DEVELOPING EOTAXIN-3 MUTANT LIBRARIES FOR GAG-RELATED FUNCTIONAL STUDIES

Alexandra Pum and Andreas J. Kungl

Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, University of Graz, Universitätsplatz 1, A-8010 Graz

Background

Eosinophilia is associated with some inflammatory gastrointestinal disorders such as eosinophilic gastritis (EGE), eosinophilic esophagitis (EoE) and colitis. Eosinophilic infiltrates are increased in gastric biopsies of patients with EGE[1] and in esophageal biopsies from patients with EoE[2]. The CCR3-eotaxin axis is crucial for the recruitment and accumulation of eosinophils in the target tissues of eosinophilic disorders[3]. Eotaxin is a chemokine family with 3 members: eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26). Healthy esophagus is devoid of eosinophils. Therefore, the presence of esopahageal eosinophilia is a defining pathologic feature of EoE. Eosinophil migration in EoE is mainly driven by eotaxin-3[4]. The esophagus is lined with a multilayered squamous epithelium[5] typically involving a GAG-enriched glycocalix. Eotaxin-3 must be immobilized on epithelial cells via GAGs to mediate eosinophil migration[6].

To get a closer insight into the eotaxin-3/GAG axis and its involvement in eosinophilia, we started a site-directed mutagenesis (SDM) study of eotaxin-3 to identify the binding site(s) of this chemokine towards glycosaminoglycans, since we regard them as co-receptors for Eotaxin-3. This knowledge enables us to find out by biophysical methods the specificity and affinity of the eotaxin-3/GAG interaction. Based on this structural information we employed our dominant-negative mutagenesis technology[7] in order to create protein-based antagonists of eotaxin-3/GAG interaction. In addition, we generated a panel of mutants with enhanced and decreased GAG-binding affinities (‘GAG agonists /knock-in’ and ‘GAG-antagonists /knock-out’). Cell mobilization and migration assays allow us to study the influence of GAGs by comparing chemotaxis induced by wild type eotaxin-3 versus its mutants with altered GAG-binding properties as well as dominant negative mutants.

Workflow

01 Protein Structure Analysis and Engineering
Of wildtype eotaxin-3

02 Primer Design
Custom designed oligonucleotide primers to confer a desired mutation in a double-stranded DNA plasmid

03 PCR
Exponential amplification, treatment with kinase, ligase, DpnI (phosphorylation, ligation and template removal)

04 Transformation in *E. Coli* Top 10
For plasmid amplification and storage

05 Sequencing
Determining the nucleic acid sequence of the isolated mutated plasmid DNA

06 Recombinant protein expression in *E.Coli* BL21
Allows high-efficiency protein expression of the underlying DNA, achieved by IPTG addition.

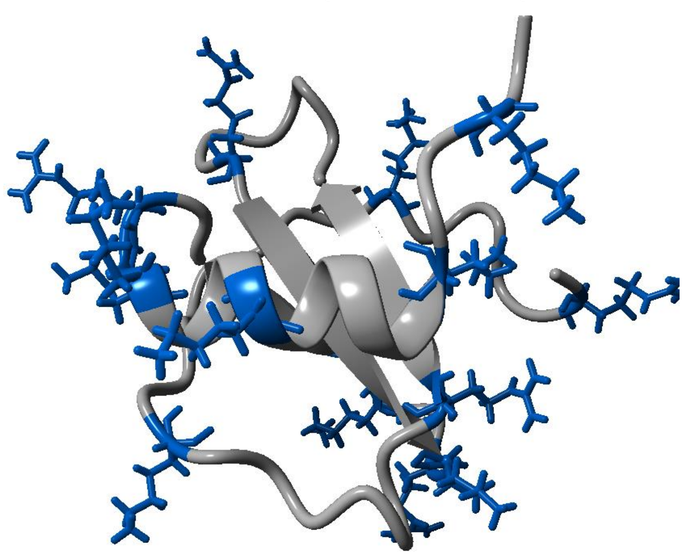
07 Purification
Processes for isolation of the expressed protein from cells: *cell lysis, solubilisation, shock dilution, FPLC, rpHPLC, formulation*

08 Characterization / Binding Studies / Migration Assays
Purity (SDS PAGE + silver stain), Mass spectrometry, Secondary structure (CD), Binding Studies (IFT, SPR), Immunological Assays (Chemotaxis)

Eotaxin-3 Mutants

Protein modification	Mutation	Localization
CCL26 Wildtype	None;	-
GAG knock-out	K60A	α-helix /point mutation
GAG knock-out	K55A K56A	α-helix /double mutation
GAG knock-out	R54A K55A K56A	α-helix /triple mutation
GAG knock-out	R54A	α-helix / point mutation
GAG knock-out	ΔP53-L71	α-helix /truncation
GAG knock-out	K44A	β-sheet /point mutation
GAG knock-out	K47A	β-sheet /point mutation
GAG knock-in	Q59K	α-helix /point mutation
GAG knock-in	T51K	β-sheet /point mutation
Dominant-negative	Δ8 Q59K	N- terminal; α-helix

1 MTRGSDISKT 11 CCFQYSHKPL 21 PWTWVRSYEF 31 TSNSCSQRAV
41 IFTT**KR**GKKV CTHP**RRK**WVQ **K**YISLLKTPK 71 QL



Secondary structure of wildtype CCL26 from UniProt Q9Y258
Side chains of modified positions are visible and highlighted in blue

1 Lwin, Thida; Melton, Shelby D.; Genta, Robert M. (2010): Eosinophilic gastritis: histopathological characterization and quantification of the normal gastric eosinophil content. In: Modern Pathology 24, 556 EP -. DOI: 10.1038/modpathol.2010.221.
2 Guarino MP, Cicala M, Behar J. Eosinophilic esophagitis: New insights in pathogenesis and therapy. World J Gastrointest Pharmacol Ther. 2016;7(1):66-77.
3 Fulkerson PC, Rothenberg ME. Targeting eosinophils in allergy, inflammation and beyond. Nat Rev Drug Discov. 2013;12(2):117-29.
4 Davis BP, Rothenberg ME. Mechanisms of Disease of Eosinophilic Esophagitis. Annu Rev Pathol. 2016;11:365-93.
5 Rosekrans et al. Esophageal development and epithelial homeostasis. Am J Physiol Gastrointest Liver Physiol. 2015; 309(4):G216-28.
6 Yuan et al. Membrane-bound eotaxin-3 mediates eosinophil transepithelial migration in IL-4-stimulated epithelial cells. Eur. J. Immunol. 2006; 36: 2700–27142700
7 Adage T, Piccinini AM, Falsone A, et al. Structure-based design of decoy chemokines as a way to explore the pharmacological potential of glycosaminoglycans. Br J Pharmacol. 2012;167(6):1195-205.