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Serum albumin as a probe for testing the selectivity of irreversible cysteine protease inhibitors: The case of vinyl sulfones

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ABSTRACT

Vinyl sulfones are used for drug design of irreversible inhibitors of cysteine proteases since they are able to alkylate cysteine thiols inside the catalytic pocket of this class of enzymes. Some authors have reported the lack of reactivity towards glutathione as sufficient evidence of the selectivity of such a mechanism. Herein, we demonstrate that some simple molecules containing a vinyl sulfone moiety are not thiol-specific alkylants since they react with some albumin nucleophiles including side chains of Cys34 and His146. Such side-reactions are not desirable for any drug candidate since they limit serum stability, bioavailability and they possibly trigger toxicity mechanisms. *In silico* predictions, indicate that the compounds tested share similar structural features with reported inhibitors of cysteine proteases, as well as similar poses around the main albumin nucleophiles. Altogether, the data suggest that albumin is better than glutathione for the setup of early *in vitro* tests probing the selectivity of cysteine protease inhibitors.

1. Introduction

Cysteine proteases (CPs) are widely studied pharmaceutical targets. Specifically, cysteine protease inhibitors have been developed for the treatment of several diseases including cancer [1,2], inflammation [3] and both viral [4] and parasite infections [5–8]. Starting from the eighties, one of the most studied inhibition mechanisms is the covalent binding to the cysteine into the catalytic pocket of CPs, which leads to an irreversible inactivation of the enzyme [9,10]. Therefore, the first approach for the synthesis of cysteine protease inhibitors was to link a warhead (necessary for inhibition) to a natural substrate of CPs (necessary for target recognition). To date, many different moieties have been tested as warheads in order to design molecules with improved potency and selectivity for specific targets [11].

Vinyl sulfones and other Michael acceptors are included in the long list of alkylating agents used as warheads for the design of CPs inhibitors [12]. Some authors have claimed that such moieties are specific alkylants of CPs since they need the catalytic machinery of CPs for activation [11]. Such selectivity is important to minimize any interaction with endogenous targets, which potentially leads either to toxicity or to pharmacokinetic issues. So far, the selectivity of vinyl sulfones has been proven *in vitro* by demonstrating their stability towards serine proteases and small circulating thiols (*e.g.*, glutathione) [13,14]. Furthermore, *in silico* studies have suggested that such a selectivity lies in the differences of the catalytic mechanisms between serine and cysteine proteases [15]. Despite these data, some warnings about the lack of selectivity and potential toxicity of vinyl sulfones have been given in the past [16,17]. Moreover, few authors have reported some pharmaceutical strategies for the development of vinyl sulfone prodrugs delivering protected alkylants into blood cells, where a specific catalytic mechanism releases vinyl sulfones [18,19]. This approach circumvents potential toxicity due to covalent binding with unspecific targets. In this context, *in vivo* experiments aimed at the development of compounds against blood parasite infections have shown some toxic effects of vinyl sulfones in animal models [20,21]. The reason for such an unanticipated toxicity could lie in the poor prediction of *in vitro* tests that challenge the selectivity of vinyl sulfones by using glutathione. In fact, such an approach is biased by two main factors. The first is the absence of the cofactors enhancing the nucleophilic-

Abbreviations: CP, cysteine protease; PVS, phenyl vinyl sulfone; PPS, 1-penten-1-yl phenyl sulfone; MSS, mesityl styryl sulfone; CTS, 1-cyclohexenyl *p*-tolyl sulfone; CH_3CN, acetonitrile; H_2O, water; HCOOH, formic acid; ESI, electrospray ionization; NSI, nanoelectrospray ionization; AGC, automatic gain control; CID, collison induced dissociation; Cys, cysteine; Lys, lysine; His, histidine; HSA, human serum albumin; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; SIC, single ion chromatogram; FWHM, full width at half maximum peak height.

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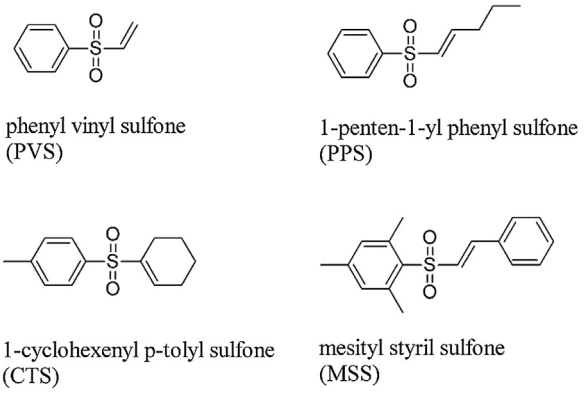

Fig. 1. Structures of the vinyl sulfones tested.

ity of small thiols *in vivo* (*e.g.*, Glutathione *S*-transferases) [22]. The second is the speculation that vinyl sulfones selectively react with thiols and not with other nucleophiles.

Herein, we challenge the selectivity of model vinyl sulfones towards human serum albumin (HSA). The use of a protein is not a new approach since Choi et al. have already proposed the use of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for *in vitro* evaluation of vinyl sulfones selectivity [23]. Such an enzyme has a thiol-dependent catalysis mechanism, which is different compared to the catalytic mechanism of CPs. Therefore, an alkylation of GAPDH give strong evidence of the non-selectivity of alkylants towards CPs.

In this context, we propose the use of albumin since it has other advantages over GAPDH. First, albumin is cheaper than GAPDH. Second, albumin has already been reported as a strong scavenger of electrophiles, owing to the presence of a self-activated thiol (*i.e.*, Cys34) but also many non-thiolic nucleophiles [24–27]. Third, we noticed that independent *in silico* models show largely superimposable mechanisms explaining the reactivity of CPs and albumin towards Michael acceptors [11,15,26,28]. Specifically, both for albumin and for CPs the increased nucleophilicity of cysteine thiol is explainable by the stabilization of the corresponding thiolate anion, owing to the interaction with a neighboring imidazole ring of a histidine.

2. Material and methods

2.1. Chemicals and reagents

Human serum, triethylammonium bicarbonate buffer (TEAB), tris(2-carboxyethyl) phosphine (TCEP), dimethyl sulfoxide (DMSO), phenyl vinyl sulfone (PVS), 1-penten-1-yl phenyl sulfone (PPS), mesityl styryl sulfone (MSS), 1-cyclohexenyl *p*-tolyl sulfone (CTS), formic acid (Mass Spectrometry Grade) and acetonitrile (Chromasolv®, LCMS grade) were from Sigma-Aldrich (Milan, Italy). Trypsin/Lys-C Mix (Mass Spectrometry Grade) was from Promega (Milan, Italy). Purified water (18.2 mΩ × cm resistivity at 25 °C) was prepared with a Milli-Q purification system (Millipore, Vimodrone, MI, Italy).

2.2. Incubation of vinyl sulfones in human serum

Fig. 1 shows the structures of the compounds tested. Each compound was dissolved in DMSO and diluted in the same solvent to the desired concentration. Each compound was tested separately by spiking 2 μL of vinyl sulfone in 200 μL of human serum. The 2 μL aliquots spiked into the serum were prepared accordingly to reach a final concentration of the tested compound equal to 0, 10, 100 or 1000 μM in the serum.

Table 1
SRM transitions used for vinyl sulfone serum time course monitoring.

Compound	Precursor ion (*m*/*z*)	Fragment ions (*m*/*z*)	Collision energy (%)
PVS	169.07	97.09, 125.04	20
PPS	211.02	125.01, 143.01	20
MSS	287.11	103.10, 166.97	20
CTS	237.09	81.15, 139.01	20

The temperature was kept at 37 °C throughout the incubation time and both serum and vinyl sulfones solutions were pre-warmed at 37 °C for 10 min before starting the reaction. Aliquots of 20 μL of human serum were sampled at the beginning of the incubation (kinetic starting points) and after 5, 15, 30, 60, 120 and 180 min. The aliquots were chilled to −80 °C right after sampling and stored at that temperature until use. Corresponding blanks were prepared by spiking vinyl sulfones in 10 mM phosphate buffer at the same concentrations and in the same reaction conditions reported above. Blanks were sampled only at the beginning of the incubation and after 30, 60, 120 and 180 min. Human serum samples were prepared in triplicate, while blanks were prepared as single replicate.

2.3. Time course of vinyl sulfones in human serum

The analytic platform was composed of a Surveyor LC system, which was connected to a TSQ Quantum Ultra mass spectrometer through a Finnigan IonMax electrospray ionization (ESI) source assembled with a low flow stainless steel emitter (Thermo Fisher Scientific, Rodano, MI, Italy). Before the analysis, serum aliquots were diluted five fold into pure acetonitrile and kept for 15 min at 4 °C in an ice bath to allow proteins precipitation. Samples were then centrifuged for 10 min at 18,000*g* and the supernatant were further diluted 5 fold in water containing 0.1% formic acid, placed in clear glass vials and kept at 4 °C in the autosampler compartment. The sampling program was a 50 μL-partial-loop injection. Chromatographic separation was performed on a Phenomenex Sinergy Max-RP column (150 mm × 2 mm i.d., 80 Å pore size, particle size 4 μm) working at 37 °C at a flow rate of 200 μL/min. The elution of the peptides was performed by a multi-step binary gradient, solvent A was water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. The gradient started with an isocratic flow at 15% solvent B for 0.4 min, followed by a linear ramp up to 95% solvent B in 1.5 min, followed by an isocratic flow at 95% solvent B for 5 min and 3 min for re-equilibrating the column to the initial conditions before the next analysis. The flow coming from the column was sprayed directly into the mass spectrometer by the ESI source operating as follows: positive ion mode, spray voltage of 3.5 kV, capillary temperature of 300 °C, sheath gas 45% and auxiliary gas 10%. During the chromatographic separation, the mass spectrometer was operating in multiple reactions monitoring (MRM) to scan the transitions reported in Table 1, which were previously optimized to allow the maximum sensitivity and selectivity of detection for vinyl sulfones.

The other instrument settings were: Q1 and Q3 power 0.4 amu, Q2 gas pressure 1.5 Torr, scan with 0.4 amu, scan time 50 ms, skimmer offset 10 and microscan set to 1. Full instrument control and extraction of peak areas used for quantitation were provided by Xcalibur software (version 2.0.7, Thermo Fisher Scientific, Rodano, MI, Italy).

2.4. Intact protein analysis of serum albumin

The analyses were performed by an automated loop injection method, developed on the same analytic platform as previously reported. The analyses were performed on aliquots of serum incubated with 1 mM vinyl sulfones and sampled at the beginning of the