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Bacterial surface display library screening by target enzyme labeling: Identification of new human cathepsin G inhibitors

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Abstract

The aim of this study was to establish a new tool for screening surface displayed peptide libraries based on the idea that cells expressing an enzyme inhibitor at the surface can be specifically labeled by the target enzyme. For this purpose peptide P15, exhibiting a K_i value of 0.25 μM toward human cathepsin G, was expressed on the *Escherichia coli* cell surface by the use of Autodisplay. Purified cathepsin G was coupled to biotin and incubated with cells expressing the inhibitor. After addition of streptavidin–fluorescein isothiocyanate, these cells could be clearly differentiated from control cells by whole-cell fluorescence using flow cytometer analysis. To determine whether this protocol can be used for the sorting of single cells, a mixed population of cells with and without inhibitor was treated accordingly. Single cells were selected by increased fluorescence and sorted using fluorescence-activated cell sorting (FACS). Single cell clones were obtained and subjected to DNA sequence analysis. It turned out that the bacteria selected by this protocol displayed the correct peptide inhibitor at the cell surface. The protocol was then used to screen random peptide libraries, expressed at the cell surface, and a new lead structure for human cathepsin G ($IC_{50} = 11.7$ μM) was identified. The new drug discovery tool presented here consists of three steps: (a) surface display of peptide libraries, (b) selection of single cells with inhibiting structures by using the inherent affinity of the target enzyme, and (c) sorting of single cells, which were labeled by FACS.

Keywords: Autodisplay; Peptide libraries; Labeling; Screening; Cathepsin G; FACS; Drug discovery

Over the past decade combinatorial chemistry and the synthesis of compound libraries have become versatile tools used to create molecular diversity [1,2]. Using these approaches, a high number of variable compounds that can be subsequently screened on variants of a distinct specified feature have been obtained. Among other applications, this strategy proved to be successful in the identification of new leads and potential drug candidates [3]. A major challenge in this strategy is the structure determination of the variant that has been selected from the library. Depending on the complexity of the library applied, it requires either highly sophisticated logistics during synthesis or fastidious analytics [4]. Although limited to polypeptides, display libraries offer the advantage that every variant displayed at the surface carries an internal label—the DNA sequence—which reveals the sequence and hence the (primary) structure of the variant by simple and low-cost methods. In this respect phage display has become a versatile and effective method to identify new ligands for pharmaceutically important receptors and to find new potent lead structures [5]. In phage display a recombinant peptide is expressed as a fusion with the coat protein of a filamentous bacteriophage. Phages with positive variants exhibit an increased affinity, e.g., to the target enzyme or the target receptor under investigation. This affinity can be exploited to enrich bacteriophages displaying the peptides of desired properties. This enrichment usually requires several rounds of binding, followed by amplification of the phages and was named "panning" [6].

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Our aim was to combine the general idea of phage display with the advantages of a cellular surface display system in *Escherichia coli*. Cells have the advantage of being self-replicative, whereas phages need a two-step replication consisting of infection of host cells and subsequent purification. Moreover cells can be subjected to high-throughput screening methods such as fluorescence-activated cell sorting (FACS)[2] [7], whereas phage particles in general are too small for these purposes [8]. Additionally, the fact that an inhibitor shows high affinity to its target enzyme (and vice versa) can be exploited in cellular surface display to specifically label cells bearing an inhibiting structure at the surface, namely by binding of the respective target enzyme. The labeling can then be used for the high-throughput screening of a library consisting of cells displaying different inhibitor variants at their surface. It is important to note that for this approach each cell needs to display only one unique variant but in high numbers. In the first step toward the aim of this study, a system has to be established that allows the expression of peptide inhibitors in high numbers at the surface of *E. coli*. The peptide must be able to fold to its active conformation at the surface and finally it needs to be accessible for the target enzyme to use this binding affinity for selection.

Human cathepsin G is a serine proteinase that accumulates in the human azurophil granule of neutrophils [9]. During normal phagocytosis cathepsin G participates in the proteolytic degradation of the engulfed particles, e.g., bacteria [10]. Under certain circumstances as found in inflammatory diseases, the granule and its constituents can be released to the extracellular milieu. In this situation, the proteinase can participate in local destruction of connective tissue proteins. Therefore, in addition to human leucocyte elastase, cathepsin G is assumed to be an important target in the treatment of chronic inflammatory diseases such as rheumatoid arthritis or pulmonary emphysema [11]. During inflammatory episodes inhibitors could alleviate the damage by inactivation of excessive cathepsin G released from activated neutrophils. More recently, cathepsin G was also reported to activate matrix metalloproteinase-2, playing a role in tumor invasion [12]. Furthermore, peptides derived from the human C-reactive protein, whose serum concentration increases during inflammation [13], have been reported to inhibit cathepsin G. The most active derivative was peptide 15 (P15, EILIFWSKDIGYSFT) with a K_i of 0.25 μM [14].

In the present study, we chose P15 as a model inhibitor and cathepsin G as a model target enzyme to establish a new labeling procedure and drug discovery system using bacterial surface display.

[2] *Abbreviations used:* TBS, Tris-buffered saline; FCS, fetal calf serum; PBS, phosphate buffered saline, FACS, fluorescence-activated cell sorting; CTB, cholera toxin β-subunit; FITC, fluorescein isothiocyanate.

Materials and methods

Bacteria, plasmid, and culture conditions

Escherichia coli strain UT5600 (F$^-$ *ara*14 *leu*B6 *azi*-6 *lac*Y1 *pro*C14 *tsx*-67 *ent*A403 *trp*E38 *rfb*D1 *rps*L109 *xyl*-5 *mtl*-1 *thi*1 Δ*omp*T-fepC266) [15] was used for the surface display of peptide 15 (P15) and the β subunit of cholera toxin (CTB) [16]. Plasmid pJJ942 which directs CTB to the cell surface has been described earlier [17]. Bacteria were routinely grown at 37 °C in liquid medium with vigorous shaking (200 rpm). The liquid medium consisted of Luria–Bertani (LB) broth with 20 mM 2-mercaptoethanol and 100 mg ampicillin per liter [18].

Recombinant DNA techniques

For the construction of an artificial gene for the surface display of peptide P15 two oligonucleotides, DZ019 (5′-TCG AGT ACC CGT ACG ACG TTC CGG ACT ACG CTG AAA TCC TGA TCT TCT GGT CCA AAG ACA TCG GTT ACT CCT TCA CCC CGC-3′) and DZ020 (5′-GGG GTG AAG GAGT AAC CGA TGT CTT TGG ACC AGA AGA TCA GGA TTT CAG CGT AGT CCG GAA CGT CGT ACG GGT AC-3′), were designed and synthesized (MWG-Biotech, Munich, Germany). The oligonucleotides were dissolved to a final concentration of 100 pmol/μl in water; 10 μl of each solution was mixed and incubated at 96 °C for 5 min followed by a 5-min incubation on ice. This resulted in hybridization of the oligonucleotides and yielded a DNA double strand with *Xho*I/*Sac*II restriction site compatible ends at the 5′ and the 3′ ends, respectively (Fig. 2). The double-stranded DNA fragment encoding P15 and the epitope (HA) of a commercially available monoclonal antibody was inserted into plasmid pJJ942 and digested with *Xho*I and *Sac*II. Restriction of plasmid pJJ942 with *Xho*I and *Sac*II resulted in the elimination of the CTB-encoding DNA fragment. Therefore, the resulting new plasmid pDZ15HA is basically identical with pJJ942, but the CTB encoding DNA region is replaced by a fragment encoding P15 and the HA epitope. The HA epitope was useful to perform immunoblotting and whole-cell immunofluorescence experiments by the use of a specific monoclonal antibody.

Outer membrane preparation

Escherichia coli cells were grown overnight and 1 ml of this culture was used to inoculate 20 ml fresh LB medium. Cells were incubated at 37 °C with vigorous shaking (200 rpm) for about 5 h until an OD_{578} of 0.7 was reached. Subsequently, cells were harvested and outer membranes were prepared according to the rapid isolation method of Hantke [19].

SDS–PAGE and western blot analysis

Outer membrane preparations were diluted (1:2) with sample buffer (100 mM Tris–HCl, pH 6.8, containing 4%