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Full Paper

Design and Evaluation of the Performance of an NMR Screening Fragment Library

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The design of a suitable library is an essential prerequisite to establish a fragment-based screening capability. Several pharmaceutical companies have described their approaches to establishing fragment libraries; however there are few detailed reports of both design and analysis of performance for a fragment library maintained in an academic setting. Here we report our efforts towards the design of a fragment library for nuclear magnetic resonance spectroscopy-based screening, demonstrate the performance of the library through analysis of 14 screens, and present a comparison to previously reported fragment libraries.

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Introduction

Over the past 17 years fragment-based drug design (FBDD) has grown to become an established method for drug discovery with the first approved drug developed through fragment screening (vemurafenib) reaching the clinic in mid-2011.[1,2] The fragment-based approach entails screening of small compounds with most reported libraries containing compounds with an average molecular mass of ~200 Da (Table 1). This has many advantages, one of which is that relatively small fragment libraries (100s–1000s of compounds) are able to sample a relatively broad chemical space.[3–5] This in turn means that smaller screening libraries can be used to identify 'hit' compounds against a particular target. Fragment screening is typically undertaken at relatively high concentration as the small size of the fragments usually dictates that they interact weakly with their targets. This imposes several constraints on the types of compounds that are suitable for inclusion in the screening collection. For a general screening library, diversity, solubility, purity, and suitability for the proposed screening technique/s are key considerations.[6–8] Fragments that are identified as hits in a primary screen must subsequently be elaborated to increase their potency. This can be achieved through a range of different strategies that have been exemplified in several recent reviews of successful FBDD campaigns.[9–11] In all cases a suitable library of fragments and a robust means of identifying their interactions with target proteins are required in order to select the best fragments for further development. Here we detail the approach we have taken to design and evaluate the Monash Institute of Pharmaceutical Sciences (MIPS) fragment library for NMR-based screening and analyse its performance in 14 screens.

Methods

Design and Comparison of the MIPS Fragment Library

Most fragment libraries are designed to comply with the 'rule of three',[12] which is a set of guidelines describing some of the desirable biophysical property profiles of fragments. In addition, for a general screening library the fragments should ideally be chemically and structurally diverse, possess appropriate solubility for the screening approach to be employed, not contain chemically reactive functional groups, and be amenable to chemical elaboration so that screening hits can be developed into higher affinity compounds. These were the main principles that were employed in designing the MIPS fragment library. The fragment library was assembled from a compilation of available compounds from a selected set of commercial suppliers. The initial compound set was processed with the Ligand Prep tool in SybylX2.0 (Tripos, www.tripos.com) to remove duplicate entries, metals, compounds with isotopic labels, and to strip salts. Further processing by Ligand Prep was used to remove compounds that were deemed unsuitable for inclusion in the screening library, specifically Pan Assay Interference compounds (PAINS)[13] which have been observed to hit numerous unrelated targets across a variety of screening formats. In addition compounds containing reactive and undesirable functional groups as previously described by Hubbard et al.[14] and Shivanyuk et al.[15] were removed. The cleaned set of compounds was then filtered according to a relaxed 'rule of 3'[12] (MW <300, number of rings $= 1$ to 3, number of rotatable bonds $= 1$ to 4, and calculated LogP (CLogP) ≤ 3.5) and clustered using Unity fingerprints in Dataminer version 1.6 (Tripos)

Table 1. Comparison between properties of reported libraries and MIPS library properties

MW, molecular weight; HAC, heavy atom count; CLogP, calculated logP; HBD, Hydrogen bond donors; HBA, hydrogen bond acceptors; Fsp[3], fraction of sp^3 carbons from total carbons; NRotB, number of rotatable bonds; PSA, polar surface area

Library	Compounds	MW[A]	HAC[A]	CLogP[A]	HBD[A]	HBA[A]	Fsp[3][A]	NRotB[A]	Rings[A]	PSA[A]
Monash Institute of Pharmaceutical Sciences 2012	1137	206.0	14.0	1.40	1.0	2.5	0.34	2	1.8	47
Vernalis 2004[7,18]	1275	187.9	13.3	0.5	1.2	3.1		2.2	1.4	
Vernalis 2008[7]	1063	193.2	13.6	0.4				2.2	1.6	
ZoBio/Pyxis[19]	2000	217.5		1.2	1.3	2.7		1.9		53
Pfizer[6]	2592	205.3[B]		1.0[B]	1[B]	3[B]		2[B]		57[B]
Novartis[8]	2826	~240[B]		~2[B]	1[B]	3[B]				
GSK Kinase[20]	1064	150–175[B]		1–2[B]	1[B]	2[B]				
Evotech[21]	20000	~220[B]		~2[B]	1[B]	3[B]		3[B]		~50[B]
Philipps University Marburg, Klebe et al.[22]	364	224	15	1.58	1.3	3.7		1.7		52
BioFocus[23]	~1300	198		1–2[B]	1[B]	3[B]		2[B]		
3D frag consortium[13]	200		14–16[B]		1[B]	3[B]	0.2–0.4[B]	2[B]		40–60[B]
Hubbard set[24]	200	174.9	12.2	1.38	0.5	2.1		1.5	1.5	43
Abbott[25,26]	~10000	210–220		1.5						
Abbott HFE[27]	1357	~160[B]		1[B]	1[B]	2[B]			2[B]	40[B]
Old AstraZeneca[28]	600–40000			1.35						
New AstraZeneca[28,29]	20342		15.8	1.41						
AstraZeneca NMR[29]	1200		13.7	1.59						
deCODE[30]	1329	182.5		0.96	1.5	2.6		2.7	1.7	119
Merck[31]	1280	211		1.4						40
Erlanson and literature survey[32–34]	480–10000	Includes Merck, Vanderbilt, Ariad, Genentech, Emerald, U Cambridge, CSIRO, ZoBio, Amgen-^{19}F, Heptares, Elan, Roche, Selcia, GSK, Schering–Plough								
Astex[9,35–37]	327–1600		14							
Plexxikon[38]	20360	120–350								

[A]Average of the library unless otherwise stated.
[B]Median value.

with a difference cut-off of 0.15 to facilitate the selection of a structurally diverse screening set. The compound with the lowest molecular weight from each cluster was selected as the representative compound for the cluster. The representative compounds were reviewed by experienced medicinal chemists to identify and remove any molecules that were deemed undesirable but had passed earlier filters. Finally, to enable a rapid assessment of structure–activity relationships of hit fragments, a search was conducted for each representative compound to identify commercially available analogues that were structurally related (2D Tanimoto similarity >70 % or 2D substructure) but not significantly different in size. This was assessed from the heavy atom count (HAC) for the compound with a change in HAC of no more than three permitted. Compounds that had at least 10 commercially available analogues satisfying the similarity and ΔHAC criteria were selected. Of these molecules, 1592 compounds were available from suppliers in adequate quantities (>9 mg) and were purchased. The fragments were initially dissolved in 100 % [D_6]DMSO at a concentration of 200 mM. A sample was prepared at a fragment concentration 1 mM in phosphate buffered D_2O (50 mM phosphate, pD 7.4) and 1 % [D_6]DMSO for analysis by ^{1}H NMR spectroscopy. NMR spectra were analysed manually to confirm identity and purity and assess solubility based on the intensity of peaks in the NMR spectrum. Those that were judged to be <90 % pure, or where the NMR spectrum was inconsistent with identity or solubility at 1 mM were discarded, resulting in 1137 fragments. Compounds were grouped into screening cocktails based on chemical shift diversity such that at least one resonance from each compound was unique and resolved in the ^{1}H NMR spectrum of the mixture. Cocktails of six compounds were prepared for NMR screening, giving 190 mixtures that were dispensed and stored in 2×96 well plates. Physicochemical properties of compounds in the MIPS library were calculated with Instant JChem 5.10[16] and the distribution of properties was compared with the physicochemical properties of other fragment libraries in the literature (Table 1).

Screening

Initial screening was performed by recording a saturation transfer difference (STD)-NMR experiment for each cocktail in the presence of the target protein (1–10 μM). STD-NMR is commonly employed in FBDD as it is an efficient way to screen small libraries for weak binding to a target. STD-NMR relies on the transfer of magnetisation from a larger molecule to its bound ligand, and it is very efficient at detecting ligand binding even if the K_D is in the μM range or weaker.[17] Screens were undertaken at 600 or 800 MHz on spectrometers equipped with a cryogenically cooled probes. Where possible, screens were carried out in phosphate buffer prepared in >90 % D_2O, to minimise spectral interference from either buffer signals or the H_2O resonance, and at a pH close to 7.0 and temperature of 10°C. Other buffer conditions were used where necessary as dictated by the stability of the target protein. The resulting data were processed in Topspin (Bruker Biospin) and analysed manually. STD signal intensities in the spectra were qualitatively classified as strong, medium, or weak based on the relative intensity of signals observed in the low-field region (>5.0 ppm). Relative intensities were based on the most intense STD signal (I_{max}) identified across all the STD spectra for a particular target.

A positive STD signal was categorised as strong where the intensity was >50 % I_{max}, medium where the intensity was >25 % I_{max} and <50 % I_{max}, or weak where the intensity was <25 % I_{max}. If the fragment contained no resonances in the low-field region of the ^{1}H NMR spectrum, the aliphatic region (<4.5 ppm) was analysed and such fragments were considered hits if any positive STD signal was observed. No attempt was made to categorise aliphatic STD signals due to the potential for either direct excitation by the on-resonance saturating pulse in the STD experiment and/or interference from overlapping protein resonances. Validation of primary hits from the STD-NMR screening of mixtures was performed on a target-specific basis with single compounds using a variety of methods including competition Carr–Purcell–Meiboom–Gill/STD-NMR, surface plasmon resonance (SPR), ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) NMR, high concentration assay, and/or thermal shift assay, as appropriate. Due to the varied methods of validation, the follow up data were not included in the current analysis of library performance, which is based on the primary STD-NMR screens only. Full or partial screens of the MIPS fragment library have been conducted for 14 targets and analysis has been conducted for each hit set with respect to the compounds screened. Of the compounds in the MIPS library, 96.6 % have been screened against 10 or more targets while the remaining 3.4 % have been screened against at least eight targets. Hit compounds from the targets where only part of the library was screened had a similar property profile to hit compounds identified in screens against the full MIPS library. The targets screened include enzymes, DNA-binding proteins, and protein–protein interaction targets.

Results and Discussion

Evaluation of Library Properties

The performance of the library was evaluated by comparing hit rates from different screens as well as cross-checking the identity of the hits obtained in each screen. To further understand the distribution in chemical space of a typical fragment screening hit, property profiles of the hits from each screen were compared with the property profile of the overall library, as has been reported for other fragment libraries.[7,34] Fig. 1 shows the property profile of the full MIPS fragment library. For comparison, property profiles of the hits for each of the 14 screens were calculated individually and then averaged to give the overall profile of hits obtained from the MIPS library. The property profiles for 149 fragment hits compiled from the literature by Ferenczy and Keseru[39] were also calculated and are presented for comparison. Similar analyses for several commercially-available fragment libraries have also been collated and are available online,[40] but are not discussed here in detail. In addition to comparing the property profiles of the MIPS library, its hits and the literature hits, an additional comparison was undertaken. This analysis was undertaken to investigate the chemical diversity of the MIPS library with respect to enumerated and reported fragment space. Here, enumerated fragment space is represented by a random 5 million compound subset of GDB17[41,42] and reported fragment space by a filtered PubChem dataset (MW 110–300, H-donors ≤4, H-acceptors ≤6, XLogP <3.5, ~9.77 million compounds). A 50 million compound subset of GDB17 was downloaded from the Reymond group website http://www.gdb.unibe.ch/ and a random 5 million subset was used for analysis. To simplify the representation of the property profiles, a principal component analysis (PCA) of 10 physicochemical properties (molecular weight = MW, heavy atom count = HAC, hydrogen bond acceptors = H-acceptor, hydrogen bond donors = H-donor, polar surface area = PSA, calculated LogP = CLogP, calculated LogD at pH 7.4 = CLogD(7.4), number of rotatable bonds = NRotBonds, ring count, fraction sp^3 = Fsp[3]) was conducted on the filtered PubChem dataset in R v2.15.2. All descriptors for the PubChem dataset were recalculated with Instant JChem v5.10, scaled by the standard deviation, and then the PCA performed. This gave three components (PC1–3) corresponding to factors representing lipophilicity, size, and flexibility, respectively (Fig. 2). The same scaling and factors were then applied to subsequent datasets for comparison of principal components. Principal moment of inertia (PMI) plots (Fig. 2) were generated following the procedures of Sauer and Schwarz.[43] 3D structures were generated using Corina and PMI calculated in CDK descriptor calculator. Heat maps were plotted in GNUplot v4.6 with custom scripts. Properties profiles were generated in Excel. The distribution of physicochemical properties across the MIPS library is similar to that published for other diversity-oriented libraries suggesting that a similar basis for library design has been employed in each case.

Hit Rate Performance of the Library

Hit rates for initial screening with the MIPS library (22.5 %, Table 2) are higher than many of those reported in the literature, perhaps indicative of the high false-positive rate observed with STD screening. This is reflected in the more typical hit rates obtained from secondary screening. In our experience ~50 % of STD hits identified in the primary screen are subsequently confirmed by the second method. This is consistent with the high false-positive rate reported previously for STD-NMR.[44–46] As an example, hits from many of the STD-NMR screens were subsequently validated by recording ^{15}N-^{1}H HSQC NMR spectra of the target protein (100 μM) in the presence of each individual hit fragment (1 mM). Fragments that resulted in observable changes in the chemical shift of peaks in HSQC spectra of the target protein were considered to be validated hits. In the case of one target protein, the hit rate from the STD screens was 19.0 %; following validation by HSQC the hit rate fell to 9.9 %. Another screen was validated by recording SPR data for the interaction between the target protein and fragments (200 μM) on a Biacore T200 SPR instrument. Fragments that produced a sensorgram consistent with a 1 : 1, specific binding[47,48] interaction and gave a significant response (>2 RU) were considered as validated hits. The initial hit rate from the STD screens in this case was 46.6 %, which following validation by SPR, fell to 25.6 %. Taken together this suggests that the false-positive rate in our STD-NMR screens is ~50 % bringing the average validated hit rate to 11.3 %. A hit rate of 11.3 % is still slightly above the average but within the range seen in published studies. It has previously been observed that hit rates can vary depending upon the stringency of the criteria used to classify hits.[49,50] We have deliberately chosen criteria that result in a larger number of hits progressing through early screening. For example we do not require selectivity over a second protein or competition with a known ligand for validation of an initial hit.

Property Profile of Library, Hits, Common Hitters, and Literature Compounds

The MIPS library was designed so that the fragments populate a region of chemical space where their properties are suitable for

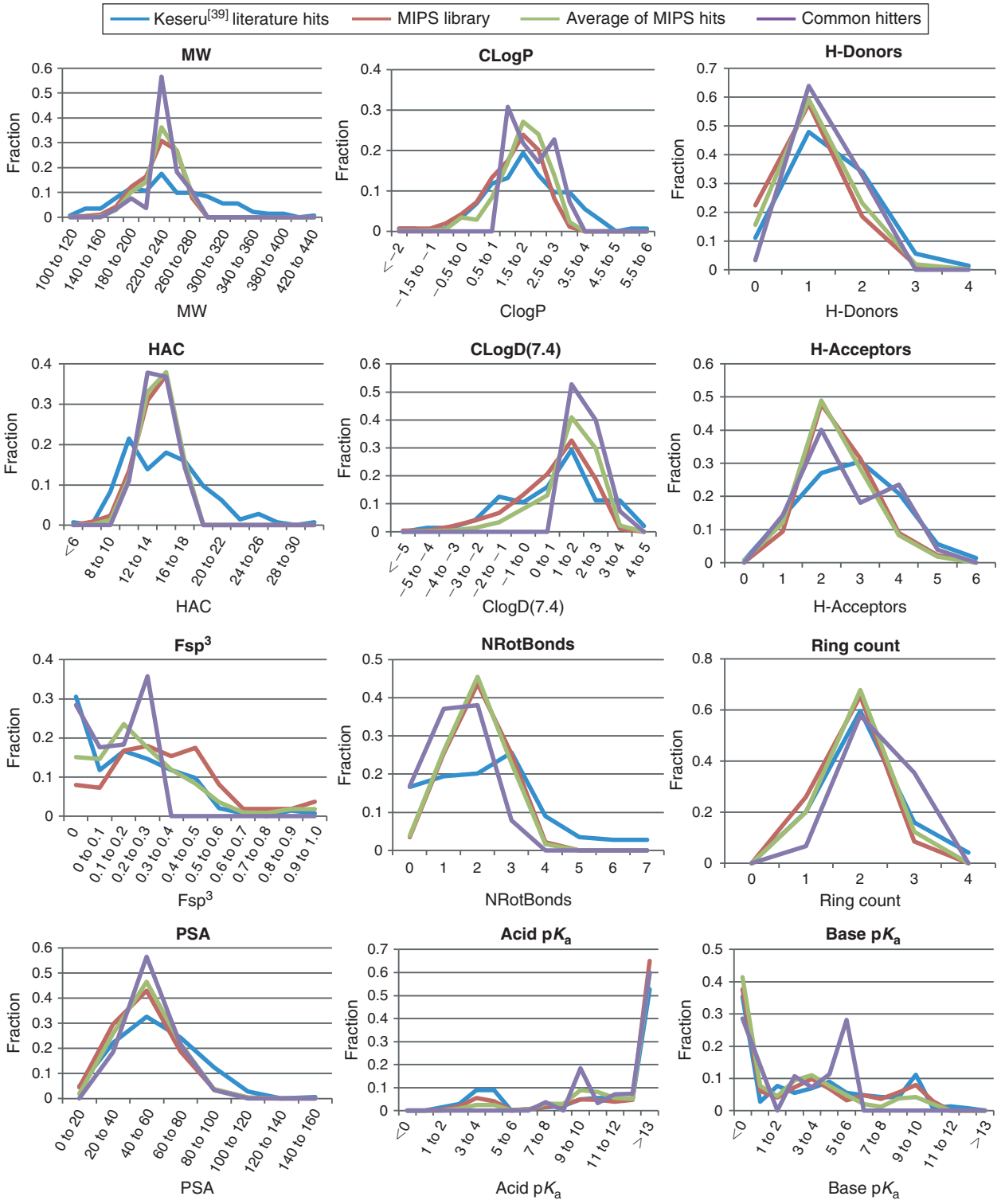

Fig. 1. Distribution of physicochemical properties of datasets. Monash Institute of Pharmaceutical Sciences (MIPS) library (red), average of MIPS hits (green), common hitters (hit in >90 % of screens, purple), and literature hits[39] (blue).

development into high affinity ligands of their target. Ideally, the property profiles of the fragment hits from the screens should reflect those of the entire library (i.e. screening does not bias results towards a particular physicochemical property such as size or lipophilicity). In addition, 24 fragments were found as hits in more than 90 % of the screens (see Supplementary Material for full details) carried out with the MIPS library; comparison of the properties of these 'common hitters' may help identify particular properties of fragments that are linked to promiscuity. As property profiles vary between libraries a comparison between the property distribution of the entire MIPS library, the average properties of fragments hits by STD-NMR in our screens, fragments that have been identified as 'common hitters', and reported fragment hits[39] provides an internal and external reference for analysis of the library (Fig. 1). Several properties were calculated to assess size (MW, HAC), polarity

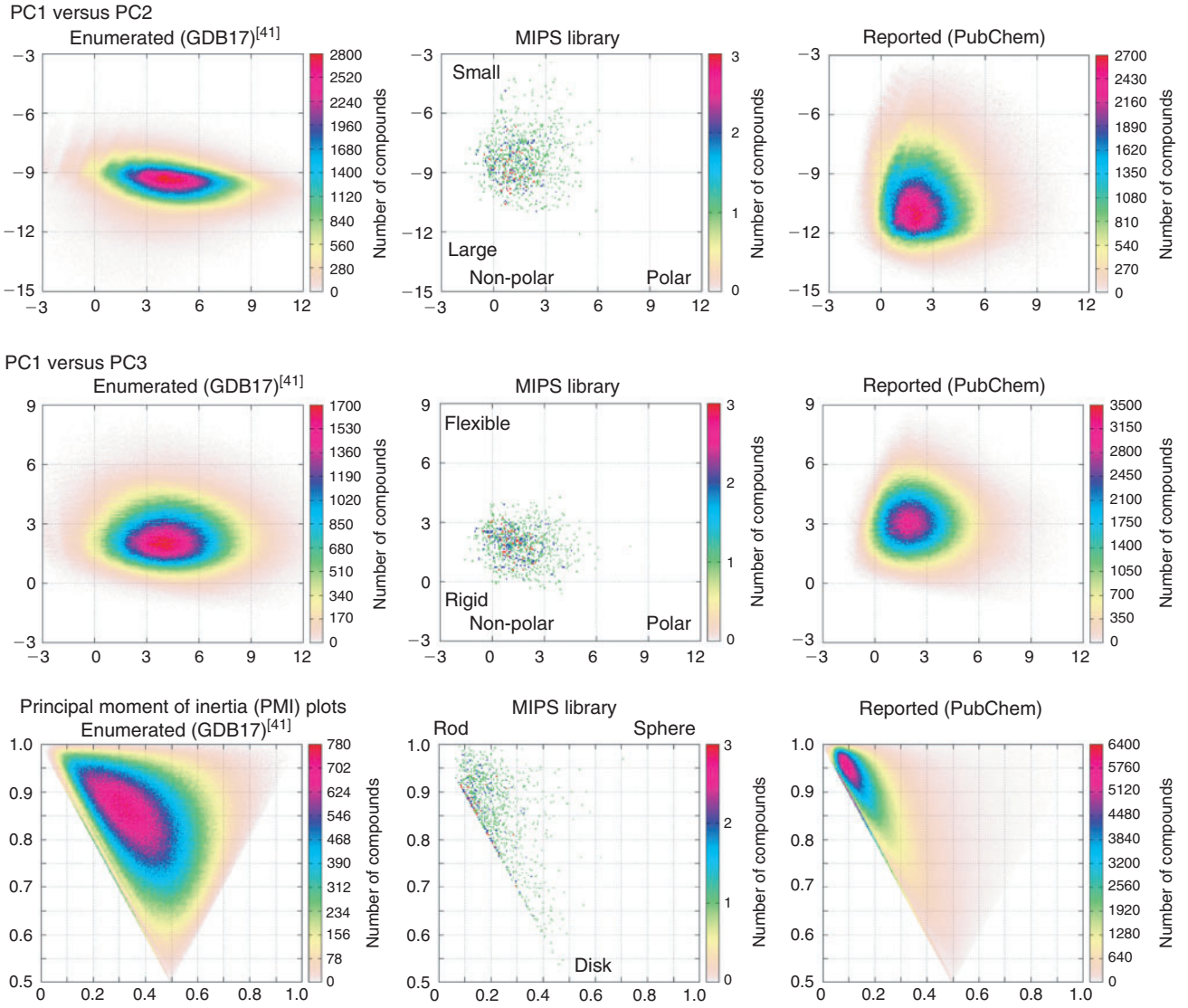

Fig. 2. Principal component analysis (PCA) and principal moment of inertia (PMI) analysis of datasets. PMI plots calculated according to Sauer et al.[43] Distribution shown by gradient coloured from white to red by number of compounds.

(H-acceptor, H-donor, PSA, CLogP, CLogD(7.4)), flexibility (NRotBonds, ring count, Fsp[3]), and ionisation (acid pK_a, base pK_a). The distribution of MW, H-Donors, H-Acceptors, HAC, ring count, polar surface area, and NRotBonds are all similar between the MIPS library and hits, indicating that screening identifies a broad range of compounds from the library without a significant bias. The physicochemical property profiles of the hits does show that compounds with higher CLogP, CLogD (7.4), and lower Fsp[3] are slightly more likely to be hits. This has been noted previously in the literature and has been linked to non-specific or promiscuous binding.[53] An analysis of the common hits across all screens showed a similar bias in CLogP, CLogD (7.4), and Fsp,[3] further supporting the observation of a correlation between promiscuity and these properties. Indeed if the common hitters are removed from the hit data, the comparison of profile of hits and the library is more favourable. Interestingly there are no common hitters with acid pK_a <7 or base pK_a >6, indicating that only partially ionisable on non-ionised compounds are identified as common hitters in our library.

Comparing the library and hit profiles with those calculated from a list of literature-reported fragments compiled by Keseru et al.[39] the most obvious difference is in the wider spread of the size of compounds (MW and HAC) in the literature data, which leads to slightly broader distributions of H-Donors, H-acceptors, and rotatable bonds. Relatively similar profiles can be seen for the number of rings, CLogP, and CLogD(7.4) in all sets of compounds. However, there is also a larger proportion of low Fsp[3] compounds in the literature set which may be due, in part, to the relatively recent development of the idea of 'escaping flatland'.[16]

Comparison to Reported and Enumerated Fragment Space

Comparison of the MIPS library with respect to datasets representing all possible enumerated chemical space (GDB17) and reported fragment chemical space (filtered PubChem) allows for analysis of the current limitations and coverage of the MIPS fragment library. Due to the large size of these datasets PCA and PMI[43] analysis was performed. Plotting the distribution of compounds in each of the datasets shows the MIPS library has a higher proportion of smaller compounds than both reported and enumerated fragment space, which is expected. This is due to the exponential nature of the number of possible

Table 2. Comparison of hit rates between MIPS library and literature reports

CMPG, Carr–Purcell–Meiboom–Gill; HCS, high concentration screening; HSQC, heteronuclear single quantum coherence; MIPS, Monash Institute of Pharmaceutical Sciences; SPR, surface plasmon resonance; STD, saturation transfer difference; Water-LOGSY, water-ligand observed via gradient spectroscopy

Library	Average hit rate [%]	Max [%]	Min [%]	% of library as hits in any screen	Screening method
MIPS (14 screens)	22.5	47.0	4.3	73.7	STD-NMR
MIPS validated ~	11.3	23.5	2.2	36.9	HSQC NMR or SPR validated
Vernalis[7](12 screens)	3.2	7.3	0.4	29	STD, Water-LOGSY, CPMG NMR, and competitive versions
AstraZeneca[28](12 screens)	3.3				STD/Water-LOGSY NMR, HCS, HSQC NMR
AstraZeneca[4](7 screens)	7	33	0.2		HCS and NMR
VU university[49](5 screens)	4.4	8.6	0.4	16	>50 % radioligand displacement at 10–30 μM
Novartis[8]	2–8	30	3		CPMG, Water-LOGSY NMR, and validation screen
Abbott[26](23 screens)	0.23	0.95	0		Heteronuclear NMR
Pfizer[6,25](13 screens)	6.9	12.58	2.82	33–44	STD-NMR
GSK Kinase[20](30 screens)	26.2	62.2	2.2	81.8	HCS, 30 % inhibition at 400–667 μM
Genentech[51](13 screens)	7.5	14.9	2.6		SPR
Misc. Pharma[25]				33	
Vertex SHAPES[52]	10–20				NMR

compounds with respect to size; hence the distributions of filtered PubChem and GDB17 compounds are weighted towards the higher MW/HAC compounds. This is exemplified by the fact that in GDB17 ~65 % of compounds have HAC of 17 while in our library 68 % of compounds have between 13 and 16 HAC. Finding the most ligand efficient compounds (those with the highest affinity with respect to size) requires screening a library with a diverse size-range of compounds, as confirmed by the profiles of hits (Fig. 1).

Relative to GDB17 both the MIPS library and the filtered PubChem dataset have a higher proportion of non-polar compounds, consistent with the previous comments of Ruddigkeit et al.[41] While the MIPS library only contains commercially available compounds it may be advantageous to include a higher proportion of polar compounds in future library designs, similar to the Vernalis libraries (CLogP average of 0.5). The filtered PubChem dataset also has a higher proportion of flexible compounds compared with GDB17 and the MIPS library; however the reasons for this differ, as GDB17 has a large proportion of complex constrained saturated rings reducing flexibility while the MIPS library has a large proportion of flatter aromatic cores.

Another way to visualise compound space is by shape; principal moment of inertia (PMI) plots[43] provide a size independent measure of compound shape. PMI plots classify compounds on the basis of their three principal moments of inertia, identifying 'disk-like', 'rod-like', or 'sphere-like' shapes and can be used to produce triangular shaped plots with each vertex corresponding to a perfect disk, rod, or sphere (Fig. 2). Here the shape distributions differ greatly with GDB17 having a higher proportion of sphere-like compounds relative to both the filtered PubChem dataset and the MIPS library, consistent with previous reports.[41] Common hitters do not appear to occupy any particular regions of this plot (data not shown) and the MIPS library covers a similar space to the filtered PubChem dataset despite shape distribution not being used as a constraint during library design. The lack of more three-dimensional (sphere-like) compounds in the literature has been noted previously.[41,54] Our intention in future libraries is to include more compounds with diverse shapes, while achieving a balance between the challenges of decreased commercial analogue availability and decreased synthetic tractability for more three-dimensional molecules and potential benefits in novelty, diversity, and investigation of sparsely characterised or screened fragment space.

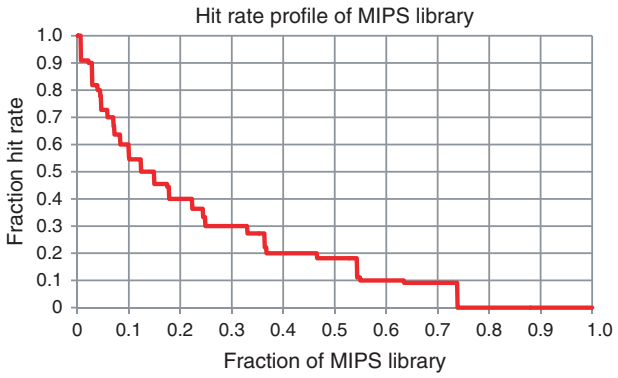

Fig. 3. Hit rate profile of the Monash Institute of Pharmaceutical Sciences (MIPS) library in initial STD-NMR screen.

Common Hits and Non-hits

Analysis of the library performance and evaluation of common hitters and compounds that have not been classified as a hit in any screen may highlight properties or scaffolds that may be undesirable, and compounds that should be excluded from future libraries. An analysis of the proportion of screens in which each fragment was classified as a hit is presented in Fig. 3. Common hitters have a fractional hit rate close to 1.0, whereas compounds that have not hit any target have a fractional hit rate of 0. While it has been noted above, and in the literature, that common hitters are often more lipophilic, analysis of the structures that are common hitters in our library also shows some substructures appear frequently. For example, in the MIPS library seven fragments with a 2-amino thiazole substructure appear in the top 24 common hitters (fraction hit rate >0.9), and these are often validated by a further screening technique (^{15}N-^{1}H HSQC NMR or SPR). Though this substructure has not been regarded as a PAIN it is present in several PAINS[13] and thiazoles have been generally reported as being less developable

than other substructures.[55] However, there are also several reported examples where 2-aminothiazoles have been developed into potent hits.[56–58] Without strong negative evidence, we have not yet removed members of such compound classes from our library but their high hit rate is noted during further selection and development. From all 14 screens 26.2 % of fragments failed to hit any target (Fig. 3), however analysis of these compounds did not yield any discernible common attributes.

Conclusion

A fragment library suitable for NMR screening was designed and assembled based on a combination of criteria described in the literature, and the performance of this compound set has been evaluated. Analysis of the screening results indicates it performs similarly to other libraries reported in the literature and an internal comparison of physicochemical property profiles for hits and the full MIPS library indicate the desired distribution of properties. Comparisons to enumerated fragment space (GDB17) and reported fragment-like molecules (filtered PubChem) highlight that one of the current challenges in assembling a diverse fragment library is the bias towards flat disk or rod shaped compounds in the commercially available compound set.

Supplementary Material

The Supplementary Material containing the chemical structures of the fragments that were identified as frequent hitters in the screens reported here can be found on the Journal's website.

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