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Physicochemical profile of macrolides and their comparison with small molecules

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ABSTRACT

Macrolides are stereospecific macrolactones of high molecular weights. Herein, 600 mostly semi-synthetic macrolides are compared with 50,000 small non-macrolide synthetic molecules in terms of measured physicochemical properties in order to assess the drug-likeness and developability chances of macrolides. The pre-selected set of diverse macrolides is comprised mostly of derivatives of clarithromycin and azithromycin cores. Lipophilicity (CHI logD), affinity for immobilized artificial membranes (CHI IAM), human serum albumin (HSA) and α_1-acid glycoprotein (AGP) plasma protein bindings (PPB), DMSO precipitative solubility as well as artificial membrane permeability (AMP) have been determined by high-throughput screening methods. It has been found that macrolides and small molecules have similar lipophilicity profiles, though macrolides show weaker PPB and have better solubility than small discovery molecules. However, macrolides are poorly permeable and have high affinity for immobilized artificial membranes signifying their strong interaction with biological phospholipids. In order to retain the drug-like profile, the design of novel macrolide molecules should be focused on optimisation of macrolide cores, that is macrolactone moiety with sugars and other small substituents avoiding large substituents and flexible linkers such as in conjugate derivatives.

1. Introduction

Macrolides are large complex molecules consisting of 8- to 38-membered macrolactone with a number of stereocenters. As drugs, they have mostly been used for anti-infective indications, typically as bacteriostatic antibiotics [1]. Certain macrolides, such as rapamycin and azithromycin have also been used in oncology [2,3] and anti-inflammatory area [4,5]. In natural macrolides, macrolactone is commonly substituted by hydroxyl groups, one or more deoxy sugars, usually cladinose and desosamine, and generally relatively small substituents generated by post-polyketide modifications [6,7]. However, in semisynthetic derivatives, structural modifications range from relatively small changes [7,8] to introductions of large substituents [9–15].

Due to high molecular weight (greater than 500) and complexity [16], macrolides are usually not included into training sets for generation of either simple rules for drug and lead-likeness [17–22] or various sophisticated absorption, distribution, metabolism, elimination and toxicity (ADMET) models [23,24], derived by using calculated structural and physicochemical properties. These models also work only when no active transport takes place, while macrolides have been reported to interact often with cellular membrane transporters [25–27]. Hence, common rules like Lipinski's Rule of 5 and commercial software used for *in silico* analysis of compound libraries are in general inappropriate for macrolides. For such untypical molecules structure-activity (property) models and rules should be generated separately [28].

In this study we demonstrate that, similarly to other classes of natural compounds, macrolides have drug-like physicochemical

Abbreviations: ADMET, absorption, distribution, metabolism, elimination and toxicity; AGP, α_1-acid glycoprotein; AMP, artificial membrane permeability; ANOVA, one-way analysis of variance; aring, number of aromatic rings; CHI, chromatographic hydrophobicity index; CHI IAM, CHI immobilized artificial membrane; CLND, chemi-luminescent nitrogen detection; DMSO sol, dimethyl sulfoxide precipitative solubility; HSA, human serum albumin; HTS, high-throughput screening; (lip)hba, number of (Lipinski) H-bond acceptors; (lip)hbd, number of (Lipinski) H-bond donors; MW, molecular weight; PK, pharmacokinetic; Pos/Neg, number of positively/negatively charged atoms; rb, number of rotatable bonds; tpsa, topological polar surface area.

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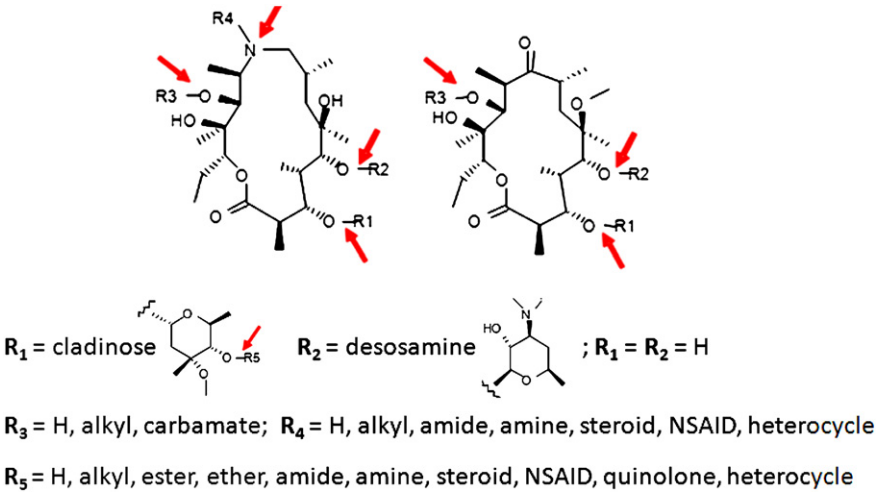

Fig. 1. Typical macrolide cores and subtituents used in the study.

properties [29–31]. Specific pharmacokinetic (PK) profile of many macrolides, like high tissue distribution and poor bioavailability are related to specific physicochemical properties [28,32,33].

Relationship between physicochemical profile of a compound and its metabolism and PK behaviour is generally recognized [34]. Lipophilicity and ionization of molecules influence most of ADMET properties. Analysis on ~30,000 diverse small molecules demonstrates that ADMET properties generally deteriorate with either increasing molecular weight, logP or both, while ionization state has either a beneficial or detrimental effect depending on the parameter considered [35,36]. Solubility and permeability underlie FDA's Biopharmaceutics Classification System (BCS) guidance for predicting compound intestinal absorption [37,38]. Additionally, unfavourable physicochemical properties like high lipophilicity or low solubility may cause problems in HTS experiments [39].

The aim of this study was to characterize the physicochemical property space of macrolides, compare it to the physicochemical space of in-house small discovery molecules and identify the similarities and dissimilarities that might affect the drug-like properties and developability chances of macrolides. We performed extensive physicochemical measurements for about 600 macrolide and 50,000 small synthetic research in-house compounds. HPLC-based high-throughput (HT) measurements were used to determine lipophilicity (the chromatographic hydrophobicity index logD – CHI logD) [40–42], artificial membrane permeability (AMP) [19], phospholipid affinity (CHI IAM) [43] and plasma protein binding (PPB) [44] to human serum albumin (HSA) and α_1-acid glycoprotein (AGP). Solubility was measured from DMSO stock solutions using precipitative method (DMSO Sol) [45].

The 600 diverse, mostly semisynthetic macrolide molecules were pre-selected from the corporate compound database by filtering and clustering procedure [46–49]. The most of the representative macrolides were derivatives of macrolide cores with anti-infective activity (Fig. 1) [1]. About 60% of the compounds were derivatives of azithromycin and clarithromycin (Fig. 2) [9–14]. There were also representatives of tylosin and roxythromicin derivatives as well as ketolides. Almost 55% of the representative macrolides were conjugates of the specified macrolide cores with quinolones [9–14] or steroid and nonsteroidal anti-inflammatory drugs joined by (hetero)alkyl linkers of variable lengths at various positions (Fig. 1).

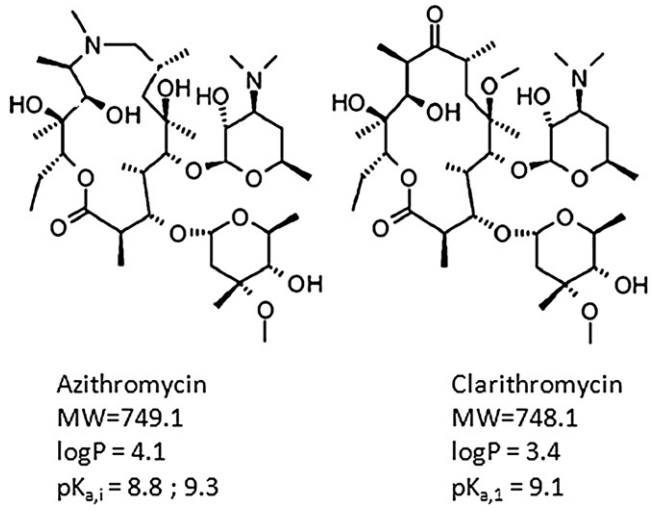

Fig. 2. Chemical structures of two commercially available macrolide drugs and their molecular weights (MW), octanol/water partition coefficients (logP) and the lowest acid-base equilibrium constants (pK$_{a,1}$) [28].

Table 1
Mean values (±standard deviations) of simply calculated structural descriptors[a] for the three compound sets: All considered macrolides, macrolide cores only and all small compounds.[b]

Feature	Macrolides	Macrolide cores	Non-macrolides
MW	951.6 ± 192.8	701.2 ± 160.2	448.1 ± 112.6
hba	12.7 ± 3.1	10.8 ± 3.3	3.2 ± 1.8
liphba[c]	17.6 ± 4.0	13.4 ± 3.8	6.5 ± 2.6
hbd	4.7 ± 1.8	4.0 ± 2.3	1.4 ± 1.3
liphbd	5.1 ± 1.8	4.1 ± 2.4	1.5 ± 1.2
pos	1.7 ± 0.8	1.0 ± 0.6	0.5 ± 0.6
neg	0.4 ± 0.5	0.0 ± 0.2	0.2 ± 0.4
rb	12.6 ± 4.7	7.0 ± 3.4	6.0 ± 3.1
tpsa (Å^2)	227.5 ± 48.3	176.6 ± 54.8	80.1 ± 35.5

[a] Molecular 2D structural descriptors were calculated by the in-house programes [19,28,36].
[b] Regarding each of the descriptors, the means of the three compound sets are mutually different at p-value ≤ 0.05.
[c] Liphba is defined as a sum of Ns and Os and liphbd as a sum of NHs and OHs. Hba and hbd were calculated as described in reference [19].

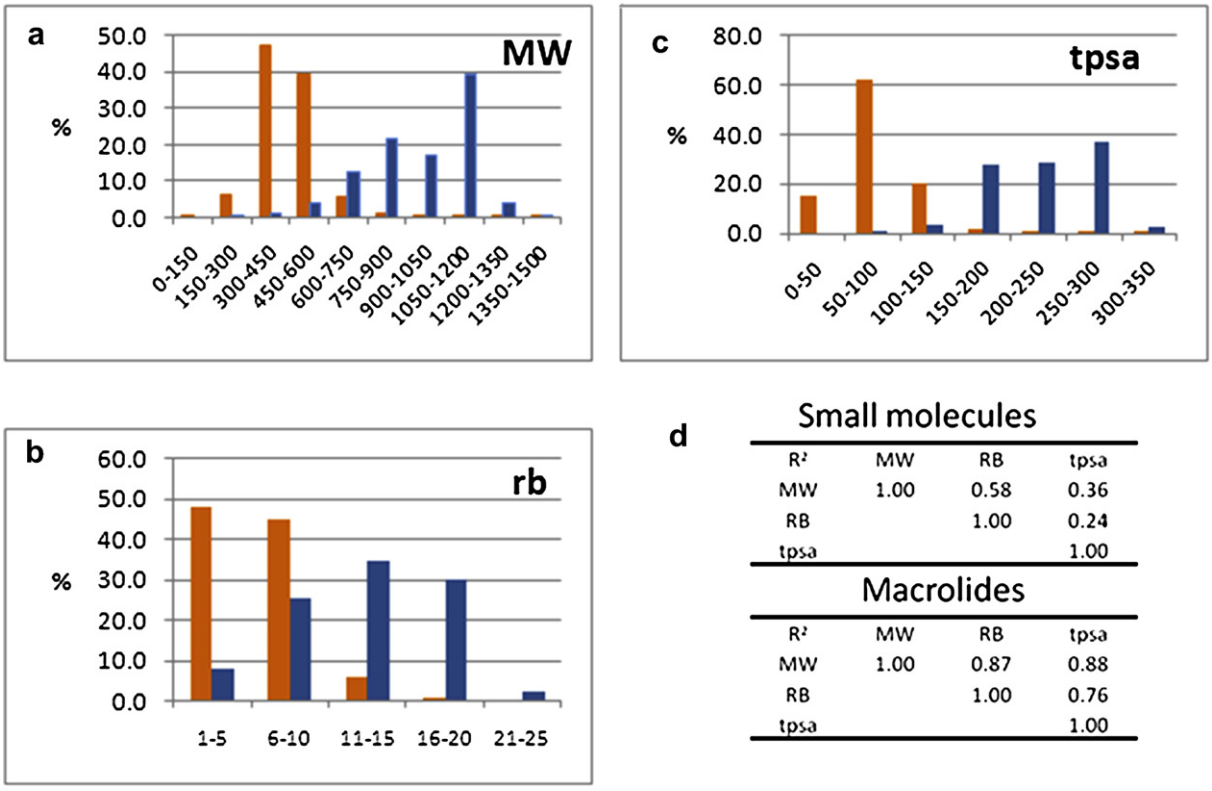

Fig. 3. Distributions of the calculated structural descriptors (a) MW, (b) rb and (c) tpsa (Å^2) for the macrolides (blue) and small compounds (orange). (d) Cross-correlations between MW, rb and tpsa in terms of square of Pearson's correlation coefficient R^2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The small discovery research compounds analysed in this study were obtained from around 300 projects each covering two to three chemical series. The small molecules were generally synthetized in accordance with simple drug (lead) -likeness rules. There was no need to cluster small molecule set as the physicochemical data have been available owing to the high-throughput screening efforts.

The macrolide selecting procedure and experimental physicochemical measurements are described in details in Experimental

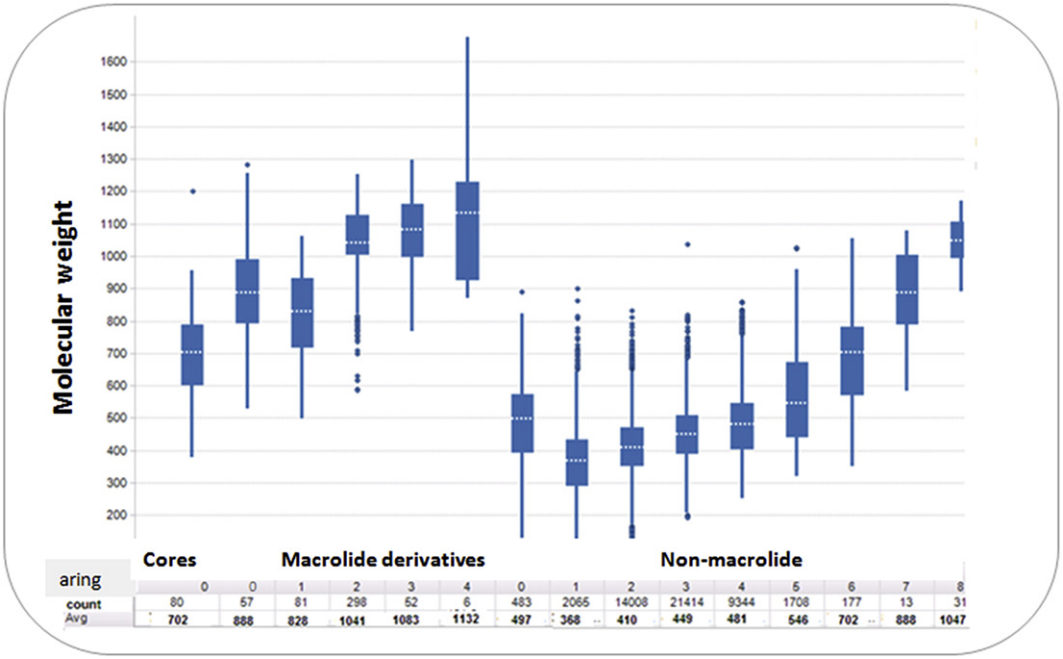

Fig. 4. The mean molecular weights (MW) of the macrolide cores, macrolide derivatives as well as small compounds collected from about 300 projects. The MW means were calculated for compound groups based on the number of aromatic rings (aring). The blue rectangular box contains the 50% of the data, the white line is the mean and the lines show the spread. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

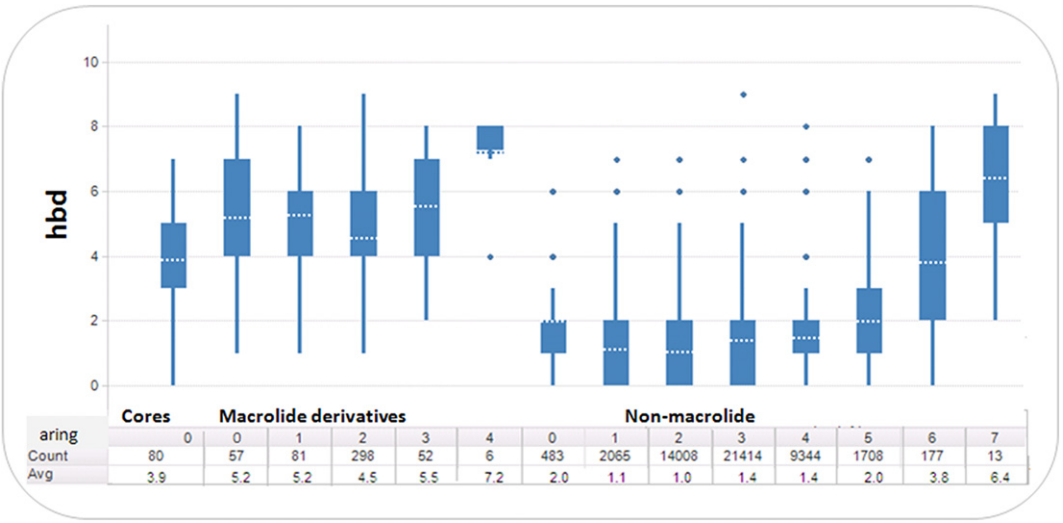

Fig. 5. The mean number of H-bond donor moieties (hbd) for groups defined by number of aromatic rings (aring), separately for macrolide cores and derivatives as well as non-macrolide research compounds. The box plot layout is described in Fig. 4.

protocols. Statistical data analysis was done by SpotFire DecisionSite [50] (descriptive statistics) and JMP® programs (one-way analysis of variance (ANOVA)) [51].

2. Results and discussion

Structural differences between macrolides and small synthetic molecules are illustrated in Table 1 and Figs. 3 to 5 by comparing the mean values and distributions for a series of simply calculated molecular descriptors often used in molecular profiling. Differences in molecular weight (MW), numbers of H-bond acceptor (hba) and donor (hbd) atoms [19], numbers of positively (pos) and negatively (neg) charged atoms, number of freely rotatable bonds (rb) and topological polar surface area (tpsa) [52] were established by ANOVA at the 5% significance level followed by pairwise Tukey–Kramer HSD *post hoc* test (Table 1) [51].

Macrolides are larger than typical drug discovery molecules (Table 1, Figs. 3 and 4). These macrocyclic molecules are among largest naturally derived drugs [53]. In terms of molecular weight, for illustration, 73% of in-house non-macrolide molecules designated as "small", have MW $\leq$ 500 (Fig. 2) and almost all satisfy the Rule of 5 [17]. In difference, only 1% of the considered macrolides have MW $\leq$ 500 and they satisfy the Rule of 5. These 9 macrolides belong to the "light" macrolide subset containing macrolide cores. The box plot in Fig. 4 shows MW distribution with number of aromatic rings (aring) for the three sets of the analysed compounds: macrolide cores, macrolide derivatives and non-macrolide small research compounds.

Macrolide cores are composed of rigid natural or re-arranged macrolactone scaffolds with or without sugar rings and eventually relatively small (hetero) (a)cyclic substituents [30]. Due to distinct chemical and physicochemical profiles, macrolide cores are characterised in separate columns in Tables 1 and 2 as well as in the shown box plots. They constitute 14% of the macrolide set. The rest of the diverse macrolide set corresponds to core derivatives with additional rings like aromatic, quinolone [9–14] or steroid moieties joined with macrolide cores through (hetero)alkyl linkers of variable lengths at various positions (Fig. 1). By conjugation of cores with various fragments, the additivity principle was satisfied regarding a range of properties. For example, the parameters rb and tpsa correlate better with molecular size than in the case of the small compounds (Fig. 3(d)).

All macrolides are more abundant in both H-bond acceptors and donors than small chemical compounds (Table 1), but also other natural products [53], mainly due to numerous OH-groups. However, the number of H-bond donor groups is below 5 in accordance with the Lipinski rule of 5 for one third of all macrolides and two thirds of macrolide cores (Fig. 5). Considering charge, most of investigated macrolides contain amino nitrogens in desosamine and/or azithromycin macrolactone rings and they are positively charged at physiological pH [28].

Although the macrolides are structurally very different from small molecules (Table 1, Figs. 3–5), they share similar physicochemical space (Table 2, Figs. 6 and 7). In difference to structural parameters, physicochemical properties can be used to compare drug-like properties of macrolides with those of the small molecules.

Regarding lipophilicity, macrolides and small molecules have similar ranges as illustrated by the pie charts in Fig. 6 and the box plot in Fig. 7 showing distribution of CHI logD means of macrolide cores and remaining macrolides as well as small discovery compounds grouped according to aring number. The macrolides comply the Lipinski Rule of 5 regarding not only the number of H-bond donor groups (Table 1, Fig. 5), but also lipophilicity (Table 2, Fig. 7) [31]. This supports previously reported observation that lipophilicity and H-bond donating capacity can be generally used to describe drug-likeness [31].

Concerning PPB, macrolides have weaker binding affinity to HSA and AGP than non-macrolide small compounds (Table 2,

Table 2
Mean values (±standard deviations) of experimentally determined physicochemical parameters for the three compound sets.

Physchem property	Macrolides	Macrolide cores	Non-macrolides
CHI logD	2.5 ± 1.2	1.8 ± 1.2* [a]	2.5 ± 1.1
HSA%	76.5 ± 20.5	51.9 ± 24.5	90.4 ± 12.3
AGP%	77.1 ± 16.0	60.0 ± 20.5	82.9 ± 15.1
CHI IAM	62.7 ± 20.5*	40.7 ± 19.3	42.9 ± 10.8
DMSO Sol (μmol/L)	177.3 ± 112.6	271.1 ± 113.0	189.2 ± 188.7
AMP[b]	39%/51%/9%	21%/57%/21%	10%/32%/58%

[a] At the 0.05 level of statistical significance, an asterisk denotes the significantly different set. In other properties, means between all considered compound sets are mutually different.

[b] Compounds are classified as low, medium and high permeable if AMP is <10 nm/s, 10–200 nm/s and >200 nm/s, respectively.

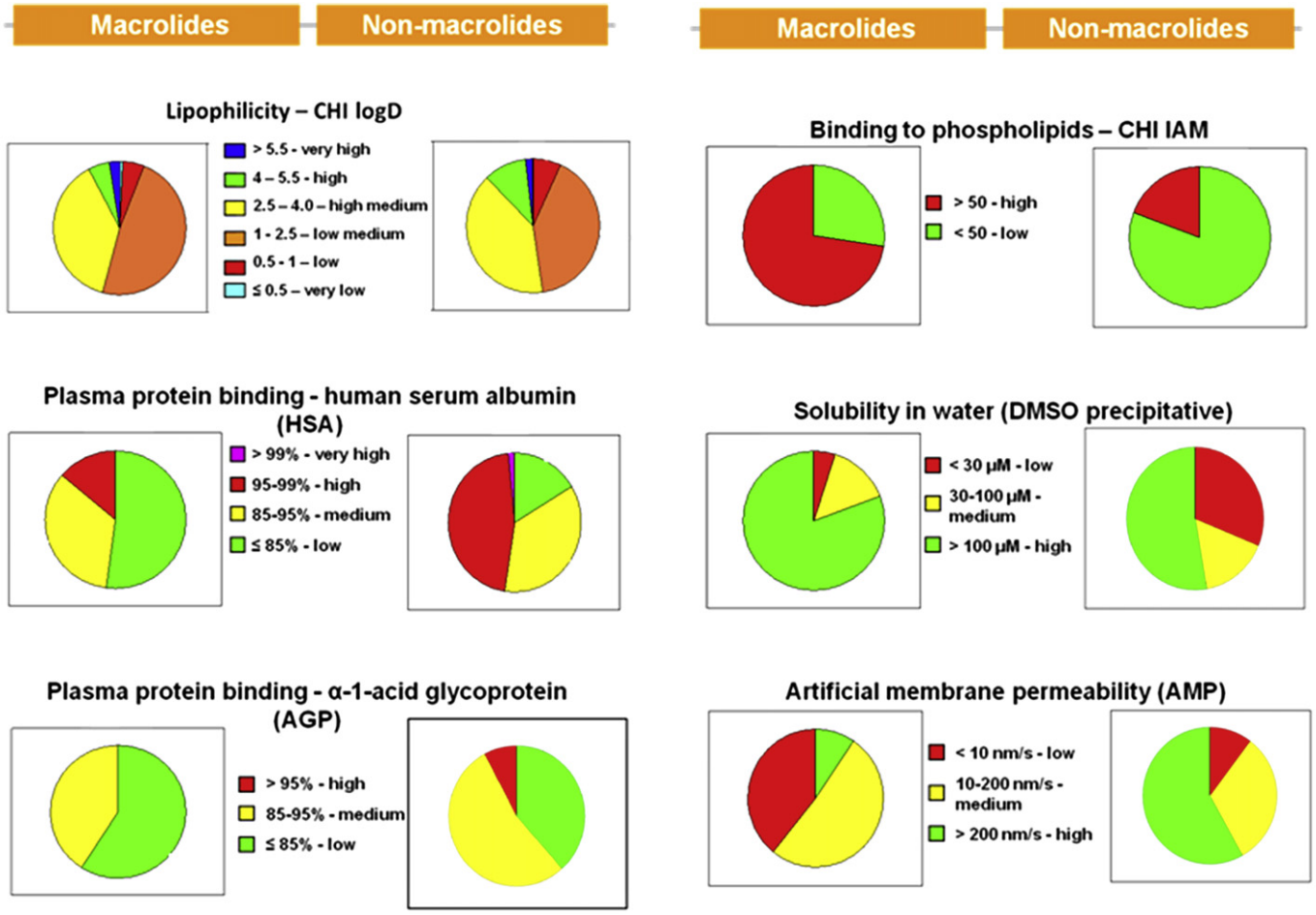

Fig. 6. Physicochemical properties of macrolides vs. small non-macrolide compounds.

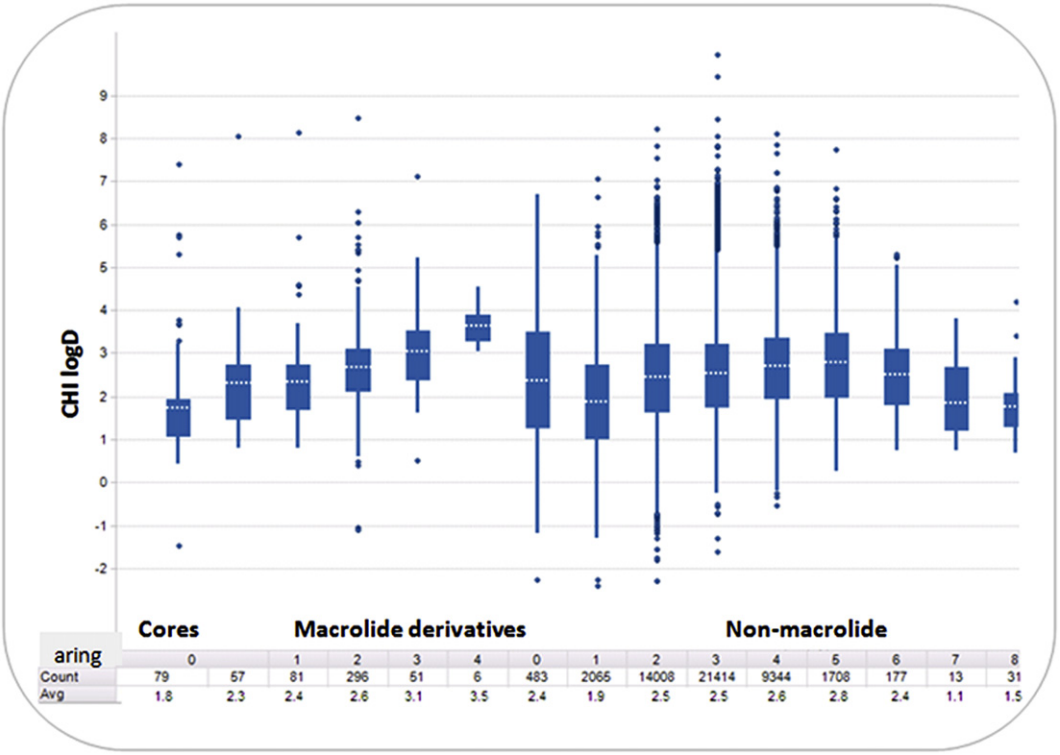

Fig. 7. The means of CHI logD lipophilicity measured at pH 7.4 for the macrolide cores and derivatives as well as ~50K small compounds. The box plot layout is described in Fig. 4.

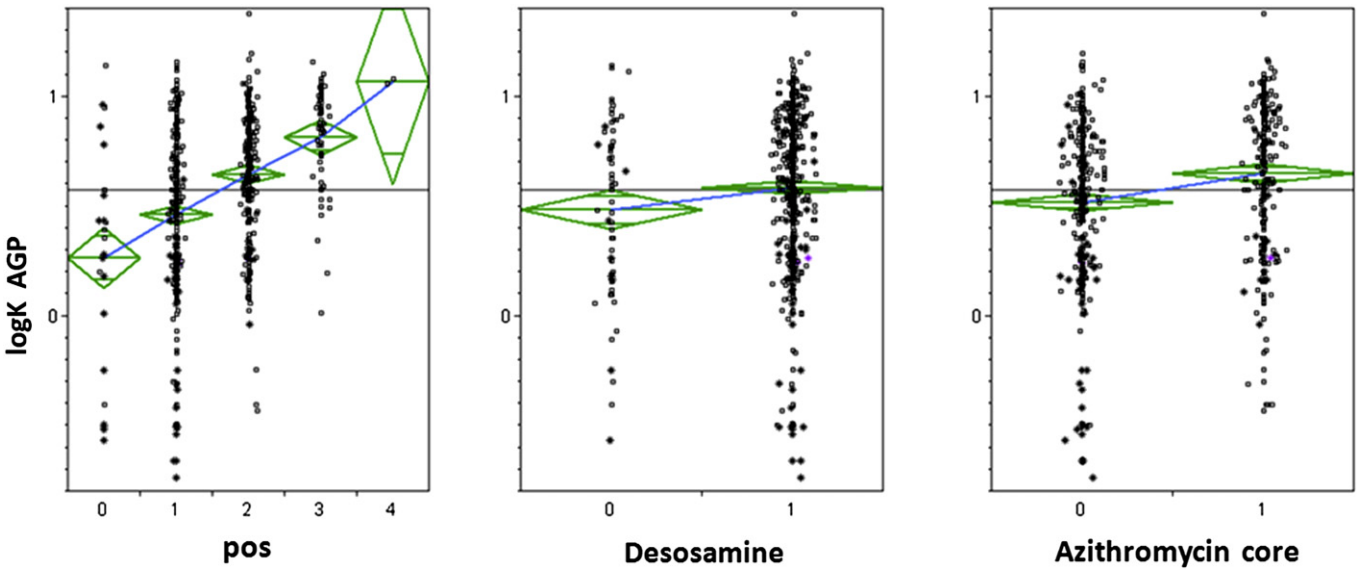

Fig. 8. Statistically significant variation of the normalized AGP binding affinity [35] of macrolides with a number of positive charges (pos) and presence (1) or absence (0) of the desosamine and azithromycin fragments within the macrolide (ANOVA, 5% significance level, followed by pairwise Tukey–Kramer HSD test). Mean diamond describes the group mean and 95% confidence interval. The black horizontal line corresponds to the total sample mean.

Fig. 6). Only 13% of macrolides bind to AGP stronger than 90% and only one macrolide molecule bind to AGP more strongly than 95% (AGP% = 97). The AGP binding affinity of macrolides generally increases (p-value ≤ 0.05) with the positive molecular charge (pos) coming not only from azithromycin core and/or desosamine sugar nitrogens but also from protonable nitrogen(s) in substituents (Fig. 8). In difference to AGP affinities, HSA affinities of azithromycin derivatives are not, in average, stronger in comparison with other macrolide derivatives. The bindings of macrolides to both plasma proteins increase with the molecular lipophilicity and with the number of aromatic rings and halogen atoms (Fig. 9).

Macrolides are more soluble in aqueous buffer, but considerably less permeable through artificial membrane (AMP) than small discovery molecules (Fig. 6). The permeability of the macrolides was noticed to be affected by similar factors as the permeability of non-macrolides. AMPs of macrolides and non-macrolides decrease with the increases of the molecular size and the number of H-bond donor groups as it is demonstrated in Fig. 10. Macrolides have lower mean permeability than the non-macrolides belonging to the same MW bin.

Macrolides show low AMP permeability due to their interaction with membrane phospholipid components [54]. This is also manifested in large CHI IAM binding. We observed that highly

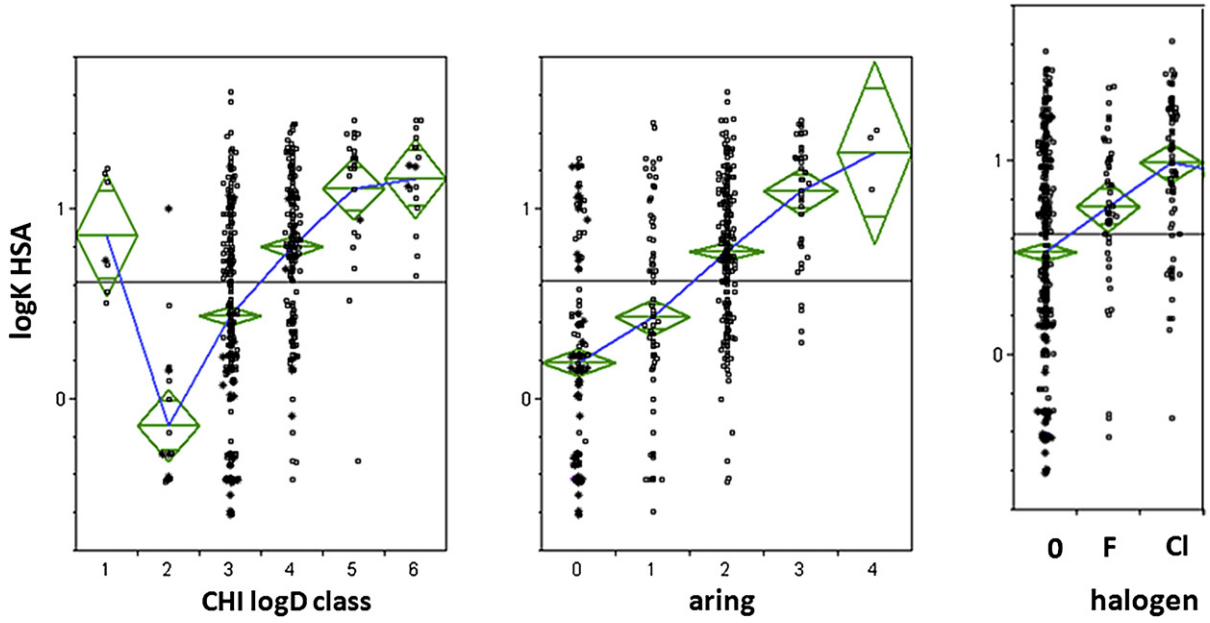

Fig. 9. Statistically significant variations of the normalized HSA binding affinity of macrolides with the change of molecular lipophilicity (classes defined in Fig. 6), number of aromatic rings (aring) and halogen atoms. Similar associations were observed for logK AGP values. See Fig. 8 for the plot layout explanation.

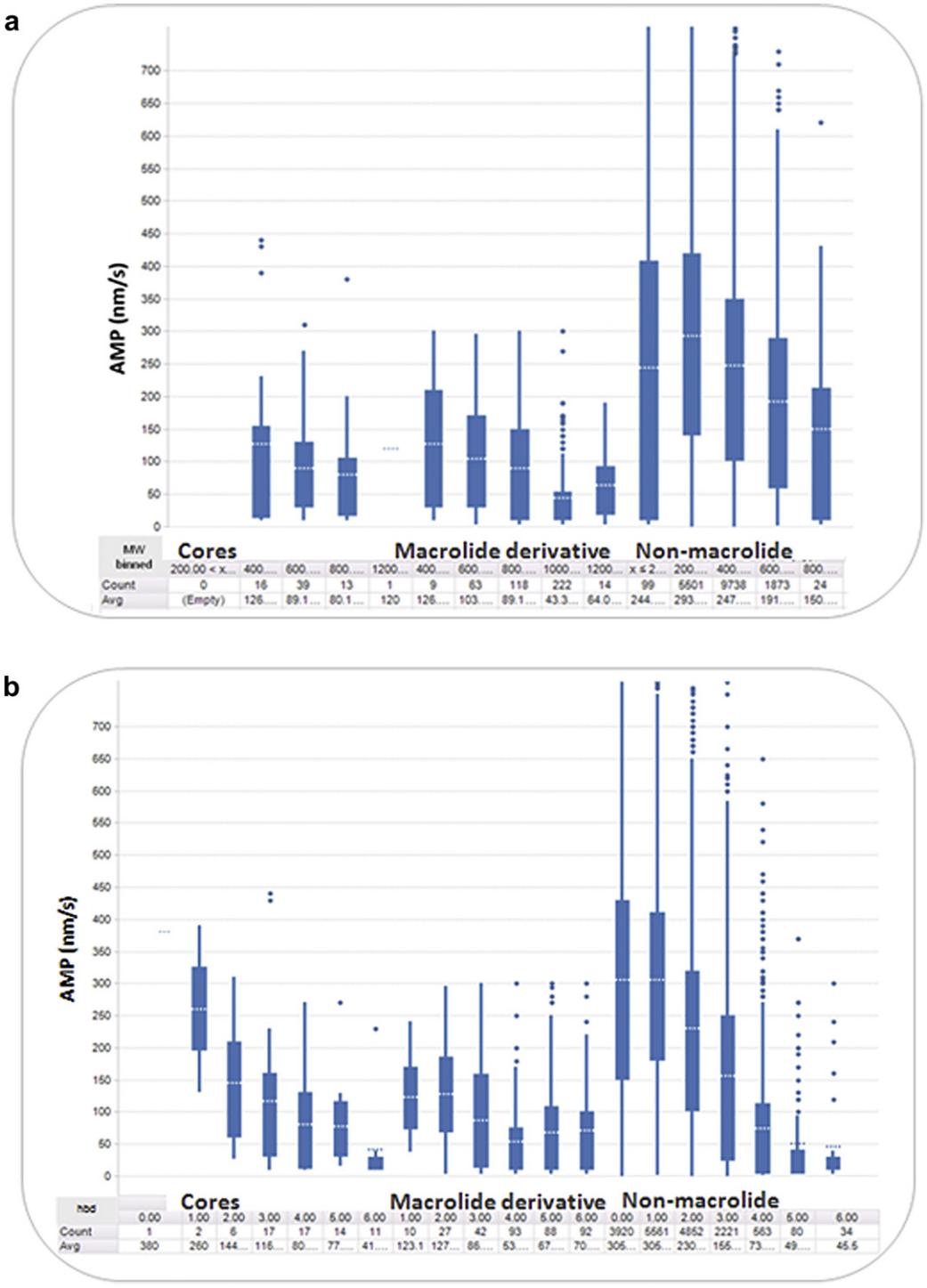

Fig. 10. Dependence of permeability AMP of the macrolide cores and derivatives as well as non-macrolides on (a) The molecular size (MW) and (b) The number of H-bond donors (hbd). The box plot layout is described in Fig. 4.

permeable macrolides (AMP > 200 nm/s) have significantly lower CHI IAM values than other macrolides of poor and moderate permeability. In Fig. 11 it can be seen that the macrolides have lower mean permeability than the non-macrolide compounds within the same bin of CHI IAM values. For the macrolides the AMP and CHI IAM values both increase, in average, with the molecular lipophilicity. However, AMP and CHI IAM of the macrolides change oppositely with the increase of the number of positively chargeable groups (pos) and/or the number of H-bond donor group (hbd) (Fig. 10). AMP decreases, while CHI IAM increases significantly with such modifications.

Extensive CHI IAM binding means that compounds bind strongly to phosphatidyl choline and cellular membranes causing extensive tissue distribution and large volume of distribution [55,56] that are characteristics of the macrolides [32,33]. Such a physicochemical profile is also associated with a risk for

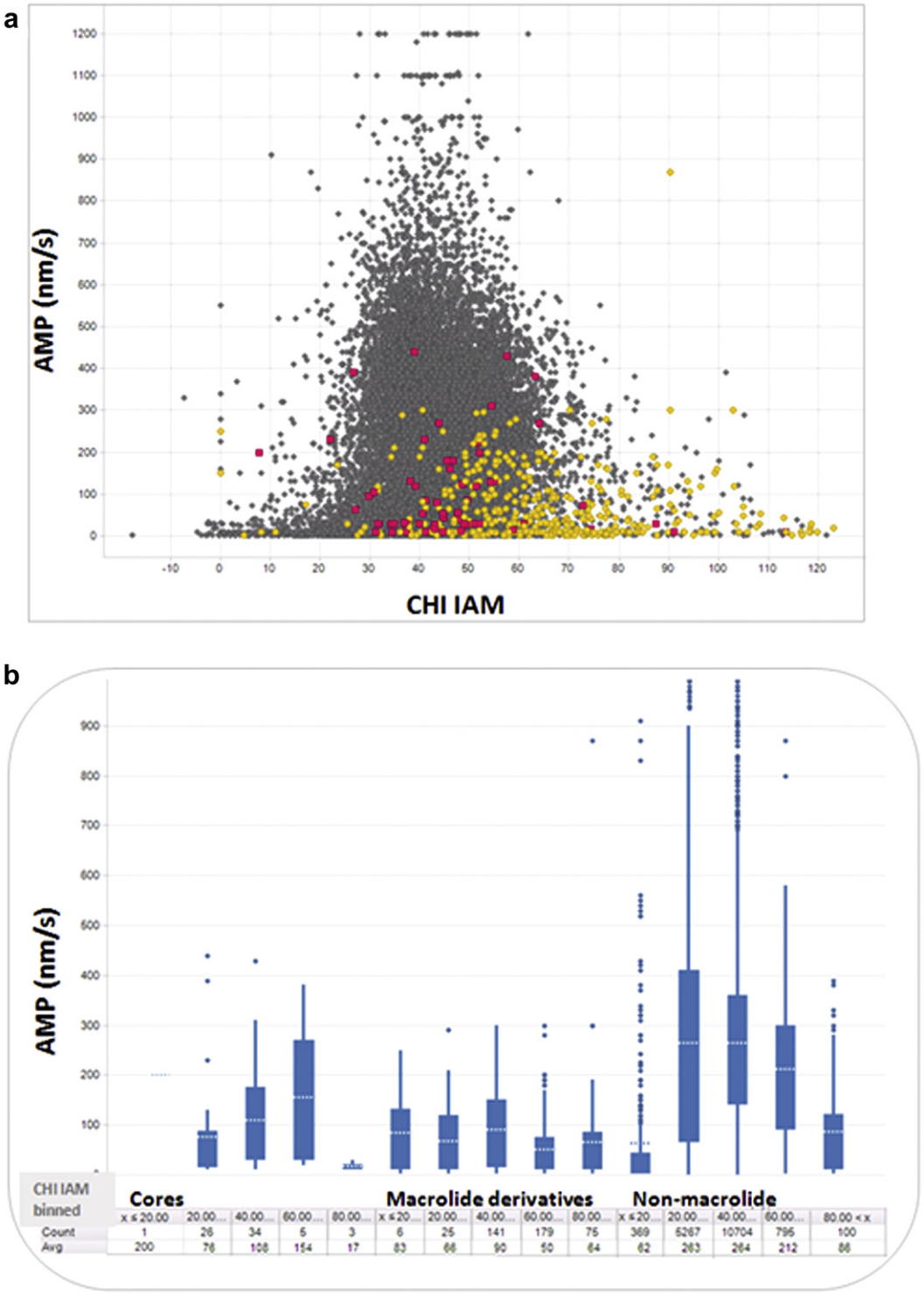

Fig. 11. (a) The scatter plot of the measured permeability (AMP) as the function of phospholipids binding (CHI IAM) (macrolide cores – red, macrolide derivatives – yellow, non-macrolides – grey). (b) The mean AMP values based on the binned CHI IAM values. The box plot layout is described in Fig. 4 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

phospholipidosis [56] as well as with poor permeation across the gastrointestinal membrane and low oral bioavailability [38]. In addition, molecules with good bioavailability generally have (1) 10 or fewer rotatable bonds and (2) polar surface area (tpsa) less than 140 $Å^2$ (or 12 or fewer H-bond donors and acceptors) [19]. While 92% of non-macrolides have such structural features (Fig. 3), only 8% of macrolides satisfy these criteria and they all are macrolide cores. The good oral bioavailability of a macrolide suggests that it is probably actively transported.

Regarding drug-likeness, macrolide cores have, in average, better physicochemical profile than the other macrolide derivatives and small discovery molecules, as well (Table 2). By adding

functional moieties to the macrolide core the molecular complexity [16] increases and also the drug-likeness of macrolides declines. Increase in size and molecular complexity resulted in greater lipophilicity, more pronounced PPB and phospholipids binding, lower solubility and permeability (Table 2). Similar trend has also been observed during lead optimization phase in the small molecule chemical space [57]. Improving potency at the expense of increasing molecular complexity often deteriorates physicochemical and ADMET profile of molecules [21,58].

The performed physicochemical analysis indicates that in order to increase diversity within macrolide chemical space and stay within drug-like space at the same time, focus should be on designing novel macrolide cores with relatively small but significant structural modifications influencing their biological features [7,15] rather than on synthesis of large conjugated derivatives [9–14]. The supporting example of this is the case of structurally related macrolides FK506, rapamycin and ascomycin that show different cellular effects and have different therapeutic applications [7]. Chemical modifications to improve therapeutic properties of the macrolide lead molecule, should be aimed at identifying key pharmacophore features and slightly modifying the structure in order to increase binding to specific macromolecular targets and/or improve ADMET profile. As an example, azithromycin was synthesised by incorporation a methyl-substituted nitrogen atom into the 14-membered lactone ring of erythromycin that resulted in stronger ribosome binding, wider anti-bacterial spectrum and better PK profile [8,32]. The physicochemical profiling of macrolides may provide valuable information in guiding structural modifications during activity or PK optimisation.

3. Conclusions

In summary, the extensive measurements of physicochemical properties performed for macrolides and their comparison with data determined for small molecules demonstrate that they cover similar physicochemical space. Exploring simple high-throughput physicochemical measurements is hence generally recommended practise ensuring molecules stay within the drug-like space. Natural-like macrolide cores composed of macrolactone with small substituents have been found to have more drug-like physicochemical characteristics (*e.g.* higher solubility and membrane permeability and lower plasma protein binding) than the larger macrolide derivatives like conjugates with quinolones [9–14]. They can be recommended as the first choice in expanding macrolide chemical diversity [7] as well as in optimizing molecular recognition with a biological target [58]. The measured physicochemical properties were used in models for predicting cellular accumulation and retention of macrolides [28]. They show similar trends with changing simple molecular properties like size, number of aromatic rings or H-bond donating atoms as small synthetic molecules.

4. Experimental protocols

4.1. Representative set of macrolides

Macrolides registered in the corporate compound database (4800 compounds) were represented as canonical SMILES using *cansmi* program [49]. *Make_parent* program [49] was used to transform salts, neutralise molecules, and uniform all charge-separated systems (*e.g.* to convert [N+]-[O−] into N═O). Number of molecules was reduced to 3658 compounds by filtering out those with MW > 1300 and/or reactive functional groups [46] inadequate for HTS. Having in mind (1) redundancy typical for corporate databases (similar compounds generated for specific targets/programs in lead optimization phase), (2) specificity of macrolide structure (*e.g.* size of the macrolide ring, number and type of sugars) and (3) the size limit of targeted representative set (app. 600 compounds) which should represent diverse chemical space of macrolides, compound selection was done by combining substructure- and Daylight fingerprint-based approaches. In the first step, the set of 3658 macrolides was divided into subsets using three substructural features as dividing conditions: (1) size of the macrolide ring, (2) number of sugars, and (3) presence of aromatic atoms in molecule. Molecules in subsets obtained in such a way were described by 8192-bit fixed-size Daylight fingerprints with paths from 2 up to 20 bonds encoded in it to capture small structural differences between large macrolides [47]. Under these conditions all macrolides were represented by fingerprints with not more than 50% of *on* bits in them. In the next step, molecules within each subset were clustered by *dbclusp* [48] program using Tanimoto coefficient (TC) as a measure of similarity. TC value of 0.75 was generally used; only for a few highly populated subsets TC was set to 0.8 to make clusters more specific. Clusters were represented in the representative set with 161 compounds; centroids not available in the compound repository were replaced with the closest available cluster member. All available singletons (281 compounds) were selected in the representative set. Additionally, representative set of macrolides was enriched with 20% randomly chosen compounds and compounds interesting to our drug discovery programs. SMILES, *cansmi*, *make_parent* were used with Daylight Toolkit [49].

4.2. High-throughput physicochemical measurements

The HTS physicochemical properties for at most 637 macrolides and at most 49386 non-macrolide compounds were determined in the following ways.

4.2.1. Chemi-luminescent nitrogen detection (CLND) solubility assay

GSK in-house kinetic solubility assay: 5 μl of 10 mM DMSO stock solution diluted to 100 μl with pH 7.4 phosphate buffered saline, equilibrated for 1 h at room temperature, filtered through Millipore Multiscreen HTS-PCF filter plates (MSSL BPC). The filtrate is quantified by suitably calibrated flow injection Chemi-Luminescent Nitrogen Detection [45]. The standard error of the CLND solubility determination is ±30 μM, the upper limit of the solubility is 500 μM when working from 10 mM DMSO stock solution. CLND solubility was determined for 85.2% of macrolides and 44.8% of small molecules.

4.2.2. Artificial membrane permeability (AMP) assay

The donor cell contained 2.5 μl of 10 mM sample solution in pH 7.05 phosphate buffer. To enhance solubility, 0.5% hydroxypropylcyclodextrin (encapsin) has been added to the buffer. The artificial membrane is prepared from 1.8% phosphatydilcholine and 1% cholesterol in decane solution. The sample concentration in both the donor and acceptor compartments is determined by LC-MS after 3 h incubation at room temperature [19]. The permeability (logPapp) measuring how fast molecules pass through the black lipid membrane is expressed in nm/s. The average standard error of the assay is around ±30 nm/s that can be higher at the low permeability range. This assay is different from the commercially available PAMPA assay offered regarding the thickness of the membrane, which is in our case much thinner and can be considered as phospholipid bi-layer formed in the pores of the filter, that is why it is called “black lipid membrane”. AMP values were measured for 83.2% of macrolides and 34.9% of non-macrolides.

4.2.3. HPLC based lipophilicity assay

The Chromatographic Hydrophobicity Index (CHI) [40] values were measured using reversed phase HPLC column (Luna C18, 50 × 3 mm), (Phenomenex, UK) with fast acetonitrile gradient at starting mobile phase of pH = 7.4. The mobile phase was a mixture of 50 mM ammounium acetate solution (adjusted to pH 7.4 by cc ammonia) and acetonitrile with 1.0 ml/min flow rate. The acetonitrile concentration was increased from 0 to 100 % using a 2.5 min linear gradient. Then it was kept at 100% for an additional 0.5 min before returning back to 0% within 0.2 min. Allowing re-equilibration of the column the total cycle time was 4.0 min. As many of the macrolides have no UV chromophore, the chromatographic peaks were monitored by mass spectrometry. CHI values are derived directly from the gradient retention times by using a calibration line obtained for standard compounds [41]. The CHI value approximates to the volume % organic concentration when the compound elutes. The CHI scale has been transformed to log *D* scale (logarithm of octanol octanol/water distribution coefficients) based on the data of 98 diverse compounds [42] using the following formula: CHI logD = 0.0525*CHI-1.467. The average error of the assay is ±3 CHI unit or ±0.25 CHI logD. The CHI values were determined for 94.4% of macrolides and 100% of small molecules.

4.2.4. Plasma protein binding (PPB) assay

Chemically bonded Human Serum Albumin (HSA%) and α-$_1$-acid glycoprotein (AGP%) HPLC stationary phases (Chiral Technologies, France) were used for measuring compounds' PPBs, applying linear gradient elution up to 30% iso-propanol. The run time was 6 min including the re-equilibration of the stationary phases with the 50 mM pH 7.4 ammonium acetate buffer. Mass spectrometric detector was used to monitor the elution of the chromatographic peaks. The obtained gradient retention times were standardised using a calibration set of mixtures as described in the reference [44]. The average standard error of the assay depends on the binding strength and kinetics of the compounds. It ranges from ±5% in the medium binding range which reduces to 0.1% to binding above 99% with fast kinetic. HSA% values were determined for 89.5% of macrolides and 100% of non-macrolides, while AGP% values were obtained for 91.7% of macrolides and 66.7% of non-macrolides.

4.2.5. Phospholipid binding assay (CHI IAM)

Compounds binding to immobilised artificial membrane (IAM) has been measured using commercially available IAM PC DD (Regis Analytical, West Lafayette, USA) HPLC column. Applying acetonitrile gradient up to 70% retention times of the compounds were converted to Chromatographic Hydrophobicity Indices (CHI IAM) using a calibration set of compounds as described in literature [43]. The CHI IAM values were detected for 84.9% of macrolides and 100% of small molecules.

Conflict of interest

The authors declare there are no conflicts of interest.

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