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RESEARCH

Identification of Protease Inhibitors by a Fast Fluorimetric Assay

Nunzianna Doti · Domenico Raimondo · Marco Sabatella · Menotti Ruvo

Abstract Anomalous protease activities are associated with many diseases. Great efforts are paid for selecting specific protease modulators for therapeutic approaches. We have selected new modulators of enzyme activity by an homogeneous assay based on a doubly labeled small peptide used as substrate of trypsin. The substrate incorporates the fluorophore 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) at one end and an EDANS-quenching moiety (Dabcyl, (4-(4-dimethylaminophenylazo)-benzoic acid)) on the other end. Following cleavage by trypsin, the peptide-EDANS product is released interrupting the fluorescence resonance energy transfer effect and yielding bright fluorescence, which can be detected using excitation wavelengths at 335–345 nm and emission wavelengths at 485–510 nm. The method optimized, tested by detecting the strong inhibiting effect of α1-antitrypsin on trypsin activity, has been developed on 384 multi-well plates in a volume of 10 μL, using an automated platform. From the screening of a chemical library, four compounds that inhibit trypsin activity with IC_{50}s in the micromolar range have been identified. Interestingly, the most active compound (M4) shows a chemical structure recapitulating that of other more potent inhibitors with thiourea and halogenated centers. Molecular docking studies show that M4 is a competitive inhibitor recognizing most residues within or nearby the catalytic pocket.

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Abbreviations

FRET	Fluorescence resonance energy transfer
HTS	High throughput screening
EDANS	5-[(2-Aminoethyl)amino]naphthalene-1-sulfonic acid
Dabcyl	(4-(4-Dimethylaminophenylazo)-benzoic acid

Introduction

Serine proteases (SP) belong to a large family of protein-cleaving enzymes playing an essential role in processes like blood coagulation, apoptosis and inflammation [1]. SP signaling pathways are tightly controlled at transcriptional as well as post-translational levels by the temporally and spatially controlled expression and activation of a number of specific endogenous peptide-protein inhibitors/modulators [2]. SP activity deregulation is generally due to failures of the signaling system and leads to severe disorders which must be therefore treated by supplementing the patients with the defective protease or specific inhibitor [1–3]. Consequently, proteases have emerged as promising targets for drug discovery for a wide variety of human diseases, including cancer, neurodegeneration, ischemic diseases, inflammation and infectious diseases [4–6]. Very well-known examples of therapeutic application of SPs are the intravenous administration of tissue plasminogen activator (t-PA) to improve clinical outcomes in patients with acute ischemic stroke [7], and intravenous infusion of recombinant Factor IX (FIX) for the prophylaxis and treatment of

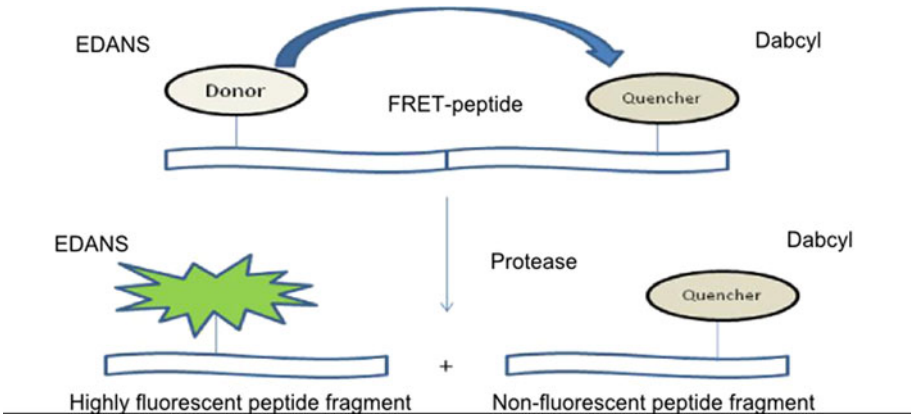

Fig. 1 The principle of FRET-based enzyme assays. In the intact FRET peptide, fluorescence is quenched. After cleavage into two separate fragments by trypsin, fluorescence is restored and can be detected as a function of enzyme and substrate concentration

hemophilia B patients [8]. On the other hand, a canonical example of therapeutic treatment by using SP inhibitors is the administration of α1-antitrypsin, the most potent endogenous elastase inhibitor, which is defective in individuals carrying the homozygous ZZ mutation and whose reduced blood concentration leads to the onset of emphysema [9].

Excessive proteolysis can be prevented or modulated by blocking the appropriate proteases also by synthetic inhibitors, therefore this area is widely explored by pharmaceutical companies which need an ever increasing number of lead compounds for developing more effective and safer drugs. Screenings are often carried out on thousands or millions of chemical compounds, therefore, under these circumstances, traditional techniques like gel-based assays, filter-binding assays, or enzyme-linked immunosorbent assays (ELISAs) must be replaced by assays amenable to automation in order to simplify and speed-up laborious and time-consuming steps such as washings and separations.

Well-established and widely used assay techniques matching these needs are scintillation proximity (SP and flash plate), fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), and Alpha screen assays. All of these are homogeneous mix-and-measure techniques that allow the one-pot addition of all reagents without the need for separation steps. In screening large chemical libraries, cost pressure is an issue that drives the field towards automatization and miniaturization. The 96-well format is no longer able to meet the needs of industrial research. 384- or 1536-well microplates have become standard, and to minimize reagent costs, assay volumes have been downscaled to 3–15 μL.

There are several examples of FRET-based assays that generate an "activated" fluorescence signal upon proteolysis. FRET-based peptide substrates of enzymes are highly versatile and sensitive tools for probing protease specificity and activity [10]; these are particularly useful for serine proteases since they have a requirement for specific amino acids on both sides of the cleavage site for substrate recognition.

One of the most commonly used pairs of fluorophores and quencher is EDANS and Dabcyl, owing to the excellent spectral overlap between the emission spectrum of EDANS and the absorbance spectrum of Dabcyl. The principle of FRET is the transfer of energy from an excited fluorescent donor to a suitable quenching acceptor molecule (Fig. 1). The energy transfer efficiency decreases exponentially by the distance between the two moieties, thus by cleavage of the interlinking peptide chain, the donor and the acceptor become separated, leading to an increase in fluorescence intensity, which is proportional to the rate of hydrolysis of the substrate peptides.

In this study, we have used trypsin as a model to develop one of these assays, because trypsin and trypsin-like proteases are good pharmacological targets due to their key role as mediators in a wide variety of human diseases. Trypsin was initially isolated from the pancreatic juice of animals, but it was later identified in many different tissues. Trypsin plays pivotal roles in food digestion as well as in cellular signal transduction mediated through proteolytic activation of proteinase-activated receptors (PARs) activation PARs [11]. Extrapancreatic trypsin and trypsin-like serine proteases have been detected in the brain, where they are involved in neural development, plasticity, and brain neurodegeneration [11].

We have identified a set of new inhibitors of trypsin by the screening of molecular repertoires using an automated FRET-based assay. The selected inhibitors have IC_{50}s in the low-medium micromolar range and, based on a molecular docking study, are predicted to have a competitive mechanism of inhibition.

Materials

Protected amino acids and activating agents for the synthesis of peptide substrates were from Novabiochem

(Laufelfingen, Switzerland and GL Biochem, Shangai, China). Piperidine, di-isopropylethylamine (DIEA), Dabcyl-OSu (4-(4-dimethylaminophenylazo)-acid-N-hydroxy-succinimide ester), sequence-grade porcine trypsin, acetonitrile and other unspecified reagents were from Sigma-Aldrich. Dimethylformamide (DMF) and trifluoroacetic acid (TFA) were from Romil, Dublin, Ireland). α1-antitrypsin was a kind gift of Prof. Charles Dinarello of the University of Colorado, Denver.

Methods

Synthesis of Fluorescent Substrates

Peptide substrates chosen for this study, bearing at the N- and C-terminal ends a pair of FRET donor/acceptors, Dabcyl-GGKGG-E(Edans) and Dabcyl-GGGGG-E(Edans), were synthesized using the Fmoc-chemistry.

Peptides were assembled by the solid phase method on a RINK amide resin following the standard Fmoc/tBu approach. HBTU and HOBt were used as coupling agents. Fmoc-Gly-OH and Fmoc-L-Lys(Boc)-OH were used to assemble the peptide chain after introduction of the C-terminal Fmoc-L-Glu(EDANS)-OH derivative. Fmoc deprotection was afforded by treatment with 40 % piperidine in DMF. After incorporation of the first residue, a Kaiser test was performed to prevent the generation of C-terminally deleted peptides. Following incorporation of the N-terminal Fmoc-Glycine, the protecting group was removed by extensive treatment with 40 % piperidine in DMF, then Dabcyl was incorporated onto the N terminus using a solution of Dabcyl-OSu in DMF at alkaline pH. Peptides were removed from the solid support by treatment with a solution of TFA/water (95:5, v/v) for 2 h at room temperature, then they were precipitated by adding cold diethyl ether, purified by semi-preparative RP-HPLC and finally characterized by LC–MS to assess purity and identity. MW (943.2 amu for the substrate and 872.3 amu for the Lys → Gly negative control). Peptide stock solutions were prepared at high concentration and stored frozen until use.

Assay Set-up

In order to set up the experimental conditions for the automated assay, several preliminary experiments were carried out at different enzyme-substrate ratios. All experiments were performed in 384 low-volume-well plates (well volume 20 μL) in a total volume of 10 μL. Experiments were run as quadruplicate and reported as averaged values ± standard deviation (SD).

Instruments for Liquid Handling and Plate Detection

A automated MICROLAB® STAR Liquid Handling Workstation from Hamilton Robotics (Bonaduz, Switzerland) was used to develop the screening assay. The workstation includes eight independent pipetting channels, several positions for sample tubes and plates, the barcode identification for samples, microplates, reagents and carrier and a robotic arm to connect the workstation to the Biotek Senergy 2 multi-well reader (Winooski, VT, USA) equipped with suitable optical filters. The liquid handler was implemented with the Total Aspiration and Dispense Monitoring (TADM) system, with the Liquid Level Detection system and the Tip Attachment (CO-RE) system. In all procedures, the Monitored Air Displacement (MAD) system was used. All workstation functions and integrated third-party devices were controlled by the Venus software (Hamilton Robotics, Bonaduz, Switzerland).

Automation

For assays on full 384-well plates, a trypsin solution (2.2 mL) in Reaction Buffer (Tris–HCl 20 mM, DTT 10 mM, pH = 8) was prepared at a concentration twice compared to the final concentration used in the assay. The peptide substrates were prepared by diluting stock solutions up to a concentration 4-fold higher than that of the final solution used in the assay. Solutions of inhibitors (200 μL) were prepared in 96 well plates (mother plates) at a 4 × concentration in the Reaction Buffer.

The procedure consisted of just few steps:

(1) Dilution of compounds from the stock solution (in DMSO) in the reaction buffer in the mother plate.
(2) Dispensing 2.5 μL of buffer or inhibitor solution into 384-wells of white OptiPlate-384
(3) Addition of 2.5 μL of peptide substrate or negative control
(4) Addition of 5.0 μL of diluted enzyme
(5) Incubation for 20 min at room temperature (RT) in the dark
(6) Reading over time fluorescence, using a 330 ± 80 nm excitation filter and a 460 ± 40 nm emission filter

Z′-Factor Calculation

Commercial porcine trypsin, at final concentration of 120 nM, was added in 384-well plates in which substrates were already dispensed following the protocol reported before, and fluorescence was recorded after 20 min. Z′-factor was calculated as per Zhang et al. [12].

The assay was performed also in the presence of increasing amounts of DMSO to simulate the experimental conditions whereby compounds from stock solutions in this solvent are submitted to screening. The chosen concentrations of DMSO were 1, 2, and 5 % which simulate dilutions of 5.0 mM stock solutions in neat DMSO (a worst case, since most compounds are typically soluble in this solvent at higher concentrations) up to 50, 100, and 200 μM. The Z′-Factor was determined at 0, 1, 2, and 5 % DMSO concentration on 120 independent data points for each condition.

IC_{50} Determination

Dose-dependent assays with the known inhibitor α1-antitrypsin and with the hit inhibitors, were performed at concentrations ranging between 1 and 100 nM (α1-antitrypsin) and between 1 and 200 μM (selected inhibitors). For all these assays, each data point was in quadruplicate. Experimental data were fitted with GraphPad Prism, vers. 4.00, GraphPad Software (San Diego, California).

Molecular Modeling

Molecular docking simulations were performed using AutoDock version 4.2 [13]. The 1.2 Å crystal structure of porcine pancreatic trypsin was downloaded from the Protein Data Bank (PDB ID: 2A31) [14]. The ligand and solvent molecules were removed from the crystal structure to obtain the docking grid. The three-dimensional (3D) structure of the ligand was built by means of MarvinSketch (http://www.chemaxon.com/marvin/sketch/index.jsp) and minimized into the optimal conformation. The AutoDockTool was applied to prepare ligand in docking format. Gasteiger atomic charges were assigned and the flexibility of the molecule was determined using the AutoDock module AutoTors. All torsion angles of the ligand were defined so that they could be explored during the docking process. The protein coordinates were fixed during docking simulations, while the ligand was flexible and moved on the grid as implemented in AutoDock. Non polar hydrogens, including their partial charges, were merged to parent atoms. The atomic solvation variables were assigned by the AutoDock module AddSol and the grid map representing the protein in the docking process was calculated by means of AutoGrid. Docking experiments were carried out by selecting the grid to be large enough to include the entire trypsin protein and in order to provide sufficient space for ligand translational and rotational movement. As a consequence, the dimension of grid map was 110 Å × 118 Å × 126 Å with a grid-point spacing of 0.375 Å. Grid searching was performed using the Lamarckian genetic algorithm. Two hundred independent docking runs were performed to find the best conformation and orientation of ligand, based on the binding energy and the number of energy evaluations was set to 25 × 106. Resulting conformations differing by less than 1.0 Å in positional root-mean-square deviation (rmsd) were clustered together. The conformation with the lowest free energy of binding was taken as the representative of the most populated cluster and it was used to analyze ligand placement in the binding site. Binding free energy (ΔG) and binding constants (Ki) were calculated and estimated within the AutoDock scoring function. Detailed analyses of the ligand–receptor interactions were carried out and final coordinates of the ligand and receptor were saved as pdb files. The obtained complex was used for interpretation of the potential bioactivities of the ligand. Molecular graphics images and structure preparation were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (UCSF Chimera visualization system for exploratory research and analysis [15].

Results

Design, Synthesis, and Characterization of Serine Proteases Substrate

A peptide Dabcyl-GGKGG-E(EDANS), hereafter named only G2KG2, used as substrate of trypsin and a peptide Dabcyl-GGGGG-E(EDANS), hereafter only G5, used as negative control in the assays, were easily prepared in high yield and with high purity (>95 % by LC–MS). The N-terminal Dabcyl dye is an acceptor of the fluorescence emitted at 460 nm by the C-terminal EDANS, introduced on the side chain of a glutamate residue, when it is excited at a wavelength of about 340 nm. The intact molecule is therefore internally quenched, but EDANS fluorescence is readily restored upon cleavage within the peptide sequence. The presence of the lysine confers to this substrate a strong selectivity for enzymes like trypsin, thrombin or plasmin [16]. The peptide length and sequence were chosen in order to have an optimal distance between the two fluorophores, thus a maximum quenching effect, and at the same time to maximize chain flexibility, which renders the internal lysine more accessible to proteolysis by enzymes (Fig. 1). A peptide where the central lysine was replaced by glycine was also designed and prepared and was used as negative control. Typical yields for the synthesis of both peptides were in the order of about 10 %. We tested the FRET-assay for linearity, dynamic range, sensitivity, and stability using porcine trypsin and the synthetic substrates.

Results showed that the enzyme efficiently accepted the EDANS/Dabcyl-labeled peptide as a substrate. Hence the assay could be used to detect trypsin activity.

Evaluation of Trypsin Activity

In order to set up the optimal assay conditions, time course experiments were performed with substrate concentrations between 1 and 8 μM and with enzyme/substrate ratios of 1:100, 1:50, and 1:25. The reaction was monitored for at least 120 min, although plateaus were reached much earlier, about 20 min. Complete cleavage of the substrate was assessed also by LC–MS analysis of the products (not shown). The negative control was used at the highest concentration in each experiment. Results are summarized in Fig. 2a–c, where time courses for all the experiments are reported. As shown in Fig. 2d, the reaction was linear for about 15 min, quite fast and virtually complete after 25–30 min under all experimental conditions. As expected, a higher fluorescence emission was generated at increasing substrate concentrations and enzyme-substrate ratios. Optimal conditions for the assay, in terms of signal-to-noise (S/N) values, reaction time and amount of substrate were identified as follows: substrate concentration 6.0 μM, trypsin 120 nM (1:50 enzyme:substrate molar ratio), reaction time 20 min. Under these conditions, an overall signal of around 3000 relative fluorescence units (RFU) (Fig. 2b) was consistently detected. Considering the reaction volume, 240 pmol (226 ng) of substrate and 4.8 pmol (110 ng) of enzyme were used to generate a quadruplicate data-point.

Assay Procedure Automation and Validation

The assay procedure described for the manual experiments, was next automated using an integrated platform comprising a 8-channel Starlet station for liquid handling, a robotic arm and a multi-well plate reader (Biotek). Optimal assay performances were evaluated in terms of final Z′-factor with and without added DMSO. To assess the intra-plate reproducibility and select the optimal conditions for the screening of random libraries dissolved in stock solutions in DMSO, Z′-Factors in the presence of different DMSO amounts were determined on 120 individual data points for each condition. As shown in Fig. 3, an excellent Z′-factor of 0.881 was determined without DMSO and in the presence of 1 % solvent, whereas a progressive decrease of the value was observed increasing the DMSO concentration. However, also the worst value obtained in the presence of 5 % DMSO (0.76) was sufficiently high to enable optimal performances in the subsequent screenings. To further validate the automated assay, a dose–response experiment was carried using α1-antitrypsin as known inhibitor and results are shown in Fig. 4. It can be seen that

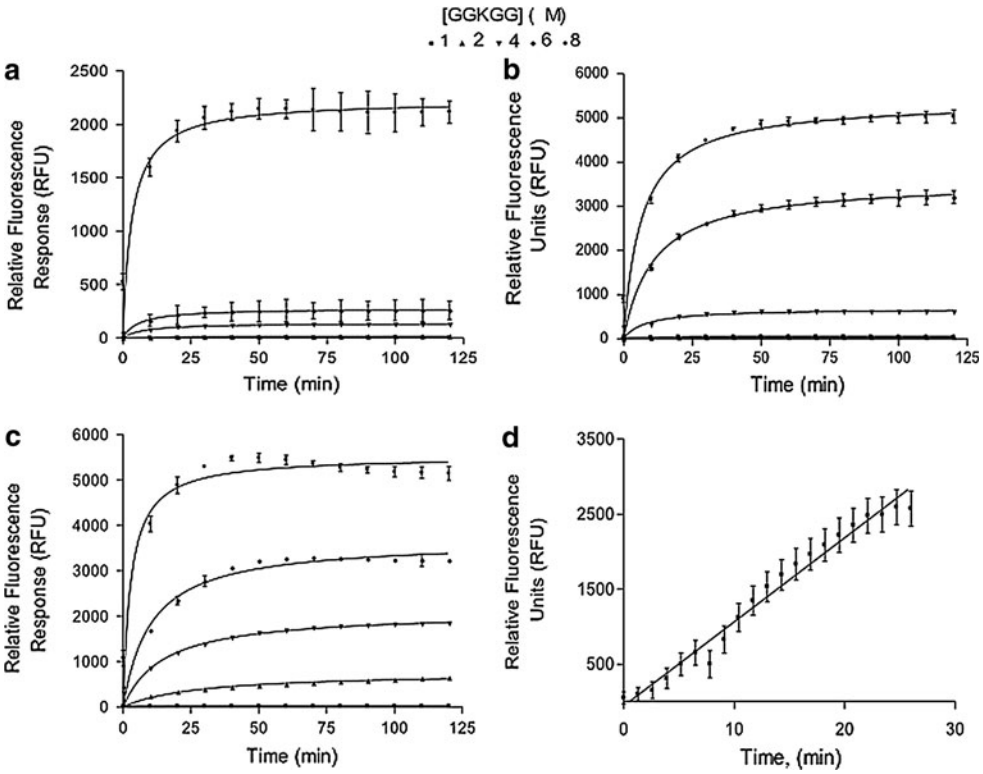

Fig. 2 Assay set-up. The peptide substrate was incubated with trypsin in low-volume (10 μL) 384-well plates at different concentrations for 120 min. Fluorescence emitted at 460 nm was monitored upon excitation at 330 nm. A fluorescence signal is generated when the substrate is cleaved. **a** Time- and dose-dependent assays carried out using the peptide substrate at different concentrations and the trypsin at 1:100 molar ratio respect to substrate. **b** Time- and dose-dependent assays using trypsin 1:50 molar ratio respect to substrate. **c** Time- and dose-dependent assays using trypsin 1:25 molar ratio respect to substrate. **d** Time-dependent assay (∼30 min) using trypsin 1:50 molar ratio and 6 μM of substrate

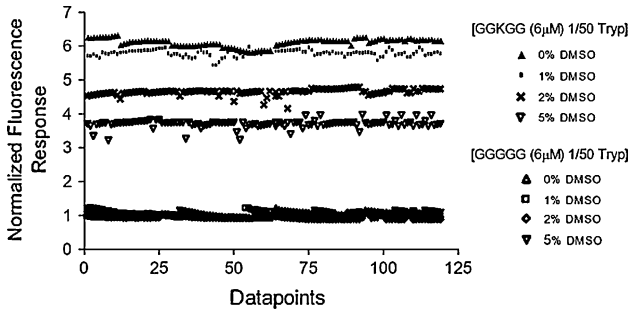

Fig. 3 Z′-Factor. Determination of the Z′-Factor for the automated assay under the optimized conditions of signal-to-noise ratio (S/N). The factor has been determined on 94 data points. The same assay performed on 94 data points in the presence of 1, 2, and 5 % DMSO. The conditions were: 10 μL reaction volume, 6 μM of peptide substrate, 120 nM trypsin (1:50). The reaction was conducted at RT for 20 min

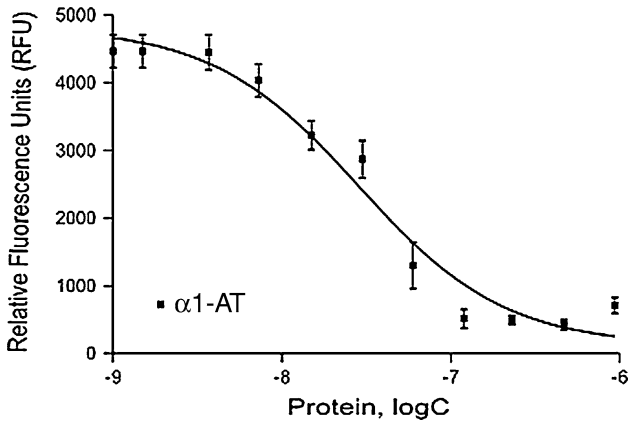

Fig. 4 Assay validation. α1-antitrypsin inhibition of trypsin activity. In a 10 μL reaction volume, 6 μM of peptide substrate, 120 nM trypsin and increasing amounts of α1-antitrypsin were used at RT for 20 min

quadruplicate data points were all very reproducible with very low SD and an apparent IC_{50} of 29 nM was determined for this covalent inhibitor.

Library Screening and Hit Identification

The optimized assay was finally used to screen a small, unbiased library of small molecules to select some hits working as trypsin inhibitors, which could be further optimized in the search of more potent and selective compounds. The library contained 500 commercial small molecules (MW < 500 Da) arrayed in 96-well plates as single compounds at 5 mM in DMSO. Each plate contained 94 compounds and two wells with pure DMSO, which were used as negative (no substrate) and positive controls (no inhibitors, 100 % substrate hydrolysis). Compound quality was ensured by the vendor as being greater than 90 % pure, with provided quality control data; this was further verified by HPLC or HPLC–MS analysis on around 20 % randomly sampled compounds. Plates were manipulated by the liquid handler and samples directly diluted into the 384-well assay plate. The automated screening was performed at three different concentrations of small molecules (50, 100, and 200 μM) using 6 μM of substrate and 120 nM trypsin. The reaction was conducted for 20 min at room temperature (RT) in the dark. Data were manipulated to average quadruplicates and to calculate standard deviations.

In Fig. 5a, data obtained from one of the plate tested using the compound at 100 μM are reported as bar plot with included ± SD. From this screening, a number of positive hits were identified and those affording more than 60 % inhibition at 100 μM were selected. Four compounds, denoted as M1, M2, M3, and M4 were submitted to a dose–response assay, together with a negative control. Inhibition curves for the best 4 compounds are reported in Fig. 5b, whereas in Fig. 5c their structures are shown. It is shown that, while the negative control showed no inhibition, the four positive hits reduced the activity in a dose–response manner. Compound M4, (E)-*N*′-(5-bromo-2-oxoindolin-3-ylidene)morpholine-4-carbothiohydrazide, exhibited the lowest IC_{50} (52 μM).

Docking Experiments of M4 into the Trypsin Structure

Docking experiment of M4 into the trypsin structure very clearly showed that the compound can bind into the S1 specificity subsite of the trypsin active site (Fig. 6a). Ser195, His57, and Asp102 (chymotrypsin numbering used) form the catalytic triad of this serine protease. Interestingly, the experiment produced few (ten) putative binding modes and a clustering of solutions (166 out of 200 solutions essentially identical) was found near the catalytic site. The best-docked conformation, i.e. the one with the lowest estimated free energy value, is also the conformation representative of the most populated cluster and we used it to analyze ligand placement in the binding site. We have measured the predicted binding constant (Ki) derived from AutoDock algorithm for this conformation resulting a binding free energy of −8.8 kcal/mol and a theoretical binding constant equal to 311.76 nM. Docking results showed that M4 compound fits well the active binding site of trypsin and it interacts with the catalytic amino acids Ser195 and His57. In particular, the NH group belonging to the indolinonic cycle can establish two hydrogen bonds with both the His57 Nε2 and the Ser195 hydroxyl group (Fig. 6b). Furthermore these interactions are present in all poses of the best cluster and they are stabilized by another hydrogen bond between the thiocarbonylic sulfur atom of M4 and nitrogen atom of Gly216 backbone. In this binding

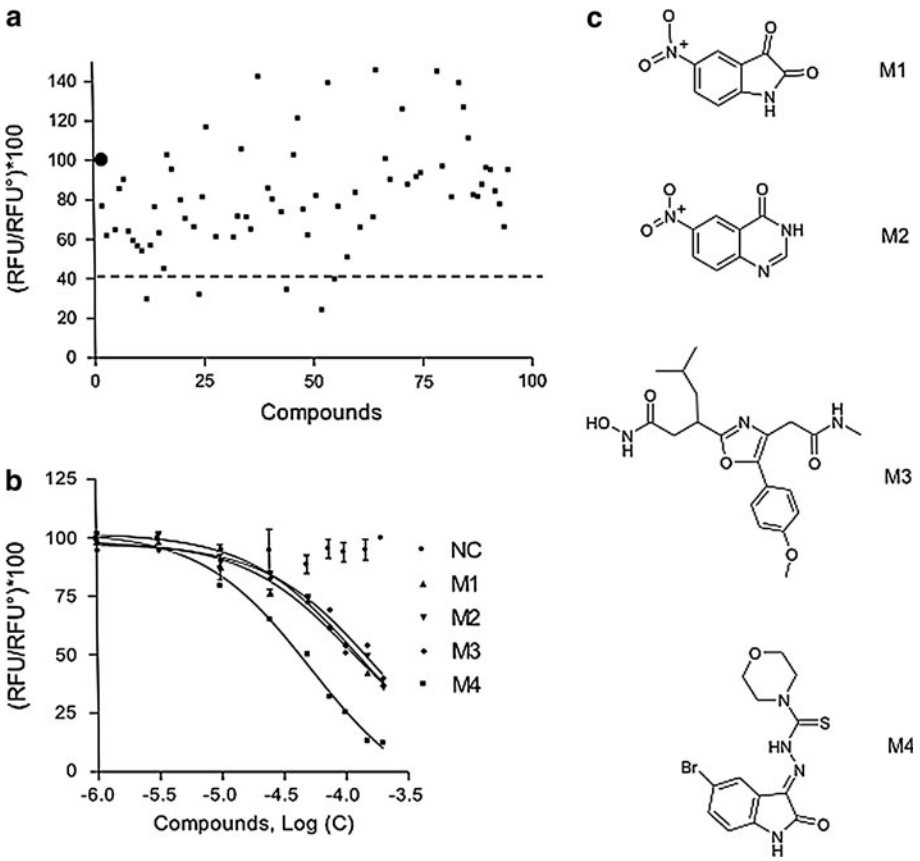

Fig. 5 Inhibition experiments. **a** Plot of data deriving from the screening of 94 compounds (one 96-well plate) using the automated assay. The assay was performed with compounds at 50, 100, and 200 μM (here only data from those at 100 μM are shown). The inhibition effect of compounds were shown by plotting (RFU/RFU°)*100, where RFU is the average relative fluorescence units from the quadruplicate for a given competitor and RFU° is that determined without competitors. The symbol *filled circle* correspond to 100 % of trypsin activity. **b** Dose-dependent assays with four selected hits, denoted as M1, M2, M3, M4, and a negative control (NC), selected among those having no activity at 200 μM. Data were fitted and the IC_{50} determined by non-linear regression analysis. The most active compound, exhibiting the lowest IC_{50} was M4. **c** Structure of molecules M1, 5-nitro-2,3-dihydro-1H-indole-2,3-dione (IC_{50} = 116 μM); M2, 6-nitro-3,4-dihydroquinazolin-4-one (IC_{50} = 121 μM); M3, *N*-hydroxy-3-[5-(4-methoxyphenyl)-4-[(methylcarbamoyl)methyl]-1,3-oxazol-2-yl]-5-methylhexanamide (IC_{50} = 171 μM); M4, (E)-*N*′-(5-bromo-2-oxoindolin-3-ylidene)morpholine-4-carbothiohydrazide (IC_{50} = 52 μM). Dose-dependent inhibition of selected compounds were showed by plotting (RFU/RFU°)*100 versus the logarithm of molar concentration of added molecule

mode, the docked compound fully occupy the narrow and well-defined catalytic pocket (Fig. 6c) with also other Van der Waals contacts to the enzyme (Gly96, Asn97, Leu99, Gln192, Cys191, Ser214, Trp215) (Fig. 6b). These in silico predictions are in line with experimental findings, indicating that M4 should slot into the trypsin active site cleft. Furthermore, potentially significant hydrophobic and hydrogen bonding interactions involving catalytic residues have also been indicated by docking studies.

Discussion

We have identified new trypsin inhibitors developing a simple mix-and-read HTS assay. The assay is based on a new peptide substrate bearing a well-known FRET pair (Dabcyl-EDANS), which upon cleavage by the target enzyme, gives rise to an intense and persistent fluorescence signal proportional to the extent of cleavage. Although many different fluorescent substrates for trypsin and other serine proteases are commercially available, the one we report here, can be very easily prepared using a straightforward solid phase method. Furthermore, the use of FRET-based substrate probes, due to the specific combination of wavelengths that generates or inhibits fluorescence, strongly suppresses interferences by compounds having similar spectroscopic properties. Also in the presence of compounds having absorbing spectra similar to that of the acceptor fluorophore, an effective fluorescence suppression or enhancement occurs only at very high concentrations. Given its simplicity, the assay could be rapidly adapted to the screening of inhibitors of other enzymes and for this purpose only the internal peptide sequence should be redesigned and reaction parameters optimized.

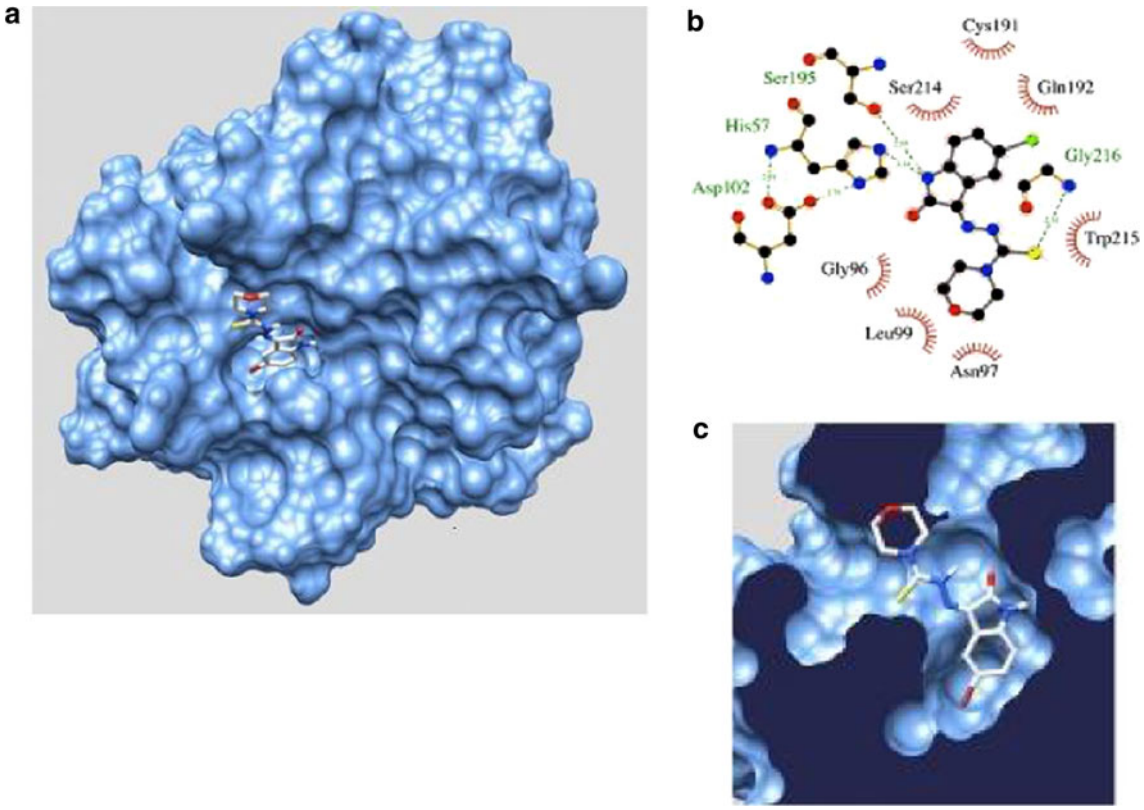

Fig. 6 Docking experiments. **a** Binding mode of compound M4-docked in the active site of porcine trypsin showing the full occupancy of S1 specificity subsite. **b** M4 compound interactions into the active site shown are those mediated by hydrogen bonds and by hydrophobic contacts. Hydrogen bonds are indicated by *dashed lines* between the atoms involved, while hydrophobic contacts are represented by an *arc* with spokes radiating towards the ligand atoms they contact. The contacted atoms are shown with *spokes* radiating back. **c** A protein *surface slab view* to get a better view of the ligand binding mode inside the S1 specificity pocket

The assay has been also scaled to a high throughput format using an integrated platform whereby all reagents and compounds submitted to screening are handled in complete automation. All steps related to dilution of compounds and reagents, of assay preparation and fluorescence reading have been indeed performed using an automated station and the associated devices.

Automation has been first validated performing the dose–response inhibition by α1-antitrypsin (a well-known suicide trypsin inhibitor) and assessing that very low SD values are achieved under the assay conditions. Next, a library of 500 commercial small molecules has been screened, determining Z′-factors under different conditions. Remarkably, all compounds have been tested in one day, since the screening of one single plate with 94 compounds takes about one hour, including the operations of plate preparation, incubation and reading. With this throughput and assuming 20 working days in a month, virtually 10.000 single compounds per month could be tested in quadruplicate.

Our test screening has led to the identification of four active hits, reported in Fig. 5c. Among these, the most active M4 contains a thiourea moiety on one side and an indole ring on the other, two sub-structures involved in strong interactions with the catalytic groups of Ser195 and His57 and with the amide group of Gly216. Notably, several serine protease inhibitors have a triazole urea core, which mostly resemble the indolic and thiourea groups of M4. We could therefore deduce that the interactions predicted by the docking study, especially the one involving the indolic nitrogen, could be conserved with other similar inhibitors, and could involve one or more nitrogen atoms harbored by known triazole [17] or tetrazole inhibitors [18, 19]. Although we have not structurally investigated compounds M1, M2 and M3, we can hypothesize that, at least the dihydro-indolinonic and dihydroquinazolic rings of M1 and M2, could fit well within the trypsin catalytic pocket, likely establishing similar interactions with the serine and histidine side chains.

In conclusion, we have identified new trypsin inhibitors automating a FRET-based screening assay. The method is very reproducible and robust with an optimized Z′ factor calculated over more than 120 data points of 0.881. When applied to 384-well plates with a 10-μL reaction volume,

the time for assaying 94 different compounds in quadruplicate is 20 min. Compounds identified from an unbiased library of small molecules, have IC_{50}s in the μM range and structures similar to those of other organic serine protease inhibitors.

Since new probes for other proteases can be rapidly accessed by simply changing the internal peptide sequence and the assay procedure can be easily automated, the approach we propose can be readily extended to the screening of inhibitors of other pathologically relevant peptidases.

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