Targeting furin activity through in silico and in vitro drug repurposing strategy for SARS-CoV-2 spike glycoprotein cleavage repression

Bruno O. Villoutreix (bruno.villoutreix@inserm.fr)
Univ. Lille, Inserm, Institut Pasteur de Lille

John Creemers
KU Leuven

Yannick Léger
Univ. Bordeaux

Geraldine Siegfried
Univ. Bordeaux

Etienne Decroly
Aix Marseille Univ.

Serge Evrard
Bergonié Institute

Abdel-Majid Khatib (majid.khatib@inserm.fr)
University Bordeaux

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Abstract

In December 2019, a new coronavirus was identified in the Hubei province of central china and named SRAS-CoV-2. This new virus induces COVID-19, a severe respiratory disease with high death rate. The spike protein (S) of SARS-CoV-2 contains furin-like cleavage sites absent the other SARS-like viruses. The viral infection requires the priming or cleavage of the S protein and such processing seems essential for virus entry into the host cells. Furin is highly expressed in the lung tissue and the expression is further increased in lung cancer, suggesting the exploitation of this mechanism by the virus to mediate enhanced virulence as shown by the higher risk of COVID-19 in these patients. In this study, we used structure-based virtual screening and a collection of about 8,000 unique approved and investigational drugs suitable for docking to search for molecules that could inhibits furin activity. Sulconazole, a broad-spectrum anti-fungal agent, was found to be of potential interest. Using Western blot analysis, Sulconazole was found to inhibit the cleavage of the cell surface furin substrate MT1-MMP that contains two furin cleavage sites similar to those of the SARS- CoV-2 spike protein. Sulconazole and analogs could be interesting for repurposing studies and to probe the yet not fully understood molecular mechanisms involved in cell entry.

Introduction

In December, 2019, a new virus belonging to the human coronavirus family was identified in the Hubei province of central china and named SRAS-CoV-2[1]. Although the identity of the source of zoonotic infection is not yet confirmed, it is likely that this emerging virus result from a recombination between coronavirus from bat and pangolins. SRAS-CoV-2 mediates severe human respiratory disease with high death rate[2]. According to WHO Coronavirus Disease 2019 situation report of March 29, 2020, up to 634 835 patients worldwide, in 202 countries, or territories had tested positive for COVID-19 with 33,529 people deaths. These values are still increasing and to date there is no vaccine or validated antiviral treatments.

The coronaviruses are a group of enveloped viruses with positive-sense RNA. These viruses belong to the family of Coronavirinae, order Nidovirales[3] comprising of four genera namely alpha, beta, delta, and gamma[2]. These viruses are responsible for a wide range of neurological systems, liver, hepatic and respiratory acute and chronic diseases. Prior to present crisis, only six human coronaviruses (HCoVs) have been known to mediate infection in human and induce respiratory diseases[2,3]. Of these, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are the highly pathogenic coronaviruses able to infect the lower respiratory tract. The other four Coronavirus namely HCoV–229E, OC43, NL63, and HKU1 are associated to upper respiratory infections and common cold[2,3]. Most enveloped viruses encode for viral envelope glycoproteins, synthetized in an immature polyprotein precursor. These proteins require proteolytic cleavage before they can mediate viral entry into host cells. In many aspects, viruses take advantage of cellular proteases for this key function[4]. Indeed, during viral infection, the reliance on particular proteases is a determinant

factor for viral infection and spread. While several viruses mediate local infections due to the limited expression of their host proteases in a small number of cell types and/or specific tissues such as the case of the low pathogenic avian influenza A viruses, the highly pathogenic virus uses furin-like enzymes that are ubiquitously expressed to cleave influenza A virus hemagglutinin (HA) leading to high viral spread and in turn cause higher rates of mortality[5]. Thus, the ability of viruses to exploit furin-like enzymes affects the cell tropism and the virus pathogenicity. Previously, furin-like proteases or pro-protein convertases (PCs) were reported to be involved in the conversion to their bioactive forms of a large majority of secretory proteins synthesized as inactive protein precursors. These include growth factors, receptors, adhesion molecules, matrix metalloproteinases and viral envelope glycoproteins[6,7]. Precursors are usually cleaved at the general motif (K/R)- $(X)_n$-$(K/R)^-$, where n= 0, 2, 4 or 6. To date, one or more of the seven known pro-protein convertases (PCs) family has been implicated in these processes, namely, furin, PC1, PC2, PC4, PACE4, PC5 (and its isoform PC5-B), and PC7[7–9]. Previous studies however showed that viral glycoproteins activation including those of several coronaviruses is mediated by secreted furin-like enzymes that proteolytically process monobasic or multi-basic cleavage sites[4].

Proteolytic cleavage of viral envelope glycoprotein by furin-like enzymes into a functional binding virus receptor and a fusogenic transmembrane protein is central for the mediation of virus cell entry and infectivity of the dengue virus[8], respiratory syncytial virus (RSV)[9], HIV[10], human papilloma virus[11] and Chikungunya[12]. Although the viral glycoproteins are processed at specific cleavage site, the subcellular localization of the cleavage by furin-like enzymes and the time course of the cleavage vary between viruses. Furthermore, proteolytic activation of viral glycoproteins can occur at different steps of the viral replication cycle due to the ability of these glycoproteins to transit thought the Golgi network during virus production where converting enzyme like furin are enriched. Some viral envelope proteins can also meet several furin-like enzymes in the extracellular space or during the virus entry into the endosome where the envelope protein can be processed. In coronavirus, the viral glycoprotein responsible for cell entry is the spike (S) protein[13-17]. It is processed at two different cleavages sites[13] by different proteases that drive the viral tropism. The S protein is synthetized as a protein precursor transiting through the endoplasmic reticulum-Golgi apparatus intermediate compartment (ERGIC). For some coronaviruses such as MERS-CoV and probably SARS- Cov-2, which contain a furin-like cleavage site between S1 and S2, the protein can be cleaved into S1 and S2 in the Trans Golgi Network (TGN) in cells expressing high level of furin. This priming process can also involve cell surface proteases belonging to the transmembrane protease/serine subfamily member (TMPRSS) family, which is highly, expressed in the lungs[18]. The two viral subunits resulting from priming have distinct functions. The SARS- CoV (1 & 2) S1 subunit contains the angiotensin-converting enzyme 2 (ACE2) receptor binding domain. The S2 subunit ensures membrane fusion after a second proteolytic cleavage at a the S2’ cleavages site, upstream of the fusion peptide. The fusion which releases the nucleocapsid inside the infected cells, depends on a conformational change of the S2 protein subunit and occur either at the plasma membrane or in the endosome depending of the protease availability. Sequence analysis of the spike protein of the coronaviruses, MERS- CoV[19], HCoV-OC43[20] and HCoV-HKU1[21] reveal the presence of a canonical furin-like cleavage site between S1/S2 and

at the S2' cleavage site[22] **(Figure-1)** . Similar furin-like cleavages sites were identified in the SARS-CoV-2 spike protein sequence[14] **(Figure-1)**. Thereby, the high expression of furin and other furin-like enzymes found in human lung, liver and brain tissues[7,23] may be exploited by the SARS-CoV-2 for the activation of S protein leading to enhanced infection, virulence and spread of the virus. Compounds interfering with the cleavage of the SARS-CoV-2 S protein processing could be a valuable antiviral approach. In the current report, we used structure-based virtual screening and several structural bioinformatics tools[24] to identify approved and investigational drugs as putative inhibitors of furin.

Methods

Structure-based virtual screening

In order identify approved drugs or advanced molecules acting against furin, virtual screening computations were carried out. We first generated a hand-curated database of approved and investigational drugs (small molecules). Over 20,000 non-unique approved and investigational compounds (experimental molecules were not included initially as we were interested in molecules that are approved or have entered clinical trials) were first downloaded from the last released of DrugBank[25], DrugCentral[26], and SWEETLEAD[27]. 17449 additional molecules were extracted from Wikipedia Chemical Structures using utilities implemented in the DataWarrior package[28]. Molecules were flagged using our FAF-Drug server[29] to remove compounds with inorganic atoms and molecules with unwanted toxicophores as reported in a previous study but keeping molecules even if they could not be found in commercial vendor catalogs[30]. Salts and duplicates were also removed. Manual inspection took place, further guided by the DataWarrior Drug-likeness scores and several others computed physicochemical properties. Further, only molecules that could be docked with a reasonable chance of success were kept (e.g., docking accuracy decreases when molecules are too flexible, thus we kept molecules with less than 20 rotatable bonds and with a MW below 900 Da). We obtained a final collection of about 8,000 molecules acting in different therapeutic areas in 2D that were generated in 3D and protonated using the Surflex tools[31]. All compounds were docked with the 2019 version of Surflex-Dock[32] (pgeom option to explore in depth the catalytic site) into the furin X-ray structure co-crystallized with a peptide-like inhibitor[33] (PDB entry 5jxh) or co-crystallized with a small chemical compound[34] (PDB entry 5mim). The protein structures were prepared with Chimera[35] (water molecules, unwanted heteratoms and inhibitory molecules were removed) while exploration of the protonation states of the titratable residues was performed with our server PCE[36]. A short energy minimization of the 3D protein structures was then carried out priori to virtual screening.

Effect of identified molecule on cell surface furin substrate cleavage

Cells were incubated with the selected molecule and the maturation of MT1-MMP, a cell surface substrate that contains two cleavage sites of furin similar to the ones of the S protein[7], was analysed in cell lysates that were subjected to immunoblotting analysis as previously described[6,7].

Results

Of the identified molecules with high and intermediate Surflex docking scores (e.g., an estimation of binding affinity) and favourable non-covalent interactions in the catalytic site of furin as judged by interactive structural analysis, Sulconazole, a broad-spectrum anti-fungal agent, was unexpected (as not a known protease inhibitor) and found to be of potential interest. Sulconazole is thought to inhibit the fungal cytochrome P-450 isoenzyme C-14-alpha demethylase. At high concentration (often around 100 microM and above), Sulconazole was found to aggregate in some assays while several closely related antifungal analogues such as Fluconazole were not[37]. This drug and several related analogs do not however seem to be promiscuous at reasonable concentration. The molecule was indeed found to inhibit specifically a protein-protein interaction involving the WW domains of cellular ubiquitin ligases of the Nedd4 family and the PPxY motif of the adenoviral capsid protein VI[38]. Further, the molecule could also interact with other protein targets as it has been shown to impede rhodopsin (GPCR) dimerization in a dose-dependent manner[39] and to moderately inhibit the activity of heme oxygenases[40]. A potential binding pose for Sulconazole in the catalytic site of furin is shown in **Figure 2**. The docked compound and a co-crystallized inhibitory peptide (PDB entry 5jxh) displaying a basic residue (eg., arginine) at the P1 position can be seen (**Figure 2**). As Sulconazole does not display a positively charged group at this position, we thought to investigate different proteases such as to gain additional knowledge over our docked poses. We for instance used the crystal structure of human coagulation factor Xa serine protease (SP) domain in complex with the approved anticoagulant drug Rivaroxaban[41]. Many proteases have substrates or inhibitors with a positively charged P1 residue that makes favourable interactions with the negatively charged D189 (chymotrypsinogen numbering) at the bottom of the S1 specificity pocket **(Figure 3)**. Rivaroxaban displays at this position a chlorothiophene moiety that interacts strongly with a Tyr residue (Y228), and as such a highly basic P1 group such as amidine (arginine-P1 mimetics) is not required, enabling high potency and good oral bioavailability in contrast to molecules having a positively charged P1 group. By comparison, the 4-chlorophenyl-P1 moiety of Sulconazole could bind to the S1 pocket and replace the positively charged benzamidine group seen in the X-ray structure of furin complexed with a peptide-like inhibitors by making favourable interactions with the aromatic residue W291 of furin. This could mimic similar interactions seen between FXa and Rivaroxaban **(Figure 3)**. Further, the imidazole P2 moiety of Sulconazole could also have electrostatic interactions with the conserved D154, somewhat like the arginine P2 residue of the peptide co-crystallized with furin. In addition, the peptide hydrophobic valine P3 residue of the inhibitor co-crystallized with furin would be here replaced by the hydrophobic 2,4 dichlorophenyl P3 moiety of Sulconazole. A binding score between Sulconazole and furin was re-computed after energy minization with different tools including the MolDock package[42] and found to be around -143 kcal/mol (dominated by favourable steric interactions with a small contribution from electrostatic interactions) and about -200 kcal/mol between furin and the modified peptide (the predicted score is better as the peptide is much larger than Sulconazole and makes

many more interactions) while the score between FXa and Rivaroxaban using the same protocol was found to be around -152 kcal/mol (again Rivaroxaban is bigger than Sulconazole and makes more contacts). Scores between targets cannot be directly compared, but by taking into account these values and the structural analysis mentioned above, we expected that Sulconazole could inhibit furin.

To evaluate the ability of Sulconazole to inhibit cellular furin substrate maturation, we directly analysed in cells the cleavage of a well-established furin substrate MT1-MMP[7] that contains two cleavage sites for furin using Western blot analysis. As illustrated in **Figure 4**, Sulconazole inhibits the cleavage of MT1-MMP, as assessed by the accumulation of its unprocessed form (63 KDa) and reduction of the mature form (60 KDa).

Several approved, investigational and experimental Sulconazole analogs are known and some are shown in **Figure 5**. These compounds could be further investigated with regard to the inhibition of furin and the cleavage of the SARS-CoV-2 S protein.

Discussion

The recent pandemic of the SARS-CoV-2 proves the complexity of responding to infection diseases. Although considerable progresses were made in various fields of medicine and virology, the emergence of COVID-19 revealed that we were not prepared to take rapid actions so as to reduce the impact of the emerging infection. There are growing evidences that the proteolytic activation of fusion proteins used by coronaviruses for their entry into host cells participate to the virus spreading and favours the virus dissemination in different cell types and species[43]. The SARS-CoV-2 and the previously reported MERS-CoV viruses, that are to date the only viruses with two optimal furin cleavage sites (**Figure 1b**), reinforce seriously this concept and highlights the implication of the proteases expressed by a cell type as a decisive factor during viral infection[44]. Like MERS-CoV, SARS-CoV-2 S protein contains furin cleavage sites in the S1/S2 domain and S2’ domain suggesting the S2' site in the emergence and virulence of COVID-19 and its tropism.

The potential clinical and pharmacological role of the furin-like enzymes has fostered the development of both peptide- and protein-based PC-inhibitors[7,45,46]. In various preclinical studies, the most promising protein-based specific inhibitors of PCs were reported to be attributed to a1-antitrypsin Portland also known as a1-PDX[47], an a1-antitrypsin variant, and the individual convertases-pro-segment based inhibitors[48]. a1-PDX, was first shown to be a potent inhibitor of furin-mediated cleavage of HIV gp160[49], but subsequently demonstrated to also inhibit all the furin-like enzymes involved in processing within the constitutive secretory pathway[50,51]. Other studies further showed that endogenous inhibition of precursor convertases by a_1-PDX reduces the maturation of the surface glycoproteins of infectious pathogens[52]. Interestingly, exogenous addition of a1-PDX potentially inhibits the furin- dependent processing of HCMV gB, thus reducing the titer of infectious human cytomegalovirus more effectively than currently used antiherpetic agents[53]. The reported furin inhibition by the external application of a1-PDX occurs since

furin is localized to the trans- Golgi network (TGN) and cycles to the cell surface, where it could meet a1-PDX, and back via endosomal compartments[53]. In addition to the implication of furin in the activation of viral glycoproteins required for various viral infections, elevated expression of furin was reported for a range of human cancers including lung cancer and suggested to constitute a significant prognostic factor independent of other conventional clinicopathological ones[7]. Interestingly, patients with cancer showed higher risk of COVID-19 than individuals without cancer with poorer outcomes from COVID-19[54], suggesting that the enhanced expression of furin in cancer patients may strongly contribute to the massive activation of S proteins leading to rapid patient's health deterioration. Silencing of furin was recently employed to treat patients with cancer using an autologous tumor-based strategy consisting of a plasmid that encodes granulocyte-macrophage colony-stimulating factor (GMCSF) and furin shRNA. This vaccine was found to be efficient during phase I and II clinical trials in patients with cancer[55]. These treatments were not associated thus far with adverse effects but are difficult to administrate and monitor. As such small drug-like molecules inhibiting furin would be very valuable.

Bioinformatics studies were reported to provide crucial information about the interaction between viruses and the infected cells and to assist in the choice of drug and vaccine candidates for potential antiviral treatments. These include the HIV epidemic, H1N1 influenza virus pandemic; the Zika, the Nipah and Ebola epidemic (reviewed in[56,57]). Such computational procedures can for example assist the selection of virus proteins that may possibly constitute interesting targets. In the face of drastically rising drug discovery costs, strategies focusing on reducing development timelines such as drug repositioning are also relevant. It is indeed known that small molecule drugs can bind to about 3 to 6 targets on average, opening new avenues to rapidly identify potential novel treatments[58,59]. Molecular modelling techniques and virtual screening can definitively assist drug repositioning endeavours. Thus, facing the pandemic COVID-19 situation and the lack of validated treatment or vaccine, we decided to use different bioinformatics approaches and our novel collection of about 8,000 approved and investigational compounds to search for novel potential furin inhibitors. The anti-fungal agent Sulconazole was identified after structural analysis and was further found to inhibit the maturation of a major cell surface furin substrate in human cells. Here, we suggest that the inhibition of furin could be of high interest in SARS-CoV-2 infection. Although Sulconazole was identified to inhibit furin activity in human cells, direct effect of this agent or analogs will now need to be assessed on S protein processing and SARS-CoV-2 infection to validate this hypothesis.

Declarations

Contributors

AMK and BV designed the study and YL performed the western blotting analysis. AMK and BV analysed the data. Figures and schematic representations were drafted by AMK, BV, GS, SE and ED. The manuscript was drafted by AMK, BV, GS, ED and SE and the final version validated by all authors.

Declaration of interests

All authors declare no competing interests.

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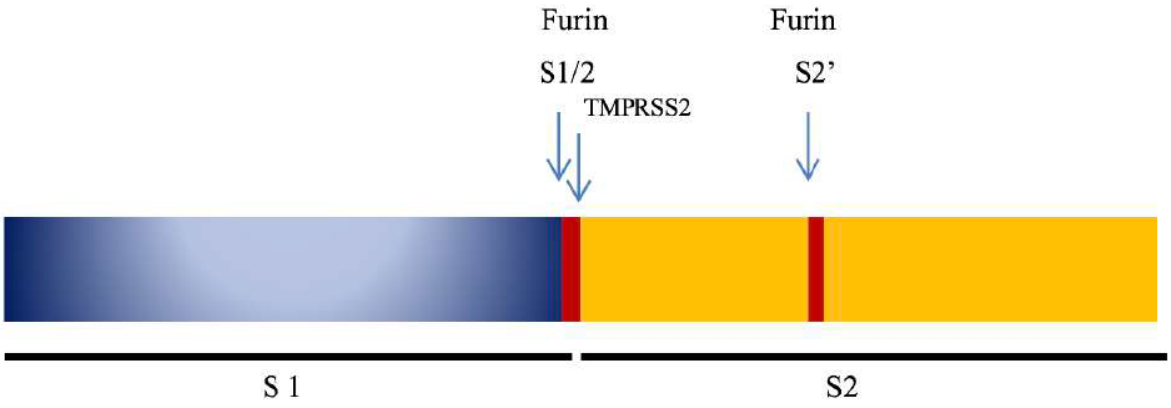
Figures

a



b

Coronavirus	Accession number	S1	S'2
SARS-CoV-2 (Covid19)	MN996531.1	^{678}TNSP*RRAR*SVASQSIIAYTMS^{697}	^{811}KPS*KR*SFIED LLFNK^{825}
SARS-CoV)	NP_828851.1	^{662}TVSLLRSTSQKSIVAYTMS^{681}	^{791}LKPTKRSFIEDLLF^{804}
MERS-CoV	AFS88936.1	^{744}TLTP*RSVR*SVPGEMRL^{759}	^{882}GS*RSAR*SAIEDLLF^{895}
HCoV–229E	BAL45637.1	^{559}S IIAVQPRNVSYDSV^{574}	^{682}SGSRVAGRSAIEDILF^{697}
HCoV–OC43,	AAA03055.1	^{751}SKN*RRSR*GAITTGYRF^{766}	^{896}ECSKASSRSAIEDLLF^{911}
HCoV-NL63,	AAS58177.1	^{863}RSSRIAGRSALEDLLF^{878}	^{863}RSSRIAGRSALEDLLF^{878}
HCoV-HKU1	AAT98580.1	^{753}SSS*RRKRR*SISASYRF^{768}	^{897}PHCGSSSRSFFEDLLF^{912}

Figure 1

a) Schematic representation of the coronavirus spike (S) envelope glycoprotein. The S protein is composed of two subunits, the S1 receptor-binding subunit, and the S2 fusion subunit that contains furin cleavage sites, S1/S2 and S2' (arrows). (b) The furin cleavage sites of the human coronaviruses known

to mediate infection in human and their furin cleavage site compared to the one of SARS-CoV-2. Note that only SARS-CoV-2 and MERS-CoV show optimal furin cleavage sites.

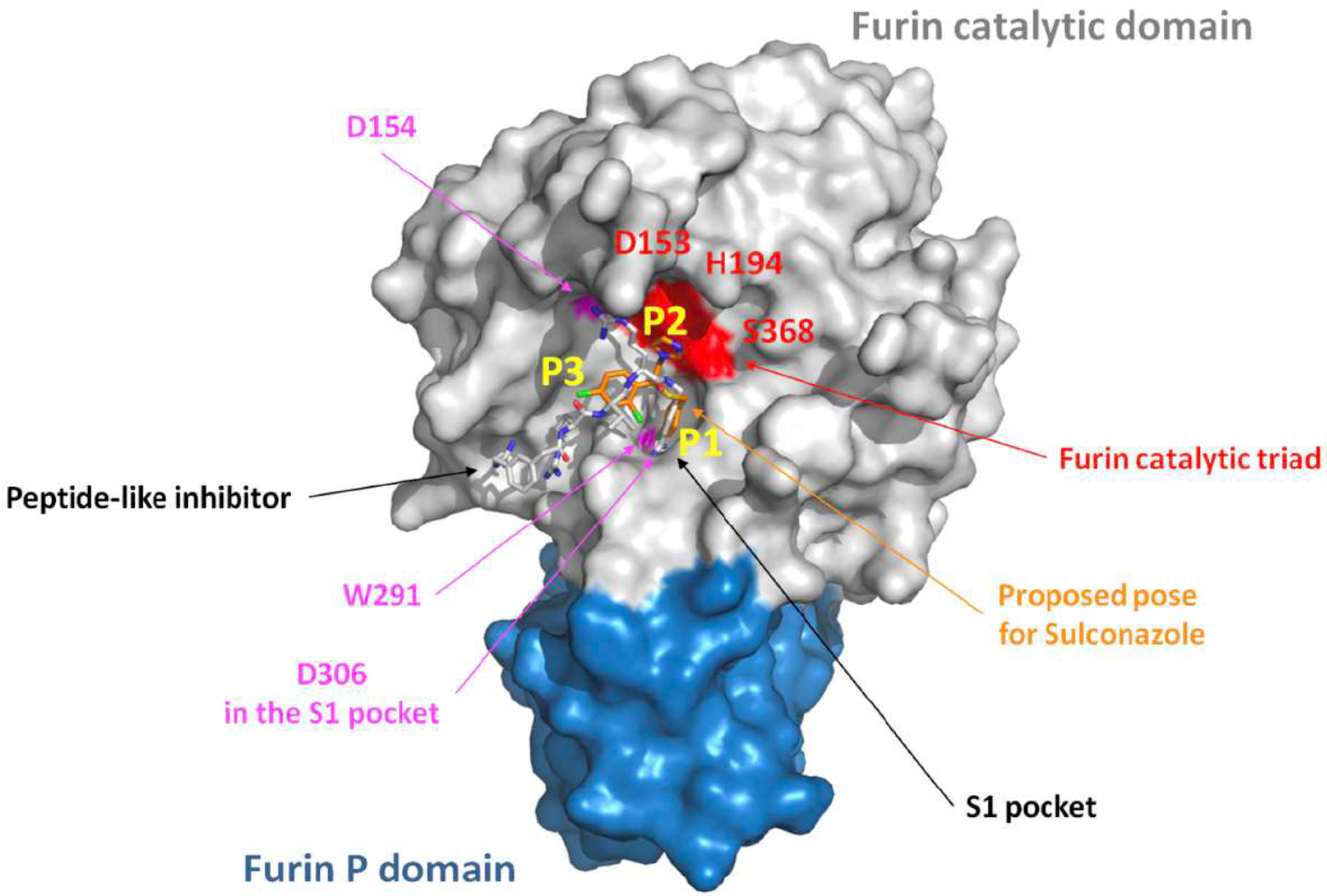


Figure 2

Virtual screening and drug repurposing strategies. The molecular surface of furin is displayed (blue and grey). The catalytic triad residues (D153, H194, S368) and specificity pocket (that includes D306) are labelled for orientation. A peptide co-crystallized with the enzyme is also shown (white, stick representation). The proposed pose for Sulconazole (orange) in the active site is also presented. The P1 group of Sulconazole interacts with the S1 pocket of furin. The other Sulconazole groups are labelled P2 and P3. Possible interactions could also involve D154.

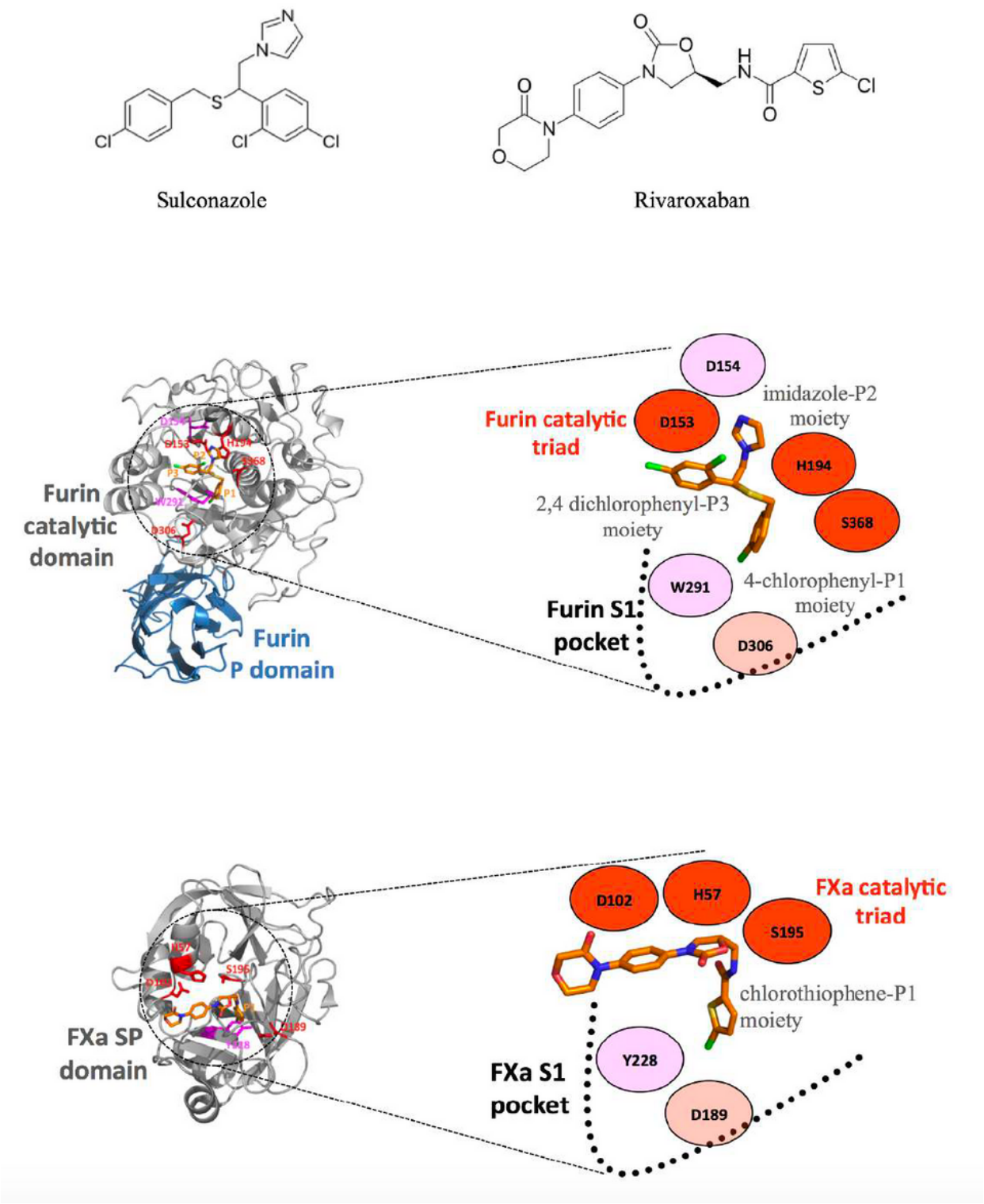


Figure 3

Analysis of the Sulconazole docked poses. All the approved compounds were docked into the experimental 3D structures of furin. Among the docked molecules, interactive analysis and investigation of binding affinity scores pointed toward a possible inhibition of furin by Sulconazole. However, compared to traditional peptide inhibitors and substrates of furin, the expected P1 residue of Sulconazole is not positively charged. We compared about 100 predicted binding modes of Sulconazole with the co-

crystallized Rivaroxaban molecule co-crystallized in the catalytic site of coagulation factor Xa and we propose that Sulconazole could fit into the catalytic site of furin in the orientation shown in the figure (see text for additional comments).

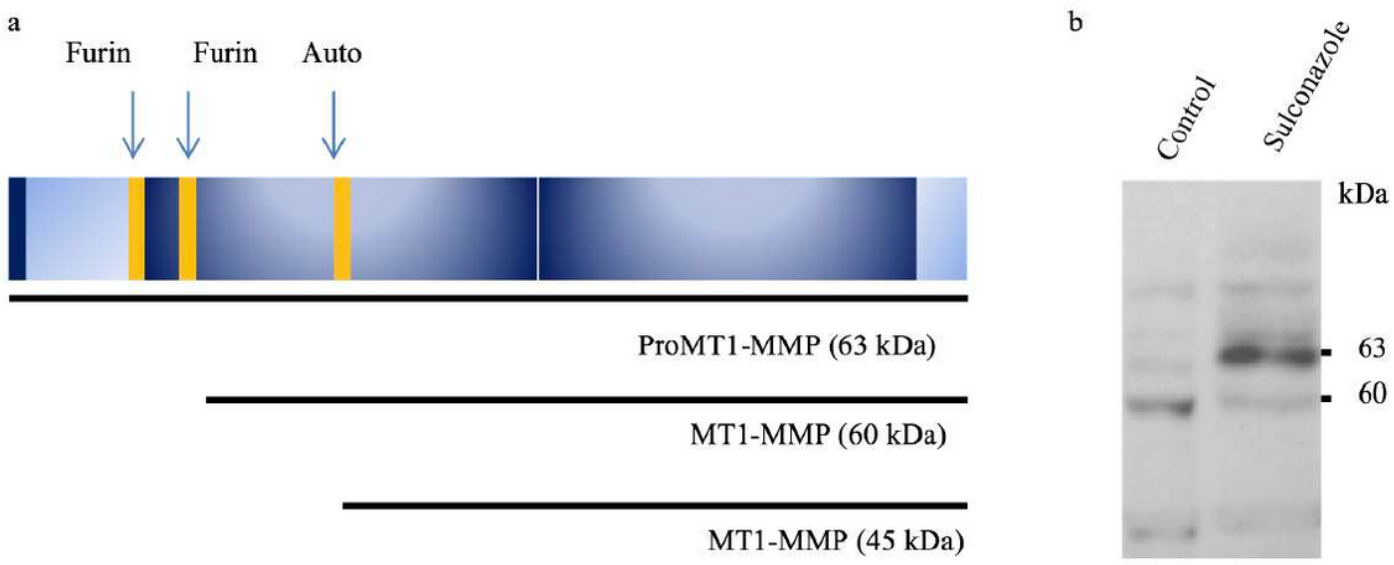


Figure 4

Effect of Sulconazole on furin substrate cleavage. (a) Schematic representation of the structural aspects of the human furin cell surface substrate pro-MT1-MMP (63 kDa) and its furin-processing sites at the RRPR922 (1) and RRKR1112 (2). The mature protein (63 kDa) can be further auto-catalytically cleaved into a 45-kd C-terminal form. (b) Inhibition of the furin protease activity in the cells in the presence of Sulconazole (10 □M), as demonstrated by the repression of MT1-MMP maturation and the accumulation of its unprocessed form (ProMT1-MMP) and shown by western blot using specific antibody.

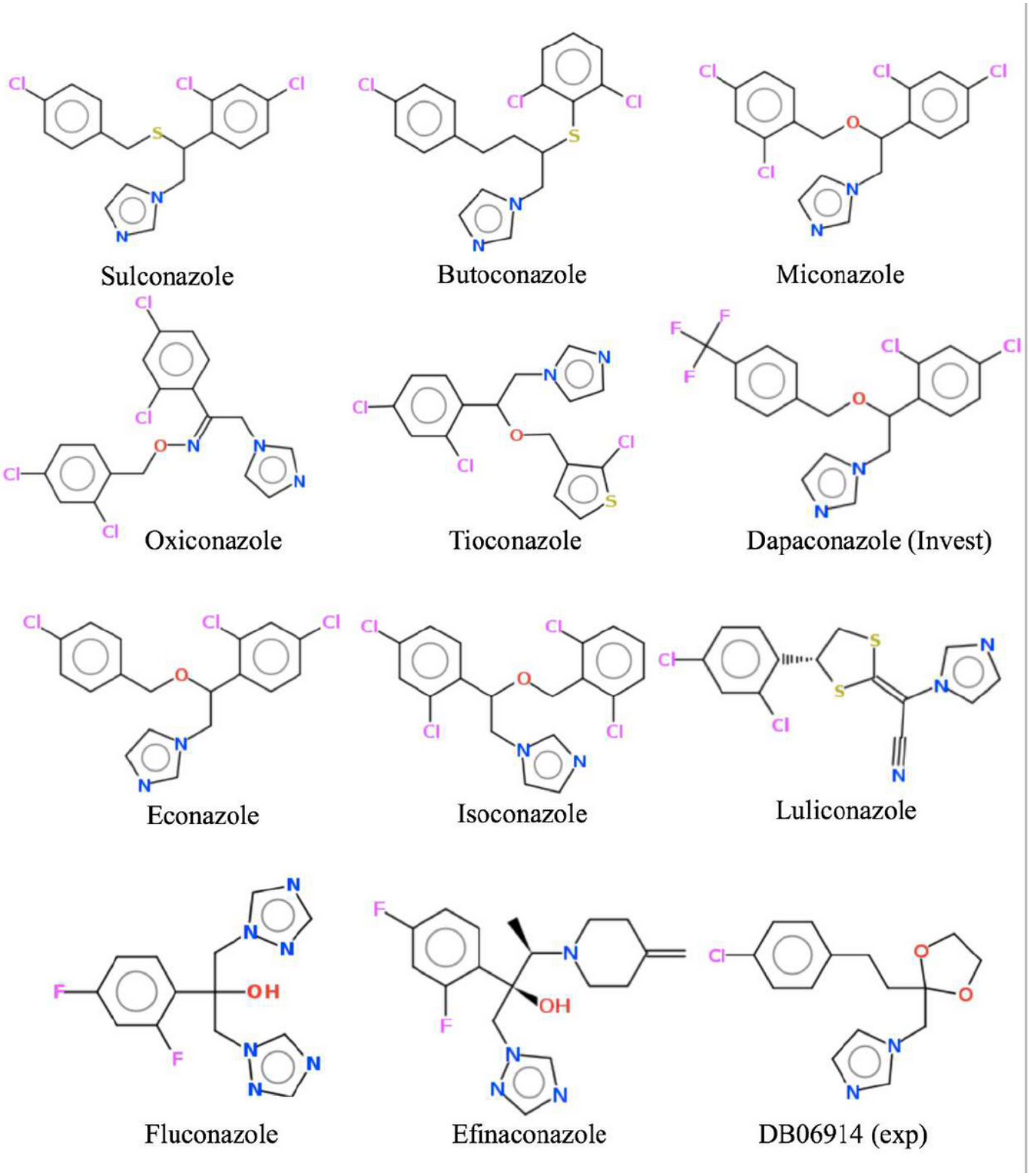


Figure 5

Sulconazole analogs. Molecules similar to Sulconazole may also inhibit furin. Molecule DB06914 corresponds to an experimental compound reported in DrugBank. “Exp” means experimental compound and “Invest” indicates investigational compound.