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An affinity selection–mass spectrometry method for the identification of small molecule ligands from self-encoded combinatorial libraries Discovery of a novel antagonist of *E. coli* dihydrofolate reductase

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Abstract

The NeoGenesis Automated Ligand Identification System (ALIS), an affinity selection–mass spectrometry (AS–MS) process consisting of a rapid size-exclusion chromatography stage integrated with reverse-phase chromatography, electrospray mass spectrometry, and novel data searching algorithms, was used to screen mass-encoded, 2500-member combinatorial libraries, leading to the discovery of a novel, bioactive ligand for the anti-infective target *Escherichia coli* dihydrofolate reductase (DHFR). Synthesis of the mass-encoded, ligand-containing library, discussion of the deconvolution process for verifying the structure of the ligand through independent synthesis and screening in a small mixture (sub-library) format, and ALIS–MS/MS techniques to assign its regioisomeric connectivity are presented. ALIS-based competition experiments between the newly discovered ligand and other, known DHFR ligands, and biological activity assessments with stereo- and regioisomers of the hit compound confirm its DHFR-specific biological activity. The method described requires no foreknowledge of the structure or biochemistry of the protein target, consumes less than 1 μg protein to screen >2500 compounds in a single experiment, and enables screening of >250,000 compounds per system per day. These advantages highlight the potential of the ALIS method for drug discovery against genomic targets with unknown biological function, as well as validated targets for which traditional discovery efforts have failed.

Keywords: Affinity selection–mass spectrometry; Combinatorial chemistry; Multidimensional chromatography

1. Introduction

The number of human and bacterial proteins identified as possible targets for small molecule therapy of human disease is increasing profoundly, partly as a result of whole genome sequencing efforts and advances in proteome analysis. At the same time, new paradigms in chemical synthesis are inspiring unparalleled creativity and technical sophistication in the construction of libraries of candidate therapeutic compounds [1]. As the number of targets implicated in disease processes grows and the ingenuity of combinatorial chemists evolves, the need for generic and efficient techniques to identify biologically active lead compounds from large chemical libraries is becoming urgent [2].

Affinity selection–mass spectrometry (AS–MS) has emerged as an attractive technique for studying protein–ligand interactions and screening biomolecular receptors

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against pools of potential small molecule ligands [3]. Such methods directly identify bound components by virtue of their molecular weights or collision-induced fragmentation patterns and therefore, in contrast to biochemical assays that yield a secondary readout of activity, the incidence of false positives based on "bulk effects" and non-specific binding [4] is very low. Due to the exquisite sensitivity of modern MS technology, AS–MS techniques consume only minimal amounts of a purified biomolecular receptor. These techniques do not require an independent biochemical assay for activity assessment; ligands thus identified can be followed up in cellular or other advanced disease models to evaluate phenotype-level effects. Also, pure affinity selection methods allow potential ligands to query all protein surfaces and not just the "active site," enabling the discovery of ligands which act through allosteric binding and other mechanisms.

AS–MS screening methods have been implemented using a number of hardware configurations, and all include the following steps: (1) the receptor is contacted with a pool of potential ligands, (2) resulting receptor–ligand complexes are separated from non-binding mixture components, and (3) ligands are identified by MS or MS/MS. Affinity chromatography [5–9] and surface-plasmon-based [10,11] AS–MS techniques are operationally the simplest to execute and enable the screening of large compound libraries against immobilized targets. However, these techniques require modification of the receptor structure to attach the target to a surface, possibly masking small molecule binding sites. Also, issues of non-specific binding to the stationary phase and the integrity and stability of the heterogeneous target can complicate the analysis.

Since its first report in 1991 [12], the direct analysis of non-covalent biomolecule–ligand complexes, particularly using electrospray ionisation-MS (ESI-MS), has become a very active and fruitful area of study [13] and has been successfully applied to screening compound pools for small molecule ligands [14,15]. This technique enjoys the advantage of having all reaction components free in solution; however, as a generic library screening method it suffers the limitation that resulting receptor–ligand complexes must remain stable under the conditions of ESI-MS. Furthermore, the method is not tractable using high concentrations of salts and non-volatile buffers that are often required for proper biopolymer folding and stability.

To circumvent these issues, implementations of AS–MS have been developed where receptor–ligand complexes are isolated from non-binding components in a first separation stage and subsequently dissociated and identified in a second stage by hyphenated MS techniques such as reverse-phase chromatography–MS (RPC–MS). Several variants of such multidimensional chromatography–MS affinity selection methods, both integrated and stepwise, have been reported, and include gel permeation "spin-column"–MS [16–20] size-exclusion chromatography–MS (SEC–MS) [21–23], ultrafiltration–MS [24,25], and affinity capillary electrophoresis–MS [26–28].

We report herein an optimized, integrated SEC–RPC–MS technique, dubbed the Automated Ligand Identification System (ALIS), for rapidly and directly identifying non-covalent chemical ligands to protein targets from large combinatorial mixtures. A schematic representation of the ALIS affinity selection–mass spectral method is shown in Fig. 1.

Combining a soluble protein and a mass-encoded small molecule library in a physiologically relevant buffer leads to the formation of a complex of the protein with any suitable library member. The complex is separated from non-binding library members by a rapid, low temperature (<30 s at 4 °C) SEC step. A rapid SEC separation insures that even weakly bound ligands (K_d < 10 μM) with moderate dissociation rates (k_{off} < 0.1 s^{-1}) are captured for identification as possible lead structures. The SEC band containing the complex is immediately transferred to a reverse-phase chromatography column. The column is maintained at 60 °C and pH < 2 to promote dissociation of ligands from the complex. The dissociated ligands are eluted into a high-resolution mass spectrometer for analysis, and automated software algorithms [29] search the mass spectral data to identify the ligands by virtue of their molecular weight. The ALIS method allows interrogation of a protein surface without modification of its structure, does not require immobilization of the protein or the small molecule pool against which the target is screened, and consumes only sub-milligram amounts of a purified, soluble protein to screen

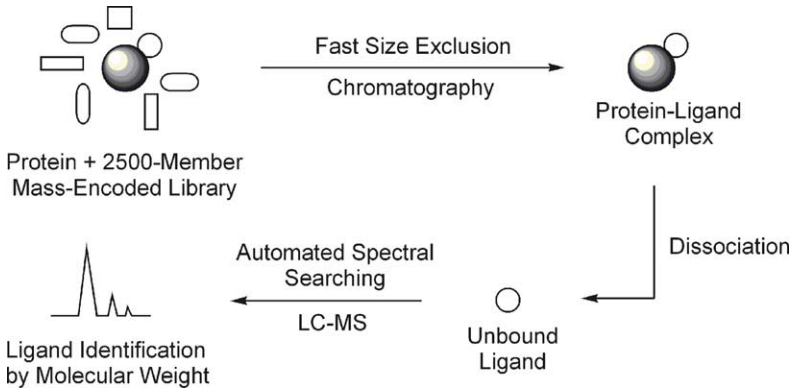

Fig. 1. Diagram of the Automated Ligand Identification System (ALIS) affinity selection–mass spectrometry method.

many millions of compounds. The method is demonstrated by the discovery of (*S*)-**1**, a bioactive antagonist of *E. coli* dihydrofolate reductase (DHFR), a known target for antibacterial drug therapy.

2. Experiment

2.1. Instrument setup

Fig. 2 shows a diagram of the integrated SEC–RPC–MS hardware used for ALIS. Size-exclusion chromatography is performed at 4 °C using 50 mM pH 7.5 phosphate buffer containing 100 mM NaCl. The eluant from the SEC column [30] is passed through a first UV detector where the band containing the protein–ligand complex is identified by its native UV absorbance at 230 nm. After a pause to allow the band to leave the first detector and enter a valving arrangement, the protein–ligand complex peak is automatically transferred to an RPC column [31]. Ligands are dissociated from the complex and trapped at the head of the RPC column, where they are desalted and eluted into a high-resolution mass spectrometer for analysis using a gradient of 5–95% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 5 min. The SEC eluant stream continues to pass through a second UV detector positioned after the transfer valve. By comparison of the two UV signals, the timing of the protein–ligand complex transfer from SEC to RPC can be monitored and optimized as shown in Fig. 3.

A Waters Q-TOF high-resolution quadrupole-time-of-flight mass spectrometer (Manchester, UK) was used in this study with ionization performed from a nebulized capillary at 3.5 kV at a desolvation temperature of 200 °C and with 30 V "cone" and 1.8 V skimmer (extraction lens) settings. Tandem mass spectrometry (MS/MS) spectra were obtained at 35 eV using argon as the collision gas.

2.2. Library synthesis

The libraries investigated in this study were designed and synthesized such that each member is constructed from building blocks chosen to maximize the diversity of shape and functionality [32], while software algorithms minimize the amount of mass redundancy present at both the library synthesis and library pooling stages [33]; as such, each library member is self-encoded by its molecular weight [34,35]. For example, the bifunctional epoxy ester core (±)-**2** was reacted with building blocks **3**–**18** to yield solution-phase library NGL127A443 containing nominally 512 substitutionally and stereochemically unique compounds, 82% having a molecular weight unique to 0.050 amu (Figs. 4 and 5) [36] as follows: To a solution of bifunctional epoxy ester core **2** (100 mg, 0.28 mmol) in DCM/THF (3 mL each) at 24 °C was added a solution of building blocks **3**–**18** (0.019 mmol each) and DIEA (98 μL, 0.56 mmol) in DCM/THF (3 mL each). The reaction was stirred for 2 h then a second portion of building blocks **3**–**18** (0.019 mmol each) as a solution in DCM/THF (3 mL each) and an Yb(III) catalyst solution (100 μL of a 120 mg solution in 1.5 mL THF) and DIEA (50 μL, 0.28 mmol) were added. The mixture was heated to 45–50 °C for 24 h then cooled. Amberlite (100 mg) was added and the reaction stirred for another 1 h at 24 °C then filtered and concentrated to yield solution-phase library NGL127A443 as a slightly yellow film. LC–MS analysis indicated that 94% of the expected masses were found. This library was combined with four similar libraries to yield a mixture of nominally 2560 compounds for ALIS screening [37].

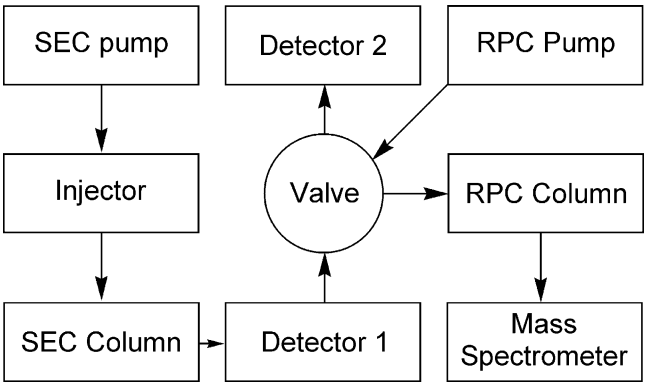

Fig. 2. Schematic of the instrument configuration used in ALIS.

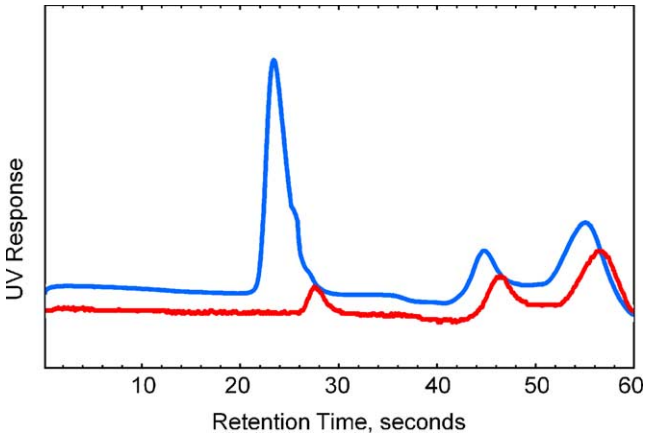

Fig. 3. UV detector responses from the SEC eluant path showing the DHFR protein–ligand complex eluting at 24 seconds and unbound library components eluting later. Upper trace: detector 1, before protein peak transfer to RPC–MS; lower trace: detector 2 showing components not transferred to RPC–MS.

2.3. Synthesis of (*S*)-**1**

To bifunctional epoxy ester core (*S*)-**2** (100 mg, 0.28 mmol) in DCM/THF (3 mL each) at 0 °C was added 1-(3-chlorophenyl)-piperazine (59 mg, 0.30 mmol) and DIEA (98 μL, 0.56 mmol). The reaction was allowed to warm to room temperature for over 2 h and was diluted with DCM. The reaction was then transferred to a separatory funnel and rinsed with 1N citric acid and water. The or-

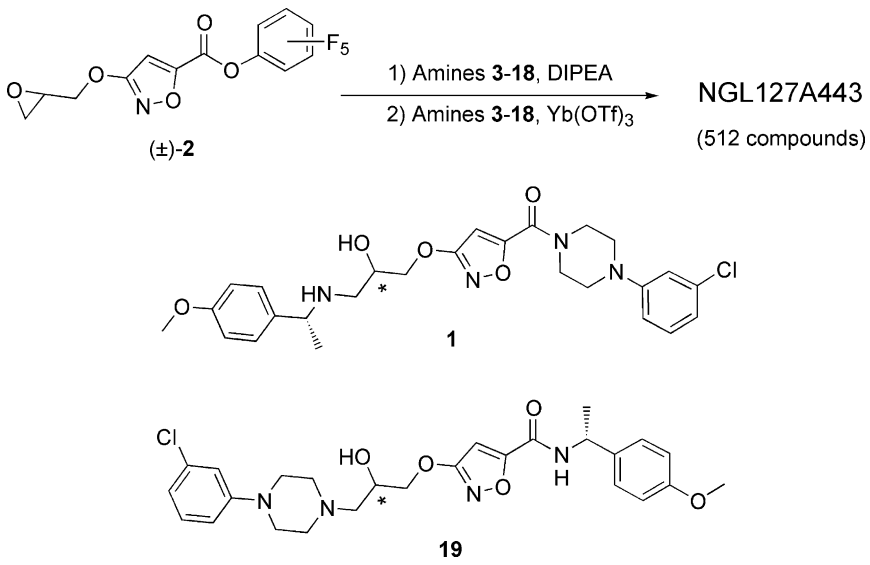

Fig. 4. Synthetic scheme for mass-encoded library NGL127A443 containing isobaric positional isomers **1** and **19**.

ganic layer was dried over sodium sulfate and concentrated to give a clear oil. The intermediate was dissolved in DCM/2-propanol (3 mL:1 mL) to which was added (*R*)-1-(4-methoxyphenyl)ethylamine (50 μL, 0.40 mol), Yb(III) catalyst solution (100 μL of a 120 mg solution in 1.5 mL THF), and DIEA (50 μL, 0.28 mmol). The mixture was heated to 45–50 °C for 24 h then concentrated and purified by flash chromatography (SiO_2, 1–5% MeOH/DCM) to give a clear oil, which was freeze-dried to a hygroscopic powder (81 mg, 56%). HPLC and HPLC–MS analysis indicated >95% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.17 (d, 1H, J = 8.2 Hz), 7.15–7.10 (m, 3H), 6.81–6.70 (m, 4H), 6.34 (s, 1H), 4.20 (dd, 1H, J = 3.9, 10.9 Hz), 4.13 (dd, 1H, 5.9, 10.9 Hz), 3.94 (m, 1H), 3.80 (apparent t, 4H, 5.1 Hz), 3.73 (s, 3H), 3.73 (m, 1H), 3.19–3.16 (m, 5H), 2.68–2.58 (m, 2H), 1.41 (d, 3H, J = 6.6 Hz). MS calculated for $C_{26}H_{32}ClN_4O_5$ $[M + H]^+$ 515.2061, found 515.1640. ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.28, 164.60, 159.80, 156.55, 151.60, 135.03, 130.16, 129.64, 120.40, 118.82, 116.50, 114.49, 112.44, 112.40, 99.85, 72.41, 68.02, 58.80, 55.19, 49.47, 48.83, 46.18, 42.61, 23.42.

2.4. ALIS sample preparation

AS–MS analysis were conducted by incubating 2500-member libraries at 2.5 mM cumulative compound concentration with 5 μM *E. coli* DHFR [38] in a final volume of 2 μL pH 7.5 phosphate buffer containing 2.5% DMSO and 100 mM NaCl. As such, 2 pmol of each library component (at 1.0 μM/component) and 10 pmol (0.18 μg) protein were

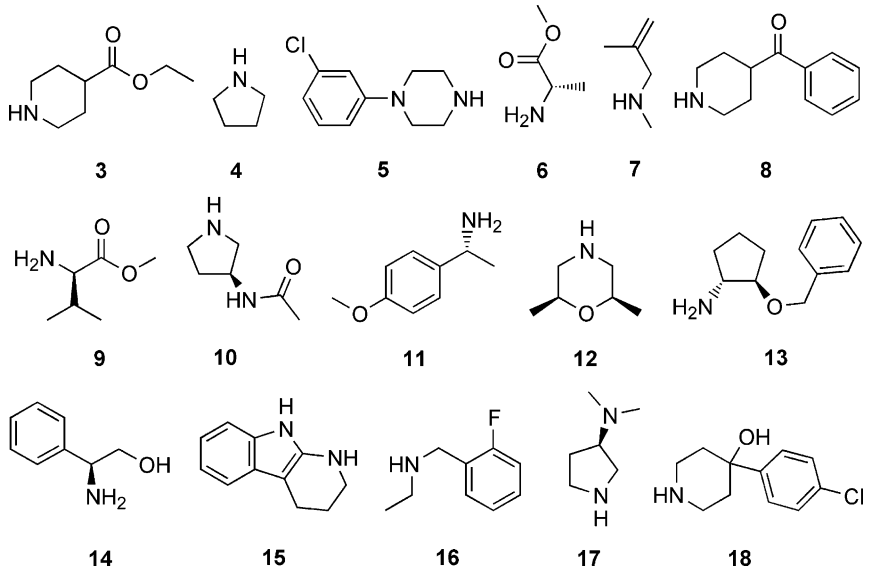

Fig. 5. Amine building blocks used in the synthesis of library NGL127A443.

used in a single analysis. The use of excess protein relative to each library member minimizes competition between multiple binders in a given library. Typical sample preparation protocol is as follows: to 1 μL of a DMSO solution of 100 mM 2500-member library was added 19 μL pre-warmed (37 °C) pH 7.5, 50 mM phosphate buffer containing 100 mM NaCl and 0.1 mM dithioethrythritol. The resulting solution was mixed by repeated pipetting and centrifuged at 10,000 × *g* for 10 min. A 1.0 μL aliquot of the supernatant was added to 1.0 μL of a 10 μM solution of purified DHFR in pH 7.550 mM phosphate buffer containing 100 mM NaCl and 0.1 mM dithioethrythritol. Samples were incubated at room temperature for 30 min and then chilled at 4 °C pending AS–MS analysis. Discrete compound screening and competition experiments were prepared identically except that compound stock concentrations in DMSO were adjusted such that the final DMSO concentration in each protein-containing sample was 2.5%.

2.5. Determination of antibacterial activity

Protocols for the measurement of MIC_{50} for the evaluation of antibacterial activity followed standard methods [39]. Briefly, purified (*S*)-**1**, antibiotic controls, or vehicle DMSO was subjected to serial dilution into miller-LB growth medium in a manner that placed 0.1 mL aliquots of medium-plus-vehicle or medium-plus-antibiotic into each well of a sterile polystyrene 96-well plate. To aid compound dissolution, medium-analyte mixtures were warmed to 37 °C for 8 h with agitation. Bacterial cells [*E. coli*: ATCC 47092] were grown in to mid-log phase at 37 °C. The culture was then diluted 1:10,000 into pre-warmed growth medium. Aliquots of diluted bacterial culture (0.1 mL) were then combined with the pre-aliquotted medium-analyte mixtures to yield 0.2 mL inoculated cultures containing no more than 2.5% DMSO. The plated inoculate series were then grown for 24 h at 37 °C with orbital shaking. Bacterial cell growth was quantified by measuring absorbance at 600 nm. IC_{50} for the inhibition of bacterial growth was defined as the concentration of drug at which cell growth was half maximal at 24 h. For comparison, trimethoprim showed an IC_{50} of 0.6 μg/mL under these conditions.

3. Results and discussion

Library NGL127A443, screened as a mixture with four other 500-member libraries, yielded a monochlorinated DHFR ligand at *m/z* 515.24, corresponding to an $[M + H]^+$ ion with a monoisotopic molecular weight of 514.23 amu. No signal for this ion was evident in an ALIS control experiment with DHFR in the absence of the screening library (Fig. 6A–C). Table 1 shows a portion of the membership of the 2500-member screening library; only one of the five combined libraries contains a monochlorinated member within 0.05 amu of the measured molecular weight. An independent affinity selection experiment confirmed that the ligand originated from library NGL127A443.

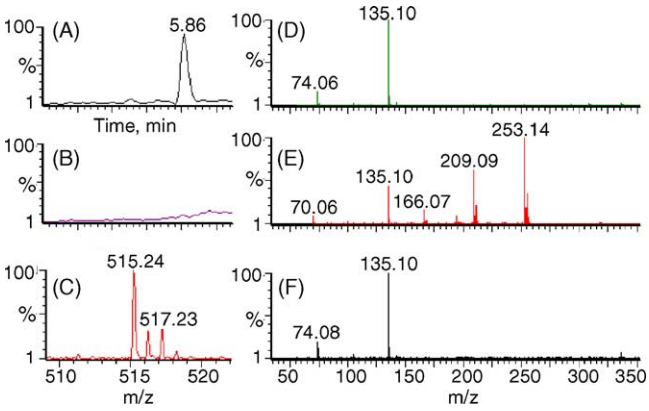

Fig. 6. (A) Extracted ion chromatogram (XIC) of *m/z* 515.2 $(M + H)^+$ from an ALIS experiment with DHFR and NGL127A443. (B) XIC of *m/z* 515.2 from control experiment (no library). (C) Mass spectrum of the region near *m/z* 515.2 underlying the XIC peak in (A). (D) LC–MS/MS spectrum of the early-eluting isomer **1**. (E) LC–MS/MS spectrum of the late-eluting isomer **19**. (F) ALIS–MS/MS spectrum of **1** from an ALIS experiment with DHFR and the NGL127A443 sub-library.

To verify the structure of the ligand from library NGL127A443, independent synthesis of a small mixture (sub-library) containing positional isomers **1** and **19** was performed using the bifunctional template **2** and amine building blocks **5** and **11**. ALIS screening of this sub-library returned the expected signal at *m/z* 515.2, thereby identifying

Table 1
A portion of the membership of the ALIS screening library, composed of NGL127A443 (library 3 in this table) and four other libraries, which yielded DHFR ligand **1** (entry 11)

Entry	EMW	Formula	Library				
			1	2	3	4	5
1	511.2220	$C_{29}H_{29}N_5O_4$			♦		
2	511.2318	$C_{27}H_{33}N_3O_7$	♦				
3	511.2318	$C_{27}H_{33}N_3O_7$		♦			
4	511.2431	$C_{26}H_{33}N_5O_6$				♦	
5	511.2482	$C_{28}H_{34}N_3O_5F$			♦		
6	511.2642	$C_{23}H_{37}N_5O_8$	♦				
7	511.2795	$C_{27}H_{37}N_5O_5$					♦
8	512.2383	$C_{25}H_{32}N_6O_6$		♦			
9	512.2999	$C_{28}H_{40}N_4O_5$		♦			
10	513.2475	$C_{27}H_{35}N_3O_7$			♦		
11	514.1983	$C_{26}H_{31}ClN_4O_5$			♦		
12	514.2791	$C_{27}H_{38}N_4O_6$					♦
13	514.2791	$C_{27}H_{38}N_4O_6$		♦			
14	515.1823	$C_{26}H_{30}N_3O_6Cl$			♦		
15	515.2631	$C_{27}H_{37}N_3O_7$			♦		
16	515.2631	$C_{27}H_{37}N_3O_7$					♦
17	515.2631	$C_{27}H_{37}N_3O_7$				♦	
18	515.2631	$C_{27}H_{37}N_3O_7$		♦			
19	516.1940	$C_{26}H_{30}ClN_4FO_4$			♦		

Compounds of similar exact molecular weight (EMW) are distributed among the five pooled libraries to minimize mass overlap and simplify hit deconvolution.

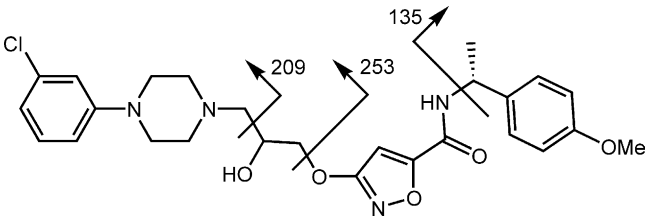

Fig. 7. Assignment of the fragment ions of **19** confirming its identity as the late-eluting isomer by LC–MS/MS (Fig. 6E).

the ligand as either compound **1** or its positional isomer **19**. While correlation of the observed mass to the corresponding library structure could allow immediate synthesis of the hit compound directly from library screening data, independent verification of the hit structure using this sub-library synthesis and screening strategy eliminates the possibility of false positives due to binding by library side products which are isobaric with library components. The sub-library strategy also enables deconvolution of hits where direct structure assignment is not possible due to mass overlap between library components.

LC–MS/MS and ALIS–MS/MS experiments were conducted with the sub-library to determine the regioisomeric connectivity of the building blocks in the ligand. The positional isomers **1** and **19** in the sub-library were readily separated by RPC and yielded diagnostic MS/MS fragment ions (Fig. 6D and E); the observed fragmentation of the late-eluting isomer is consistent with the structure of **19** (Fig. 7) The fragmentation pattern of the ligand obtained in an affinity selection–MS/MS experiment with the NGL127A443 sub-library matched that of the early-eluting isomer, identified as structure **1** (Fig. 6F).

Independent synthesis of the carbinol stereoisomers (*R*)-**1** and (*S*)-**1** were conducted by amide coupling of **5** to stereochemically pure epoxy esters (*R*)-**2** and (*S*)-**2** followed by epoxide opening with **11** in the presence of $Yb(OTf)_3$. ALIS experiments with the individual diastereomers revealed that (*S*)-**1** was preferably bound to the protein target; independent experiments established a dissociation constant (K_d) of 15 μM for (*S*)-**1** and 40 μM for (*R*)-**1**.

The antibiotics methotrexate, pyrimethamine and trimethoprim are well-characterized DHFR inhibitors [40]. Competitive ALIS experiments with (*S*)-**1** and these drugs were conducted by combining 5 μM DHFR with 80 μM (*S*)-**1** in the absence (control) and presence of 80 μM of each drug (Fig. 8). Competitive binding was observed for all three drugs, with the magnitude of the suppression of binding by (*S*)-**1** correlating with the affinity of each competitor [41]. These results are consistent with (*S*)-**1** binding in the DHFR active site, though more weakly than the known competitors.

Biological activity assessments conducted against *E. coli* [42] indicate that (*S*)-**1** inhibits bacterial growth with a mean IC_{50} of 29 μg/mL. The mean IC_{50} of the diastereomer (*R*)-**1** was determined to be 98 μg/mL. These IC_{50} values correlate well with the observed affinities and competitive binding

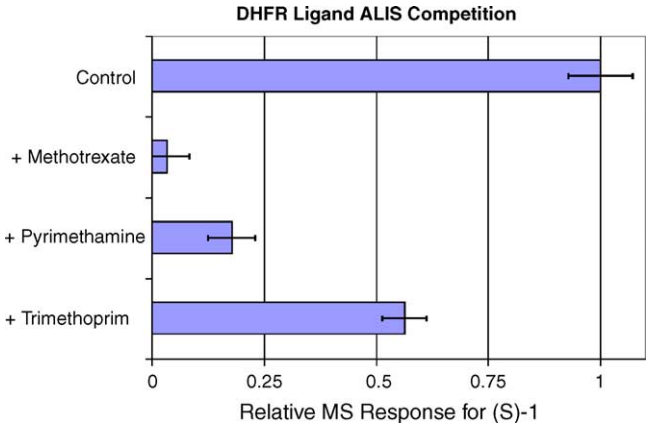

Fig. 8. Effect of added DHFR ligands on the recovery of (*S*)-**1** from ALIS competition experiments.

results. The regioisomeric control compounds (*S*)-**19** and (*R*)-**19** showed no measurable bacterial growth inhibition, which is consistent with ALIS binding experiments and suggests that the biological activity of (*S*)-**1** results from specific interaction with DHFR.

4. Conclusions

The discovery of DHFR inhibitor (*S*)-**1** demonstrates that the ALIS process is an efficient method for identifying novel, bioactive lead compounds from large combinatorial library mixtures. This process is applicable to a broad range of soluble targets and requires no structural information about the target for its success. A single ALIS experiment containing over 2500 compounds is complete in under 10 min, allowing more than 250,000 compounds to be screened from a single 96-well plate of libraries per day. Only 10 pmol (0.5 μg) of protein is consumed per sample, and the ALIS screening campaign for *E. coli* DHFR against 1500, 2500-member libraries, representing >3,500,000 compounds, consumed a total of 1.0 mg protein. Further investigations will include ALIS-based optimization of (*S*)-**1** for its DHFR-binding and antibacterial properties in a mixture format [43] as well as biochemical studies of its mode of action.

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