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Weak affinity chromatography as a new approach for fragment screening in drug discovery

Minh-Dao Duong-Thi [a,1], Elinor Meiby [a,1], Maria Bergström [a], Tomas Fex [b], Roland Isaksson [a], Sten Ohlson [a,*]

[a] School of Natural Sciences, Linnaeus University, SE-391 82 Kalmar, Sweden
[b] Astra & Zeneca AB, SE-431 83 Mölndal, Sweden

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ABSTRACT

Fragment-based drug design (FBDD) is currently being implemented in drug discovery, creating a demand for developing efficient techniques for fragment screening. Due to the intrinsic weak or transient binding of fragments (mM–μM in dissociation constant (K_D)) to targets, methods must be sensitive enough to accurately detect and quantify an interaction. This study presents weak affinity chromatography (WAC) as an alternative tool for screening of small fragments. The technology was demonstrated by screening of a selected 23-compound fragment collection of documented binders, mostly amidines, using trypsin and thrombin as model target protease proteins. WAC was proven to be a sensitive, robust, and reproducible technique that also provides information about affinity of a fragment in the range of 1 mM–10 μM. Furthermore, it has potential for high throughput as was evidenced by analyzing mixtures in the range of 10 substances by WAC–MS. The accessibility and flexibility of the technology were shown as fragment screening can be performed on standard HPLC equipment. The technology can further be miniaturized and adapted to the requirements of affinity ranges of the fragment library. All these features of WAC make it a potential method in drug discovery for fragment screening.

We are now witnessing a paradigm shift in drug discovery where large (>50,000 compounds) high-throughput screening campaigns are abandoned in favor of limited screens of a few thousand small organic fragments. Fragment-based drug discovery/design (FBDD)[2] [1–4] is based on the concept of finding small fragments (<300–350 in molecular weight) that show weak but highly efficient binding to a target molecule, for example, a protein receptor. These low-affinity binders, typically 10–0.01 mM in affinity and with a hit rate of 1–5%, are then later in the drug discovery process grown, merged, or linked into a high-affinity drug lead substance which ultimately will result in a potential drug candidate. In addition, it has been recently suggested that a weak binder in itself can be a drug candidate due to its dynamic behavior and "mild" influence on a receptor [5].

FBDD holds great promise as evidenced by the emerging number of lead compounds in clinical trials that have their origin from fragments [2,6]. The beauty of FBDD as compared to HTS is that a few thousand fragment structures can cover a major part of the chemical structure diversity space. Moreover, fragment screening can give higher hit rates and produce hits with higher ligand efficiencies. A distinctive characteristic of a hit from a fragment library is that it shows weak binding of millimolar to micromolar in K_D. One major challenge with the screening of weakly binding fragments is the difficulty in detecting the interaction bound at equilibrium, which must be screened at high concentrations [7]. However, there are now a number of biophysical methods available including NMR [8], mass spectrometry, X-ray crystallography [8], and surface plasmon resonance (SPR) [9], with their *pros* and *cons*, that can be used to detect fragments and quantify their binding in terms of affinity and kinetics. In addition functional assays including binding to intact cells [10] as well as virtual screening procedures [11] offer important tools in the fragment finding process.

The purpose of this work is to introduce a complementary method based on weak affinity chromatography (WAC) [12] for fragment screening. This technique represents a simple and robust procedure and it is based on weak zonal affinity separations of analytes such as small molecules. WAC has previously been used mainly for analysis and characterization of carbohydrates [13,14]

* Corresponding author. Fax: +46 480 446262.
E-mail address: sten.ohlson@lnu.se (S. Ohlson).

[1] These authors contributed equally to this work.

[2] *Abbreviations used*: 3-ABA, 3-aminobenzamidine; 4-ABA, 4-aminobenzamidine; AmAc, 10 mM ammonium acetate, pH 7.0; API-ES, atmospheric pressure ionization-electrospray; DAD, diode-array detector; DMSO, dimethyl sulfoxide; EIC, extracted ion chromatogram; FBDD, fragment-based drug discovery; MS, mass spectrometry; PB, 0.2 M sodium phosphate buffer, pH 7.0; PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4; PMSF, phenylmethanesulfonyl fluoride; SIM, selected ion monitoring; SPR, surface plasmon resonance; TIC, total ion current; WAC, weak affinity chromatography.

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and for chiral separations [15]. Recent work in our laboratories has shown the potential to use WAC for drug screening on albumin as a transport carrier [16,17]. In this work we have selected thrombin and trypsin as a model and as a relevant target for drug discovery. They are two highly homologous protease proteins, where thrombin is an important target for developing anticoagulation drugs in cardiovascular diseases. The WAC technique was evaluated with a small collection of amidine fragments. These amidines serve as an arginine mimetic binding to aspartic acid residues deep in the S1 pocket of trypsin and thrombin [18]. Immobilization of trypsin and thrombin in microcolumns was conveniently carried out by *in situ* binding procedures. Fragment screening was achieved on a standard HPLC platform with UV diode array and mass spectrometry (MS) detection, on equipment frequently used in the pharmaceutical industry. Fragments were screened in the millimolar to 10 μM affinity ranges and specificity was demonstrated by reversible and irreversible inhibition of the target proteins. To investigate opportunities for high-throughput screening, mixtures of compounds were analyzed and detected by MS. It was shown that fragment screening can be conveniently performed by affinity chromatography procedures and thus may serve as another valuable tool for fragment screening in drug discovery.

Materials and methods

Chemicals including fragment collection

A collection of 20 amidine fragment compounds with documented binding to thrombin was obtained from AstraZeneca, Mölndal, Sweden. This collection was complemented with aniline, benzylalcohol, and benzamide (Sigma Aldrich, St. Louis, MO). Table 1 shows structure and numbering information of the fragment collection. All fragments contained a substituted aryl group. The molecular weights of the fragment were in the range of 93–307 Da with an average of 198 Da. Melagatran was received from Astra Zeneca and it was used to inhibit the active sites of the trypsin column. The fragments were typically dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich) at 10 mM and further diluted with water to working concentrations. Human thrombin (2400 IU/mg) was received from Octapharma, Stockholm, Sweden. Bovine trypsin (8300 IU/mg), phenylmethanesulfonylfluoride (PMSF), periodic acid, sodium cyanoborohydride, and ethanolamine were purchased from Sigma Aldrich. BOC-Phe-Ser-Arg-AMC (I-1400) was acquired from Bachem, Bubendorf, Switzerland.

Preparation of affinity columns

In situ immobilization of thrombin, trypsin, and ethanolamine was carried out on three stainless-steel microcolumns (35 × 2.1 mm) packed with spherical silica particles (5 μm diameter, 300 Å pore size; Kromasil, Bohus, EKA Chemicals, Sweden) which had been silanized into diol-substituted silica according to standard procedures [1].

The direct immobilization was performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) based on reductive amination and the procedure was a modification of a previously published method [13]. For immobilization of trypsin, thrombin, and ethanolamine, the diol silica column was first rinsed with water (>20 column volumes) and then it was oxidized into aldehyde-silica by 10 × 100 μl injections of 100 mg/ml periodic acid at 0.1 ml/min for 1 min and then stopped for 12 min before next injection. The total reaction time was 2 h and it was performed at 22 °C. The column was then rinsed with 0.2 M sodium phosphate buffer pH 7.0 (PB) (>20 column volumes). In the next step 7 × 100 μl (trypsin) or 10 (thrombin) × 100 μl (10 mg/ml target protein with 10 mg/ml sodium cyanoborohydride, all in PB) was injected at a flow rate of 0.05 ml/min (in PB) onto the column where the pump was stopped for 2 h between each injection. The ethanolamine column was prepared in an identical way as the trypsin/thrombin columns (with 7.1 mg/ml ethanolamine). The total reaction time was 14–24 h and the reaction was performed at 12 °C. The eluate from the immobilization reaction was collected and the yield of bound trypsin or thrombin was determined indirectly from the absorbance at 280 nm of the eluate and the protein solution applied onto the column.

Screening of fragment collection

Screening was performed on an Agilent 1200 series HPLC system equipped with a diode array multiple wavelength detector (DAD) and a single quadrupole mass spectrometry detector (Agilent Technologies). UV detection was performed routinely at 214 nm. On MS detection, fragments were ionized by electrospray in atmospheric pressure (API-ES) in positive mode with fragmentor at 90 V. Drying nitrogen gas flow was 10.5 L/min at 250 °C and nebulizer pressure was 25 psig. Mass spectrometry signal acquisition was set at selected ion monitoring (SIM) on sample target masses. Retention times were based on peak apexes of UV traces, total ion chromatogram (TIC), or extracted ion chromatogram (EIC). Chromatograms were analyzed with the Agilent ChemStation version B.04.01 chromatography data system.

On UV detection 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), was used as mobile phase and on MS detection 10 mM ammonium acetate, pH 7.0 (AmAc), was used as mobile phase. Both mobile phases were run at 0.2 ml/min at 22 °C. Fragments were injected in duplicate either as singles at 0.1 mM (2 μl) with 1% DMSO in water or as mixtures (1% DMSO in water) of 0.009 mM of each compound. The fragment collection was screened on the trypsin and the thrombin column, respectively, and on the reference columns (diol and ethanolamine silica columns, respectively). In addition inhibited trypsin and thrombin columns were used as reference columns (see procedures for inhibition below). Between analyses columns were stored at +4 °C in PBS.

Inhibition of trypsin/thrombin columns

Reversible inhibition of trypsin column with melagatran

Reversible inhibition of the trypsin column was achieved by injection of 100 μl 10 mM melagatran in PBS at a flow rate of 0.05 ml/min. Further, to ensure as complete inhibition as possible, a 20 μl pulse of 10 mM melagatran was applied onto the column before each injection of fragment in duplicate. In addition the mobile phase was supplemented with 100 nM melagatran. Otherwise the conditions for screening were the same as above. The fragment compound 3-aminobenzamidine (3-ABA) was used as a control to monitor the level of inhibition. This substance was injected onto the inhibited trypsin column at regular intervals throughout the screening. Complete inhibition was defined as the minimum retention time of 3-ABA after exposure of trypsin column with melagatran as stated above. The inhibited trypsin column was recovered by elution of melagatran with 50 mM glycine, pH 3.1, for 90 min (0.2 ml/min).

Permanent inhibition of trypsin and thrombin columns with PMSF

Permanent inhibition of the trypsin and thrombin columns was achieved by 15 (trypsin) and 12 (thrombin) × 100 μl injections of 5.6 mM PMSF (20% methanol in PBS) at a flow rate of 0.05 ml/min. The fragment collection was then screened under the same conditions as described above for noninhibited trypsin and thrombin columns. 3-ABA was used as control substance similarly as

Table 1
Fragment structures with K_D- and IC_{50} values.

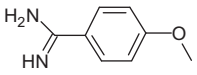
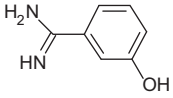
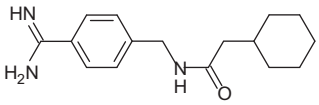
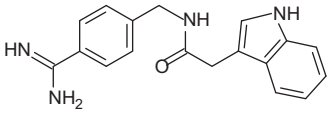
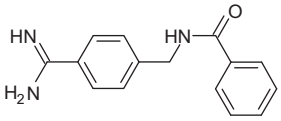
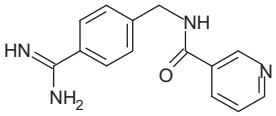
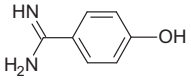
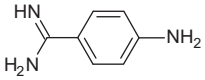
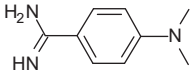
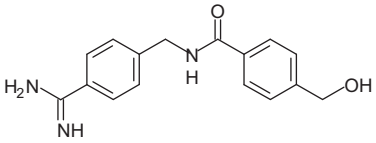
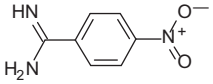
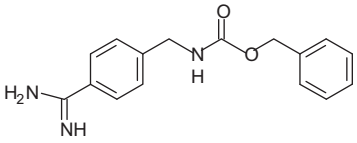
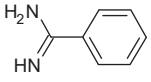
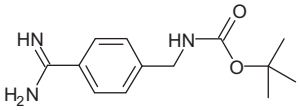
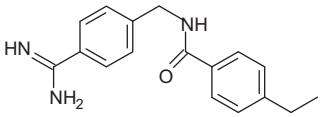
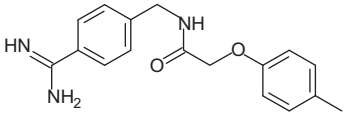
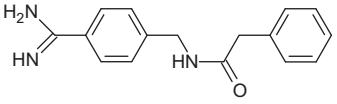
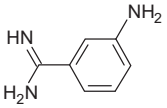
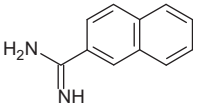
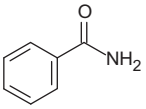
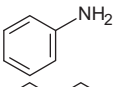
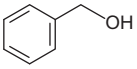
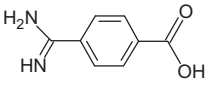
Compound	Structure	K_D (μM, thrombin)	K_D (μM, trypsin)	IC_{50} (μM, trypsin)
05		980	230	
06		160	66	
13		45	11	3.2
15		34	NA	1.8
16		120	33	11
17		190	38	51
29		170	100	
36		80	32	
42		640	300	
45		350	46	14
53		–	850[a]	
65		8.4	11	
68		330	83	
70		51	74	
71		64	21	
75		67	16	5.0

Table 1 (*continued*)

Compound	Structure	K_D (μM, thrombin)	K_D (μM, trypsin)	IC_{50} (μM, trypsin)
76		54	6.7	1.9
86		200	69	
96		140	22	
97		–	–	
98		>700	–	
99		–	–	
100		–	170[a]	

–, No binding, NA, not applicable. Substance (15) was retained on the column and could not be eluted as a visible peak.
[a] Retention of substance on inhibited column was hidden in the DMSO front and therefore there is a certain error in apparent K_D.

shown above. Complete inhibition was defined as the minimum retention time of 3-ABA that was obtained on additional injections of PMSF. The retention time of 3-ABA was measured at regular intervals throughout the screening to monitor inhibition.

Data analysis

The apparent dissociation constant, K_D (mM), of the fragments with regard to their binding to the active site of trypsin and thrombin was estimated according to [13,19]

$$K_D = B_{tot}/t'_R F, \quad (1)$$

where t'_R is the net retention time (min), B_{tot} is the total amount of active sites on the affinity column (nmol), and F is the flow rate (ml/min). The t'_R was calculated according to

$$t'_R = t_{R,\,active} - t_{R,\,inhibited} \quad (2)$$

where $t_{R,inhibited}$ is the retention time of the analyte (min) on the inhibited reference column and $t_{R,active}$ is the retention time of the analyte (min) of the same analyte on active trypsin/thrombin column. Retention times were measured as the apex of the peak. Eq. (1) is valid when $(1/K_D)\,C_a \ll 1$, where C_a (mM) is the concentration of the analyte in the column.

Enzyme assay of trypsin inhibition

Trypsin enzymatic activity was determined by a fluorescence assay that measures the inhibition of substrate consumption on administration of a fragment compound. In brief, trypsin (1 ng/ml) activity and inhibition were studied with the substrate BOC-Phe-Ser-Arg-AMC (30 μM) in 50 mM Tris–HCl, pH 7.4, 40 mM sodium chloride, 20 mM calcium dichloride, 0.1% Tween 20, and 1.2% DMSO. Fragment compounds in a suitable concentration range were added before initiation of substrate conversion. The enzyme reaction was allowed to proceed for 10 min at ambient temperature when it was stopped by addition of 1 M sodium acetate buffer, pH 4.5. The change in fluorescence was read at an excitation wavelength of 380 nm and at an emission wavelength at 460 nm. IC_{50} values were determined from response curves at different concentrations of tested compound.

Results and discussion

Characterization of trypsin/thrombin columns and of fragment collection

The target proteins, trypsin and thrombin were immobilized on high-performance silica supports which had previously been silanized with a hydrophilic coating to minimize unwanted nonspecific binding to a naked silica surface and to introduce functional groups that can be used for binding target proteins. The proteins were covalently bound by reductive amination at high concentration, 45 mg/ml column volume (236 nmol) for trypsin and 37 mg/ml column volume (120 nmol) for thrombin, respectively. They were immobilized *in situ* by binding directly onto packed activated diol-silica columns with high reaction yield (80% and 91% for trypsin and thrombin, respectively). The consumption of immobilized proteins was in the range of 5 mg and it can be further reduced by working with smaller units such as capillary columns with one order of magnitude less volume. *In situ* immobilization is a convenient procedure that can be carried out on any standard HPLC platform. The number of active sites on each column was estimated to be 50% of immobilized protein content. This is an assumption but it can be regarded as a reasonable approximation in reference to other WAC studies with immobilized proteins [13,20,21].

The small fragment collection used in this study included 20 amidine derivatives which contain charged basic groups that bind to the S1 pocket in trypsin and thrombin [22] (Table 1). They were

selected to represent binders with a wide range of weak affinities in the millimolar to micromolar range. Three molecules (benzamide, benzylalcohol, and aniline) were also part of the study to represent nonbinders to trypsin and thrombin. In fragment screening as well as in high-throughput screening with larger libraries, DMSO is the most accepted solvent for many reasons including its capacity to dissolve substances and miscibility with water [23]. The collection was dissolved individually in DMSO and it was used in the screening experiments as singles and mixtures at 1% DMSO in water.

Fragment screening by zonal weak affinity chromatography

Characteristics of fragment screening

Fig. 1 demonstrates typical chromatograms on a thrombin column of screened substances from the fragment collection with both UV and MS detection. The first peak in each chromatogram represents the unretarded DMSO and the fragments are retained according to affinity and active amount of target protein on the column (see Eq. (1) and discussion below). With UV detection physiological buffers can be selected such as in this case PBS (pH 7.4) but with MS detection compatible volatile buffers need to be used and in our studies ammonium acetate (AmAc) at pH 7.0 was used. Fragment screening was performed isocratically with no change in mobile phase conditions. High repeatability was obtained throughout the screenings where retention times of a duplicate sample typically varied less than 0.5% between injections.

The presence of DMSO in the samples restricted the potential to detect substances using UV detection at very weak affinities with K_D in the millimolar range as they were partly coeluting with the DMSO peak. Retarded fragments are clearly detected at sample concentrations of 0.1 mM at 214 nm and considerably less (at least one order of magnitude) by MS detection for most fragments. The consumption of fragments is in the range of nanograms per injection. However, detection of peaks is deteriorating the more retarded they are because of the band spreading (considerable dilution of substances) at longer retention times. The peaks in the chromatograms were quite symmetrical with some tailing and peak apex was therefore used as a reasonable measure of retention. For convenience operations were carried out at room temperature (22 °C) but using other temperatures such as 37 °C is of course an option.

After months of operation including approximately 200 injections under varying conditions, the columns showed some deterioration as evidenced by the loss of retention (13% for trypsin and 26% for thrombin) of the control substance 3-ABA as measured using PBS as mobile phase and UV for detection. Degradation of the columns may be attributed to instability and aggregation due to, e.g., protease (autolysis) action and further to leakage of protein under operation at ambient temperatures and/or when stored at 4 °C. The loss in retention can be controlled and compensated by calibration against an internal standard such as 3-ABA.

Throughput of operation

Fragment screening usually requires high throughput as libraries of up to thousands of compounds are analyzed in one session. It is therefore desirable that screening and analysis of data can be achieved within a reasonable amount of time of a day or two. Increased throughput with WAC can be obtained basically in three ways by parallelization of immobilized target columns,

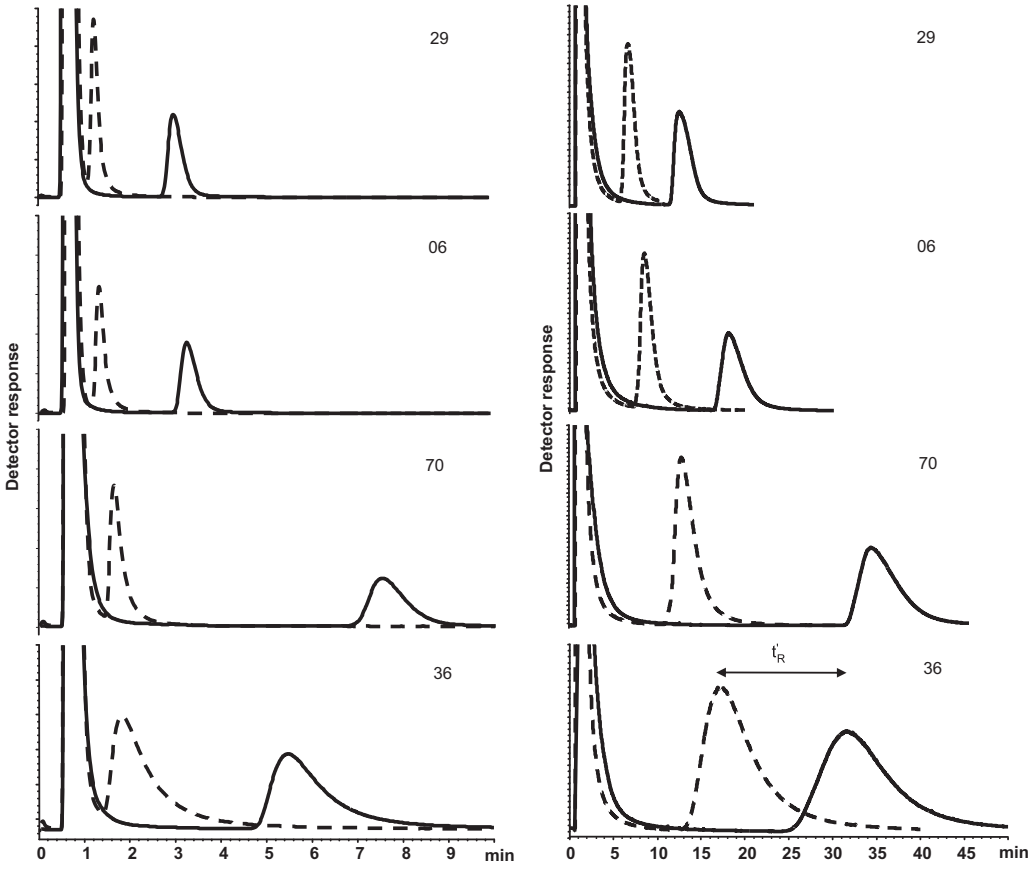


Fig.1. Chromatograms of four fragments on active (solid line) and PMSF-inhibited (broken line) thrombin columns with PBS as mobile phase (left, detected by UV) and with AmAc as mobile phase (right, TIC from MSD). The t'_R is the measurement of net retention time.

higher flow rates, and using fragments in mixtures. In an earlier study [16] we demonstrated high throughput (thousands of runs per day) by using 24 parallel columns in a chip for drug screening on albumin as a target. When using single columns as in this study higher throughput can be achieved by increasing the flow rate of mobile phase as long as the integrity of the column support can be maintained. In this study we routinely worked at 0.2 ml/min in flow rate which can be increased substantially to approximately 1.0 ml/min (data not shown).

Of higher significance is the possibility of using mixtures of fragments when employing MS. With MS detection each fragment can be followed and identified by its mass-to-charge ratio which makes it attractive for running mixtures to increase throughput and to partly characterize unknown binders in, for example, biological extracts. MS detection has been successfully used in combination with frontal affinity chromatography to detect micromolar binders from complex mixtures [24]. Single ions of individual fragments can be followed as long as they are of different molecular masses. An example of this is shown in Fig. 2 where a mixture of 11 fragments in DMSO was analyzed simultaneously by MS. Individual fragments were followed by their masses and their retentions were easily estimated by EIC. For a comparison the TIC of the mixture is also included (Fig. 2). By keeping individual fragments low in concentration, internal competition between fragments for binding sites can be minimized and retention will to a large extent be unaffected as compared to injecting them as single fragments. Identifying hits from mixtures should always be followed up by rerunning them as individual fragments to estimate any discrepancies in retention. As an example, it was less than 7% difference in retention for fragments on a thrombin column as individuals compared to the present in mixture. We have not yet optimized the use of mixtures but we certainly see the possibility of using up to a hundred substances in a mixture for one run. By using high flow rates and/or in combination with mixtures of fragments, we foresee a throughput in the range of thousands of fragments per day.

Specificity in binding of fragments

Apart from specific binding of fragment to the active sites of the protein, a number of nondesired or nonspecific binding events can occur, for example, to other areas of the protein, to the covalent linkage between protein and support, and to the surrounding environment such as the matrix in itself or remaining aldehyde groups. It can even be important to screen for cross-binding to other targets to find the most selective binders. Therefore it is important to estimate early in the screening process any nonspecific binding so false-positives can be excluded.

Binding to support and linkage was estimated with two reference columns (a diol and an ethanolamine derivatized silica column, respectively). The binding of the fragment collection in the two mobile phases was insignificant to diol silica for most members. Two fragments (71 and 96) showed minor binding to trypsin/thrombin columns (1–9% of retention on active column). No binding of fragments was seen on the ethanolamine silica column for any of the compounds in the fragment collection. These results indicate that nonspecific binding to surface and linkage is negligible.

The most appropriate reference to control any contributions from nonspecific binding is to use the immobilized target without its defined binding to the active site. The presence of immobilized protein can also modify the surface characteristics of the naked diol silica surface and influence nonspecific binding to the support

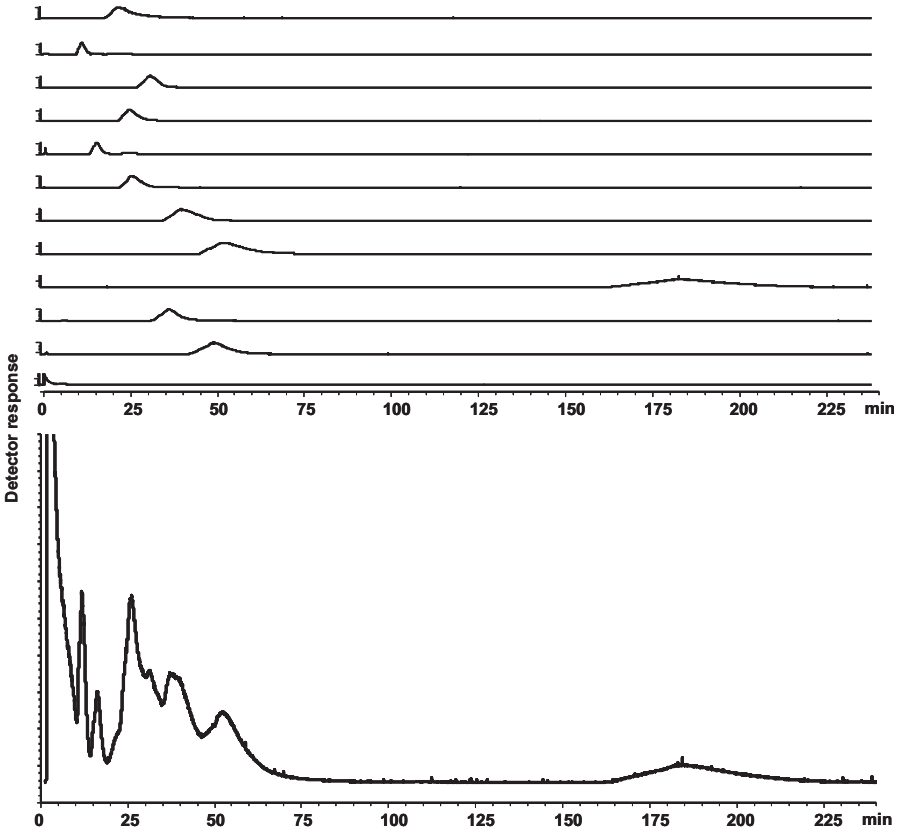


Fig.2. EIC (upper) and TIC (lower) of 11 fragments in a mixture injected on a thrombin column (AmAc). Ions in EIC in order downward are ($[M+H]^+$) of *m/z* 136 (compound 86), 137 (compound 29), 250 (compound 70), 254 (compound 16), 255 (compound 17), 268 (compound 76), 274 (compound 13), 282 (compound 71), 284 (compound 65), 298 (compound 75), 307 (compound 15), and 79 (DMSO).

matrix. In the present study on proteases, we applied two different approaches to block the active site of the proteases with both a reversible inhibitor (melagatran) and an irreversible inhibitor (PMSF). These two inhibitors bind trypsin/thrombin differently in the active site. Melagatran binds noncovalently deep into the S1 pocket of the protease at nanomolar affinity with strong binding to Asp 189 through its amidine residue [18,25], while PMSF forms a covalent bond to Ser 195 which is located at the entrance of the S1 pocket and participates in the catalytic activity of the enzyme [26]. Since PMSF is rather small it may not be able to completely block the amidines to reach down to the S1 pocket. On the other hand, a challenge with melagatran is that it is a reversible inhibitor and albeit its high affinity to target it leaks gradually from the column, leaving active sites available for binding. This can be partly circumvented by repeated injections of melagatran in-between the analysis of each sample as well as being part of the mobile phase. An advantage with a reversible inhibitor, such as melagatran, is that the immobilized target protein column can be restored by forced elution of inhibitor and reused in screening studies. Due to these concerns, PMSF was selected as the first choice for blocking target columns and it was applied to inhibit both trypsin and thrombin columns.

An example of a blocking experiment is seen in Fig. 3, where trypsin was inhibited with both melagatran and PMSF. The internal control 3-ABA lost most of its retention (>90%) on both columns. Some remaining binding activity was seen that could be attributed to nonspecific binding to other parts of the protein molecule. The presence of nonspecific binding sites, even though they are weak in affinity, may contribute significantly to the total binding. In our study, it was evident that nonspecific binding occurred to some extent for most of the fragments. The relative contribution from nonspecific binding in relation to total binding was significantly higher for fragments with lower affinities (Fig. 4). Therefore it is of vital importance to carefully evaluate the role of nonspecific binding especially when selecting binders in the millimolar range.

The higher nonspecific binding of fragments when screened on the thrombin column versus the trypsin column was partly due to the fact that the affinity of the compounds was generally higher for trypsin (Table 1). Nevertheless, in some instances the differences seem also to be characteristic of the target protein where thrombin may be more susceptible to nonspecific binding than trypsin. In other cases, the extent of nonspecific binding can be attributable to the fragment molecule. Especially fragments containing hydrophobic regions are likely to be more prone to nonspecific binding. For example, fragment 96, that contains a naphthalene group, is retarded on both the ethanolamine column and on the PMSF-inhibited trypsin/thrombin columns. However nonspecific binding of amidines to protein surfaces may not be representative for

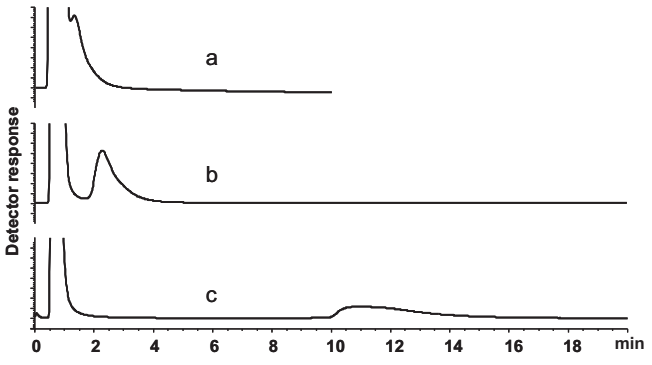


Fig.3. Retardation of compound 86 (3-ABA) on (a) the melagatran-inhibited trypsin column, (b) the PMSF-inhibited trypsin column, and (c) the active trypsin column.

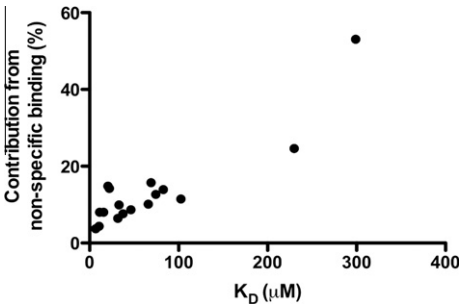


Fig.4. The contribution from nonspecific binding (measured with PMSF-inhibited column) to the total retention on the active trypsin column in relation to the apparent K_D values of the fragments.

nonspecific binding in general of a fragment library. It should also be noted that in addition to a blocked target protein, any irrelevant binding can be of interest to other protein surfaces especially those suspected for side-reactions of a potential drug.

Affinity ranking of fragments

Even though trypsin and thrombin are homologous proteins, screening of the fragment collection resulted in distinctive profiles in retention for each of the targets (see Fig. 1). Retention of a fragment is according to Eq. (1) due to the amount of active sites on the protein immobilized on the support as well as the affinity of binding of fragment to such active sites. The influence of nonspecificity as discussed above must be corrected to properly estimate the specific binding to the target (see Eq. (2)). Furthermore concentrations of the analytes must be low to estimate retention correctly.

All amidines in the library were specifically retarded with PBS as the mobile phase on the trypsin column and could be considered as hits. Fragment 15 was, however, severely retained on the trypsin column to be eluted within a reasonable time (>1 h). All amidines, except for 100 and 53, were specifically retarded on the thrombin column in PBS. Table 1 gives the estimated K_D values for trypsin and thrombin in PBS at 22 °C for the amidines in the collection. Three fragment substances (97–99) were included as reference compounds where one (aniline) showed weak binding to thrombin. As the number of active sites was approximated to 50% of immobilized protein content, the affinities should be regarded as apparent and not absolute, which should be sufficient for ranking purposes. The total amount of active sites can be determined by frontal affinity chromatography [27], but the presence of nonspecific binding and varied binding behavior of different analytes makes an accurate estimation of B_{tot} cumbersome. It can be seen from Table 1 that the affinity window was in the range of 1 mM–10 μM, that is, approximately two orders of magnitude. The lowest affinity that can be seen was in the range of 1–2 mM with MS detection. It is possible that even weaker affinities can be detected by increasing the amount of active sites on the column and by increasing the ability to distinguish retarded peaks from the DMSO peak. In this respect MS offers an advantage as the fragment's mass/charge can be followed specifically. Tighter binders (<10 μM) may be possible to detect but at the sacrifice of long retention times (>1 h). High-affinity binders in the nanomolar range are efficiently bound to the immobilized target support and they are detected by its lack of appearance in the chromatogram. By running UV detection this can be a problem as it is impossible to distinguish between a nonbinder appearing in the DMSO void peak and an adsorbed high-affinity binder. With MS detection the mass number is followed and binding versus nonbinding can be easily observed. Melagatran, as an example of a nanomolar binder to thrombin and trypsin, was adsorbed and could be efficiently eluted on the trypsin column by using 50 mM glycine buffer at pH

3.1. The experimental affinity window (retention factor (k') <100), as seen by WAC, can be regulated by the B_{tot} value and conditions of the mobile phase. For example, by using gradient elution the affinity window can be condensed.

One challenge with MS detection is that volatile buffers at low ionic strength need to be used with or without the addition of organic solvents such as acetonitrile. Therefore MS detection cannot generally be performed under physiological conditions such as with PBS used in this study for UV detection. However, when screening on thrombin, as Fig. 5 indicates, there was a good correlation between PBS and AmAc with a correlation coefficient (R^2) of 0.9893, showing that proper affinity ranking can be performed in nonphysiological buffers. Similar results were also obtained with screening on trypsin (data not shown). It is then possible to affinity rank fragments in mixtures conveniently by MS detection and confirm the hits by running chromatography under physiological conditions in, for example, PBS with UV detection. Another concern is that there will be internal competition of fragments in mixtures that will reduce binding and in turn retention to the target. By keeping concentrations low it was demonstrated that retention of fragments in mixtures did not change much as compared to single fragment injections.

Ranking fragments according to their specific affinity to a target is a valuable strategy in order to select hits, but it does not give a complete picture on how the fragments bind. The kinetic profile is also important as it address the dynamic behavior of binding and reflects more the *in vivo* situation where concentrations of target and substrates constantly fluctuate [28]. It may even be the case that it is more adequate to base selection of hits on their kinetics toward the target. As affinity is the ratio of the kinetic association and dissociation rate constants k_{on} (M^{-1} s^{-1}) and k_{off} (s^{-1}) ($K_D = k_{off}/k_{on}$), estimates of these will give full information of binding. A useful parameter is the residence time of the fragment or drug candidate on the target which is the reciprocal of the rate constant for dissociation. WAC, in its zonal format, gives under certain circumstances information on the kinetics of binding based on the spreading of the peak of a retarded fragment as was previously demonstrated [29,30] on binding of small analytes to albumin. However, it remains to be seen if kinetic information can be easily extracted from fragment screening to target especially for binders in the millimolar range.

Correlation of WAC with a bioassay

Fig. 6 shows the correlation on trypsin between apparent K_D values obtained from affinity chromatography screening in PBS and IC_{50} values determined from a functional inhibition assay. This is indeed a preliminary study and it should be regarded with caution as only a few data on IC_{50} were available. A high correlation ($R^2 = 0.9912$) could be seen (with one outlier) which indicates that the WAC method of screening can be compared with a functional biochemical assay. As the conditions in the assays were not identical we do not expect to have absolute values in full agreement but

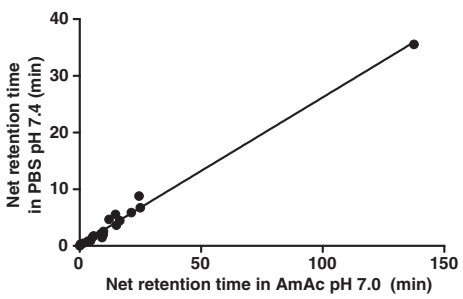


Fig.5. Correlation between net retention times for fragments when screened on the thrombin column in PBS and AmAc buffers.

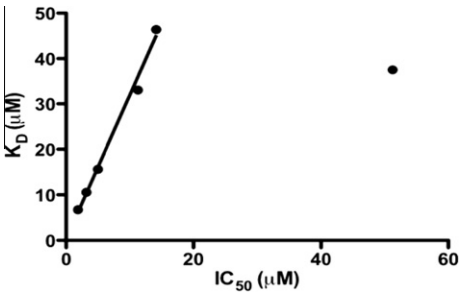


Fig.6. Correlation of fragment binding to trypsin between apparent K_D values obtained from WAC and IC_{50} values determined from an enzyme inhibition assay.

a correlation should be indicative that we are monitoring similar behavior. Confirmation of these data is needed in a larger study with as similar conditions as possible to substantiate preliminary findings. In addition cross-validation to other methods as NMR and SPR will also be desired to fully evaluate the potential of WAC as a screening method. However it is important to point out that there are also challenges with other methods to measure low affinity binders (K_D > 0.1 mM) such as with enzyme inhibition assays. In this case inhibition can only be detected with difficulty at high concentrations of fragments resulting in many false-positives.

Conclusions

A major challenge with fragment screening when applied to drug discovery is the ability to discover weak but specific binding often in the millimolar range to a target molecule. The results of this study have clearly indicated that weak affinity chromatography (WAC) can be applied to fragment screening. WAC shows promise to be a valuable complement to other techniques used for fragment screening such as functional biochemical assays, NMR, X-ray crystallography, and SPR. As WAC is based on a standard HPLC platform, it has all the inherent advantages of chromatography including high precision, robustness, and ease of operation. The consumption of fragments is low in the nanogram range for individual samples. As to the target protein, consumption in this study has been in the range of milligrams which can be further downsized by miniaturization with, e.g., chips or capillaries to at least one order of magnitude less.

WAC has also some unique advantages as it is based on separation of fragments. Components in a mixture can be individually detected and quantified in terms of, for example, affinity ranking. This could be of great importance in applications where contaminants, degradation products, and chiral compounds can be properly identified and characterized. WAC therefore opens up the possibility to directly evaluate potential binders of interest in natural mixtures such as biological extracts and in organic synthetic mixtures.

Throughput is an essential feature of fragment screening as thousands of substances should be evaluated within a reasonable time period of days. In WAC high throughput can be achieved by parallelization of separation channels such as in a chip, by utilizing high flow rates through the affinity column and/or running the fragment libraries as mixtures. In this study we used mixtures in the range of 10 substances but we anticipate that larger mixtures, maybe in the hundreds, can be used. Another major issue in fragment screening is the presence of nonspecific binding that frequently can occur especially when weak binders in the millimolar range are to be selected. It is vital to eliminate these false-positives early in the screening process so the focus can be on potential relevant hits. The appreciation of nonspecific binding can be evaluated by appropriate reference systems where, for

example, the target is devoid of its specific binding and in competition studies where known inhibitors are used.

Although WAC is a promising technology for fragment screening, further studies and developments are necessary to fully realize its inherent potential. This includes studies on diverse targets with larger libraries of fragments, optimizing throughput, minimizing the amount of target, and extending the affinity ranges to be seen for fragments.

Acknowledgments

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