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Capillary electrophoretic mobility shift displacement assay for the assessment of weak drug-protein interactions

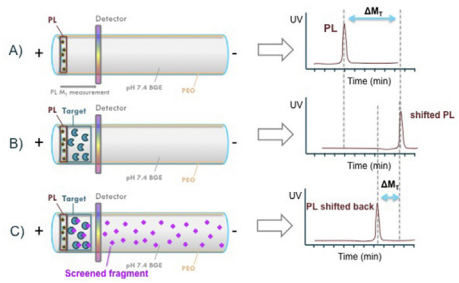
E. Farcaş [a], J. Hanson [c, d], L. Pochet [b, 1], M. Fillet [a, *, 1]

[a] Laboratory for the Analysis of Medicines (LAM), Department of Pharmacy, CIRM, University of Liege, Belgium
[b] Namur Medicine & Drug Innovation Center (NAMEDIC - NARILIS), University of Namur, Belgium
[c] Laboratory of Molecular Pharmacology, GIGA-Molecular Biology of Diseases, University of Liege, Belgium
[d] Laboratory of Medicinal Chemistry, CIRM, University of Liege, Belgium

HIGHLIGHTS

- Competitive affinity capillary electrophoresis is used in the context of fragment-based drug discovery.
- IC50 calculated are similar to the one of the literature.
- The method extends the scope of applications in comparison to the direct approach.
- The method discriminates between reversible and irreversible inhibitors.

GRAPHICAL ABSTRACT



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ABSTRACT

Only few reports describe the use of capillary electrophoresis in the context of Fragment Based Drug Discovery (FBDD). In this paper, we will present a generic, fully automated, microscale electrophoretic mobility shift displacement assay that can be used in FBDD for primary screening of weak biomolecular interactions between fragments and target protein. The accuracy and reliability of the present method was demonstrated by measuring the IC_{50} value of two known fragments inhibiting thrombin, namely benzamidine and *p*-aminobenzamidine and a relatively weak inhibitor, nafamostat. Furthermore, we built a small chemical library to evaluate the performance and the advantage of our newly developed screening-bioassay compared to the direct affinity capillary electrophoresis-binding assay. The results demonstrate the high discriminatory power of the method and above all its ability to screen neutral, negatively or positively charged molecules, as well as molecules that have no or low UV-VIS absorbance, greatly expanding the scope of the assay. Finally, we proved that this approach is able to discriminate between reversible and irreversible binders. Altogether, this work demonstrates that capillary electrophoresis could constitute an important added value in the arsenal used to screen fragments in drug discovery.

Abbreviations: % inh, percentage of inhibition; μep, electrophoretic mobility; ACE, affinity capillary electrophoresis; AGB, 4-acetamidophenyl 4-guanidinobenzoate hydrochloride; BGE, background electrolyte; BZM, benzamidine; DAPA, dansylarginine N-(3-ethyl-1,5-pentanediyl)amide; DMSO, dimethylsulfoxide; FBDD, Fragment Based Drug Discovery; HEPES-Na, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; HPAC, high performance affinity chromatography; IC_{50}, half maximal inhibitory concentration; K_i, inhibitory constant; M_T, migration time; NAFA, nafamostat; NAPAP, Pefabloc TH; NMR, nuclear magnetic resonance; PABZM, 4-aminobenzamidine; PEG 6000, polyethylene glycol 6000; PEO, poly(ethylene oxide); PL, probe ligand; PPACK, Phe-Pro-Arg-chloromethylketone; QC, quality control; RSD, relative standard deviation; SPR, surface plasmon resonance; Tris HCl, Tris(hydroxymethyl)aminomethane hydrochloride.

* Corresponding author. Laboratory for the Analysis of Medicines, Department of Pharmacy, University of Liège, B36, Tower 4, 3rd floor, Quartier Hopital, av. Hippocrate, 15, B-4000, Liège, Belgium.
E-mail address: Marianne.fillet@ulg.ac.be (M. Fillet).
[1] Equally contributed to the work.

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1. Introduction

Deciphering disease-specific mechanisms contributes to the identification of a substantial number of new pharmaceutical targets with a resulting demand on new chemical entities able to modulate them. Since drug development process is currently characterized by high attrition rates, it is needed to develop robust and cost-effective tools to provide the information required at the early stages of this process, in order to conduct a rational development of these new chemical entities.

Fragment based drug discovery (FBDD) is nowadays widely recognized as a mature approach for the discovery of new molecular entities that bind to biological targets of interest. Many new compounds issued from the FBDD are currently in clinical trials [1–6] or already commercialized [7,8]. The first step of the FBDD consists of screening a library of low molecular weight molecules (also called fragments) to identify the ones that bind to a particular target. When interesting fragments are identified, they are subsequently optimized using a structure-guided strategy such as fragment growing or fragment merging to generate high quality hits [9].

The main challenge of such approach is the detection and characterization of weak interactions. Consequently, there is a need for discriminant and reliable analytical approaches to decrease the number of false positive and false negative hits. The most frequently used analytical strategies are nuclear magnetic resonance (NMR), X-Ray crystallography, surface plasmon resonance (SPR) and high performance affinity chromatography (HPAC). These techniques can offer valuable information at the early stages of drug discovery (complex dissociation constant, binding kinetics, etc). However these approaches suffer from several drawbacks such as large amount of protein requirements (NMR), extensive assay development times since co-crystallization may require different crystal condition for each ligand (X-Ray crystallography), or the need of immobilization of the target on a support (SPR or HPAC), which could impact the pharmacological properties of the target by modifying protein integrity and conformation.

Recently, we demonstrated that an affinity capillary electrophoresis (ACE) direct binding assay could be successfully implemented in the context of FBDD due to its simplicity, reliability and ability to identify and quantitate weak interactions in near-physiological conditions [10]. The proposed method is robust and selective for weak affinity binders. However, it is not a universal approach since only positively charged molecules that absorb in UV-VIS could be screened.

To overcome these limitations, ligand binding can be studied indirectly, measuring the ability of the ligand to displace the binding of a “reporter” substance (probe ligand, PL). This approach was called competitive binding because it was the competition between the PL and the screened ligand for the same binding site that was monitored [11].

The main advantage of ACE competitive binding approach over the direct approach is the fact that all kind of fragments can be screened even if they have no net charge at physiological pH or no UV-VIS absorbance. Moreover, since the interplay between two ligands is monitored on the same target, the false positive rate of compounds that will be fished is expected to be low.

Despite the fact that competitive ACE is a generic and sensitive microscale technique that enables the screening of fragments in near-physiological environment, this approach is scarcely reported in the context of FBDD (only one article to the best of our knowledge) [12]. Austin and co-workers demonstrated the ability of competitive ACE to detect weak interactions (in the high μM to mM range) between HSP90 (a chaperone protein up regulated in response to stress, currently investigated as a therapeutic target in oncology) and small fragments [12].

The aim of our study was to develop a sensitive, robust and reliable generic displacement binding approach that could be used for the study of a heterogeneous library of fragments in order to guide the synthesis of antithrombotic agents targeting thrombin, a well-known trypsin-like serine protease from the coagulation cascade. This approach should to be able to classify the analyzed fragments according to their affinity for the target and provide, additionally, valuable information at the early stages of drug discovery, such as fragments half maximal inhibitory concentration (IC_{50}). It is noteworthy to mention that particular attention was paid to the reliability and transferability of the method to industry working in GMP conditions (qualified equipment, SOPs, QCs…). With this in mind, negative controls as well as well positive controls (well-known thrombin inhibitors) were included in the study. Furthermore, the results were compared to results obtained using the spectrophotometric activity method classically employed by the medicinal chemists.

2. Materials and methods

2.1. Reagents and materials

Benzamidine hydrochloride (BZM), 4-aminobenzamidine dihydrochloride (PABZM), nafamostat mesylate (NAFA), Pefabloc TH® B (NAPAP), L-pipecolic acid, 3-chlorobenzylamine, 4-[5-(4-chlorophenyl)-1H-pyrazol-3-yl]piperidine, N-(5-bromo-4-methyl-1,3-thiazol-2-yl)guanidine, cariporide, L-arginine, 5-(aminomethyl) indole, L-pipecolic acid, 4-Methyl-2-piperidine carboxylic acid, ximelagatran, argatroban, N-(pyridin-4-ylmethyl) guanidine, 4-aminomethyl benzamidine dihydrochloride, 6-(trifluoromethyl) pyridine-3-amidine hydrochloride, 3-aminomethyl benzamidine dihydrochloride, 3-chlorobenzamidine hydrochloride, 3,5-difluorobenzamidine hydrochloride, 4-methoxybenzamidine, 4-acetamidophenyl 4-guanidinobenzoate hydrochloride, N-(1-benzyl-4-piperidinyl)guanidine hydrochloride, Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) and poly(-ethylene oxide) (PEO) (MW 200 000 g/mol) were purchased from Sigma Aldrich (Saint-Louis, MO, USA).

Fragments Z2238944429, Z234894831, Z1211228967, Z1263820361, Z1473553993, Z234895267, Z839575938, Z234894451, Z2238944431, Z234895181, Z228583172, Z1162448363, Z394693552, EN300-249818, and Z25220620 were purchased from Enamine (New Jersey, USA). The RF2 was synthetized in Namedic (UNamur). All structures are given in Supplementary Material.

(2R,4R)-4-Methyl-piperidine-2-carboxylic acid was obtained from ABCR GmbH (Karlsruhe, Germany). Lidocaine hydrochloride and histamine dihydrochloride was purchased from Fagron (Nazareth, Belgium).

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES-Na) was obtained from Merck (Darmstadt, Germany). Sodium hydroxide and polyethylene glycol 6000 (PEG 6000) were purchased from VWR (Leuven, Belgium). Sodium chloride was provided by Acros Organics (New Jersey, USA). Dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA), Phe-Pro-Arg-chloromethylketone (PPACK), Human alpha thrombin was acquired from Haematologic Technologies (Vermont, USA). The thrombin was provided in H_2O:glycerol (50/50 v/v). All chemicals and reagents were of analytical grade. Ultrapure water was supplied by a Milli-Q equipment (Millipore, Bedford, MA, USA) and the Chromafil® syringe filters (0.20 μm) were from Macherey-Nagel (Duren, Germany).

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