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Predicting plasma protein binding of drugs: a new approach

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Abstract

In spite of the large amount of plasma protein binding data for drugs, it is not obvious and there is no clear consensus among different disciplines how to deal with this parameter in multidimensional lead optimization strategies. In this work, we have made a comprehensive study on the importance of plasma protein binding and the influencing factors in order to get new insights for this molecular property. Our analysis of the distribution of percentage plasma protein binding among therapeutic drugs showed that no general rules for protein binding can be derived, except for the class of chemotherapeutics, where a clear trend towards lower binding could be observed. For the majority of indication areas, however, empirical rules are missing. We present here an extensive list of multiply determined primary association constants for binding to human serum albumin (HSA) for 138 compounds from the literature. Correlating these binding constants with the percentage fraction of protein bound showed that the percentage data above 90%, corresponding to a binding constant below 6 μM, are of insufficient accuracy. Furthermore, it could be demonstrated that the lipophilicity of drugs, traditionally felt to dominate binding to HSA, is not the only relevant descriptor. Here, we report a generic model for the prediction of drug association constants to HSA, which uses a pharmacophoric similarity concept and partial least square analysis (PLS) to construct a quantitative structure–activity relationship. It is able to single out the submicromolar to nanomolar binders, i.e. to differentiate between 99.0 and 99.99% plasma protein binding. Depending on the system, this can be important in medicinal chemistry programs and may together with other computed physicochemical and ADME properties assist in the prioritization of synthetic strategies.

Keywords: Plasma protein binding; Human serum albumin; Albumin binding prediction; ADME modeling; Therapeutic drugs; Lipophilicity

1. Introduction

Binding of drugs to plasma proteins, mostly to serum albumin and α-acid glycoprotein, is one of many factors that influences drug disposition. It is widely accepted that the effect of a drug is related to the exposure of a patient to the unbound concentration of drug in plasma rather than total concentration [1,2]. Considering the high concentration of albumin and the wide range of effective concentrations of therapeutic drugs from nanomolar to millimolar [3], the free concentration available for therapeutic action can be effectively reduced for drugs with high binding to plasma proteins, although the affinity of drugs to plasma proteins is often less than for the receptor or enzyme targets. Drug–protein complexes in plasma also serve as drug reservoir for free drug concentration, as the drug is removed from the body by various elimination processes, and prolong the duration of drug action. Thus, the physicochemical properties, the effective concentration to be targeted and the potential side effects, influence how much binding to plasma proteins can still be tolerated and how precisely this parameter has to be tuned for a new drug entity [1,2].

Human serum albumin (HSA) [4], the most abundant protein in blood plasma (M_r 66 kDa, concentration 0.53–0.75 mM), has multiple hydrophobic binding sites (a total of eight for fatty acids, an endogenous ligand of HSA) and binds a diverse set of drugs, especially neutral and negatively charged hydrophobic compounds [3]. For drug-like compounds, two high affinity binding sites have been proposed in subdomains IIA and IIIA of HSA, which are highly elongated hydrophobic pockets with charged lysine's and arginine's residues near the surface which function as attachment points for polar ligand features [4–14]. Recently, high resolution X-ray crystal structures of HSA complexed

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Abbreviations: HSA, human serum albumin; TPR, topological pharmacophore; DFP, Daylight fingerprint.

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with two anesthetics, propofol and halothane [15], and with the widely used anticoagulant, warfarin [16], showed that by increasing the ligand concentration, ligands can occupy all known binding sites with different affinities and different pharmacological relevance. A detailed understanding of the binding mode of most drugs is, however, still missing. Instead, binding to albumin is widely assumed to be merely dominated by the lipophilicity of compounds [17].

This study elucidates on the one hand, possible constraints for the plasma protein binding of drugs implied by the therapeutic conditions. On the other hand, we analyze the effect of molecular properties, traditionally felt to influence plasma binding of drugs, in order to get new insights for the rational tuning of this molecular property and with the view to design a predictive model for albumin binding affinities of drugs. We demonstrate that lipophilicity is rather poorly correlated to HSA binding for a diverse set of molecules, in contrast to congeneric series, where lipophilicity is often found to be the dominant factor, suggesting that specific molecular recognition elements beside physicochemical parameters are essential. Based on these findings, we describe here a new, generic prediction tool for albumin binding of drugs built on a pharmacophoric similarity concept, which uses a comprehensive list of the pharmacological most relevant, first association constants to HSA for 138 compounds, which are available in the literature, as data source. With this new approach, we are able to single out the submicromolar to nanomolar binders, e.g. to differentiate between 99.0 and 99.99% plasma binding, in early stages of medicinal chemistry programs and to assist in prioritizing synthetic strategies.

2. Methods

2.1. Chemicals

(+/−)-2-(6-Methoxy-2-naphthyl)propionic acid (naproxen, M1220 TCI) were used from Isliker Albert & Co AG. Chlorothiazide (550-086-G001) ALEXIS Biochemicals. Chlorpromazine (C-904), imipramine (I-902) and nortriptyline (N-907) were purchased by Radian International LLC. Promazine (28724p) was ordered from AAPIN Chemicals Ltd. Verapamil (676777) was ordered from Calbiochem-Novabiochem Corp. Urapidil hydrochloride (193736) was bought from ICN Biomedicals, Inc. Doxycycline (000263) was purchased from Jenson Chemicals Ltd. Fentiazac (F0392) TCI, practolol (0831) and sotalol (F-0952) were ordered from Tocris Cookson Inc. 2-Propylpentanoic acid (20833-0250) was purchased from Acros Organics. Carbenoxolone (RO-22-4929/601) was available in-house. (+/−)-Lorazepam (L-1764), 3-acetylcoumarin (21,467-1), 4-acetoxybiphenyl (41,196-5), 4-chromanol (30,389-5), 4-hydroxycoumarin (H2,380-5), 5,5-diphenylhydantoin (phenytoin, 16,192-6), 5-fluorouracil (85,847-1), α-ethyl-3-hydroxy-2,4,6-triiodohydrocinnamic acid (iophenoxate, 36,104-6), acetylsalicylic acid (A-5376), bilirubin (B-4126), butyl 4-aminobenzoate (34,005-7), camptothecin (36,563-7), cefaclor (C-6895), cefadroxil (C-7020), cefamandole sodium salt (C-7145), cefoperazone sodium salt (C-4292), cefotaxime sodium salt (C-7912), cefoxitin sodium salt (C-4786), cefsulodin sodium salt (C-8145), ceftriaxone sodium salt (C-5793), cefuroxime sodium salt (C-4417), cephaloridine (C-3519), cephalothin sodium salt (C-3050), cephapirin sodium salt (C-8270), cephradine (C-8395), chlorpropamide (C-1290), clorazepate dipotassium (C-1402), coumarin (C8,555-7), dansylamide (21,889-8), dansyl-L-asparagine (D-0735), dansylsarcosine piperidinium salt (D-9631), diazepam (D-0899), diclofenac sodium (D-6899), dicumarol (M-1390), diflunisal (D-3281), digitoxin (85,173-6), etodolac (E-0516), fenbufen (F-8755), fenoprofen calcium salt hydrate (F-1517), flurbiprofen (F-8514), furosemide (F-4381), ibuprofen (28474-2), indomethacin (I-7378), indoprofen (I-3132), iodipamide (I-0639), ketoprofen (K-1751), lidocaine (L-7757), L-thyroxine (23,470-2), L-tryptophan (T-90204), norclomipramine hydrochloride (desipramine, N1280), oxazepam (O-5254), phenylbutazone (21,186-9), pregnenolone (P-9129), procaine HCL (22,297-6), quinine (Q1878), salicylic acid (24,758-8), sulfaphenazole (S-0758), sulfathiazole (29,290-7), sulindac (S-8139), suprofen (S-9894), temazepam (T-8275), testosterone (86500), tolbutamide (T-0891), warfarin (A-2250), zomepirac sodium salt (Z-2625), oxyphenbutazone hydrate (O-6128), nimesulide (N-1016), novobiocin sodium salt (N-1628), sulfobromophthalein sodium salt (S-0252), alprenolol hydrochloride (A-8676), penicillin G potassium salt (13750), bupivacaine hydrochloride (B-5274), 2-(4-chlorophenoxy)-2-methylpropionic acid (clofibric acid, 19,777-7), digitoxigenin (37020), disopyramide (D-7644), fusidic acid (F-0756), methotrexate (06564), methylorange (23,410-9), moxisylyte hydrochloride (M-5154), nafcillin sodium salt (N-3269), nalidixic acid (15,854-2), nicergoline (N-7889), L-noradrenaline (norepinephrine, A-7257), 5-ethyl-5-phenylbarbituric acid (phenobarbital, 04710), (R)-(+)-propranolol hydrochloride (82065), (S)-(−)-propranolol hydrochloride (82066), sulfamethoxazole (S-7507), sulfisoxazole (S-6377), tetracycline hydrochloride (T-4062), trimethoprim (T-7883), (3-(*N*-tris[hydroxymethyl]methylamino)-2-hydroxypropanesulfonicacid) (TAPSO, T5566) were purchased from Sigma-Aldrich, Inc.

2.2. Percentage protein binding data

The source for the percentage plasma protein binding data (f_b, fraction bound to plasma proteins) of therapeutic drugs was the handbooks of Goodman and Gilman's [3] and of Dollery [18]. The percentage data were converted into an equivalent binding affinity k_A with the following formula derived from the law of mass. k_A is the binding affinity to HSA under the assumption that binding occurs

exclusively to HSA, a binary complex is formed, and an excess of albumin (concentration 0.6 mM, [HSA]) is present compared to the concentration of the drug.

$$\log k_A = \log \frac{[f_b]}{1 - [f_b]} - \log[\mathrm{HSA}] \quad (1)$$

2.3. The log D-determinations

The log *D*-measurements were performed by a newly developed in-house HTS-screening method based on shake-flask partitioning on 96-well microtiter plates. Compounds are distributed between water and octanol. Then, the distribution coefficient is calculated from the difference in concentration in the aqueous phase before and after partitioning and from the ratio of the amounts of water and octanol phases. Starting from 10 mM DMSO-stock solution, the drug molecule is dispensed in aqueous buffer (50 mM TAPSO, pH 7.4) to get a starting concentration of 0.5 mM. One part of this solution is then analyzed by UV spectroscopy (Plate reader Spectra Max 250/ (Molecular Devices). The obtained optical density is equal to the concentration of the substance before partitioning. An exact amount of 1-octanol is added, and the mixture is then incubated by shaking (2 hr). The emulsion is allowed to separate overnight (12 hr) to reach partition equilibrium. After that, the layers need thorough centrifugation at 3000 rpm for 10 min and the concentration of the substance in the aqueous phase is determined again by measuring the UV-absorption. In order to cover the lipophilicity range from −1.5 to 4.0 several octanol buffer ratios are used simultaneously. The whole procedure is completely automated. All solvent systems have to be presaturated with each other before use.

2.4. Data sets

The binding affinities ($\log k_{Lit}$) of 138 compounds to HSA are given in Table 1. This data set was extracted from the literature (see Table 1 and references therein). 25 Roche compounds with their association constants measured in-house by equilibrium dialysis were added to the training set to enrich the data set towards more lipophilic compounds (Table 2). Furthermore, a data set for HSA binding of 76 glycine/NMDA receptor antagonists from the literature was used for validation purposes, which was derived from HPLC retention volumes using a column with a stationary phase of HSA immobilized on silica gel [19].

2.5. Molecular similarity calculations

The topological pharmacophore (TPR) and the Daylight fingerprint (DFP) description of the compounds were derived from the associated programs of Moloc (Gerber Molecular Design; http://www.moloc.ch) and Daylight CIS Inc., Moloc-topological pharmacophore generator (Mtprgn) and DFP toolkit 4.7 [20], respectively. The similarity matrices were then calculated by using the Moloc-topological pharmacophore similarity program (Mtprsml), a companion program of Moloc, and the

Table 1
Compounds: physicochemical properties and HSA binding levels

Compound	MW[a]	f_b (%)[b]	log k_{Lit}	SD	N	IIA	IIIA	Lit ref.	A/B	pK_A[a]	log D (pH 7.4)
3-Acetylcoumarin	188.19		4.39	0.26	2	1		[57,58]	0		1.5
3-Carboxy-4-methyl-5-propyl-2-furan-propionic acid	240.28	99	7.11		1	1		[59]	−2	3.2/3.6	−0.59
4-Chromanol	150.19		4.83		2	1		[57,58]	0		1.31
4-Hydroxycoumarin	162.15		5.64	0.04	2	1		[57,58]	−1	4.15	−0.99
5-Dimethylamino-naphtalene-1-sulfonamide	250.34		4.82		1	1		[60–62]	0		
7-Anilinocoumarin-4-acetic acid	295.31		4.92		1			[73]	−1		
Acenocoumarin	353.35	99	5.32	0.04	2	1		[63,64]	−1		
Alprenolol	249.39	85	3.02		1			[65]	1	9.6	1.1
Amobarbital	226.31		3.66		1			[65]	0	7.96	
Aspirin	180.17	55	4.37	0.71	3	1		[66–68]	−1	3.48	<−1.5
Azapropazone	300.40	99	5.88	0.29	4	1		[69–72]	0		
Benoxaprofen	301.74	99	6.28		1		1	[69]	−1		
Benzylpenicillin	334.42	65	3.04		1			[65]	−1	2.75	−1.81
Bilirubin	584.73	99.9	7.79	0.32	6			[59,62,73–78]	−2	4.4	2.94
Binedaline	293.45	96	4.48		1			[79]	1		
Bupivacaine	288.48	95	3.88		1			[79]	1	8.21	2.41
Camptothecin	348.38	98.3	6.56	0.68	2	1		[80–84]	0	10.83	2.69
Carbamazepine	236.29	80	3.13		1			[65]	0		
Carbenoxolone	570.84	99	7.7		1			[65]	−2		1.61
Carprofen	273.73	99	6.64	0.1	2		1	[50,85–88]	−1		
Carprofenmethylester	287.76		5		1	1		[85]	0		
Cefaclor	367.83	24.7	2.11		1		1	[89]	1		−2.71

Table 1 (*Continued*)

Compound	MW[a]	f_b (%)[b]	log k_{Lit}	SD	N	IIA	IIIA	Lit ref.	A/B	pK_A[a]	log D (pH 7.4)
Cefadroxil	363.42	20	3.28		1		1	[89]	1		−3.4
Cefamandole	462.54	73	3.57	0.42	3		1	[53,89,90]	−1		−2.4
Cefazolin	454.54	87.5	4.36	0.44	5			[53,78,89–92]	−1	2.1	
Cefoperazone	645.73	91	4.54	0.29	2		1	[53,89]	−1		−2.12
Cefotaxime	455.50	38	3.1	0.16	3			[53,89,90]	−1		−2.52
Cefoxitin	427.48	76.5	3.05		1			[53,89,90]	−1		−1.89
Cefsulodin	532.58	45	2.58		1		1	[89]	−1		<−3
Ceftazidime	546.62	15.5	2.68		1		1	[89]	1		
Ceftriaxone	554.62	93.8	4.72	0.19	4	1	1	[89,92–94]	−2	3.2	−1.23
Cefuroxime	424.42	31.5	3.14	0.57	2			[53,89]	−1		−1.91
Cephalexin	347.42	14.5	3.63		1		1	[89]	1	3.2	
Cephaloglycin	405.46		2.91		1		1	[89]	1	4.6	
Cephaloridine	415.51	20	2.5	0.25	2			[53,89]	1	3.4	−1.62
Cephalothin	396.46	68	3.61		1			[53,89]	−1	2.3	−2.2
Cephapirin	423.49	62	3.31	0.08	3			[53,89,90]	−1	2.15	−1.22
Cephradine	349.44	12	2.54	0.28	2		1	[89,90]	1		−2.1
Cetiedil	349.58	74	3.99		1			[79]	1		
Chlorazepatedipotassium	332.76		4.11		1			[95]	−1		2.68
Chlorothiazide	295.73	82.3	4.61	0.18	2		1	[96–98]	1	6.75	−0.98
Chlorpromazine	318.89	97.8	4.95	0.46	2			[99–102]	1	9.2	
Chlorpropamide	276.76	96	4.98	0.47	3	1		[70,103]	−1	4.92	−0.18
Cicletanine	261.72	93.5	4.88		1		1	[104]	0	3.11	
Cimoxatone	338.39	94.5	4.61		1			[79]	0		
Clofibricacid	214.66	98	5.52		1			[79]	−1	4.46	−0.93
Clometacin	357.81	99	4.44		1			[79]	−1		
Coumarin	146.15		3.89		2	1		[57,58]	0		1.48
Dansylasparagine	365.44		5.18		1	1		[64]	−1		<−1.5
Dansylsarcosine	322.41		5.69	0.3	8		1	[59,61,62,67,105]	−1		
Desipramine	266.42	86	4.85		1			[102]	1	10.65	1.86
Diazepam	284.76	99	5.31	0.31	11		1	[68,70,106–113]	0	3.17	2.68
Diclofenac	296.16	99.5	5.9	0.23	2		1	[69,114,115]	−1	3.8	1.14
Dicoumarol	336.31		6.1	0.38	5	1		[51,116–119]	−2	6.78	0.9
Diflunisal	250.21	99	6.2	0.71	2		1	[69,120]	−1	3	−0.02
Digitoxigenin	374.57	92.7	4.53		1			[65]	0		2.43
Digitoxin	765.05	93.5	5.26	0.91	8			[83,106,108,121–125]	0		2.83
Disopyramide	325.50	89	3.66		1			[65]	1	10.4	−0.6
Doxycycline	444.48	90.5	4.42		1			[79]	−1	3.4	−0.24
Etodolac	287.39	99.1	5.03	0.75	4	1	1	[126,127]	−1		
Fenbufen	254.30	98.5	5.62	0.3	3		1	[50,69,128]	−1	4.51	0.46
Fenoprofen	242.29	99	5.67	0.48	2		1	[50,129,130]	−1	4.5	0.83
Fentiazac	329.81	99.5	5.57		1			[79]	−1	3.6	1.08
Fluindione	240.24	99.5	5.69		1			[79]	0		
Fluorouracil	130.08	11								8.02	−0.86
Flurbiprofen	244.28	99	5.95	0.52	5		1	[50,69,128,131,132]	−1	4.27	0.74
Furosemide	330.76	98.4	5.27	0.04	3	1		[133–137]	−1	3.9	−1.23
Fusidicacid	516.79	94.8	4.89		1			[65]	−1	5.35	
Halofenate	415.82	99.5	5.2		1			[137]	0		
Ibuprofen	206.31	99	5.52	0.55	10		1	[50,67,69,72,85,138–146]	−1	4.55	1.07
Imipramine	280.45	92.6	4.38		1			[102]	1	9.5	
Indomethacin	357.81	94.5	5.71	0.34	6	1		[69,147–150]	−1	4.5	0.74
Indoprofen	281.33	98	5.27	0.04	2		1	[50,151]	−1	4.6	−0.51
Iodipamide	1139.76		7.18	0.26	2	1		[64,152]	−2	3.5	−1.84
Iophenoxate	571.92		7.9		1	1		[153]	−2		1.24
Itanoxone	300.75	98.5	5.29		1			[79]	−1	5.3	
Ketoprofen	254.30	95	6.16	0.33	3		1	[50,87,154–157]	−1	4.6	−0.18
Lidocaine	234.38	67	3.74	1.94	2			[157,159]	1	7.89	1.65
Lorazepam	321.16	91.5								0.39	2.19
Mepivacaine	246.39	84	5.4		1			[158]	1	7.73	
Methicillin	380.45	39	2.96		1			[65]	−1	2.77	
Methotrexate	454.50	53	3.45		1			[79]	−2	4.7	−2.52
Methylorange	305.38		5.57		1			[65]	−1		−0.74
Moxisylyte	279.42	26	2.83		1			[79]	1	8.72	1.77
Nafcillin	414.51	89.4	4.08		1			[65]	−1	2.7	−1.25
Nalidixicacid	232.26	94	4.34		1			[79]	−1	6.12	0.22

Table 1 (*Continued*)

Compound	MW[a]	f_b (%)[b]	log k_{Lit}	SD	N	IIA	IIIA	Lit ref.	A/B	pK_A[a]	log D (pH 7.4)
Naproxen	230.28	99.4	6.2	0.9	6		1	[69,72,136,142,143,160,161]	−1	4.08	0.12
n-Butyl-*p*-aminobenzoate	193.27		4.45		1	1		[64]	0	5.38	2.82
Nicergoline	484.43	95	3.79		1			[79]	1		2.82
Nimesulide	308.33	99	5.69		1			[162]	−1		1.72
Norepinephrine	169.20	50	7		1			[65]	1	8.42	−2.97
Nortriptyline	263.41	93.5	3.51		1			[79]	1	10.11	
Novobiocin	612.69	90	5.74		1			[163]	−1	4.3	1.22
Oxazepam	286.73	98.4	4.56		1		1	[164]	0	1.8	2.26
Oxazepamhemisuccinate	386.81		5.52	0.16	2		1	[155,165]	−1		
Oxyphenbutazone	324.41	99	5.29	0.29	4	1		[67,166–169]	0	4.7	−0.02
Pbiphenylacetate	212.26		6.11		1		1	[69]	0		3.23
Pentobarbital	226.31	51.3	3.08		1			[79]	0	7.97	
Phenobarbital	232.26	50.5	3		1			[79]	−1	7.3	1.21
Phenylbutazone	308.41	97.8	5.54	0.39	13	1		[58,63,64,67,69,70,72,161, 166–168,170–173]	0	4.8	0.28
Phenytoin	252.29	91	4.07	0.23	2	1		[172,173]	0	8.33	2.22
Pipotiazine	475.72	45	3.27		1			[79]	1		
Piretanide	362.43	94	5.13		1			[79]	−1	4.1	
Pirprofen	251.73	99.8	5.75	0.23	2		1	[50,174,175]	−1		
Practolol	266.38	30	2.42		1			[79]	1	9.4	−1.26
Pregnenolone	316.53	80	4.63		1	1		[176]	0		4.22
Procaine	236.35	6	3.49		1			[158]	1	8.11	0.18
Promazine	284.45		4.93		1			[102]	1	9.4	2.18
Propranolol	259.38	94	3.58		1			[79]	1	9.6	1.19
Quinine	324.46	93	3.88		1			[177]	1	8.05	2.04
Salicylicacid	138.13	95	4.93	0.44	11		1	[75,67,69,70,106–108,148,171,178,179]	−1	2.98	−1.33
Sotalol	272.40	17.5	3.3		1			[79]	1	8.15	−1.74
Sulfaethidole	284.38		5.18		1			[180]	−1	5.65	
Sulfamethoxazole	253.30	66	3.7		1			[79]	−1	5.81	−0.76
Sulfaphenazole	314.39	95	5.3	0.38	5			[171,181]	−1	5.91	−0.07
Sulfathiazole	255.33		4.4		1	1		[70]	−1	7.1	−0.45
Sulfisoxazole	267.33	91.4	4.34		1			[79]	−1	4.62	−1.55
Sulfobromophthalein	796.04	99.9	7.23		1			[182,183]	−2		−1.39
Sulindac	356.43	93.5	5.4	1.32	3		1	[161,184,185]	−1	4.5	−0.11
Suprofen	260.32	99	5.18	0.16	4		1	[50,155,186]	−1		−0.48
Suprofenmethylester	274.35		5.09		1	1		[186]	0		
Temazepam	300.74	96.8									2.04
Testosterone	288.47	60	4.47	0.14	4	1		[176,187–189]	0		3.13
Tetracycline	444.48	50	4.64		1			[65]	0	3.3	−1.16
Thiopental	242.37	82.5	3.64		1			[79]	−1	7.5	
Thyroxine	776.87		5.77	0.35	5	1		[188,190–198]	1	2.2	3.33
Ticlopidine	263.80	96.5	3.97		1			[79]	0		
Tinoridine	316.45	92	4.38		1			[79]	0		
Tolazamide	311.44	97	4.94		1			[103]	−1	6.18	
Tolbutamide	270.38	96	6.52	1.43	4		1	[103,136,171,199–202]	−1	5.27	1.04
Triflupromazine	352.45		4.74		1			[99,100]	1	9.07	
Trimethoprim	290.36	41.5	3.03		1			[79]	0	7.12	0.39
Tryptophan	204.25	80	4.59	0.57	3		1	[203–206]	1	2.3	−1.04
Urapidil	387.54	80	3.34		1	1		[207]	1	7.1	0.99
Valproicacid	144.24	93	4.76		1			[79]	−1	4.95	0.13
Verapamil	454.67	90	3.43		1			[208]	1	8.68	2.36
Warfarin	308.35	99	5.33	0.22	20	1		[58,63,64,67,70,72,83,106, 107,117,124,125,161,171, 204,209–218]	−1	5	1.03
Zomepirac	291.75	98.5	4.28		1		1	[161]	−1		−0.42

[a] MW, and pK_A-values were derived from the MedChem [219] database.

[b] For the percentage plasma protein binding data, the handbook of Therapeutic Drugs [18] was used. MW, Molecular weight; f_b, percentage plasma protein binding; log k_{Lit}, primary association constant from the literature; SD, standard deviation of the independently determined association constants found in the literature by different methods; N, number of independent measurements of the binding constant in the literature; IIA and IIIA, compound binds preferentially to the binding pocket within domain IIA or IIIA (1 indicates binding); A/B, Acids or Bases, where zero present a neutral compound or zwitter ion, negative and positive numbers indicate a negative and positive charge at pH 7.4, respectively. pK_A, log-value of the dissociation constant; log *D*, log-value of the octanol-water partition coefficient at pH 7.4 (non-ion corrected, phosphate buffer, see Section 2).

Table 2
Roche compounds: physicochemical properties and HSA binding levels

Compound	MW	log k	kow_C log P[a]
RO1	464.56	6.17	5.64
RO2	565.56	5.47	7.55
RO3	578.59	4.69	7.52
RO4	479.52	5.32	2.35
RO5	429.57	3.69	3.43
RO6	366.24	4.58	2.67
RO7	341.45	3.38	3.82
RO8	226.68	3.77	2.89
RO9	412.47	3.95	1.56
RO10	437.37	4.29	6.2
RO11	542.44	3.95	2.71
RO12	551.62	5.26	3.07
RO13	558.66	3.53	0.05
RO14	370.53	2.8	5.41
RO15	372.81	2.99	0.04
RO16	408.34	5.69	5.96
RO17	448.95	4.03	3.82
RO18	273.24	6.56	3.13
RO19	495.64	4.12	6.27
RO20	339.48	3.89	4.61
RO21	378.31	4.74	3.85
RO22	337.38	5.39	2.4
RO23	437.47	5.21	3.74
RO24	310.33	4.76	−0.24
RO25	248.72	4.72	2.41

[a] kow_C log P, Version 1.65 from Syracuse Research Corporation. MW, Molecular weight; log k, primary association constant from equilibrium dialysis studies; kow_C log P, calculated log-value of the octanol-water partition coefficient.

Tanimoto alogrithm of Daylight [20]. The final descriptors were derived by the diagonalization of these matrices by using the main program Moloc, Gerber Molecular Design [21].

2.6. Partial least square analysis (PLS)

The PLS models were established by using the software package TSAR, Oxford Molecular Ltd. [22]. The overall predictive quality of the PLS models was determined by the use of the cross-validated correlation factor, q^2. For the perfect prediction of the data, q^2 has a value of 1, for predictions, which are not better than random, a value of 0 is obtained. The formula of r^2 (Pearson correlation factor) and q^2 is defined as follow [23,24]:

$$r^2 = 1 - \frac{\sum_{i=1}^{N}(y_{i,\text{observed}} - y_{\text{calculated}})^2}{\sum_{i=1}^{N}(y_{i,\text{observed}} - \overline{y}_{i,\text{observed}})^2} \quad (2)$$

$$q^2 = 1 - \frac{\sum_{i=1}^{N}(y_{i,\text{observed}} - y_{\text{predicted}})^2}{\sum_{i=1}^{N}(y_{i,\text{observed}} - \overline{y}_{i,\text{observed}})^2} \quad (3)$$

Furthermore, the standard error for prediction (σ_{calc}) and cross-validated predictions (σ_{pred}) were calculated. The best model is the one with the smallest σ_{pred}. The used formula is given below, where N represents the number of compounds in the data set and n is the number of PLS components used for the model [23,24]:

$$\sigma_{\text{calc}} = \sqrt{\frac{\sum_{i=1}^{N}(y_{i,\text{observed}} - y_{\text{calculated}})^2}{N - n - 1}} \quad (4)$$

$$\sigma_{\text{pred}} = \sqrt{\frac{\sum_{i=1}^{N}(y_{i,\text{observed}} - y_{\text{predicted}})^2}{N - n - 1}} \quad (5)$$

2.7. Model validations

To generate randomized similarity matrices for model validation purposes, the software SIMCA-P, Umetrics AB [25] was used. To select the subsets for the external validation of the TPR model, the largest minimum distance method [26] implemented in the software package GOLPE, Molecular Discovery Ltd. [27] was employed.

3. Results

3.1. Protein binding of therapeutic drugs

It can be estimated, that there are around 1500 frequently described drugs [28] on the market today and for many of them, the percentage of protein binding is reported [18]. The analysis of the distribution of the percentage plasma protein binding of therapeutic drugs [18]—belonging to one of the four indications: central nervous system (CNS), inflammation, renal/cardiovascular, chemotherapy—showed that 43% possess higher protein binding than 90%. Because of no obvious trend and an broad distribution of the plasma protein binding of drugs over the whole range, the therapeutics were further separated into different indication areas. Fig. 1 shows the histograms of distributions of protein binding for four different indications.

It can be seen that only for the chemotherapeutics including antibiotic, antiviral, antifungal and anticancer drugs a low level of protein binding is observable, where 77.2% of the compounds have protein binding below 90%. Surprisingly, hardly any preference for low or high binding is evident for the remaining three indications (CNS, inflammation, renal/cardiovascular). An exception is perhaps the high percentage (26.6.%) of anti-inflammatory drugs with protein levels above 99%.

To investigate whether threshold-values for plasma protein binding exist dependent on the effective concentration [3] (total concentration of a drug in plasma associated with the wanted effect) and the solubility [18] of therapeutic drugs, we studied the correlation between these parameters and the percentage plasma protein binding. The values for the solubility of the different therapeutic drugs were randomly distributed and there was no obvious correlation between the solubility of a drug and its plasma protein binding or the effective concentration. In summary, only

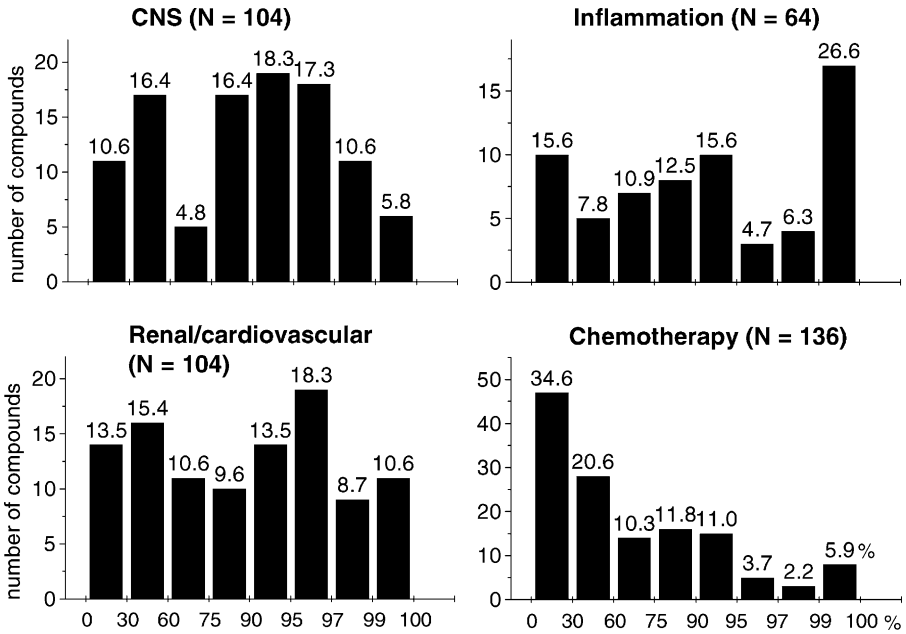

Fig. 1. Distribution of the plasma protein binding (percentage protein bound) of therapeutic drugs [18] for different indication areas. Of the chemotherapeutic drugs on the market, which include antibiotics, antiviral, antifungal and anticancer drugs, only few show high protein binding. For the other classes, no conspicuous trends are seen.

for the design of chemotherapeutics low plasma protein binding seems to be of advantage.

3.2. HSA binding levels

3.2.1. Association constants vs. percentage protein binding data

For many drugs, values of f_b have been measured, but only comparably small data sets of association constants for binding of drugs to various plasma proteins exist in the literature, probably because of the time-consuming measurements. A comprehensive search in the literature led to the compilation of 138 association constants for binding to HSA for a diverse set of compounds. Because of the multiple binding sites of HSA, a saturation for binding to HSA was rarely reported in the binding studies (see Table 1 and references therein). Therefore, for a single compound, multiple binding constants to HSA can often be found in the literature depending on the applied equation to fit the data. Due to the non-standardized calculation of association constants to HSA, we could use only the primary association constant to compare the data for the different drugs. Because for most of the drugs with medium to high binding affinity, the first association constant is well separated from the following binding constants, we are confident, that the first association constant is the most relevant one from a pharmacological point of view (see Table 1 and references therein).

Fig. 2 shows a comparison between the percentage data for plasma protein binding and the independently determined primary association constants to HSA (k_{Lit}) of 138 compounds (for more information see Table 1). The association constants reported in Table 1 and Fig. 2, present an average-value over all independently determined association constants found in the literature for one compound (at pH 7.4, 298 K and phosphate buffer) by various methods. The percentage data are reasonably well correlated with the log k_{Lit} in the range of low values, but fail to account for strong binding. Table 3 shows the relationship between the percentage data and the approximate binding constants using the formula given in Eq. (1) (see Section 2).

By choosing the percentage representation, the considerable number of strong binding compounds is presented by a very narrow interval. Taking into account the experimental error and the limited accuracy of the available

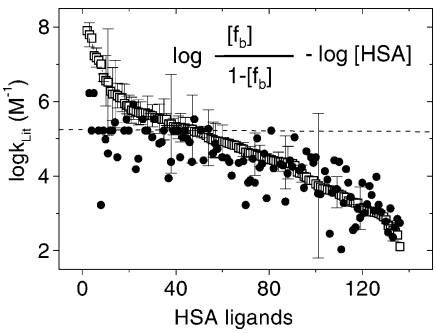

Fig. 2. Comparison between the experimentally determined primary association constants (log k_{Lit}, □) and the approximate binding affinities (●) calculated from the percentage protein binding of 138 HSA ligands. The displayed formula shows how the approximate binding affinities were calculated from the percentage data. [f_b], fraction bound to protein. A HSA concentration of 0.6 mM in plasma was used for the calculation. The dashed line indicates 99.0% plasma protein binding of the ligands showing the lack of the percentage data to differentiate among strong binders (submicromolar to nanomolar).

Table 3
Relationship between the percentage plasma protein binding and the approximate effective binding affinity and dissociation constant

Percentage plasma protein binding (%)	log k_A[a]	K_D (μM)
99.99	7.22	0.06
99.90	6.22	0.60
99.0	5.22	6.1
90.0	4.18	67
70.0	3.59	257
50.0	3.22	600

[a] This effective binding affinity k_A was calculated using the formula of Eq. (1). K_D, corresponding dissociation constant.

percentage data of drugs in the range between 99.0 and 99.99%, such data (when transformed to a logarithmic scale) cannot differentiate between drugs with association constants above 165,000 M^{-1}. Since mostly strong binding will possibly have any influence on the therapeutic effect of drugs, the recognition of high binders is essential. Therefore, a prediction model, which should be able to single out these cases, must utilize the association constant data, which accurately cover the critical range of high binding.

3.3. Correlation between binding data and the experimental determined log D-values

The binding constants to HSA span a range of six log-units. One would expect that differences in shape, lipophilicity or charge distribution of the molecules are essential for an explanation of this phenomenon. Lipophilicity is one of the most commonly used descriptor and very often thought to dominate binding to HSA [17]. In order to test this hypothesis with a homogenous data set, we have experimentally determined the octanol-water partition coefficient (log *D*) at pH 7.4 in phosphate buffer for 105 commercial drugs (see Table 1), for which the HSA association constants were available as well. For 76 compounds, log *D*-values could be successfully determined in the measurable range between −1 and +4.0, the other drugs were unstable, precipitated, or did not show sufficient UV absorption during log *D*-measurements. There is no correlation between the HSA association constants and the log *D* values for all 76 compounds as indicated by a correlation coefficient *r* of 0.2 [29]. Fig. 3 shows plots of HSA association constants for acidic (Fig. 3A) and basic (Fig. 3B) compounds against their log *D*-values. Fig. 3A shows a fair correlation for the major part of the investigated acids. However, four compounds reduce the correlation factor *r* from 0.7 to 0.4. Sulfobromophthalein and iodipamide have high binding affinities and low log *D*-values because of two acid groups and on the other end of the spectrum, phenobarbital and chlorazepatedipotassium show high log *D*-values and low binding. For the bases, there is no correlation observable ($r = 0.3$) between binding to HSA and lipophilicity. This study shows that bases and acids should be treated separately, because the

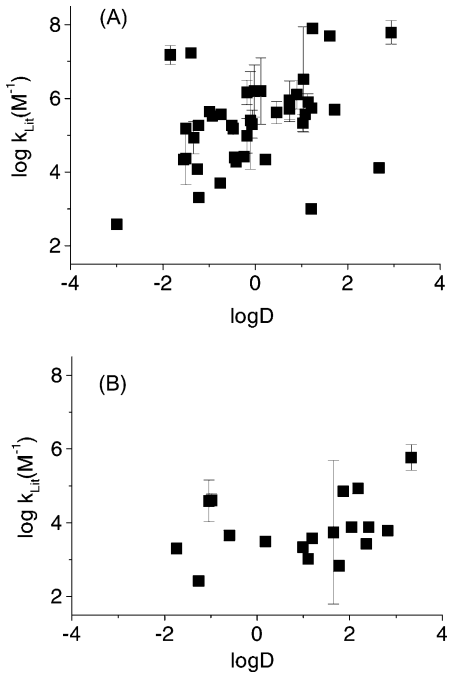

Fig. 3. Correlation between the primary association constants (log k_{Lit}) for binding to HSA and the octanol/water partition coefficients log *D* (pH 7.4, phosphate buffer). (A) Acids. (B) Bases. The Pearson correlation factor is $r = 0.4$ for the acids and $r = 0.3$ for bases.

influence of the lipophilicity on binding to HSA is larger for acids than for bases. In general, however, this parameter is too weakly correlated with binding to HSA in order to yield satisfactory predictions for diverse sets of compounds.

3.4. Prediction models: TPR vs. DFP

To estimate the potential of plasma protein binding of a drug candidate, we were interested in the development of a generic prediction tool to assist in the prioritization of synthetic strategies and to get an alert for potential high binders in virtual screening procedures. There is no *ab initio* model for calculating ADME (absorption–distribution–metabolism–elimination) properties, such as plasma protein binding, therefore molecules have to be characterized by quantitative scales (descriptors) and a quantitative structure–activity relationship (QSAR) between suitable descriptors and the biological property has to be established by statistical methods. These QSAR models are local models and are only valid within the descriptor space (interpolation), i.e. predictions outside of the descriptor space are not reliable (extrapolation).

Log *D*-values (lipophilicity) of molecules, an example of an experimental molecular property descriptor, was shown to be only partially sufficient to explain the binding to HSA. Our intention was to construct a QSAR model for the binding of drugs to HSA, the most abundant protein in blood plasma, which on the one hand yields better prediction of binding constants to this protein and on the other hand relies solely on calculated descriptors. Furthermore, it

should be applicable for structurally diverse compound classes in a virtual screening process. According to the similarity principle [30], one can expect that similar molecules, i.e. compounds with similar distribution of their pharmacophore units (hydrogen acceptor, hydrogen donor, hydrophobic parts), experience comparable interaction with a protein and, thus, also have similar binding constants. Therefore, a prediction model can be developed based on the given similarity relation. We used a new TPR description of molecules [31] and, for comparison, the well-known DFP description [32]. The TPR concept is demonstrated for the non-steroidal anti-inflammatory drug, ketoprofen in Fig. 4A. Ketoprofen is described by two hydrogen acceptors (PU1 and PU2) and two hydrophobic pharmacophore units (PU3 and PU4). The hydrogen acceptor strength values (AS) have been derived from the force field MAB [33,34] and the hydrophobic pharmacophore units are characterized by their size. The pharmacophore is further characterized by a distance matrix between pharmacophore units, which is derived from topological bond distances between all atoms of each pharmacophore unit. All this is an automated procedure described in more detail in reference [31]. The number of pharmacophore units, which are needed to represent a given molecule, is dependent on its size. Large molecules, therefore, have more pharmacophore units. The histogram of Fig. 4B illustrates the distribution of pharmacophore units for our HSA data set, which in addition to the 138 ligands from the literature, comprises 25 Roche compounds, for which equilibrium dialysis data were available in-house (Molecular weight (MW), calculated log *P* and the primary binding constant are given for the Roche compounds in Table 2). These compounds have been added to increase the diversity of our data set towards more lipophilic compounds.

As one can see in Fig. 4B, pharmacophores with four units are by far most frequent and 93% of all compounds yield less than ten units. Due to the sparse population with large pharmacophores, we restricted the model to molecules with less than 10 pharmacophoric units.

The similarity value for two pharmacophores is calculated by pairing up pharmacophore units of the same type in all possible ways. For each of these pairings a similarity value is derived from comparing donor-acceptor and hydrophobic strength values as well as corresponding elements of the distance matrices. The largest of these values yielded, after normalization with self-similarity-values of the two pharmacophores, the final similarity value in the range between zero and one. For comparison, similarity values derived from DFPs via the standard Tanimoto similarity measure were also calculated. Fig. 5 shows the hierarchical tree representation (using complete-linkage clustering) of the two similarity matrices as obtained from our set of molecules utilizing TPR and DFP description. The trees have been colored according to affinity to HSA using blue for the highest and red for the lowest values. A qualitative comparison of the two trees reveals that the description in terms of TPR leads to a better clustering of structures with similar affinity than a description in terms of fingerprints (DFP). This becomes even more evident by comparing the clustering of one subgroup of the data set, the non-steroidal anti-inflammatory drugs (in black lines) in both trees. Most of the drugs of this subgroup can be found in one branch of the TPR tree with high values of similarity, whereas in the DFP tree, they are more widely spread with low values of similarity. A branch is considered of high similarity, if it separates off from the main tree at a high similarity value. In general, it can be seen from the tree representation, that the Daylight descriptors lead to rather low similarity values, which result in extended branching near the stem of the tree.

Rather than taking its 151 similarity values against the whole set as a vector of descriptors for each compound, we represent these vectors in terms of the most relevant eigenvectors of the similarity matrix, i.e. the eigenvalues which are positive and not small compared to one. Furthermore, we scale these relevant basis vectors by the square root of their corresponding eigenvalues to ensure that the resulting components (which are our final descriptors) have

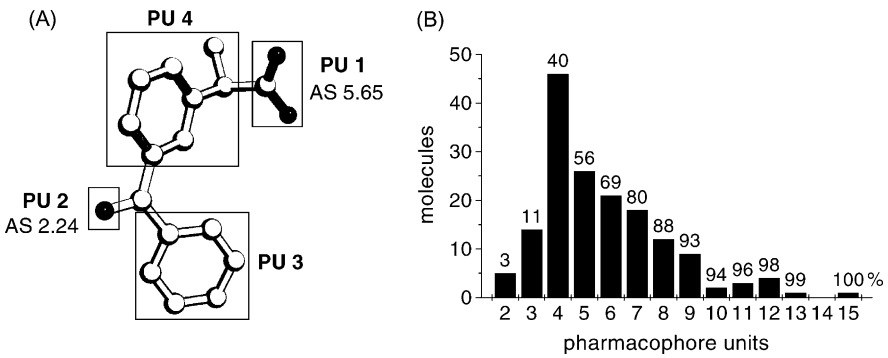

Fig. 4. (A) TPR [31] of ketoprofen as a representative of the HSA ligands. The carboxyl and carbonyl group yield hydrogen acceptor units with AS of 5.65 and 2.24, respectively (referenced to the hydrogen acceptor/donor strength of water, 2.33 (kcal $mol^{-1})^{0.5}$) [31,33,34]. The phenyl and ethylbenzyl groups are represented by the hydrophobic pharmacophore units PU3 and PU4 with size parameter values of 2.81 and 3.27 Å, respectively. The distance matrix has the elements d_{12}: 9.76 Å; d_{13}: 11.57 Å; d_{14}: 4.28 Å; d_{23}: 5.66 Å; d_{24}: 5.53 Å; d_{34}: 7.35 (effective bond distance in Angstrom units). (B) Distribution of numbers of pharmacophore units within the HSA data set (138 ligands from the literature and 25 Roche compounds).

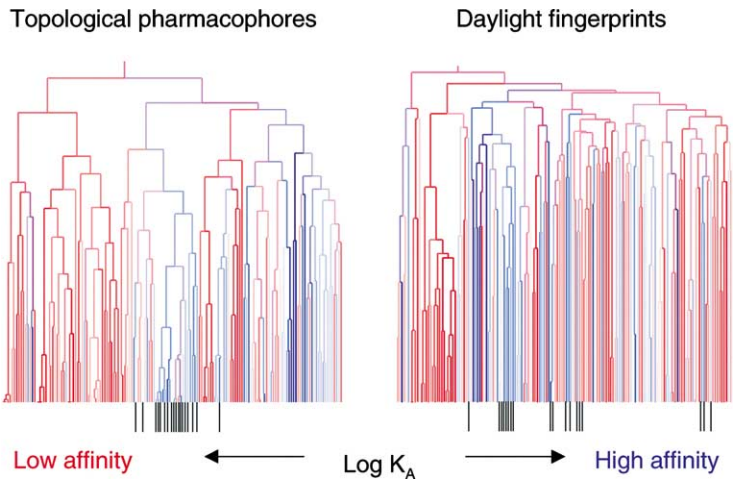

Fig. 5. Hierachical tree presentation of similarity matrices for TPR and DFP description. The trees are colored according to HSA binding affinities, log *k*-values. The TPR tree shows clustering of HSA binders of similar affinity. In particular, the non-steroidal anti-inflammatory arylpropionic acids, as indicated by black lines, are found essentially in a single branch, whereas in the DFP tree, they are wider spread and therefore categorized as more dissimilar in character.

comparable scaling [31]. Thus, the diagonalization of the two similarity matrices gave rise to 62 and 149 significant descriptors for the TPR and DFP cases, respectively. These similarity descriptors were submitted to a PLS and the statistical significance of the models were then evaluated by the Pearson correlation factor, r^2, and the crossvalidated correlation factor q^2, a measure for the overall predictive quality of the model (see in Section 2 for definition of r^2 and q^2, Eqs. (2) and (3), respectively). In the case of the DFP, r^2 reached a value of 0.76 for two PLS components, which yielded a maximum value of 0.37 for q^2. The TPR data showed a similar correlation factor r^2, but a maximum value of 0.48 for q^2 for three PLS components indicating a higher predictive power for this model. Fig. 6 shows the plot of experimental vs. predicted association constants for binding to HSA for the derived TPR model.

For the TPR model, a standard deviation for the calculated association constants (σ_{calc}) of 0.62 is obtained, which is comparable with the average experimental standard deviation of the data-set ($\sigma_{exp} \pm 0.54$) (see in Section 2 for definition of σ_{calc} and σ_{pred}, Eqs. (4) and (5), respectively.). The permutation of the *y*-variables [25] (binding data, number of permutation 100) did not yield a statistically significant model ($q^2 = -0.016$) showing that the predictive quality of the TPR model is meaningful. For the DFP model, however, the permutation of the binding constants indicated a substantial overfitting of the data due to a not reliable PLS model. In summary,

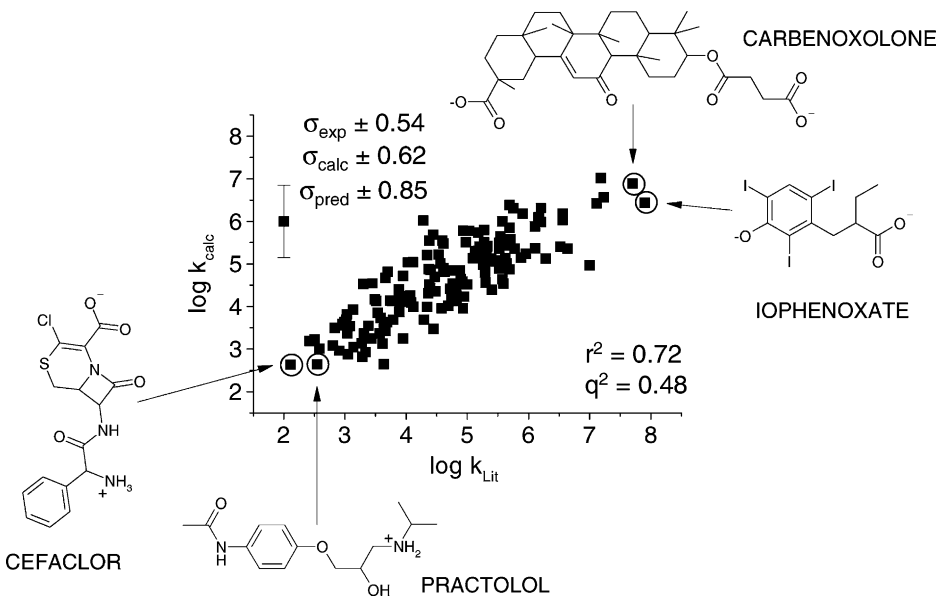

Fig. 6. Plot of experimental (k_{Lit}) vs. calculated primary association constants for the binding to HSA derived from a three-component PLS model based on TPR similarity descriptors. The average experimental standard deviation (root mean square error) of the data set is $\sigma_{exp} \pm 0.54$. The chemical structures of two typical low (cefaclor and practolol) and high binders (carbenoxolone and iophenoxate) are given.

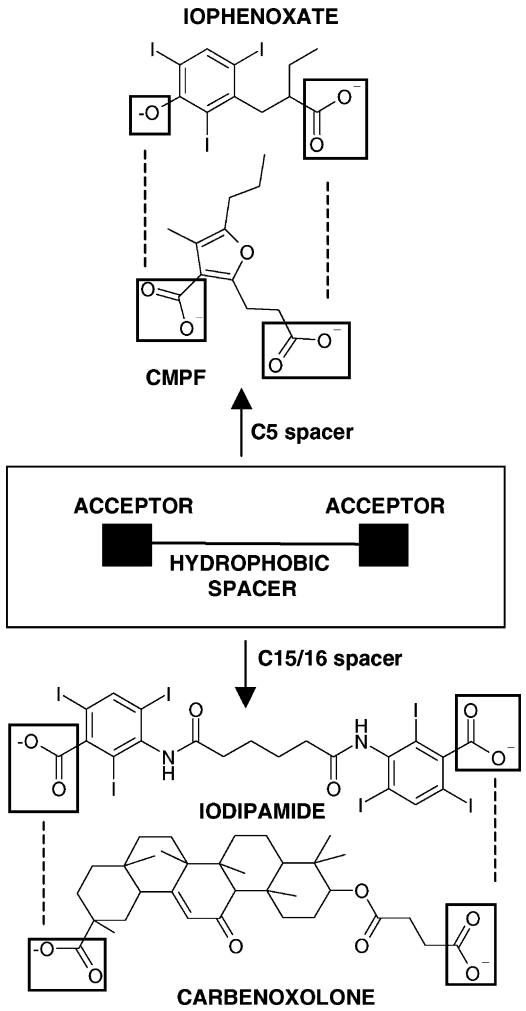

Fig. 7. Typical combination of pharmacophore units for strong binders to HSA. Iophenoxate, 3-carboxy-4-methyl-5-propyl-2-furanpropionicacid (CMPF), iodipamide and carbenoxolone have submicromolar binding constants and possess two acidic groups separated by a hydrophobic part by either 5 or 15 to 16 bond units.

the TPR model compares favorable with the one derived from DFPs. To illustrate typical low and high binders to HSA, two examples are given in Fig. 6. Cefaclor and practolol, which have binding constants in the high millimolar range, have as structural feature a cationic nitrogen in common and this is one of the criteria found for low to moderate binding to HSA. On the upper end, carbenoxolone and iophenoxate bind to HSA with submicromolar binding constants. Fig. 7 shows the typical pharmacophore combination for the high binders in this data set. All compounds, which were found to bind in the submicromolar range to HSA, have two hydrogen bond acceptor units separated by a hydrophobic spacer of either 5 or 15 to 16 bond units.

3.5. External validation of TPR model

To further validate our TPR approach, we used consistent data, e.g. HPLC retention volumes on an immobilized HSA column for a structurally homologous set of compounds [19]. For the whole data set of 76 compounds (see Table 4, group 2), for two representative subsets [26] (Table 4, subgroups 2A and 2B) and for 59 derivatives of 4-hydroxyquinolones (see Table 4, group 2s) within this data set, separate TPR models were established. Compared to the original TPR model of 151 HSA ligands (group 1), the statistical model parameter indicate a greater fit and predictivity of the TPR models for the validation data sets (see Table 4). The highest q^2-value of 0.71 (r^2 0.79) was found with the three PLS component model for the 59 derivatives of 4-hydroxyquinolones, which can be attributed to the low diversity of this data set. For external validations, the subgroup 2A was used as training set and the other subgroup 2B as test set and *vice versa*. Table 4 shows that the r^2 for the predicted data sets are in good agreement with the cross-validated correlation coefficients of the training models of 0.61. The standard errors, σ_{calc}, for the predicted test sets were ±0.4. This statistical data confirm further the significance of the TPR approach.

The prediction of the first association constants for the 76 compounds by the original TPR model of 151 HSA ligands and further correlation with the retention volumes was, however, not satisfactory giving rise to a negative r^2 and a large standard error (see Table 4). The prediction error of the TPR model ($\sigma_{pred} \pm 0.85$) is comparable to the data

Table 4
Statistical data for PLS QSARs derived from TPR similarity measures

Training	N	r^2	q^2	σ_{calc}	σ_{pred}	PLS components
Group 1 (Table 1)	151	0.72	0.48	0.62	0.85	3
Group 2 [19]	76	0.75	0.61	0.35	0.70	3
Group 2s [19]	59	0.79	0.71			3
Subgroup 2A [19]	38	0.80	0.61	0.34	0.77	3
Subgroup 2B [19]	38	0.79	0.61	0.30	0.66	2
Validation						
2A predicted by 2B	38	0.63		0.46		2
2B predicted by 2A	38	0.56		0.45		3
2 predicted by 1	76	−0.70		0.93[a]		3

[a] The predicted log *k*-values were centered to the average logarithm of the retention times before calculating the standard error σ. N, number of compounds. Correlation factor r^2 and the standard error σ_{calc} are measures for prediction of the training set, q^2 and σ_{pred} are measures for cross-validated prediction.

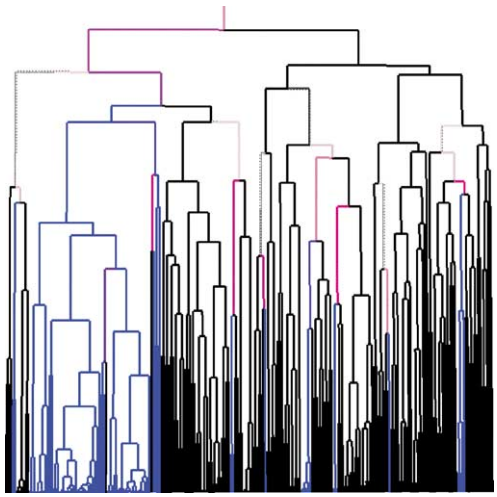

Fig. 8. Hierachical tree presentation of the similarity matrix for TPR description for all 227 compounds (Table 1, Table 2 and 76 compounds from [19]). The test set (76 compounds from [19]), indicated by blue branches, are tightly clustered together and are not very similar to compounds within the training data set (Tables 1 and 2).

range of the experimental values ($\sigma_{exp} \pm 0.69$ for the log-values of the retention volumes) which indicates that a correct prediction of the fine binding pattern within the homologous data set could hardly be expected. Furthermore, a simple, linear correlation between HPLC retention times and association constants for HSA binding was assumed. This may not be the case, which could further contribute to these negative findings. In addition, a study of the hierarchical tree representation of the whole similarity matrix for the 227 compounds showed that most of the 76 compounds were tightly clustered together and were considered to be similar to only a very small number of compounds within the training data set (see Fig. 8). Since the compounds of group 2 are not contained within the chemical property space of group 1, the prediction using the QSAR equation of group 1 is rather an extrapolation instead of an interpolation.

4. Discussion

The percentage of plasma protein binding is routinely determined by various methods [35–37] in order to estimate the potential of high protein binding for new drug candidates. The protein binding values are considered in the calculation of unbound drug concentrations across species to establish potential safety margins for human exposure and in selecting the final dosing range in human trials. For these reasons, plasma protein binding is considered to be important and a subject of concern.

Considering plasma protein binding as factor in pharmacokinetics and pharmacodynamics, strong binding to HSA can lead in rare cases to significant changes in the unbound concentration of a drug due to drug displacement interactions, disease states, or age [38]. Since only high extraction ratio drugs respond with a strong increase of the unbound concentration to displacement from HSA [39,40], this class of drugs requires in particular low binding values. Additionally, drugs, which have to be administrated in high concentration, may possibly act as displacing agents for co-administrated high extraction ratio drugs that happen to bind substantially to HSA. In most other cases, changes in the bound concentration does not give rise to changes in the unbound one and therefore no enhanced pharmacological or toxicological response occurs. Often, this fact is overlooked and the relevance of drug displacement interactions on the clinical efficacy of drugs overestimated [39–42]. Thus, discussing the relevance or the irrelevance of protein binding among different disciplines, care has to be taken to account for the particular case, one is concentrating on.

4.1. Protein binding of therapeutic drugs

4.1.1. Association constants vs. percentage protein binding data

An analysis of the fractions bound to plasma protein of therapeutic drugs (more than 500) on the market indicated an even distribution with 43% of the drugs showing values in excess of 90%. It seems to be impossible from these data to deduce general rules for optimal or threshold values. Obviously, the amount of plasma protein binding of a drug is in general not a restrictive condition for its clinical efficacy. The important parameter is the effective free plasma concentration of a drug and not its plasma protein binding *per se*, as illustrated in a recent study of a series of CNS drug candidates [19]. Comparing the distribution of protein binding of drugs within different indication areas showed that only for the class of chemotherapeutics including antibiotics, antiviral, antifungal, and anticancer drugs a clear trend towards lower binding level could be seen. This trend is most pronounced for antibiotics, of which 86% show values below 90% and for which no case of protein binding above 99% was found. This finding can be explained by the often-needed high plasma concentration for this kind of drugs (up to millimolar) to achieve multiples of the minimal inhibitory concentration (MIC) at the site of infection [43,44]. In essence, strong protein binding of antibiotics is counterproductive in terms of dosing [1].

Solely focusing on the wide range of protein binding percentage values among drugs, the question arises, whether there is any need at all for predicting protein binding, apart maybe from the chemotherapeutic indication. However, such a conclusion is misleading for two reasons. Firstly, the majority of the investigated drugs, which were categorized between 99 and 100% in our analysis, have protein binding levels of just 99.0%, but rarely above. A trivial explanation of this finding may be the detection limits of the historical analytical methods, which cannot always compare with modern methodology, such as, e.g. ion specific techniques (LC/MS/MS). More likely is the interpretation that higher binding values, corresponding to an association constant of 165,000 M^{-1} ($K_D = 6$ μM), start to become

problematic for an increasing number of cases. The examined set of existing drugs, which underwent already several steps of optimization within pharmaceutical companies, does hardly include compounds with extreme plasma protein binding properties, which probably have been filtered out in the evaluation step. Unfortunately, comprehensive data on such compounds are not easily available within pharmaceutical companies. For these reasons, it is not possible to derive more empirical rules for plasma protein binding from this analysis. This lack of empirical rules does not imply the irrelevance of predicting protein binding, but rather shows that this parameter is difficult to classify and that one has to consider the whole physicochemical and pharmacokinetic profile of the drug candidate to decide individually on the acceptable amount of protein binding. Acknowledging the indirect evidence, we nevertheless conclude that very strong binding (submicromolar to nanomolar) will influence the therapeutic effect of drugs and therefore especially the identification of such candidates is important. If an unfavorable combination of physicochemical and pharmacological properties occurs, such as low solubility and a need for a high effective concentration, the limit for the acceptable amount of protein binding shifts to lower values and even moderate binding levels may already become a critical issue. For these reasons, a prediction tool must be able to distinguish between drugs with nanomolar, micromolar and millimolar binding to plasma proteins.

In this context, a comparison between association constants for binding to HSA and independently determined percentage data for plasma protein binding, showed that the percentage data are only sufficiently accurate in the range corresponding to K_D values above 6 μM, but fail to account properly for submicromolar to nanomolar binders. Furthermore, the commonly used classification scheme of drugs into low, medium and high-level plasma binders, based on the percentage data is, in our opinion, not sufficient [36]. On the one hand, differentiation between low and medium-level binders at a threshold value of 75% (corresponding to $K_D = 0.2$ mM) does hardly assist in prioritizing any lead candidates. On the other hand, the classification of drugs with binding values above 90–95% as high-level binders, seems to be too coarse, and fails to separate micromolar binders from the most problematic cases of submicromolar to nanomolar binding.

4.2. Correlation between binding data and log D

The hypothesis, that protein binding and especially binding of drugs to serum albumin is in general non-specific, and explainable by hydrophobic interaction has wide-spread acceptance, despite of the fact that this is true only within small congeneric series of compounds [17] (and references therein), [45–51]. Some publications, however, also state that protein binding is independent [52–54] of the overall lipophilicity within such homologous series.

To investigate the role of the lipophilicity of molecules within a diverse data set, we tried to correlate the log *D*-values of 76 compounds, for which a measurement was possible, with the association constants to HSA. The found poor correlation (correlation factor of 0.2) indicates that the reported linear relationships between lipophilicity and plasma protein binding is highly biased by the used data set, and is not generally applicable for a large and diverse data set. Morris and Bruneau [17] developed a linear model relating protein binding to either experimentally determined log *P*-values for neutral and acidic compounds or to log *D*-values for basic compounds. Looking separately at acidic and basic compounds, we found that the influence of the lipophilicity on the binding to HSA is given for the major part of the acids, but still there are acids which are not following this rule giving rise to a low correlation factor ($r = 0.4$) for all the compounds. For bases, however, no correlation between binding to HSA and lipophilicity could be observed. Recently, it has been emphasized that a sigmoidal relationship between the percentage plasma protein binding and log *D*-values (pH 7.4) within a large class of different compounds (acids, bases, neutrals) exists [55]. However in substantial ranges of log *D*-values, the scatter of data spans, almost the whole range of possible plasma protein binding values. Therefore in our opinion, this is rather a demonstration, that log *D*-values alone are not sufficient to explain plasma protein binding for a diverse set of compounds. Our studies suggest that the binding to albumin is not determined by integral physicochemical properties alone, such as log *D*, but is also dependent on specific molecular recognition, such as directed hydrogen bonds, charge interactions and space-filling of binding pockets, as has been already very early demonstrated by Scholtan [45,46] and Seydel *et al.* [47].

4.3. Prediction models

4.3.1. TPR vs. DFP

Here, we described an approach applying PLS based on a pharmacophoric similarity concept. For the similarity calculation, on one hand the various pharmacophoric units identified in a compound were used and on the other hand the mutual topological distances between all these units. This ensures that structural features are accounted for as much as possible on the basis of a topological structure description. This new approach was then compared to the well-established concepts of a bit-vector description (DFP description) applying the Tanimoto index as a similarity measure and further PLS analysis. This type of DFP description can only indirectly account for structural features via the occurrence of linear atom sequences up to the given threshold size (usually seven atoms). Application of the PLS analysis on the two similarity matrices led to a three-component TPR model ($q^2 = 0.48$) and to a two-component DFP model ($q^2 = 0.37$) indicating a larger overall predictive quality of the TPR model. The significance of the

TPR model was validated by a standard randomization test demonstrating the absence of a chance correlation. The use of a congeneric chemical series for external validation, which simulates the application of such a prediction tool in medicinal chemistry programs, showed that a maximum of q^2 with a value of 0.71 could be derived by the TPR method.

In spite of the wealth of data available for plasma protein binding, only few prediction models are reported for this ADME property in the literature, which deal with the prediction of this parameter beyond a relationship with lipophilicity. Recently, Saiakhov *et al.* [54] presented a multiple computer-automated structure evaluation model for plasma protein binding of diverse drugs, where the percentage of drug bound to plasma could be correctly predicted for 80% of the compounds. The data source was the percentage protein binding data (154 compounds) from Goodman and Gilman's textbook. After checking for a correlation between the octanol-water partition coefficient and the biological activity, the algorithm detects important chemical groups, so called biophores, of the data set and the accompanying modulators, chemical fragments, which enhance or diminish binding. After this step, several local QSAR models are derived to predict the potential activity of new molecules. Considering this structure evaluation model for plasma protein binding, the definition of compounds with plasma protein binding more than 32% ($K_D = 1.3$ mM) as high-level binders seems to be questionable and rather arbitrary to us. Furthermore, it might be rather difficult to interpret their biophores with their modulators, where modulators can be activating in one subset and deactivating in another. In summary, the use of percentage data, especially the used threshold for high binders, seems to be too coarse, and the application of non-generic models for the prediction of protein binding, where such a diverse set of molecules has to be covered, might be rather problematic.

Bruneau [56] have reported a generic model ($q^2 = 0.68$) based on artificial neural networks. They established a non-linear relationships between the protein binding data, an in-house data set of 216 compounds, where the first apparent binding constant was determined in 10% rat plasma by equilibrium dialysis at 298 K, and the most relevant ten descriptors. The predictive quality of this generic model is comparable to our TPR model for the homologous data set used for external validation ($q^2 = 0.61$) supporting the fact that a homogenous data set is essential for a good QSAR. No chemical structures were presented, only the mean MW of 396 ± 92 and the mean calculated octanol–water partition coefficient (C log P_{oct}) of 3.32 ± 1.57 of their data set were given. For comparison, our data set of 151 compounds is characterized by a mean MW of 335 ± 139 and a mean value for C log P_{oct} of 2.31 ± 1.95. The selected descriptors for their generic model are classical physicochemical descriptors, such as C log P_{oct}, number of hydrogen donors and pi atoms, ionization state and surface dependent descriptors. Studies to correlate the HSA binding affinity with a similar set of computed descriptors (as implemented in the software package TSAR [22]) and applying a PLS analysis did not lead to a statistically significant prediction model for our compounds (data not shown).

5. Conclusion

The studies showed that plasma protein binding of drugs is difficult to classify and no general implications for the design of leadlike compounds could be derived by the examination of the properties of existing drugs, except that for chemotherapeutics higher than 90% binding is not observed within therapeutic drugs. Thus, for most of the drugs, one has to consider the whole physicochemical and pharmacokinetic profile of the drug candidate to decide individually the acceptable amount of protein binding. It was further demonstrated that lipophilicity is not sufficient to explain plasma protein binding alone for structurally diverse compounds. Despite of the lack of general guidelines for plasma protein binding, the recognition of potential submicromolar to nanomolar binders is important in early drug discovery in order to prioritize leads to avoid later complications in selecting the initial doses in animal and human trials. The presented generic model based on a pharmacophoric similarity concept is able to single out strong plasma binders, e.g. to differentiate between 99.0 and 99.99% plasma binding, and could therefore help to prevent unpleasant surprises in the search for lead-like compounds with an acceptable metabolic and pharmacokinetic profile.

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