Novel Affinity Ligands for Chromatography Using Combinatorial Chemistry[§]

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Abstract: Spatially addressable combinatorial libraries were synthesized by solution phase chemistry and screened for binding to human serum albumin. Members of arylidene diamide libraries were among the best hits found, having submicromolar binding affinities. The results were analyzed by the frequency with which particular substituents appeared among the most potent compounds. After immobilization of the ligands either through the oxazolone or the amine substituent, characterization by surface plasmon resonance showed that ibuprofen affected the binding kinetics, but phenylbutazone did not. It is therefore likely that these compounds bind to Site 2 in sub domain IIIA of human serum albumin (HSA).

Keywords: Affinity chromatography, combinatorial chemistry, high-throughput screening, arylidene diamide library.

1. INTRODUCTION

Affinity chromatography is an established technology for the large-scale industrial purification of macromolecules [1]. The method has had particular success with the production of biopharmaceutical agents, such as monoclonal antibodies. With the burgeoning pipeline of protein drugs in development, there is an increasing need for new affinity media that demonstrate highly selective binding characteristics. Many of the current affinity ligands are proteinaceous and exhibit limited stability in the harsh basic conditions used for cleaning-in-place (CIP) procedures that are a necessary part of biopharmaceutical manufacturing. Ligands of non-biological origin may achieve greater chemical stability without sacrifice of specificity. Examples of such ligands are Cibacron Blue F3GA that has group selectivity for a large number of nucleotide-binding proteins and a number of different media types having specificity for antibody adsorption [2-6].

Historically, most affinity ligands have been discovered in a serendipitous manner, though rational design approaches have been used [5]. This is similar to the drug discovery process before the advent of high-throughput parallel synthesis and screening technologies. Small designed combinatorial libraries have been employed to facilitate the identification of affinity ligands [7-9]. In this paper, we describe the integrated and iterative application of design, automated high-throughput synthesis and screening approaches for the discovery of small molecule affinity ligands specific for human serum albumin [10, 11].

The general strategy for this project is outlined in Fig. (**1**). The strategy begins by compiling available information about known intermolecular interactions. This information is then translated into reagents compatible with available combinatorial synthetic methods. Libraries are produced using parallel solution phase chemistry and binding is measured through the use of solution phase and chromatographic evaluation. Analysis of the resulting structure-activity relationships (SAR) is used for iterative optimization and to select compounds for further evaluation. The binding characteristics of the ligands were also studied using the BIACORE system. The covalent immobilization on chromatographic media requires a reactive group in the final ligand suitable for attachment to the support material. After immobilization, the binding characteristics of the media are examined by affinity chromatography.

2. EXPERIMENTAL

2.1. Materials

All solvents and chemicals for synthesis were purchased from Aldrich or Sigma and solvents were of the highest

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[§]The work was carried out at ArQule, Inc., Woburn, MA, USA and Amersham Pharmacia Biotech, Uppsala, Sweden

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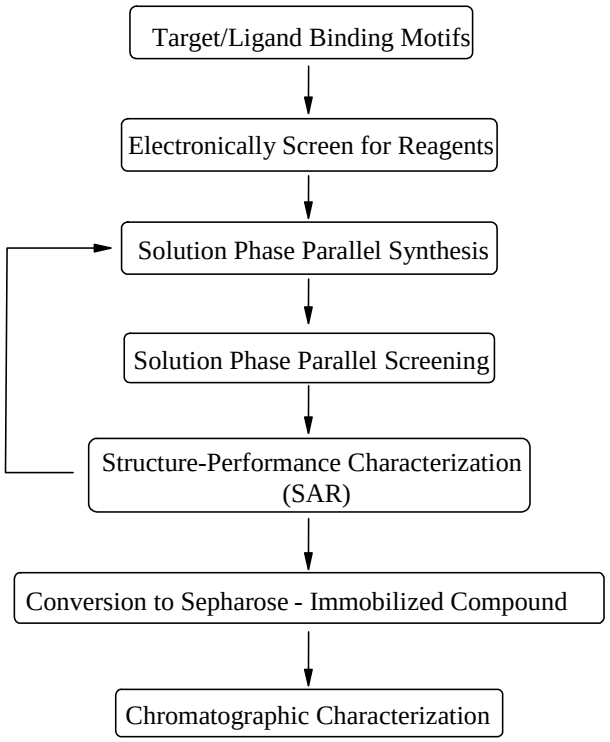


Fig. (1). Strategy for the high throughput discovery of small organic affinity ligands.

purity available. ECH Sepharose™ 4B, EAH Sepharose 4B, EAH Sepharose HP, EAH Sepharose 6FF, NHS Activated Sepharose 4FF, Cibacron Blue Sepharose, Sephasil™ C8 (4 x 10 mm) and HiTrap™ Desalting Column were obtained from Amersham Pharmacia Biotech. HiSep 2.0 cm precolumn was obtained from Supelco. Acetonitrile for HPLC (LiChrosolv, gradient grade) was obtained from Merck. Human serum albumin, (A-3782), Bovine serum albumin (A-7511), lysozyme (L-6876), transferrin (T-4515), α-lactoglobulin (L-2506) and human serum (S-7023) were all purchased from Sigma. Polyclonal immunoglobulin G (IgG) (Gammanorm 165 mg/ml) was obtained from Pharmacia Corporation and ovalbumin from Amersham Pharmacia Biotech. HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]) buffer was obtained from BIACORE AB. The syntheses were followed by LC on silica gel Merck 60 F_{254}. Flash chromatography was performed on silica gel 60 (Merck 230-400 mesh).

2.2. Instrumentation

The synthetic reactions were followed by HPLC (Shimadzu LC10) at 254 nm with Sephasil C8 (4 x 10 mm) using acetonitrile (with 0.035% trifluoroacetic acid (TFA)), water (with 0.05% TFA) and 30-100% acetonitrile as solvents (8 min). Characterizations were performed on a Jeol Eclipse-270 MHz NMR. The samples were run in a 5 mm probe and the substances were dissolved in $CDCl_3$ or DMSO-d_6. Trimethylsilane (TMS) was used as an internal standard.

The first step in the screening method was performed on a FPLC™ system (Amersham Pharmacia Biotech) equipped with a HiTrap Desalting Column. The second step was performed on a Gilson 306 HPLC equipped with a HiSep 2.0 cm pre-column. Chromatographic evaluations were performed on a SMART™ System or on an ÄKTA™ Explorer 10 (Amersham Pharmacia Biotech). Kinetic and competitive studies were performed on a BIACORE 2000. Total organic carbon (TOC) was determined on a TOC 5000 analyzer from Shimadzu. Elemental analyses performed by Mikro Kemi AB, Uppsala, Sweden.

2.3. Methods

2.3.1. Syntheses

Over 20,000 compounds were synthesized and tested using variations of Mannich [12], triazine [13], hydroxyaminimide [14, 15] and oxazolone-based arylidene diamide [16] chemistries. The generic structure of these libraries is represented in Fig. (**2**). The arylidene diamide libraries, produced the highest hit rate and thus the primary chemistry used in this work involved the formation of arylidene diamides from sets of oxazolones, aldehydes and amines.

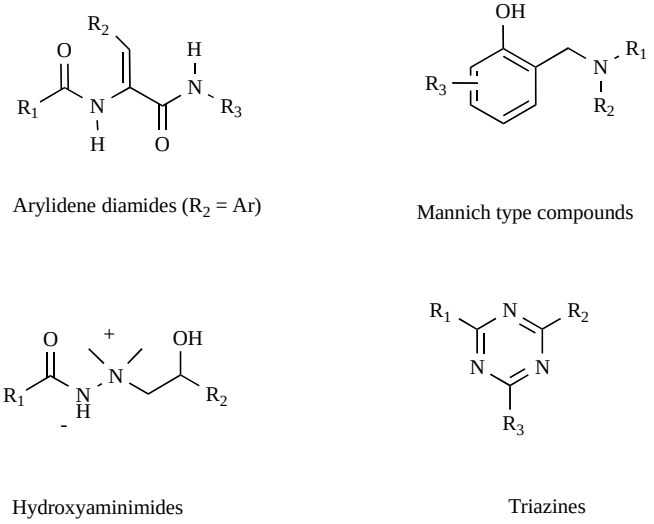


Fig. (2). Structures of the screening library compounds.

Syntheses of starting oxazolone and arylidene diamide compounds were performed as exemplified below in Fig. (**3**). For the library productions a pilot library was first synthesized and fully characterized by LCMS and NMR. In the library production the condensation and the opening of the oxazolone were done without any intermediate purification or work-up. The final libraries were characterized by LCMS. Final yields in the libraries were above 80% (HPLC).

Synthesis of 2-Thienylglycinate (I)

In a 1 L 3-necked reaction flask glycine (77.0 g, 1.03 mol) was dissolved in 600 ml water with a mechanical stirrer. NaOH (12.0 g) was added to form sodium glycinate. The reaction mixture was cooled to 5℃. 2-Thiophenecarboxylic acid chloride (100 g, 0.68 mol) was added drop wise and NaOH-solution (50%) was periodically added to keep pH around 10 during 1.5 hrs. The temperature rose to 12℃ during the addition and the solution became

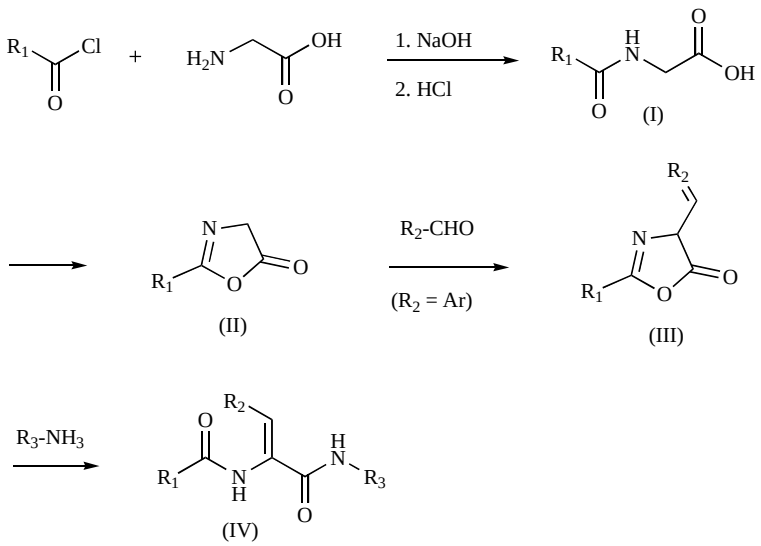


Fig. (3). Synthesis of arylidene diamides.

homogeneous. After another hour the mixture was acidified to pH 2 using conc. HCl (70 ml) and the stirring was continued for two hours. The precipitated crystals were filtered off and washed with water. The product was confirmed with NMR after drying in a vacuum oven at 60°C. ^{1}H NMR shifts: δ 4.05 (s, 2H), 7.12 (dd, 1H), 7.65 (dd, 1H), 7.71 (dd, 1H). Yield: 123 g (98%), mp 172-173°C.

Synthesis of 2-(2-Thienyl)-Oxazolone (II)

In a 2 L 3-necked flask equipped with a mechanical stirrer dicyclohexyl carbodiimide (66.8 g, 324 mmol) was dissolved in 700 ml anhydrous THF. 2-Thienylglycinate (60 g, 324 mmol) dissolved in 600 ml anhydrous THF was added drop wise during 30 min. The reaction mixture was then allowed to stir for 24 hours at ambient temperature. The mixture was cooled to 5°C and the dicyclohexylurea was filtered off. After evaporation of THF the solid product was dissolved in boiling dichloromethane and then cooled so that more dicyclohexylurea could be filtered off. The solution was evaporated and chromatographed on 400 g silica through a 15 cm wide column with dichloromethane. The first 3 L was collected and evaporated. ^{1}H NMR shifts: δ 7.14 (dd, 1H), 7.59 (dd, 1H), 7.7ppm (dd, 1H). Yield: 70% (38 g), mp 106-107°C.

Synthesis of 2-(2-Thienyl-)4-(N-Methyl-3-Indolyl)-5(H)-Oxazolone (III)

2-(2-Thienyl)-oxazolone (3.0 g, 18 mmol) was mixed with 1-methyl-indole-3-carboxaldehyde, (2.0 g, 12.6 mmol) in 12 ml toluene in a screw-cap tube. Triethylamine (0.8 ml) was added and the closed tube placed on a heating block at 70°C over night. The reaction mixture was diluted with 100 ml toluene and a small amount of acetone and extracted with 3x100 ml water. The toluene layer was dried with $MgSO_4$, evaporated to 20 ml. Crystallization occurred over night. Crystals were collected and dried in a vacuum oven at 60°C. ^{1}H NMR shifts: δ 3.93 (s, 3H), 7.17 (dd, 1H), 7.3-7.4 (m, 3H), 7.60 (dd, 1H), 7.62 (s, 1H), 7.81 (dd, 1H), 7.95 (d, 1H), 8.42 (s, 1H). Yield: 50% (2.8 g), mp 178-180°C.

General Synthesis of Arylidene Diamides (IV)

The substituted oxazolones synthesized in the last step (0.57 mmol) were mixed with different amines (0.57 mmol) in THF in screw-cap tubes. The tubes were placed on a heating block at 60°C over night (18 hrs) followed by evaporation of the solvent with heat and/or nitrogen. The products were not purified further. The raw products were analyzed with TLC, LCMS and ^{1}H NMR. Yields were typically 90-95%.

2.3.2. Immobilization

ECH Sepharose 4B, EAH Sepharose 4B, EAH Sepharose HP, NHS Activated Sepharose 4FF and EAH Sepharose 6FF, were supplied pre-swollen in ethanol (20 %). For the amino-substituted media (here exemplified with EAH Sepharose 6FF, 25 μmol amino-groups/ml) 10 ml gel was washed on a glass filter with water followed by THF until no traces of water was found in the eluate. The ligands (0.20 mmol) and dicyclohexyl carbodiimide (2.0 mmol) were dissolved in THF (10 ml) and then mixed with the gel. The suspension was rotated over night (18 hrs) at ambient temperature. The gel was washed with 100 ml water, 100 ml isopropanol, 100 ml acetonitrile and finally 100 ml water. Remaining amino-groups were blocked with a mixture of 1.7 M acetic acid and 1 M dicyclohexyl carbodiimide in dioxane. The gel was washed with 100 ml 40°C isopropanol, 100 ml THF, 100 ml acetonitrile, 100 ml 40°C isopropanol, 100 ml ethanol (95%), and then three cycles of alternating pH with 100 ml 0.1 M acetate buffer (pH 4.1) containing 0.5 M NaCl and 100 ml 0.1 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl and finally 100 ml water. Substitution levels, determined by elemental analysis, were typically in the range of 3-10 μmol/ml wet gel.

For the carboxyl substituted media (here exemplified with NHS Activated Sepharose, 16-23 μmol carboxyl-groups/ml) 10 ml gel was washed on a glass filter with water followed by THF until no traces of water was found in the

eluate. The ligands (0.10 mmol) were dissolved in THF (10 ml) and then mixed with the gel. The suspension was rotated over night (18 hrs) at ambient temperature. The gel was washed with 100 ml THF, 100 ml acetone, 100 ml water, 100 ml isopropanol, 100 ml acetonitrile, 100 ml water and then three cycles of alternating pH with 100 ml 0.1 M acetate buffer (pH 4.1) containing 0.5 M NaCl and 100 ml 0.1 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl and finally with 200 ml water.

2.3.3. Solution Phase Screening

A method for screening libraries of small synthetic molecules for their ability to bind to target proteins was developed. The method consists of three steps: 1) mixing of target and ligand, 2) removal of free ligand and 3) analysis of the target fraction for presence of bound ligand (Fig. **4**). A similar approach was used by Zuckermann, *et al*. to identify peptide binding to a specific antibody [17].

From a stock solution of the ligand (DMSO, 5 mM), 10 µl was diluted with 890 µl PBS (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0). To this solution 100 µl HSA (100 µM) in PBS buffer was added at ambient temperature (Mixture A). Excess ligand was removed by separating 500 µl of the mixture on a HiTrap Desalting Column, equilibrated in PBS buffer, at a flow rate of 10 ml/min. The column was washed with PBS buffer containing 20 % acetonitrile after each run. Analysis of the void fraction was performed on a HiSep 2 ml column equilibrated with 95:5 v/v 180 mM ammonium acetate and acetonitrile. The bound ligand was eluted with a gradient of 95:5 v/v 180 mM ammonium acetate and acetonitrile (A-buffer) and 10:90 v/v ammonium acetate and acetonitrile (B-buffer). The elution was registered at 254 and 280 nm.

2.3.4 Binding Studies Using the Solution Phase Screening Method

By using the method described above and varying the flow rate in the desalting step the dissociation rate constant for a target-ligand complex could be estimated from the time dependent dissociation of the complex. Five samples of Mixture A containing the model ligand phenylbutazone (PB) were sequentially separated on the HiTrap Desalting Column at five different flow rates respectively (0.63, 1.25, 2.5, 5 and 10 ml/min). The time between sample application and collection of the target fraction varies from 10.8 seconds at a flow rate of 10 ml/minute to 171 seconds at a flow rate of 0.63 ml/minute. Chromatograms for the reference ligand phenylbutazone are shown in Fig. (**4a**).

The analysis of ligand content in the collected protein-ligand fraction was performed by reversed phase chromatography on a HiSep column in which large molecules such as proteins are shielded from adsorption. This eliminates interference from the target protein in the ligand analysis. Fig. (**4B**) shows the analyses of the collected fractions from Fig. (**4A**). All steps in the method were automated using FPLC and Gilson HPLC Systems and a Gilson 215 liquid handler.

From the residence times (dissociation times) on the column in Fig. (**4A**) and the phenylbutazone peak areas in Fig. (**4B**), a dissociation plot was constructed and the dissociation rate constant calculated. In this case the best fit was obtained by using a model in which there are two independent binding sites on HSA for the ligand using the formula $xAT + yAT \rightarrow (x+y)A + T$ where x and y represents the two different binding sites (Fig. **4C**). The two dissociation rate constants for phenylbutazone were calculated to be 1.1×10^{-1} s^{-1} and 7.3×10^{-3} s^{-1} respectively, which are in accordance with those previously determined

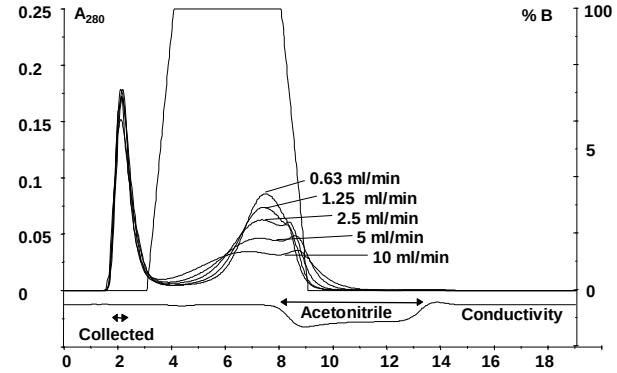


Fig. (4A). Gel filtration on HiTrap Desalting Column of HSA/phenylbutazone at different flow rates. The target fraction was collected from 2.3 ml. A small molecule showing no adsorption to the matrix would elute at ≈ 4.5 ml.

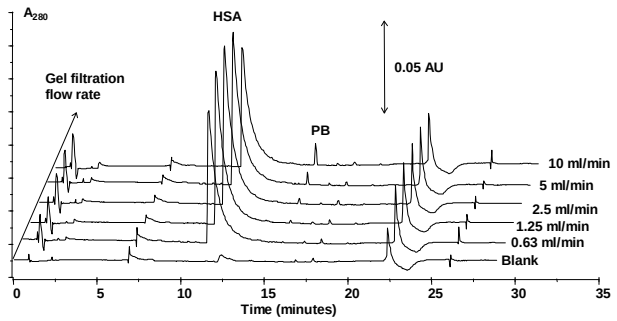


Fig. (4B). RPC analysis on the HiSep column of the HSA fraction from the gel filtration step in Fig. (**5a**).

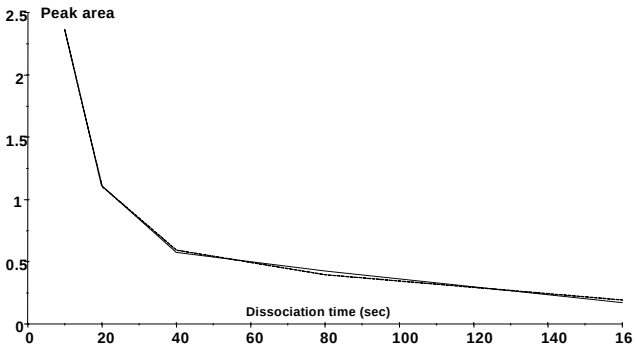


Fig. (4C). Plot of the ligand peak area as a function of the dissociation time.

with other techniques [18]. The peak areas were normalized using the HSA peak area as an internal standard. The dotted line represents the fitted values using a two-site binding model.

2.3.5. Binding Studies by Surface Plasmon Resonance

Interaction analyses based on surface plasmon resonance (SPR) were performed using a BIACORE 2000 (BIACORE AB). SPR correlates with the changes in mass on the surface of the gold film involving association and dissociation between proteins and ligands [19]. The effect is expressed in resonance units (RU). After subtraction of the background RU due to bulk refractive index change (determined by injection over a blank surface), *R*eq/ligand concentration (*C*) was plotted versus *R*eq where *R*eq is the equilibrium response (in RU) of analyte at the injected analyte concentration. The *K*a was calculated from the slope by linear least-square curve fitting using the equation: *R*eq/*C* = *K*a x *R*max – *K*a x *R*eq, where *R*max is the maximum amount of bound ligand.

For these studies each target protein was covalently immobilized onto the BIACORE CM5 sensor chip by NHS activation of carboxymethylated dextran on the surface. The immobilized levels of HSA and BSA were 8756 and 8687 RU, respectively. All the affinity measurements were done in HBS buffer (comprised of 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% BIACORE surfactant P20) with 1.1% DMSO. A ligand solution (<100 μM, in the same running buffer) was injected across the chip surface at a flow rate of 30 μl/min. The interaction was monitored at 25°C as the change in the SPR response [19]. After monitoring for 3 min, the running buffer was introduced onto the sensor chip in place of the ligand solution to start the dissociation. The affinity constant, *K*a, was determined from the equilibrium binding level (*R*eq) by Scatchard plot analysis.

2.3.6. Chromatographic Evaluation

The prototype gels and Cibacron Blue Sepharose were packed in HR 5/5 columns (1 ml) in distilled water and equilibrated with PBS buffer for protein separations and with 50 mM citric acid, 0.1 M Na_2HPO_4, pH 7 for the serum separations on the SMART system before use.

500 μl of the target molecule (HSA, 3 mg/ml) and the test proteins (3 mg/ml of human IgG, lyzosyme, ovalbumin, β-laktoglobulin and transferrin) were applied at a flow rate of 500 μl/min (150 cm/hrs). The elution was performed using 20 mM citrate buffer, pH 3. The chromatograms were registered at 280 nm.

500 μl Human serum, diluted 4 times with PBS buffer, (corresponding to 5 mg HSA) was applied to the Cibacron Blue Sepharose column (reference column) and the prototype column at a flow rate of 500 μl/min (150 cm/hrs). Both columns were eluted with 50 mM KH_2PO_4 with 1.5 M KCl, pH 7. The prototype column was further eluted with 20 mM citrate buffer at pH 3.0.

2.3.7. Stability Studies

Samples of free ligands and ligand-substituted gels were incubated for one week at 40°C in aqueous solutions at pH 2 and 14 for the free ligands and at pH 2, 3, 11, 12, and 13 for the immobilized ligands respectively. 0.5 ml water at the respective pH was added to 100 μl of the wet gel. The mixture was stored in a capped tube for a week. The supernatant (0.2 ml) was diluted with water (4 ml) to determine any leakage products in the interval 0.5-5 ppm. The carbon leakage from the gels was analyzed using a TOC analyzer [20]. The analyses for the free ligands were performed by ^{1}H- and ^{13}C-NMR to determine degradation products. 5 mg of each substance were weighed into two NMR-tubes and dissolved in 0.01 M DCl (DMSO) and 1 M KOD (DMSO) respectively and were kept in a thermo stated water bath for a week. ^{1}H NMR spectra were recorded after 2, 4 and 7 days respectively.

3. RESULTS AND DISCUSSION

3.1. Screening Results of Mapping Arrays and Design of Directed Libraries

Although hits were identified from every chemotype, the arylidene diamide libraries produced the largest number of strongest binding compounds. All subsequent directed libraries were produced using this chemistry (Fig. **3**). Frequency tables were constructed from the primary screening binding data, recording how often the various R1, R2 and R3 fragments were present in the hits. This is exemplified in Fig. (**5**). The frequency was normalized for the number of times the component appeared among all compounds in the library. It is clear from these plots that certain components occur in hits with significantly greater frequency than others. When correlated with structure, certain trends emerge. In general, the same 3-4 oxazalones consistently appear but this position does not appear as sensitive to structural variation as the others. This may be a function of the limited structural diversity of available oxazolones. However, variation of amine and aldehyde component lead to pronounced differences in hit rates. Large, aromatic aldehydes appear to work well (typically found in 10% or more of the hits). 2-Phenyl or 3-alkyl branched amines are also frequently found.

Using this initial SAR information, a 1600 member directed library was constructed using four oxazolones (R1), 10 heterocyclic aldehydes (R2), and 40 amines (R3) as shown in Fig. (**3**). to increase the selectivity of the ligands heterocyclic aldehydes at R2 were included since the SAR indicated that large aromatic ring systems increased the binding properties. Only the 4 top scoring oxazalones were included from the parent libraries. To thoroughly test the SAR at R3 with a focused R1, R2 combination a diverse set of 40 pre-qualified amines was used (the building blocks are listed in Fig. (**6**)).

The screening of this directed library resulted in several confirmed hits. Compound (**1**), Fig. (**7**) displayed exceptionally strong binding towards HSA. A second series of directed libraries comprising a total of more than 400 compounds were then designed to explore immobilization possibilities. The SAR information is displayed by the various R-groups in Fig. (**5**). Variation of the oxazolone did not significantly affect binding. However, two aldehydes

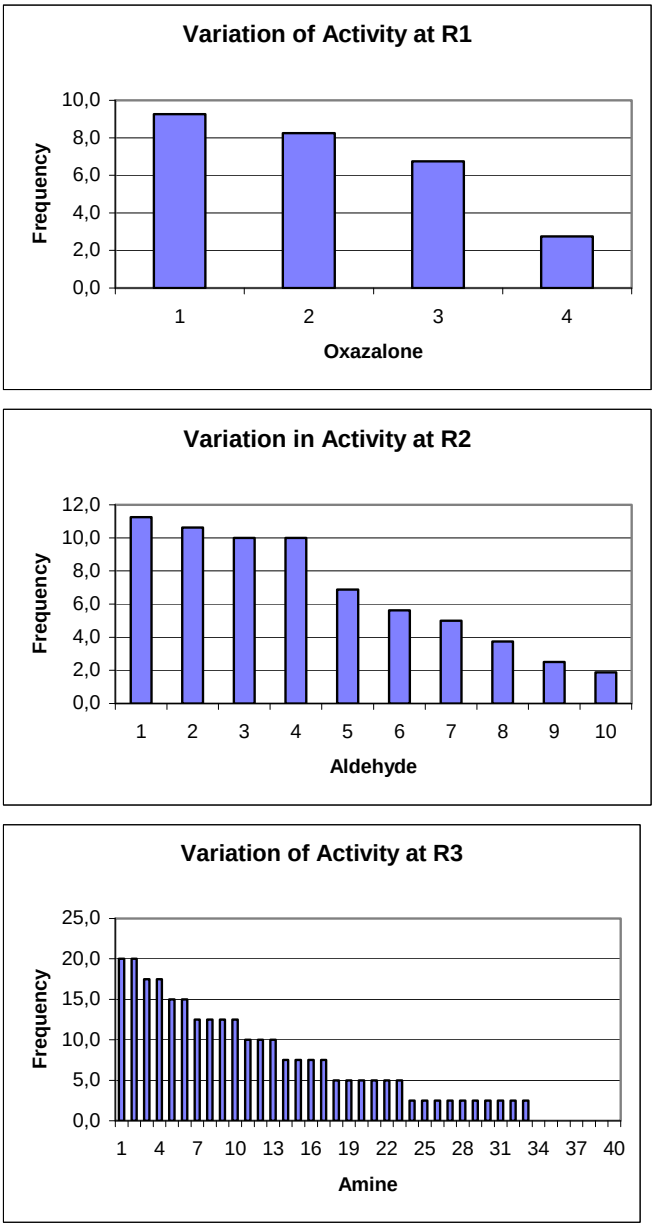


Fig. (5). Frequencies of occurrence of various R1, R2, R3 groups among hits for the directed arylidene diamide library.

(1-methyl-3-indolecarboxaldehyde and 6-methyl-2-pyridine-carboxaldehyde) were prevalent among the best compounds, as was a single amine (N-methyl-ephedrine). It should also be noted that separate tests of the various starting materials and intermediates such as oxazolones condensed with aldehydes or amine-opened oxazolones without the aldehyde component did not yield any compounds that exhibited measurable HSA binding.

Several attempts were made to introduce functional groups such as $–NH_2$, $-CH_2NH_2$ (both protected as carbamates), -CN, -COOH, $-CH_2NO_2$, and $-CH_2Cl$ into the R1 ring system for matrix immobilizations. The oxazolone was then condensed with a set of aldehydes and the resulting substituted oxazalone ring opened with ephedrine. Most of the ligands carrying functional groups at R1 showed no binding activity. The only effective compound contained a methyl amino group in the 4-position of the benzene ring of the phenyloxazolone component. Compound (**1**) could not be immobilized through the hydroxyl function of the ephedrine part in good yields. Immobilization of the ligand through a *para*- hydroxyl group in the phenyl ring of the ephedrine component resulted in an inactive ligand.

The introduction of handles for immobilizations in R2 of the molecule was restricted to the indole structure by adding N-allyl- and N-(4-chlorobutyl)-groups to the indole nitrogen. The structures used were 2-methyl and 5-methoxy-indoles. Both the types of N-substituted derivatives were found to be weak HSA-binders when immobilized to various activated Sepharose materials.

Several of the possible ephedrine analogues were tested as ephedrine mimetics but most of these compounds also exhibit undesirable pharmacological activity. To avoid this problem a sub-library containing (S)- and (R)-N-methylphenylalanine were combined with phenyl- and thiophene-oxazolones that had been reacted with 1-methyl-3-indolecarboxaldehyde and naphthaldehyde. Compounds (**2**) and (**3**), Fig. (**8**), in this series showed binding affinity comparable to compound (**1**). Compound (**4**), the methyl-ester of corresponding compound (**2**), was also synthesized to estimate potential changes in affinity upon immobilization. A summary of the various replacement amines used to explore SAR and immobilization at R3 is shown in Fig. (**9**). Each of these amines was reacted with the adduct between phenyl- or thienyloxazolones and 1-methyl-3-indolecarboxaldehyde.

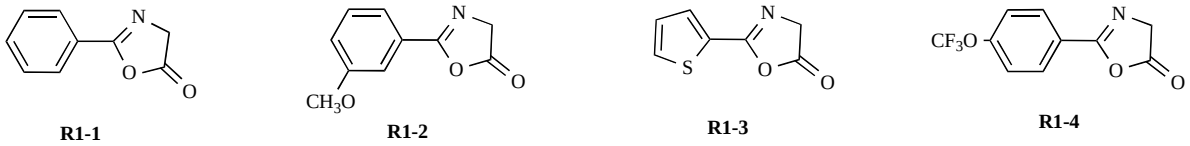
The binding characteristics of ephedrine, N-methylphenylalanine and phenylalaninol containing ligands did not appear to be dependent on the absolute configuration around the chiral centers. Both (S)- and (R)-phenylalaninol was found to bind less well than ephedrine and N-methylphenylalanine compounds, so some sensitivity in the position of the hydroxyl group can be inferred. In most cases, N-methylation significantly enhanced binding. Both enantiomers of the N-methylphenylalanine exhibited the same binding towards HSA as compound (**1**), and thus all immobilizations were performed using the (S)-isomer.

Sensitivity to changes in the geometry of the central R2 component was explored by performing a reduction of the carbon-carbon double bond for compound (**2**). One of the diastereomers formed was inactive and the other one showed only weak activity. Due to the poor activity the absolute configuration of the two different stereoisomers was not determined. The presence of this double bond and the concomitant conformational restrictions appear to confer significant contributions to the total energetics of binding.

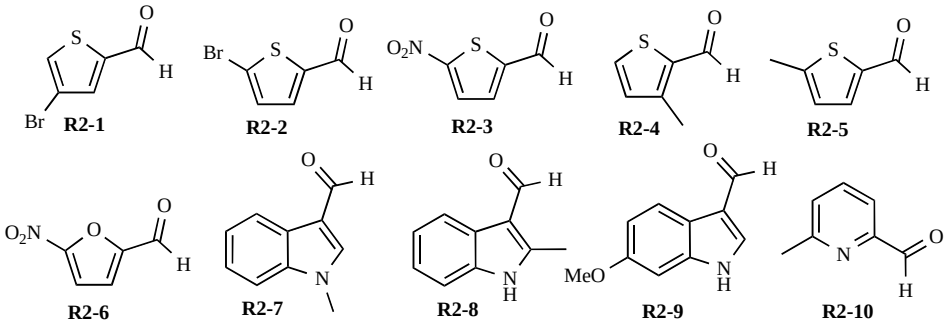
3.2. Binding Stoichiometry and BIACORE Binding Studies

The HSA binding characteristics of compound (**1**) was studied by determining the dissociation rate constant using different flow-rates in the screening gel filtration step as described in Methods. Compound (**1**) was found to bind at a 2 to 1 ratio (ligand:HSA) under saturating conditions. To

a) Oxazolones (R1)



b) Aldehydes (R2)



c) Amines (R3)

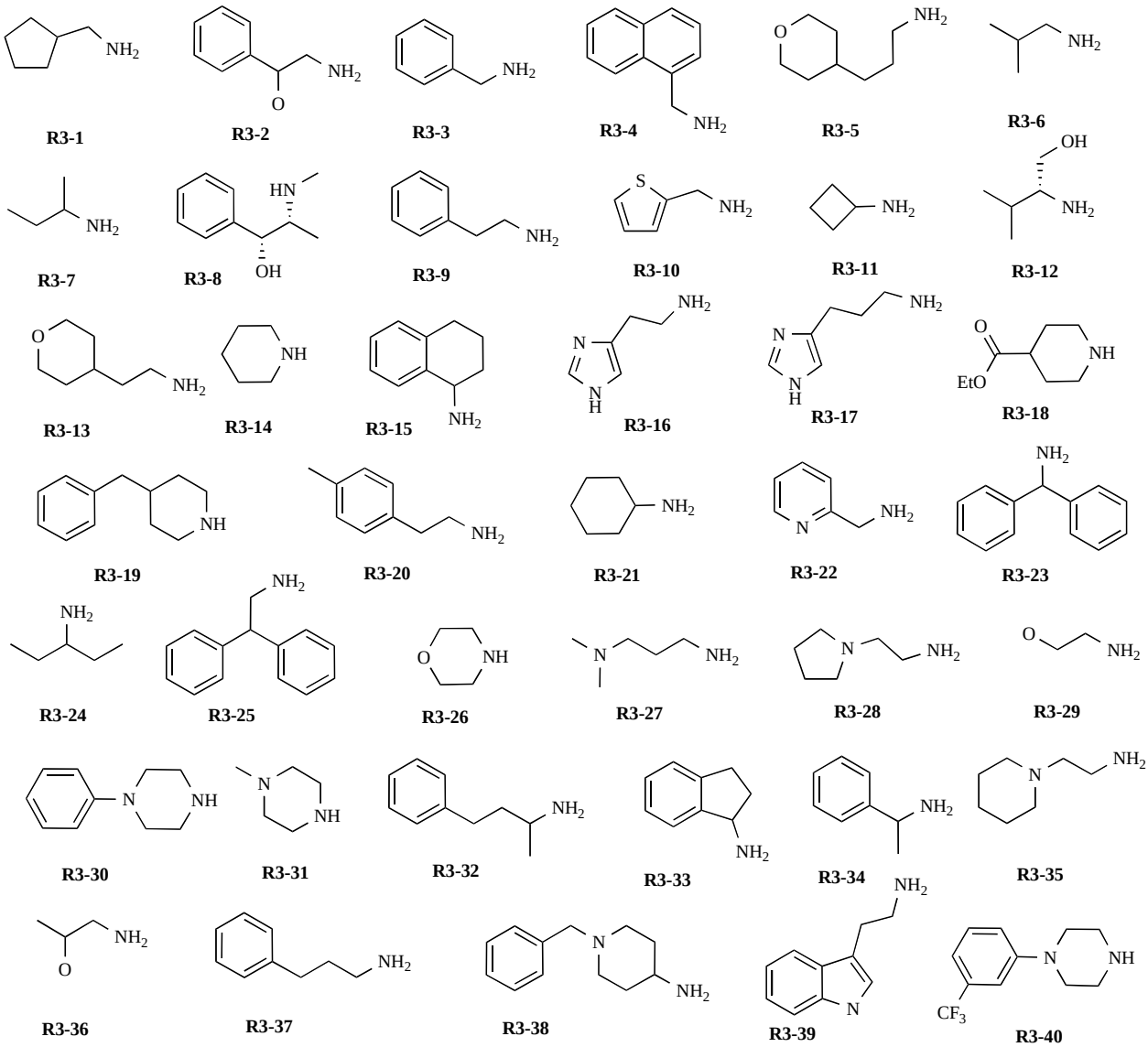


Fig. (6). Composition of the 1600 member arylidene diamide array. a/ Oxazolone building blocks, corresponding to R1. b/Aldehyde building blocks, corresponding to R2. c/Amine building blocks, corresponding to R3.

investigate the effect of the replacement of ephedrine with *N*-methyl-phenylalanine in more detail, interactions of 1 and its (S)-*N*-methyl-phenylalanine analogue 2 were analyzed by BIACORE using immobilized HSA. As shown in Fig. (**10A**, **B**), both compounds (**1**) and (**2**) clearly interacted with immobilized HSA. Scatchard plot analysis shows marked curvature for both compounds, which suggests the presence of plural binding with different affinities. Because of this curvature, two values for *K*a were calculated using either lower ligand concentrations (0.4-1.8 μM for higher *K*a) or higher ligand concentrations (14-56 μM for lower *K*a). Though both of these *K*a values tend to decrease by the replacement of ephedrine with *N*-methyl-phenylalanine, they are still fairly similar (2-3x10^6 M^{-1} as higher *K*a and 7-12x10^4 M^{-1} as lower *K*a).

The effect of free carboxyl group of compound (**2**) was also analyzed by comparing the interaction of compound (**2**) and its methyl ester, compound (**4**). As shown in Fig. (**10A**), this protection causes significant decrease in resonance signal when the ligand concentration is high. Interestingly, the Scatchard plot obtained for compound (**4**) is fairly linear (Fig. **10B**). The lower *K*a observed commonly for both compounds (**1**) and (**2**) is decreased, while the higher *K*a is

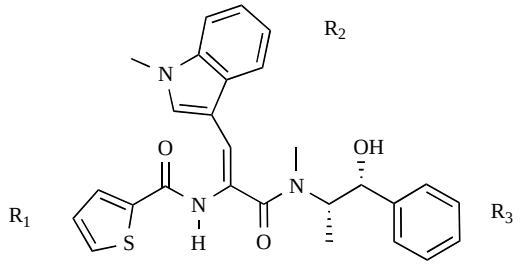
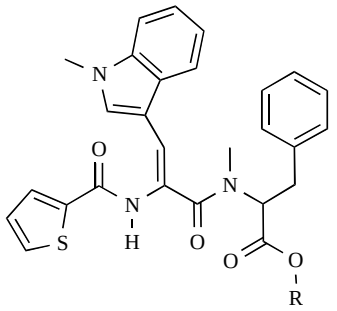


Fig. (7). Compound (**1**), lead compound, having highest binding capacity from the 1600 member directed library.

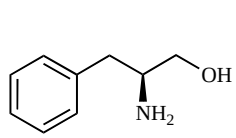


(2) R = H, (S)-N-Methylphenylalanine

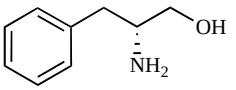
(3) R = H, (R)-N-Methylphenylalanine

(4) R = Me, (S)-N-Methylphenylalanine

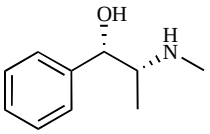
Fig. (8). Compounds having N-methyl-phenylalanine as analogues to ephedrine at R3.



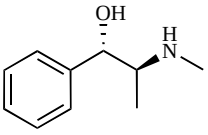
(S)-phenylalaninol



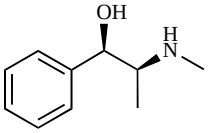
(R)-phenylalaninol



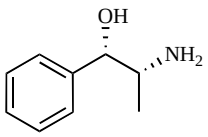
(1S, 2R)-(+)-Ephedrine



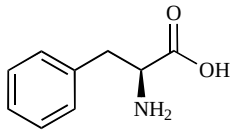
(1S, 2S)-(+)-Ephedrine



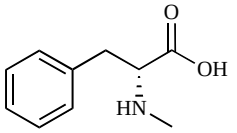
(1S, 2S)-(-)-Ephedrine



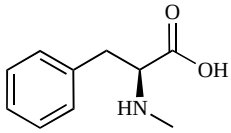
(1S, 2R)-(+)-Phenyl propanolamine



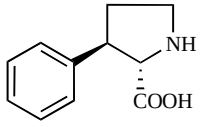
(S)-phenylalanine



N-Methyl-(R)-phenyl alanine



N-Methyl-(S)-phenyl alanine



(2S, 3R)-3-Phenyl-pyrrolidine-2-carboxylic acid

Fig. (9). Structural analogues used for the variation at R3.

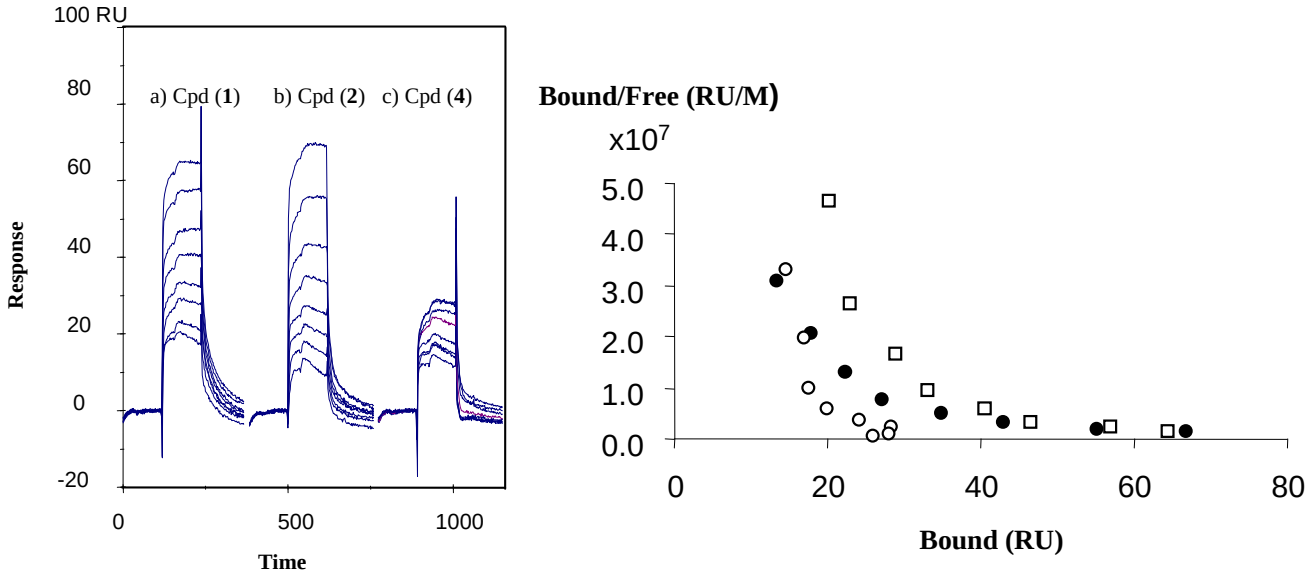


Fig. (10). Interaction analysis of ligand (**1**), its *N*-methyl-phenylalanine analogue (**2**) and methyl ester of (**2**), (**4**) with immobilized HSA by SPR. (**A**) Sensorgrams showing the interaction of ligand (**1**) (a), analogue 2 (b) analogue 4 (c) with immobilized HSA. Each compound was injected onto the HSA-immobilized surface at concentrations of 0.44, 0.88, 1.75, 3.5, 7, 12, 28 and 56μM. (**B**) Scatchard plot obtained for each interaction; i.e. ligand 1 (□), analogue 2 (●) and analogue 4 (○).

not significantly altered. It may therefore be possible that presence of the free carboxylate contributes to the binding energy of the lower affinity binding site. Since protection of free carboxyl group did not affect the higher affinity interaction, the usage of this functional group for the attachment to a gel is justified.

3.3. Competitive Studies

SPR was used to establish the protein-ligand interaction sites. The ligands were immobilized onto the sensor surface using standard conditions. Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) and ibuprofen ((*R,S*)-α-methyl-4-(isobutyl) phenyl acetic acid) are known to bind to binding sites 1 and 2 of HSA, respectively [21]. Essentially fatty acid free (approx. 0.005 %) and globulin free (approx. 99 %) HSA (62.5 μg/ml) was injected over the ligand substituted surface at a constant concentration with the two binders in series of increasing concentrations respectively. The response will be affected by the known ligand concentration if competition occurs between the immobilized ligand and the known binder.

As can be seen in Fig. (**11**), only ibuprofen inhibited the binding of HSA with immobilized compound (**5)** under the conditions used. A weak response could be found even at high concentrations of ibuprofen, which could be explained by the fact that oxazolones were found to bind to two binding sites on HSA in the screening studies and in other BIACORE studies. It has been established that ibuprofen binds at site 2, although it has been reported that it also interacts with a further unknown binding site, which is neither site 1 nor site 3 [18]. The same results were obtained in competitive studies using immobilized compound (**2**).

3.4. Selectivity Studies

The selectivity of potential HSA ligands can be rapidly estimated by measuring the differential binding to HSA and the highly homologous protein BSA (Bovine Serum Albumin). As shown in the sensorgrams in Fig. (**12A**) and the Scatchard plots in Fig. (**12B**), compound (**1**) clearly interacts with immobilized HSA, while it shows only slight increase in resonance signal with immobilized BSA. Once ephedrine is replaced with N-methyl-phenylalanine in compound (**2**), it was found to bind both HSA and BSA (Fig. **12A**, **B**). However, the compound (**4**) again showed good selectivity toward HSA (Fig. **12A**, **B**). This further supported the validity of replacing ephedrine with N-methyl-phenylalanine and immobilizing *via* the carboxyl group. Although immobilization of protein might lead to loss or reduction of binding activity, the *K*a obtained for phenylbutazone and ibuprofen by this method were comparable with previously reported values. As shown in the Scatchard plot in Fig. (**12B**) compound (**4**) displayed approx. 25 times higher selectivity versus HSA as compared to BSA.

3.5. Affinity Chromatography

The immobilizations of the ligands were performed on the various amino-substituted gels to achieve substitution degrees between 3 and 10 μmol ligand/ml wet gel. The spacer arms used were between 6 and 12 atoms long. Several different types of agarose matrices were also used.

Ligand (**2**) immobilized on EAH Sepharose™ 4B (ligand density 3 μmole/ml wet gel) was used for selectivity tests by applying the test proteins onto the column in PBS. A small amount of binding was observed for α-lactoglobulin but not for any of the other test proteins (polyclonal IgG, ovalbumin, lysozyme, transferrin).

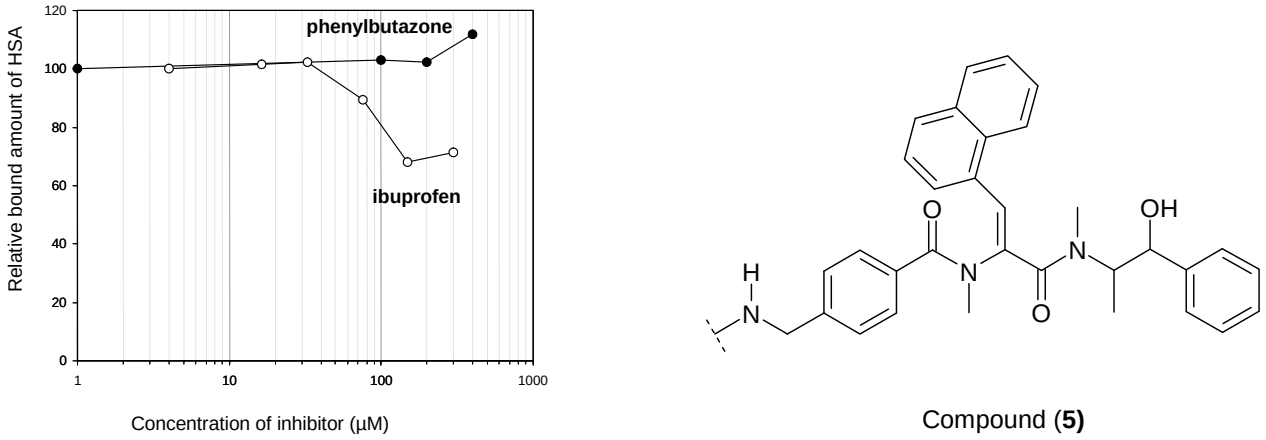


Fig. (11). Inhibitory effect by ibuprofen and phenylbutazone on the interaction of HSA with immobilized compound **5**. HSA was introduced onto the compound 5-immobilized surface at a concentration of 62.5 μg/ml with different concentrations of ibuprofen or phenylbutazone.

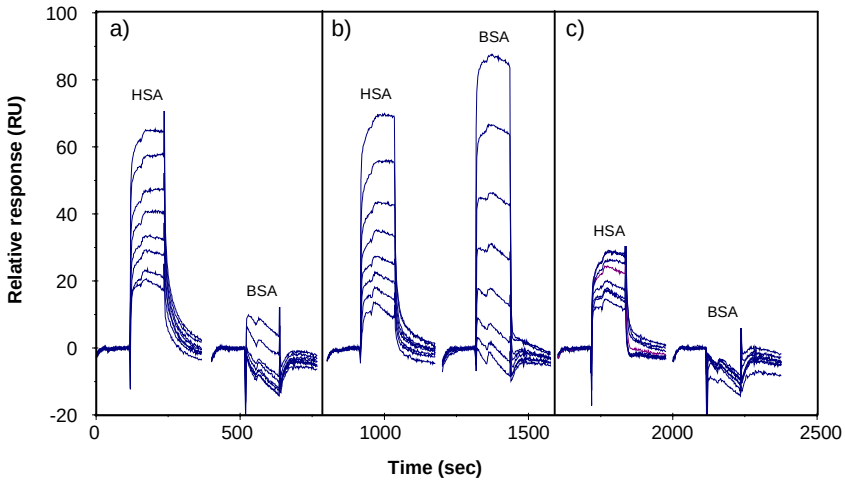


Fig. (12A). Comparison of the ability of immobilized ligands to differentiate between HSA and BSA on the BIACORE system. Sensorgrams showing the interaction of ligand (**1**) (a), analogue (**2**) (b) analogue (**4**) (c) with immobilized HSA and BSA. The concentrations used for each ligand are the same as shown in Fig. (**10**).

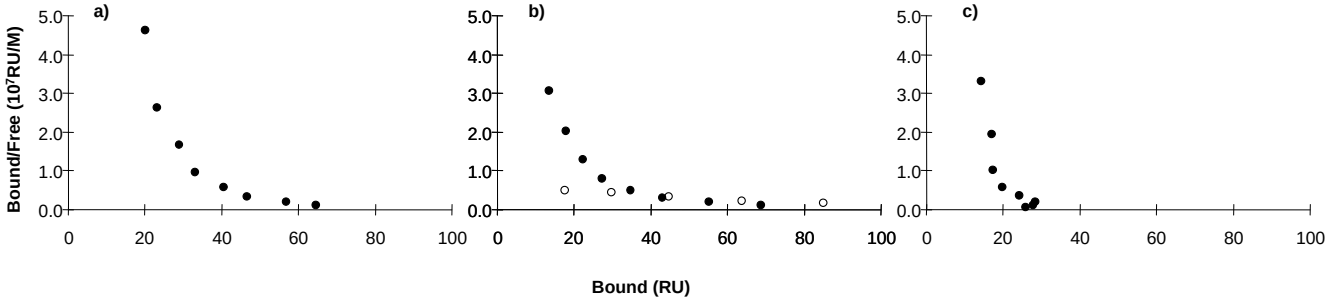


Fig. (12B). Scatchard plots obtained for ligand (**1**) (**a**), analogue (**2**) (**b**) and analogue (**4**) (**c**) with immobilized HSA (●) and BSA (○).

To test the binding characteristics for this ligand the separation of human serum was performed using the standard conditions for serum separation on Cibacron Blue Sepharose. Binding was performed with 50 mM citric acid and 100 mM Na_2HPO_4, pH 7, and the elution was performed using high salt conditions (50 mM KH_2PO_4, 1.5 M KCl, pH 7.0) in the first step. In a second step the ligand (**2**) column was eluted using 20 mM citric acid buffer, pH 3.0.

It is evident from the chromatograms obtained with ligand (**2**) that a small part of the bound serum protein is eluted under high salt conditions; however, the major part is eluted using acidic buffer. This indicates different binding modes for the new ligand as compared to the dye ligand. In the primary screening method we could also observe that the interaction involved two binding sites on the protein (Fig. **4C**). The dynamic binding capacity for HSA at 10% flow

through and a substitution level of 5 μmole/ml gel was 10 mg/ml gel.

3.6. Stability Studies and Stereochemistry

When any oxazolone is reacted with aldehyde, isomers are formed around the double bond (22). The Z/E-ratio was typically 9:1 as was confirmed by HPLC and ^{1}H NMR. After the opening of the oxazolone with the amine, the Z/E-ratio is still 9:1. This is in accordance with previous work [22]. Initially some isomer pairs were separated by crystallization and the double bond configurations were established using ^{1}H- and ^{13}C-NMR. Generally the major isomer was active in the screening versus HSA, but the minor one was not. In the libraries the 9:1 mixtures were used for screening without separation.

The stability of the ligands was studied using enforced cleaning-in-place (CIP) conditions (pH 2 and 14, 40^{o}C, 7 days). Samples were taken regularly and the possible degradation was studied by ^{1}H- and ^{13}C-NMR. In general it was found that all arylidene diamide compounds studied are stable toward strongly basic conditions, whereas acidic conditions are somewhat less tolerated.

Compound (**1**) is stable at pH 3-14 but is partly hydrolyzed at pH 2. The hydrolytic products were detected after 2 days and increased in concentration but not in complexity. After 7 days 30% of the R3 component had been hydrolyzed, but no further degradation products could be established.

4. CONCLUSIONS

The results clearly demonstrate that combinatorial chemistry is a promising technology for the identification of affinity chromatography ligand candidates. The analysis of the screening results was greatly facilitated by the use of systematic reagent layout in a spatially addressable array format. The ability to computationally screen structural databases of commercially available reagents and virtual libraries led to rapid identification of patentable lead compounds for HSA affinity matrices [23]. The capability of computationally screening virtual and physical combinatorial libraries provided mechanisms for rapidly expanding the SAR information in an efficient manner.

Compound (**2**) is clearly acceptable as an affinity ligand, although further process optimization would be required to produce a commercially viable product. The ligand demonstrated reasonable selectivity and dynamic binding capacity, achieved reasonable purity from a complex feedstock and showed low salt elution characteristics. The ligand also worked with samples of whey, yeast and *E. coli* extracts, which had been spiked with recombinant HSA.

However, in the course of this project, it became clear that the solution phase screening of solution phase libraries has limitations for affinity chromatography ligand discovery. Hits found with this procedure must be converted to the appropriate solid phase structure. Of course binding may be diminished or eliminated as a result of the immobilization. A more expeditious approach would involve the use of solid phase screening technology together with either (a) solution phase syntheses with active groups for post-synthesis immobilization, or (b) direct solid phase syntheses.

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