (**C**) warfarin binding region is situated behind quercetin binding region; (**D**) warfarin and quercetin binding regions are independent of each other.

Our results—that flavonoids and warfarin do not share the same binding region—are consistent with results published by Zsila et al. [28] and Dufour and Dangles [21]. Further support comes from crystallographic structures obtained by Petitpas et al. [38] and research done by Yamasaki et al. [43,45] stating that the IIA binding site can accommodate additional ligands in the warfarin vicinity. The difference in flavonoid fluorescence can be attributed to conformational and possible size changes of the IIA binding site caused by warfarin. This causes changes in flavonoid binding site and flavonoid F_{max}, without a significant impact on their binding constant. Difference in flavonoid fluorescence at 92% HSA saturation can be explained by a more pronounced conformational change of the binding site, but the possibility of a secondary warfarin molecule bound at its LAS at higher warfarin concentrations directly influencing flavonoid fluorescence intensity cannot be excluded [27].

Results published by Di Bari et al. [23] and Poór et al. [24,46] state that quercetin, as well as some other flavonoids, can displace warfarin from its binding site even at lower concentrations. Di Bari et al. stated that the decrease in intensities of quercetin induced circular dichroism (ICD) bands suggests its displacement from HSA. Additionally, Poór et al. stated that the decrease of polarization values corresponds to higher rotational freedom of the free drug, i.e., displacement of warfarin from its albumin complex. As shown by Yamasaki et al. [43,45], binding of a second ligand can influence the mobility of the first ligand and affect ICD and fluorescence anisotropy without displacement involved; changes in fluorescence anisotropy or induced circular dichroism do not unequivocally suggest ligand displacement. Additionally, the saturation transfer difference nuclear magnetic resonance (STD-NMR) technique used by Di Bari et al. is characterized by the dependence of signal intensity on the proximity of ligand hydrogen atoms to HSA hydrogen atoms. Signal intensity depends on the inverse sixth power of the ligand-HSA distance and the relative signal intensity of quercetin in presence of warfarin was ~40% of its intensity without warfarin. Therefore, binding of warfarin does influence the distance

between quercetin and HSA, but the results do not explicitly show displacement. They can also suggest conformational changes.

2.2.2. Simultaneous Binding of Warfarin and Flavonoid Glycosides

Additional measurements were performed under the assumption that warfarin would obstruct binding of flavonoid glycosides more easily. Flavonoid glycosides have lower binding constants and are bulkier so they are expected to interact with warfarin more easily than their aglycon counterparts. As with aglycons, two different behaviors were observed: (a) quercetin derivatives (all substituted at position 3) experience a slight decrease in F_{max} with little or no impact on the c_{max}, except with HSA being 92% saturated (Figure 2C, data for the other quercetin derivatives are not shown due to similarity) and (b) luteolin derivatives (substituted at positions 6 and 7) experience no decrease in F_{max} nor c_{max}, again with the exception of the HSA being 92% saturated (Figure 2D, data for isoorientin are not shown due to similarity). Substituents at position 3 influence the fluorescence intensity as they participate in the large conjugated system connecting the B ring with the 4-oxo group, but have almost no effect on the interactions with warfarin. Substitution at positions 6 or 7 has no effect on flavonoid fluorescence nor on interactions with warfarin. As with quercetin, the only interaction observed was at 92% HSA saturation. This can be explained by the binding of warfarin to a LAS [38,39,44] causing allosteric interactions or energy transfer with flavonoids and formation of a less fluorescent ternary complex or by a direct competitive interaction. Also, as with quercetin and luteolin, it is evident that warfarin has a higher influence on quercetin than on luteolin glycosides, emphasizing the influence of the 3-OH group on fluorescence intensity.

2.3. Molecular Modeling of the Quercetin-HSA and Quercetin-3-O-glucuronide-HSA Complexes

The comparison of docked (*R*)- and (*S*)-warfarin with the crystallographic structure of (*R*)-warfarin bound to HSA (Protein Data Bank (PDB) entry 2BXD) showed high similarity of ligands' positions (Figure 4) and validating the appropriateness of this approach. In warfarin vicinity, there are four positively charged residues, Lys-199, Arg-222, His-242, and Arg-257, and three residues that are not charged, Tyr-150, Leu-238, and Leu-260. Warfarin also forms three hydrogen bonds, with Tyr-150, Arg-222, and His-242.

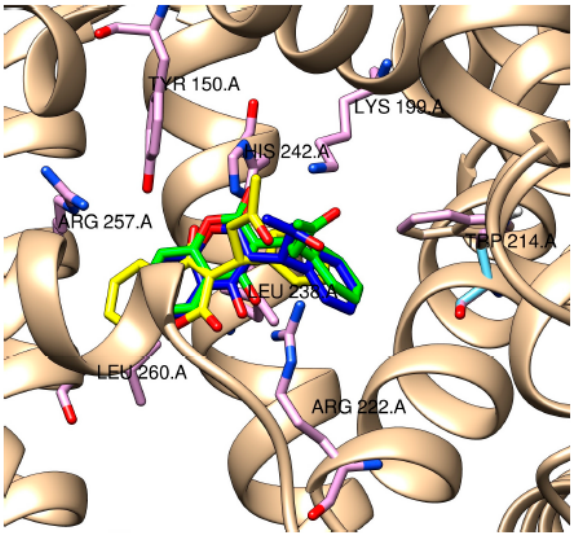

Figure 4. Overlay of crystallographic structure of (*R*)-warfarin (yellow) with docked (*R*)-warfarin (blue) and (*S*)-warfarin (green).

A docking summary for quercetin species can be found in Table 2. From all the shown clusters, only clusters number 1 and 3 of both 7- and 4′-quercetin anions and cluster 4 from 3,4′-fluorescent quercetin anion show overlap in binding positions with warfarin, but these clusters are formed from a very low number of runs (3 and 2 for quercetin anion at position 7, 1 and 1 for quercetin anion at position 4′, and 3 for fluorescent quercetin anion at positions 3 and 4′, respectively) and thus are unlikely. For all the other runs, for both non-fluorescent and fluorescent species, quercetin binding site does not overlap with that of warfarin and is located at the hydrophilic entrance of the IIA subdomain (Figure 5).

Table 2. Summary of quercetin species docking.

Quercetin Anion at Position 7			Quercetin Anion at Position 4′		
Cluster Rank	Number of Runs	Binding Energy (kcal/mol)	Cluster Rank	Number of Runs	Binding Energy (kcal/mol)
1.	3	−4.79	1.	1	−4.68
2.	1	−4.42	2.	19	−4.38
3.	2	−4.22	3.	1	−4.30
4.	4	−4.20	4.	2	−4.16
5.	12	−4.12	5.	9	−4.11
6.	23	−4.07	6.	9	−4.08
7.	3	−3.94	7.	2	−3.69
8.	14	−3.94	8.	8	−3.68
Fluorescent quercetin anion at positions 3 and 7			**Fluorescent quercetin anion at positions 3 and 4′**		
Cluster Rank	Number of Runs	Binding Energy (kcal/mol)	Cluster Rank	Number of Runs	Binding Energy (kcal/mol)
1.	43	−4.66	1.	5	−4.42
2.	1	−4.61	2.	18	−4.04
3.	4	−4.60	3.	13	−3.87
4.	7	−4.24	4.	3	−3.82
5.	4	−4.20	5.	1	−3.81

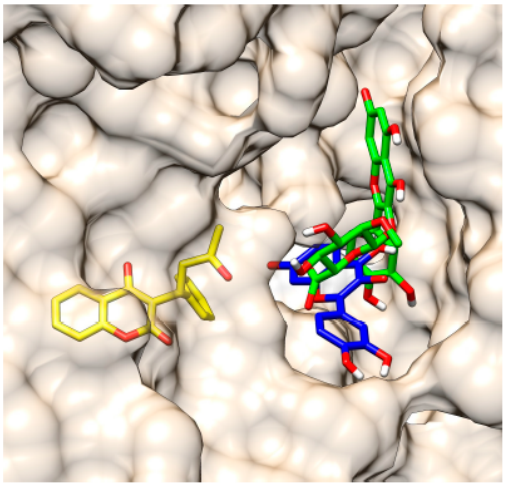

Figure 5. Overlay of crystallographic structure of (*R*)-warfarin (yellow) with docked fluorescent species of quercetin (blue) and quercetin-3-*O*-glucuronide (green).

On the other hand, the binding site of quercetin-3-*O*-glucuronide does not overlap with the binding site of warfarin in any of the runs, but overlaps with the quercetin binding site. The number of runs per cluster for quercetin-3-*O*-glucuronide (Table 3) is lower than that of quercetin, which can be explained by a greater flexibility of the glucuronide ligand, as well as by the spaciousness of the

HSA IIA entrance which is able to accommodate it in various conformations. The binding sites of all the clusters is located at the mouth of the IIA subdomain (Figure 5), with the aglycon moiety of anionic and fluorescent anionic species being rotated by approximately 180°. The glucuronic moiety of the molecules is located in the same plane, but in the case of fluorescent anionic species, it is rotated by 90° and protrudes towards the protein surface (Figure 6), which can be the cause of weaker binding (Table 3).

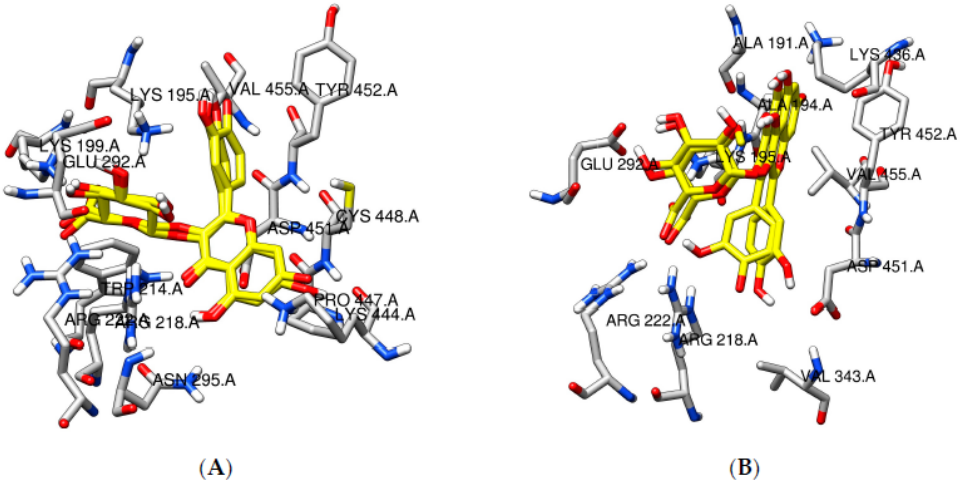

Figure 6. Comparison of docked positions of quercetin-3-*O*-glucuronide species (yellow) with nearby amino acid residues. (**A**) quercetin-3-*O*-glucuronide anionic species; (**B**) fluorescent quercetin-3-*O*-glucuronide anionic species. Nitrogen atoms are colored blue, oxygen atoms red, hydrogen atoms white, and HSA carbon atoms grey.

Table 3. Summary of quercetin-3-*O*-glucuronide species docking.

Quercetin-3-*O*-glucuronide Anion at Position 7			Quercetin-3-*O*-glucuronide Anion at Position 4′		
Cluster Rank	**Number of Runs**	**Binding Energy (kcal/mol)**	**Cluster Rank**	**Number of Runs**	**Binding Energy (kcal/mol)**
1.	4	−5.02	1.	5	−6.09
2.	5	−4.76	2.	3	−4.89
3.	1	−4.62	3.	2	−4.78
4.	4	−4.49	4.	4	−4.74
5.	2	−4.49	5.	6	−4.52
6.	9	−4.45	6.	1	−4.16
7.	5	−4.45	7.	4	−4.02
8.	1	−4.35	8.	4	−3.99
Fluorescent quercetin-3-*O*-glucuronide anion at position 7			**Fluorescent quercetin-3-*O*-glucuronide anion at position 4′**		
Cluster Rank	**Number of Runs**	**Binding Energy (kcal/mol)**	**Cluster Rank**	**Number of Runs**	**Binding Energy (kcal/mol)**
1.	13	−4.21	1.	5	−4.42
2.	2	−4.07	2.	18	−4.04
3.	11	−4.06	3.	13	−3.87
4.	1	−4.05	4.	3	−3.82
5.	3	−3.95	5.	1	−3.81

Docking results of (*R*)- and (*S*)-warfarin in order to locate warfarin's LAS (Table 4) both showed (almost unanimously for all runs for both isomers) a high preference for a binding region located in the vicinity of Ala-191, Lys-195, and Tyr-452, which are amino residues that also interact with flavonoid glycosides in their binding to HSA but do not have high significance in flavonoid aglycone

binding (Figure 7). From the data, it can be concluded that warfarin binding to its LAS could compete with flavonoid glycosides in binding to HSA as the location of the coumarin and flavonoid AC rings overlap. This can explain lower fluorescence of flavonoid glycosides at high warfarin concentrations. On the other hand, warfarin bound to its LAS does not seem to interfere with binding of flavonoid aglycons. However, the proximity of the conjugated AC rings (see Figure 1) to the warfarin coumarin ring could explain lower quercetin fluorescence at high warfarin concentrations via energy transfer. Luteolin is less affected by this energy transfer in terms of relative change in fluorescence intensity (Figure 2). However, its absolute fluorescence is lower in all cases precisely due to the lack of the 3-OH group (see Table S2).

Table 4. Summary of (*R*)- and (*S*)-warfarin low affinity site docking.

(*R*)-Warfarin			(*S*)-Warfarin		
Cluster Rank	Number of Runs	Binding Energy (kcal/mol)	Cluster Rank	Number of Runs	Binding Energy (kcal/mol)
1.	9	−6.70	1.	27	−6.70
2.	6	−6.52	2.	6	−6.56
3.	4	−6.49	3.	5	−6.51
4.	35	−6.28	4.	15	−6.42
5.	4	−6.27	5.	2	−6.31

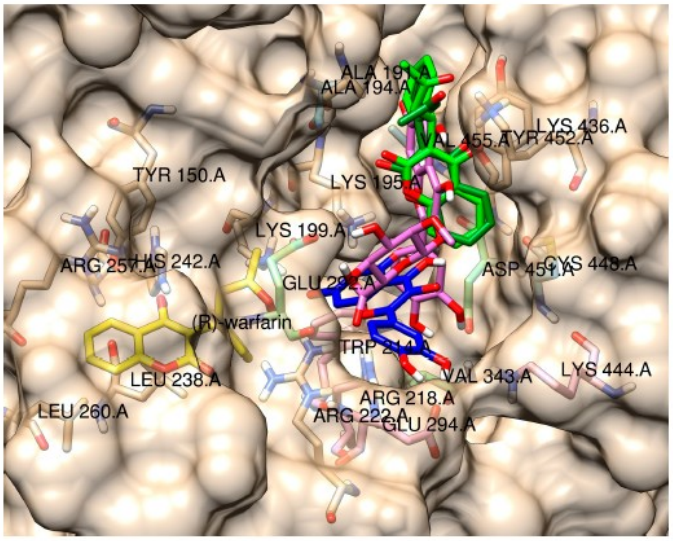

Figure 7. Comparison of crystallographic data of (*R*)-warfarin (yellow) with docked position of (*R*)- and (*S*)-warfarin at LAS (both green), fluorescent quercetin anion at positions 3 and 7 (blue), and fluorescent quercetin-3-*O*-glucuronide anion at position 7 (pink) with nearby amino acid residues.

3. Materials and Methods

3.1. Chemicals and Materials

Fatty acid free HSA and warfarin sodium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavonoids were obtained from Sigma-Aldrich (quercetin), TransMIT GmbH (Gießen, Germany) (isoquercitrin, rutin, hyperoside, cynaroside, and isoorientin), and Extrasynthèse (Genay, France) (luteolin and quercetin-3-*O*-glucuronide). All standards had a specified purity of ≥98% and have been used without further purification.

3.2. Fluorescence Measurements

HSA was freshly dissolved in Dulbecco's phosphate-buffered saline pH 7.4 (137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, 1.47 mM potassium dihydrogen phosphate) immediately before measurements. The stock solution of warfarin sodium was prepared in MeOH, while the stock solution of luteolin was prepared in MeOH:DMSO = 2:1, and of other flavonoids in MeOH:DMSO = 4:1. In all experiments, the final co-solvent concentration was held below 5%. This was done to prevent conformational changes of HSA due to changes in dielectric constant of the solvent.

Steady-state fluorescence was recorded using a thermostated Safas Xenius fluorometer (Safas Monaco, Monaco) with an integrated stirrer. All studies were performed at 25 ± 1 °C using 10 nm excitation and emission slit widths. Warfarin and flavonoid solutions were added to a 2 mL of a 6 μM (0.4 g/L) HSA solution placed in a quartz cell. Higher concentrations of HSA and ligands were not used due to poor solubility of ligands and high influence of ligand absorption. Measurements were performed in duplicate.

3.3. Determination of Binding Constants

A stock solution of a single ligand was gradually added via syringe to a 2 mL of a 6 μM HSA solution placed in a quartz cell. Measurements were conducted at the excitation wavelength of 295 nm and emission was recorded in the 310–400 nm range enabling calculations of corresponding binding constants. As flavonoids and warfarin both absorb light at 295 and 320 nm, their molar absorption coefficients (ε) were calculated at these wavelengths using a water-thermostated HP 8453 UV-visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA) at 25 ± 0.1 °C to take into account the inner filter effect. Binding constants were determined according to Dufour and Dangles [21]. A Scatchard analysis was performed assuming n identical binding sites on HSA. Values for binding constant (K), molar fluorescence intensity of the complex (f), and the stoichiometric coefficient (n) were estimated by fitting the F vs. Lt curves against Equations (1) and (2) using a least-square regression program Scientist (MicroMath, Salt Lake City, UT, USA), where F is fluorescence intensity, $[L]$ the free ligand concentration, $[L_t]$ the total ligand concentration, and c the total albumin concentration. Since all values of n were close to 1, a simple 1:1 binding model was used. In all cases, the inner filter effect was taken into account by using absorption of free ligands at excitation and emission wavelengths (ε_{ex} and ε_{em}, respectively) (Equation (3)), where F_{corr} is the fluorescence corrected for the inner filter effect, F_0 is the measured fluorescence, ε is the sum of ε_{ex} and ε_{em}, l = 0.65 is the optical path and c is the flavonoid concentration.

$$F_{corr} = fc\frac{nK[L]}{1+K[L]} \tag{1}$$

$$[L_t] = [L]\left(1+\frac{ncK}{1+K[L]}\right) \tag{2}$$

$$F_0 = F_{corr} \times 10^{-\varepsilon l c} \tag{3}$$

3.4. Fluorescence Measurements

In all experiments, the concentration of HSA was also kept at 6 μM while the fraction of HSA saturated with warfarin has been varied: 0%, 22%, 38%, 63% and 92%. The saturated fraction was calculated using the Equations (4) and (5),

$$K = \frac{[cL]}{\left[c_f\right][L]} \tag{4}$$

$$p = \frac{[cL]}{[c]} \quad (5)$$

where K is the binding constant, $[cL]$ is the concentration of HSA-ligand complex, $[c_f]$ is the concentration of unbound HSA, $[L]$ is the free ligand concentration, $[c]$ is the total HSA concentration and p is the fraction of saturated HSA. Flavonoid binding measurements were conducted at the excitation wavelength of 450 nm to maximize the fluorescence of the bound flavonoid and emission was recorded in the 466–580 nm range. Competition of warfarin and flavonoids for binding to HSA was determined based on the maximum intensity of fluorescence (F_{max}) of flavonoids and the concentration needed to achieve it (c_{max}). Additional measurements were taken at the excitation and emission wavelengths of warfarin, 317 and 380 nm, respectively, to confirm data obtained from the flavonoid fluorescence measurements.

3.5. Docking Experiments

The AutoDock 4.2.6. (The Scripps Research Institute, La Jolla, CA, USA) [47] uses dispersion, hydrogen bonding, and electrostatic and desolvatation energy components to determine the conformation of the most probable complex. It was used to locate probable quercetin, quercetin-3-*O*-glucuronide, and secondary (*R*)- and (*S*)-warfarin binding sites on the HSA molecule. Quercetin was chosen for these experiments as a representative of flavonoid aglycons, and quercetin-3-*O*-glucuronide was chosen as a representative of quercetin metabolites. The three-dimensional coordinates of HSA molecule co-crystallized with (*R*)-warfarin was obtained from the RCSB [44]. This structure was chosen because it is the only one where HSA was crystallized only with warfarin (without myristic acid) and best suits our experimental conditions. Monomer A was selected for the docking procedure and missing side-chain atoms were added. The warfarin molecule, as well as water molecules, were removed from the file used for docking of flavonoids. Hydrogens were added to the HSA molecule, all Lys, Arg, and Cys side-chains were protonated, all Asp and Glu side-chains were deprotonated, the amino and carboxy termini were charged, the His-242 side-chain was N_ε-protonated, and all other His side-chains were N_δ-protonated based on visual inspection, resulting in the net charge of -14. Due to their similarity to flavonoids, docking of (*R*)- and (*S*)-warfarin was also performed and their docking locations were determined to be the same and correspond to the location of the crystallized (*R*)-warfarin. This is in correspondence with previous research showing that (*R*)- and (*S*)-warfarin bind at the same region of Sudlow's site I. [35] Additionally, docking of (*R*)- and (*S*)-warfarin was also performed in presence of crystallized (*R*)-warfarin to locate a putative secondary binding site located in Sudlow's site I. The three-dimensional forms of the ligands were drawn and their initial geometries were optimized in HyperChem 8.0 (Hypercube, Inc., Gainesville, FL, USA), and their charge was set to represent the most abundant species at pH 7.4, calculated according to Rimac et al. [48], as well as verified at chemicalize.com. For (*R*)- and (*S*)-warfarin, anionic species were docked, while for quercetin and quercetin-3-*O*-glucuronide, two most prevalent anionic and fluorescent species [49] were docked. Afterward, ligands were read in the AutoDock software in a compatible file format and partial charges were set according to Ionescu et al. [50] As binding of flavonoids was confirmed to be at the Sudlow's site I, grid maps of size 80 × 80 × 80 Å were generated with 0.375 Å spacing centered on the coordinates of the side-chain nitrogen in Trp-214 (3.466, −10.477, −4.189) in Sudlow's site I by the AutoGrid program [47] and Lamarckian genetic algorithm (LGA) [51] was applied. The receptor molecule was regarded as rigid while all ligand single bonds were allowed to rotate freely during the Monte Carlo simulated annealing procedure. Ligand flexible docking simulations were performed with 100 runs, population size of 150, 2.5×10^7 energy evaluations, 27,000 numbers of generations, rate of gene mutation of 0.02, and rate of crossover 0.08. Root-mean-square-deviation (rmsd) of 2.0 Å was used as a criterion for cluster analysis of the docking results (in order to determine if two docked conformations were similar enough to be included in the same cluster) and several such clusters with the lowest binding energy of each ligand species were used for further comparison.

4. Conclusions

Warfarin and selected flavonoid aglycons and glycosides do not share the same binding region as the IIA binding site. Rather, their binding regions are separated and loosely dependent of each other through allosteric modulation, i.e., conformational change of HSA or through a presence of warfarin LAS. While the warfarin binding region is located inside the hydrophobic pocket of the IIA subdomain, binding regions for flavonoid aglycons and glycosides are located at the hydrophilic entrance of the IIA binding pocket, where a putative warfarin LAS is also located. The only significant interaction between warfarin and flavonoid occurs at physiologically unattainable warfarin and flavonoid concentrations, but even at those concentrations, displacement is very questionable and can be due to energy transfer.

Supplementary Materials: Supplementary materials are available online.

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Author Contributions: Ž.D., C.D. and M.B. conceived and designed the experiments; H.R. performed the experiments; Ž.D., C.D., and H.R. analyzed the data; B.Z. contributed reagents/materials/analysis tools; H.R., C.D., Ž.D. and M.B. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors as well as commercially.

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9. ŽIVOTOPIS

Hrvoje Rimac, rođen je 1. svibnja 1988. godine. U Zagrebu je završio osnovnu školu i XVI gimnaziju. Od 2006. do 2011. godine studira na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu, integrirani preddiplomski i diplomski studij farmacije. Tijekom studiranja je bio aktivan i u studentskoj udruzi studenata farmacije i medicinske biokemije (CPSA) gdje je sudjelovao i u nekoliko međunarodnih projekata. Dobitnik je Stipendije grada Zagreba, kao i Rektorove nagrade za rad „Antifungalni i antioksidativni učinci polifenola u uvjetima *in vitro*". Diplomski rad pod nazivom „Semiempirijsko optimiziranje geometrije odabranih derivata hidroksiureje" izradio je na Zavodu za farmaceutsku kemiju Farmaceutsko-biokemijskog fakulteta pod vodstvom doc. dr. sc. Željka Debeljaka te je diplomirao 8. rujna 2011. kao jedan od 10% najboljih studenata u generaciji. Godine 2011. upisuje i diplomski studij Molekularna biotehnologija na Prehrambeno-biokemijskom fakultetu Sveučilišta u Zagrebu. Diplomski rad pod nazivom „Anotacija genskih nakupina prirodnih spojeva u genomu bakterije *Streptomyces sp*. C" izradio je u Kabinetu za bioinformatiku na Zavodu za biokemijsko inženjerstvo pod mentorstvom doc. dr. sc. Jurice Žučka te je diplomirao 20. rujna 2013., također kao jedan od 10% najboljih studenata u generaciji. Zapošljava se 2012. kao asistent na Zavodu za farmaceutsku kemiju Farmaceutsko-biokemijskog fakulteta. Iste godine upisuje doktorski studij Farmaceutsko-biokemijske znanosti na Farmaceutsko-biokemijskom fakultetu. Godine 2015. upisuje poslijediplomski specijalistički studij Razvoj lijekova na Farmaceutsko-biokemijskom fakultetu. Ostvario je jedan znanstveni boravak u trajanju od tri mjeseca (rujan – prosinac 2014.) na Nacionalnom institutu za agronomska istraživanja (franc. *Institut national de la recherche agronomique*, INRA) u Avignonu, Francuska kroz stipendiju Vlade Republike Francuske, pod vodstvom dr. sc. Claire Dufour. Sudjelovao je na više međunarodnih i domaćih kongresa sa sveukupno 16 posterskih priopćenja te je održao dva javna predavanja na kongresima.

Popis znanstvenih radova (CC):

1. **H. Rimac**, Ž. Debeljak, D. Šakić, T. Weitner, M. Gabričević, V. Vrček, B. Zorc, M. Bojić, Structural and electronic determinants of flavonoid binding to human serum albumin: An extensive ligand-based study, RSC Adv. 6 (2016) 75014–75022.
2. **H. Rimac**, Ž. Debeljak, M. Bojić, L. Millerd. Displacement of Drugs from Human Serum Albumin: From Molecular Interactions to Clinical Significance, Curr. Med. Chem. 24 (2017) 1–18.

3. **H. Rimac**, C. Dufour, Ž. Debeljak, B. Zorc, M. Bojić. Warfarin and flavonoids do not share the same rinding region in binding to the IIA subdomain of human serum albumin, Molecules 22 (2017) 1153.

10. TEMELJNA DOKUMENTACIJSKA KARTICA

TEMELJNA DOKUMENTACIJSKA KARTICA

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Zavod za farmaceutsku kemiju
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INTERAKCIJE FLAVONOIDA I ODABRANIH KSENOBIOTIKA PRI VEZANJU NA HUMANI SERUMSKI ALBUMIN

Hrvoje Rimac

SAŽETAK

Flavonoidi, kao i veliki broj ksenobiotika, nakon ulaska u cirkulaciju vežu se za humani serumski albumin (HSA) u poddomeni IIA. To vezno mjesto se naziva i Sudlowljevo mjesto I, odnosno vezno mjesto I. S obzirom na veliki broj ksenobiotika koji se ovdje veže, postavlja se pitanje važnosti interakcija istiskivanja pojedinih spojeva s tog veznog mjesta. Iz tog je razloga potrebno bolje opisati svojstva vezanja flavonoida za vezno mjesto I te kliničkih interakcija s lijekovima do kojih bi potencijalno moglo doći.
U prvom dijelu istraživanja ispitivana su vezna svojstva i glavne odrednice vezanja aglikona flavonoida za vezno mjesto u poddomeni IIA HSA. Za određivanje konstanti vezanja koristila se metoda fluorescencijske spektrofotometrije, a za izračunavanje kvantno-kemijskih molekulskih deskriptora računalni program Gaussian 09. U drugom dijelu istraživanja ispitivane su interakcije između aglikona i glikozida flavonoida s odabranim lijekovima te je u tu svrhu korištena metoda fluorescencijske spektrofotometrije zajedno s metodama sidrenja pomoću računalnog programa AutoDock 4.2.6.
Eksperimentalno su utvrđene konstante vezanja 20 flavonoida, koje su iznosile između $5{,}25 \times 10^3$ i $1{,}95 \times 10^5$ M^{-1}. QSAR analizom pronašlo se više strukturnih odlika aglikona flavonoida koje su odgovorne za njihovo vezanje za vezno mjesto I: nukleofilnost i parcijalni naboj atoma C3, parcijalni naboj atoma O4, koplanarnost A i C prstena te koplanarnost AC i B prstena i HOMO i LUMO energije, od čega su najjači utjecaj imala proton-donorska i proton-akceptorska svojstva te koplanarnost prstena. Također je pronađeno da ispitivani lijekovi i flavonoidi mogu ulaziti u interakcije pri vezanju za HSA, ali je vjerojatnost njihova međusobnog istiskivanja pri fiziološkim uvjetima niska jer se ne vežu za isto vezno područje.
Utvrđena su sterička i elektronska svojstva flavonoida koja utječu na jačinu njihova vezanja na HSA. Vjerojatnost istiskivanja ispitivanih lijekova s HSA pomoću flavonoida s klinički važnim posljedicama je niska. Ispitivani lijekovi mogu i pojačavati i smanjivati intenzitet fluorescencije flavonoida vezanih za HSA, tako da je primjena fluorescencije u rutinskom ispitivanju vezanja lijekova na HSA ograničena.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 86 stranica, 27 grafičkih prikaza, 6 tablica i 149 literaturni navod. Izvornik je na hrvatskom jeziku.

Ključne riječi: humani serumski albumin, flavonoidi, lijekovi, interakcije, fluorescencija, QSAR
Mentori: **Dr. sc. Branka Zorc**, *redovita profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*
Dr. sc. Mirza Bojić, *docent Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta*
Ocjenjivači: **Dr. sc. Zrinka Rajić Džolić**, *izvanredna profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*
Dr. sc. Željan Maleš, *redoviti profesor Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*
Dr. sc. Dubravko Jelić, *viši znanstveni suradnik, Fidelta d.o.o., Zagreb*

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BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Pharmacy and Biochemistry
Department of Medicinal Chemistry
A. Kovačića 1, 10000 Zagreb, Croatia

Doctoral thesis

COMPETITION BETWEEN FLAVONOIDS AND SELECTED XENOBIOTICS FOR BINDING SITES ON HUMAN SERUM ALBUMIN

Hrvoje Rimac

SUMMARY

Flavonoids, as well as a large number of xenobiotics, after entering circulation, bind to human serum albumin (HSA) at the IIA subdomain. This binding site is also called Sudlow site I or binding site I. Considering the large number of xenobiotics which bind to this site, it is necessary to evaluate the importance of their displacement interactions from the binding site I. Also, it is important to better describe the characteristics of flavonoid binding to the binding site I and clinical interactions with drugs which could possibly occur.

In the first part of the study, characteristics of flavonoid aglycons' binding to the binding site I, as well as their most important determinants were studied. Fluorescence spectrophotometry was used to determine the binding constants and computer program Gaussian 09 was used to calculate quantum-chemical molecular descriptors. In the second part of the study, interactions between flavonoid aglycons and glycosides with selected drugs were studied by using fluorescence spectrophotometry combined with docking studies carried with AutoDock 4.2.6. computer program.

Binding constants of 20 flavonoids were experimentally determined and they ranged from $5{,}25 \times 10^3$ to $1{,}95 \times 10^5$ M^{-1}. QSAR analysis found several flavonoid features which are key determinants responsible for their binding to the binding site I: nucleophilicity and partial charge of the C3 atom, partial charge of the O4 atom, coplanarity of the A and C rings, as well as coplanarity of the AC and B rings, and HOMO and LUMO energies, with proton-donor and proton-acceptor properties and coplanarity being the most prominent ones. It was also found that the selected drugs and flavonoids can interact in binding to the binding site I, but the possibility of their mutual displacement interactions is not high as they bind to different binding regions.

Flavonoid sterical and electronic characteristics which influence their binding to HSA were determined. A small possibility of displacement of selected drugs by flavonoids with clinically important consequences was found. Examined drugs can both increase and decrease fluorescence intensity of flavonoids bound to HSA, thus the application of fluorescence in routine determination of drug binding to HSA is limited.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 86 pages, 27 figures, 9 tables and 149 references. Original is in Croatian language.

Keywords: human serum albumin, flavonoids, drugs, interactions, fluorescence, QSAR

Mentor: **Branka Zorc, Ph.D.**, *Full Professor,* University of Zagreb Faculty of Pharmacy and Biochemistry
Mirza Bojić, Ph.D., *Assistant Professor,* University of Zagreb Faculty of Pharmacy and Biochemistry

Reviewers: **Zrinka Rajić Džolić, Ph.D.,** *Associate Professor,* University of Zagreb Faculty of Pharmacy and Biochemistry
Željan Maleš, Ph.D., *Full Professor,* University of Zagreb Faculty of Pharmacy and Biochemistry
Dubravko Jelić, Ph.D., *Senior Research Associate, Fidelta d.o.o., Zagreb*

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