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Amine coupling *versus* biotin capture for the assessment of sulfonamide as ligands of hCA isoforms

Tiphaine Rogez-Florent [a], Laurence Goossens [a, b], Anne-Sophie Drucbert [c, d], Sophie Duban-Deweer [e], Perrine Six [b], Patrick Depreux [a, b], Pierre-Marie Danzé [c, d], Jean-François Goossens [a], Catherine Foulon [a, c, *]

[a] *Univ Lille, EA 7365 – GRITA – Groupe de Recherche sur les formes Injectables et les Technologies Associées, F-59000 Lille, France*
[b] *Univ. Lille, ICPAL, F-59000 Lille, France*
[c] *Univ. Lille, Plate-forme d'interactions moléculaires, F-59000 Lille, France*
[d] *CHU Lille, Centre de biologie pathologie génétique, F-59000 Lille, France*
[e] *Univ. Artois, Laboratoire de la barrière hémato-encéphalique (LBHE) - EA 2465, F-62307 Lens, France*

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This work was dedicated to the development of a reliable SPR method allowing the simultaneous and quick determination of the affinity and selectivity of designed sulfonamide derivatives for hCAIX and hCAXII *versus* hCAII, in order to provide an efficient tool to discover drugs for anticancer therapy of solid tumors. We performed for the first time a comparison of two immobilization approaches of hCA isoforms. First one relies on the use of an amine coupling strategy, using a CM7 chip to obtain higher immobilization levels than with a CM5 chip and consequently the affinity with an higher precision (CV % < 10%). The second corresponds to a capture of proteins on a streptavidin chip, named CAP chip, after optimization of biotinylation conditions (amine *versus* carboxyl coupling, biotin to protein ratio). Thanks to the amine coupling approach, only hCAII and hCAXII isoforms were efficiently biotinylated to reach relevant immobilization (3000 RU and 2700 RU, respectively) to perform affinity studies. For hCAIX, despite a successful biotinylation, capture on the CAP chip was a failure. Finally, concordance between affinities obtained for the three derivatives to CAs isozymes on both chips has allowed to valid the approaches for a further screening of new derivatives.

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1. Introduction

Human carbonic anhydrases IX (hCA IX) is a transmembrane zinc enzyme that belongs to the CA's family whose major enzymatic function is to catalyze the hydratation of carbon dioxide to bicarbonate [1]. High tumoral carbonic anhydrase IX (hCA IX) expression has been associated with poor prognosis, tumor progression and aggressiveness [2]. hCA IX is expressed in a limited number of normal tissues (mainly the gastrointestinal tract), whereas its overexpression is observed on the cell surface of a large number of solid tumors, and it is invariably associated with the hypoxic phenotype, mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1) [3]. hCA IX has been identified as a potentially important marker of hypoxia. Furthermore, hCA IX overexpression is often associated with a poor responsiveness to the classical radio- and chemo-therapies [3–5]. Development of potent, highly selective inhibitors against this target remains an unmet need in anticancer therapeutics. New sulfonamide derivatives were designed and synthetized by some of us as potential inhibitors of hCAIX [6], but also of hCAXII, as these transmembrane isoforms are to some extent overexpressed in hypoxic solid tumors [7,8]. In order to reduce side effects, selectivity of these inhibitors for hCAIX and hCAXII *versus* ubiquitous h CAII were also intended [9].

It is necessary to have a reliable method to identify hit among new derivatives and thereby to investigate the affinity and the selectivity of these derivatives for their therapeutic target, *i.e.* hCAIX and hCAXII *versus* hCAII. Many techniques to measure binding strength are available. Among them, biophysical methods like isothermal titration calorimetry (ITC), Fluorescent Thermal

* Corresponding author. Laboratoire de Chimie Analytique, EA 7365 - GRITA, Faculté de Pharmacie, 3 rue du Pr Laguesse, BP 83, 59006 Lille, France.
E-mail address: catherine.foulon@univ-lille2.fr (C. Foulon).

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shift assay (FTSA), nano-electrospray mass spectrometry (nESI-MS), nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR) have undergone a great increase in their use in the pharmaceutical industry to identify hits and leads [10]. SPR exhibits the capacity of studying the interactions between two partners in real-time. Contrarily to the previous methods, SPR is not completely label-free, since one of them is immobilized on a thin gold surface of a chip, flowed over by a buffer containing the second partner by means of a microfluidic system. The immobilization of one partner is the main drawback of the SPR, as it could compromise the activity/functionality of the protein or the access to the ligand binding site(s). The great advantage of SPR is that it not only allows to characterize a wide range of affinity (K_D = 1 mM–1 pM), but also to access to kinetic constants of the noncovalent binding process [11]. Relatively low amount of proteins, required for immobilization and characterization of whole sets of ligands, and associated with the capability of studying several interactions simultaneously with different targets immobilized on the same chip, are other key advantages of this technique. As a result, in recent years, it has gained more importance in drug discovery for the routine investigation of small molecules binding to macromolecular target [12–16].

Hence, numerous works dedicated to the study of molecular interactions involving different carbonic anhydrase isoforms are available in the literature. Whereas only one study deals with protein/protein interactions involving hCAIX immobilized on a carboxymethyl dextran chip (CM5) via amine coupling [17], several studies have been carried out to characterize the interaction of CA isoforms and small molecules. To this purpose, immobilization of bovin CAII (bCAII) [18–25], or hCAII [25,26] or hCAI [27] was also performed on CM5 sensor chips and amine coupling. Among these studies, several have demonstrated the accordance of the binding constants measured by SPR, with those measured by the orthogonal fully label-free methods, *i.e.* ITC [22,23,25,27], FTSA [25] and nESI-MS [27]. To our knowledge, only one study proposes another approach to immobilize bCAII by capture of the biotinylated bCAII on a streptavidin-based chip, included in the Biacore Biotin CAPture kit [28]. This chip, which main characteristic is its regeneration capability, contains an oligomer surface that readily hybridizes with specially prepared oligo-linked streptavidin. In this study, the authors use this regeneratable chip as a tool to optimize biotinylation of bCAII but not for affinity constants evaluation. CAP chip was besides used by Suenaga [29] to monitor influenza hemagglutinin and glycan interaction after immobilization of biotinylated glycan. They have shown that CAP-chip leads to highly reproducible results, with rate constants comparable to those measured on a classical streptavidin chip. This chip was also successfully used for numerous studies as for example for the kinetic characterization of the interaction between ADP-ribosylation Factor-1 and the Sec7 domain of Arno guanine nucleotide [30] or also the development of SPR-based assays to evaluate the impact of the glycosylation pattern of monoclonal antibodies on their interaction with Fcγ receptors (FcγR) [31]. In the latter study, the CAP chip allowed to compare two approaches for the biotinylated FcγR production, and to obtain a stable and homogeneous receptor population at the biosensor surface own to an oriented immobilization instead of a random immobilization [13,32].

The goal of our study was to develop a reliable SPR method allowing the simultaneous determination of the affinity and selectivity of sulfonamide derivatives for hCAII, hCAIX *versus* hCAXII and to compare two immobilization approaches of hCA isoforms, *i.e.* amine coupling on a carboxymethyl dextran chips and capture on a CAP chip. The immobilization strategy evaluated first (primary amine coupling) which relevance has been already demonstrated for hCAII [25,26], results in a random-orientation of the protein and then in a heterogeneity of the surface. The second strategy (capture using the biotinylated protein) leads to an orientation –controlled immobilization and then to a more homogeneous surface [13]. After optimization of the immobilization conditions, developed sensor surfaces were used to assess the affinity of three sulfonamides including a reference compound (Acetazolamide (**1**)) and two designed derivatives described earlier (**2** and **3**) (Fig. 1). Comparison of results obtained according to the both approaches, that to our knowledge had never been achieved to date, should allow to establish if the immobilization does compromise or not the activity/functionality of the protein or the access to the ligand binding site and validating the molecular binding parameters.

2. Materials and methods

2.1. Materials and apparatus

Carbonic anhydrase isoenzyme II from human (hCAII; MW = 29 kDa; lyophilized powder with purity > 80%) erythrocytes, Bovine serum albumin (BSA; MW = 66 kDa; lyophilized powder with purity > 96%), DMSO (dimethyl sulfoxide) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Solution of carbonic anhydrase isoenzyme IX isolated from human erythrocytes at 1 mg mL^{-1} in 25 mM Tris pH 7.5, 150 mM NaCl (hCAIX; MW = 42 kDa; purity > 95%) was purchased from Bio-Techne France (Lille, France). Carbonic anhydrase isoenzyme XII from human erythrocytes (hCAXII; MW = 37 kDa; powder; lyophilized powder with purity > 95%) was purchased from Interchim (Montluçon, France). Sodium dodecyl sulfate (SDS), sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium hydrogenophosphate (Na_2HPO_4), sodium dihydrogenophosphate (NaH_2PO_4) and sodium acetate ($NaCH_3COO$) were obtained from Merck (Nogent sur Marne, France). Acetazolamide (AZA) was purchased from Alpha Aesar (Schiltigheim, France). N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), ethanolamine HCl, 10% v/v surfactant P20 solution, 1X HBS-P (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v surfactant P20), CM5 and CM7 chips, as the Biotin CAPture Kit (containing CAP chips, Conjugate Biotin CAPture [CAP] solution and regeneration reagents (8 M guanidine-HCl and 1 M NaOH)) were purchased from Biacore (GE Healthcare, Velizy-Villacoublay, France). EZ-Link sulfo-NHS-LC-LC-biotin and EZ-Link pentylamine-biotin were obtained from ThermoFisher Scientific (Villebon-sur-Yvette, France). Diarylpyrazole sulfonamide derivatives **2** and **3** were prepared by some of us according to the published procedure [6]. Amicon® Ultra (0.5 ml, 10 K) centrifugal filters, were purchased from Merck Millipore (Fonteray-sous-Bois, France). Criterion TGX Gel (ref 567–1043), 0.45 μm polyvinylidene fluoride membrane (ref 162–0177), 10 X TBS (200 mM Tris, pH 7.5, 5 M NaCl), AP conjugate Substrate kit (ref 1706432) were purchased

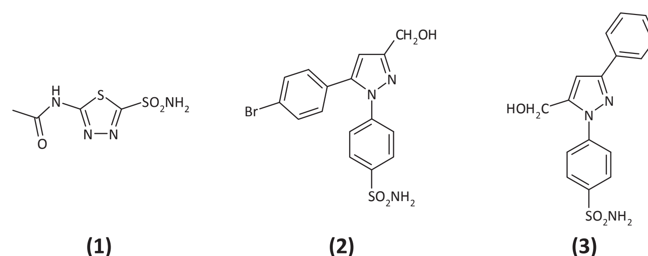

Fig. 1. Studied compounds. (1): reference compound, acetazolamide; (2): 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-*p*-bromophenyl-*1H*-pyrazole; (3): 1-(4-Aminosulfonylphenyl)-5-hydroxymethyl-3-phenyl-*1H*-pyrazole [6].

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