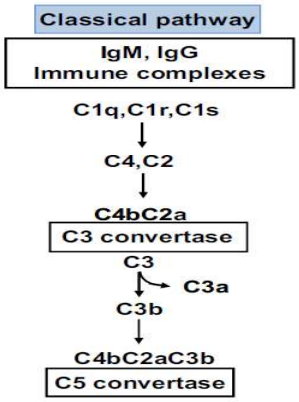
Design, selection and *in vitro* evaluation of potential, small-molecule complement C1s inhibitors

Katalin Szilágyi[1], István Hajdú[1,2], Beáta Flachner[1], Zsolt Lőrincz[1,2], Péter Gál[2], Péter Závodszky[2], Sándor Cseh[1], György Dormán[1,3]

[1]Targetex Biosciences, H-2120 Dunakeszi, Madách Imre utca 31/2. Hungary; [2]Research Centre for Natural Sciences, Institute of Enzymology, Hungarian Academy of Sciences, H-1117 Budapest, Magyar tudósok körútja 2., Hungary; [3]Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, H-6720 Szeged Zrínyi u. 9., Hungary.

Introduction

The complement system (CS) is a key component of innate immunity, which is involved in several physiologic and pathologic processes. Dysregulated or impaired complement is involved in an increasing list of human diseases (e.g. autoimmune, inflammatory, and neurodegenerative diseases etc.). CS consists of over forty protein components that are present in the blood or on cell surfaces. CS is activated by infection or by injury and activation may be prolonged or misdirected to healthy cells and can lead to inflammatory or auto-immune diseases. Complement-targeted drugs could provide novel therapeutic intervention against the above diseases. Nine serine proteases are integral elements of the CS cascade (C1r, C1s, C2, MASP-1, MASP-2, MASP-3, factor D, factor B, factor I). C1s is present as a proenzyme within the C1 molecule in complex with C1q and C1r. The activation of the C1 complex is the „classical activation pathway" of CS, which is initiated by the interaction of C1q with immunoglobulin (Ig) antigen complexes. The activation signal is mechanically transmitted by C1q to C1r dimers; activated C1r proteases then cleave and activate the C1s proenzymes. Activated C1s protease forwards the activation signal by cleaving C4 and C4b-associated C2 to form C3 convertase C4b2a, so an inhibitor that targets the C1s protease domain could block the activation of the classical pathway of CS.



Objectives

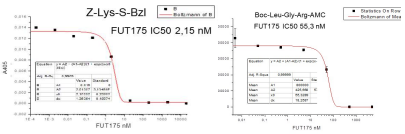
The main challenges in the C1s inhibitor discovery are the followings: 1.) the selectivity over other serine proteases (including the proteases of the blood coagulation and fibrinolysis etc.) and 2.) the very distinct, narrow bioactive chemical space of the available inhibitors. The typical architecture of a C1s inhibitor contains a heterocyclic amidine (or guanidine), however, recently additional inhibitors were identified (mostly N-heterocycles with multiple nitrogenes) lacking the amidine (guanidine) moiety. These compounds and their bioisosteric replacements served as starting points for designing a C1s focused compound library.

Protein expression and assay development

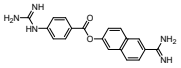
Recombinant C1s and C1r fragments CCP1-CCP2-SP containing the serine protease domain were expressed in E. Coli *BL21(DE3)pLysS* host strain (transformed with pET-17b vector) in inclusion bodies, isolated, renatured and purified as published earlier (*Kardos, 2001*). Recombinant C1s, isolated in intact proenzyme form, was activated by limited proteolysis using the recombinant, autoactivated C1r fragment. Activated C1s and C1r were separated using anion exchange chromatography.

Two end-point assays have been developed:

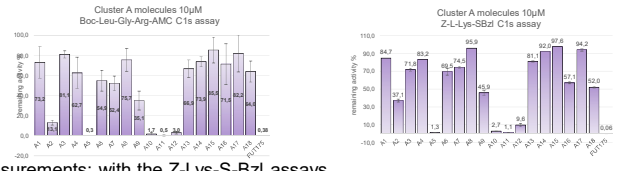
- for screening: specific, fluorescent amide substrate (Boc-Leu-Gly-Arg-AMC, the cleavage site sequence of C4) high enzyme concentration (0.2 μM), 20 mM HEPES (pH 7.4), 200 mM NaCl, 0.05% Tween20, 0.2% DMSO; 384 microplate format; 10μL enzyme + 10 μL inhibitor compounds (final concentration: 10 μM); incubation (10 min, RT), and 20 μL substrate solution (1 mM final concentration); incubation (45 min, 30°) reading the fluorescence (355/460; Perkin Elmer Wallace 1420 Victor2 microplate reader.)
- -for IC_{50} measurements: sensitive thioester substrate (Z-Lys-SBzl; DTNB) low enzyme concentration (below 1 nM), the same buffer used for screening, in a 96 well microplate format (in 100 μL). 40 μL enzyme + 10 μL inhibitor compounds (final concentration: 10 μM) were incubated (10 min, RT), and 50 μL substrate solution (1 mM final concentration) was added; incubated for 45 minutes and finally absorbance was read (405 nm) with a Perkin Elmer Wallace 1420 Victor2 microplate reader.



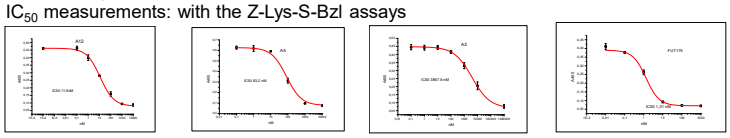
Validation of the assays: FUT175, Published IC_{50}: 2x10^{-8} M (*Aoyama,* 1984); 3.2x10^{-7} M (*Ueda, N, 2000*)



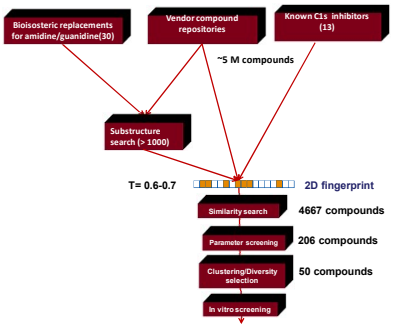
First screening: using both assays (Z': 0.689; Z': 0.704)



IC_{50} measurements: with the Z-Lys-S-Bzl assays



Generation of the C1s focused library



We first collected known C1s inhibitors and generated the bioisosters of the key recognition motifs (guanidine, amidine). Based on the resulting novel structural motifs and the structure of the known inhibitors a focused library was selected from commercial vendor libraries (5 M compounds). (InstantJChem, ChemAxon, Budapest)

An initial library was filtered for physico-chemical parameter space, cluster representation and diversity (InstantJChem, ChemAxon, Budapest)

Biological results

No	Structure	% inhibition Boc-LeuGly-Arg-AMC	% inhibition Z-Lys-S-Bzl	IC_{50} (microM)	Cluster	No	Structure	% inhibition Boc-LeuGly-Arg-AMC	% inhibition Z-Lys-S-Bzl	IC_{50} (microM)	Cluster
1		98.8	99.6	0.091	A	5		99.7	98.7	0.083	A
2		99.3	99.2	1.85	A	6		98.3	97.3	0.094	B
3		98.0	89.9	1.3	A	7		99.5	98.9	0.044	B
4		86.9	62.9	3.87	A	8		97.0	90.4	0.012	B

8 compounds out of the 50 showed > 85 % inhibition at 10 μM concentration and 5 compounds inhibited C1s < 1 μM concentration. (The IC_{50} value of the standard FUT175 compound = 1.31 nM)

Summary and conclusion

During the present study first the C1s protein was expressed, 2 inhibition assays were developed and validated. Applying 2D similarity search, bioisosteric replacements and physico-chemical parameter filtering a 50-membered target-focused library was generated and acquired from commercial vendor libraries. The compounds were screened first at 10 μM concentration and the IC_{50} values were determined for the most active compounds. Eight compounds showed excellent inhibitory activities (hit rate: 16 %). The identified hits belonged to two clusters. Although the chemotypes of the hits were previously reported, the hit analogues showed higher activity compared with the known compounds. Novel chemotypes were not identified due to the limited chemical space of the available commercial libraries and the distinct, limited bioactive chemical space of the C1s enzyme. Our future plan is to combine the 2D similarity search with 3D methods (docking, 3D similarity selection or 3D pharmacophore modelling) which would increase the probability to identify novel chemotypes.

References

Kardos, J. et al., The role of individual domains in the structure and function of a modular serine protease, C1r, *J. Immunol.* 167:5202-5208, (2001).
Aoyama, T., Pharmacological studies of FUT-175, nafamostate mesilate, *J. Pharmacol* 35:203-227 (1984).
Ueda, N., Inhibitory effects of newly synthesized active center-directed trypsin-like serine protease inhibitors on the complement system, *Inflamm. Res* 49:042-046 (2000).

TargetEx llc., Madách Imre utca 31/2., 2120 Dunakeszi, Hungary
Tel.: +36 1 279 3153; Fax: +36 1 279 3154
www.targetex.com