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Research paper

Substrate-derived triazolo- and azapeptides as inhibitors of cathepsins K and S

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ABSTRACT

Cathepsin (Cat) K is a critical bone-resorbing protease and is a relevant target for the treatment of osteoporosis and bone metastasis, while CatS is an attractive target for drugs in autoimmune diseases (e.g. rheumatoid arthritis), emphysema or neuropathic pain. Despite major achievements, current pharmacological inhibitors are still lacking in safety and may have damaging side effects. A promising strategy for developing safer reversible and competitive inhibitors as new lead compounds could be to insert non-cleavable bonds at the scissile P1-P1′ position of selective substrates of CatS and CatK. Accordingly, we introduced a 1,4-disubstituted 1,2,3-triazole heterocycle that mimics most of the features of a *trans*-amide bond, or we incorporated a semicarbazide bond (azaGly residue) by replacing the α-carbon of the glycyl residue at P1 by a nitrogen atom. AzaGly-containing peptidomimetics inhibited powerfully their respective target proteases in the nM range, while triazolopeptides were weaker inhibitors (Ki in the μM range). The selectivity of the azaGly CatS inhibitor (**1b**) was confirmed by using spleen lysates from wild-type *vs* CatS-deficient mice. Alternatively, the azaGly bradykinin-derived CatK inhibitor (**2b**) potently inhibited CatK (Ki = 9 nM) and impaired its kininase activity *in vitro*. Molecular modeling studies support that the semicarbazide bond of **2b** is more favorable than the 1,2,3-triazole linkage of the bradykinin-derived pseudopeptide **2a** to preserve an effective affinity towards CatK, its protease target.

Abbreviations: Abz, *o*-aminobenzoic acid; Ahx, 6-aminohexanoic acid; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; AMC, 7-amino-4-methyl coumarin; BK, bradykinin; BM, basement membrane; Cat, cysteine cathepsin; CA-074, *N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; CuAAC, copper-catalyzed azide/alkyne cycloaddition; DMF, dimethylformamide; DMK, diazomethylketone; Dnp, 2,4-dinitrophenyl; DTT, dithiothreitol; E-64, L-3-carboxy-trans-2,3-epoxy-propionyl-leucylamide-(4-guanido)-butane; ECM, extracellular matrix; EDDnp, *N*-(2,4-dinitrophenyl)-ethylenediamine; FRET, Fluorescence Resonance Energy Transfer; HATU, 1-[*bis*(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HCTU, 2-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium-hexafluorophosphate; HNE, human neutrophil elastase; Fmoc, fluorenylmethyloxycarbonyl; KO, knockout; LHVS, morpholinourea-leucinyl-homophenylalanine-vinyl-sulfone; Mca, (7-methoxycoumarin-4-yl)-acetyl; MMP, matrix metalloproteinase; MMTS, *S*-methyl thiomethanesulfonate; NMP, 1-methyl-2-pyrrolidone; Nva, norvaline; Odanacatib, (2*S*)-*N*-(1-cyanocyclopropyl)-4-fluoro-4-methyl-2-([(1*S*)-2,2,2-trifluoro-1-{4′-(methanesulfonyl)-[1,1′-biphenyl]-4-yl) ethyl] amino) pentanamide; THF, tetrahydrofuran; PMSF, phenylmethylsulfonyl fluoride; Z, benzyloxycarbonyl; PTFE, polytetrafluoroethylene; RP-HPLC, reversed phase high performance liquid chromatography; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; WT, wild type.

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1. Introduction

Human cathepsins (Cat) K and S are cysteine proteases that belong to the C1 family, clan CA family of cysteine proteases, which has eleven members in human cells [1]. These enzymes share close three-dimensional structures and thus have the same catalytic mechanism and overlapping substrate specificity [2]. They are primarily involved in end-stage degradation of endocytosed proteins within acidic lysosomal and/or endosomal compartments [3,4]. However, these proteases are also involved in more discrete cleavages including sequential processing of thyroglobulin, TGF-β1 and the MHC-II associated chaperone invariant chain (Ii) as well as roles in bone remodeling, keratinocyte differentiation and cell-adhesion molecule shedding [5–7]. Moreover, the ability of CatK and CatS to cleave extracellular matrix constituents including collagens, fibronectin and laminins [8–10], highlights that their activity is not confined to the lysosome; indeed they have been also been detected in other compartments such as secretory vesicles, mitochondria and the extracellular medium [11,12]. Frequently, the localization of these proteases in these alternative locations is linked to their overexpression and/or dysregulation, making them putative targets for the development of new therapies [13]. CatS has been identified as a relevant target for the treatment of autoimmune diseases [14] as well as neuropathic pain [4]. CatK, predominantly expressed in osteoclasts, is a critical bone resorbing protease and is considered as druggable for the treatment of osteoporosis and bone metastasis [3,15].

Among possible strategies for developing reversible competitive protease inhibitors is conversion of a peptide substrate into a stable inhibitory molecule. This could be achieved by the introduction of a non-hydrolyzable peptide bond mimic at the P1-P1′ position, while retaining the main characteristics of the parent substrate that interact with proteases on both sides of the cleavage site. For example, methyleneamino (so-called “reduced amide bond”) or hydroxyethylene surrogates have been frequently utilized as non-cleavable pseudo-peptide bonds [16]. Nevertheless, their introduction often leads to a notable decrease in affinity [16], probably because of rather poor mimicry of the *trans*-amide bond. In this context, we thought to explore the 1,4-disubstituted 1,2,3-triazole cycle that mimics the geometric, steric and electronic features of the *trans*-amide bond, and can likewise participate in hydrogen bonding and dipole–dipole interactions [17–25]. These derivatives were compared to their azaGly-containing peptide counterparts where the alpha carbon of the glycyl residue at P1was replaced by a nitrogen atom (i.e. incorporation of a semicarbazide bond) [26]. We introduced the mimetic moieties at the P1-P1′ position of two known peptide substrates of CatS and CatK; namely Gly-Arg-Trp-His-Pro-Met-Gly-Ala-Pro-Trp-Glu-D-Ala-D-Arg; derived from a substrate previously designed for quantification of CatS activity in antigen-presenting cells [27], and Abz-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Tyr-3-NO2, a bradykinin-derived substrate of CatK [28,29].

We report herein the synthesis of these four substrate-derived peptides (**1a**, **1b**, **2a**, **2b -** see Fig. 1 for their structures) and their ability to be effective inhibitors of cathepsins S (**1a, 1b**) and K (**2a, 2b**). After *in vitro* analysis against a panel of proteolytic enzymes and determination of the kinetic constants, their inhibitory properties were evaluated on some suitable experimental models, including cell lysates from wild-type *vs* CatS-deficient mice. AzaGly-containing peptides (**1b**, **2b**) powerfully inhibited their respective target proteases in the nanomolar range, while triazolopeptides (**1a**, **2a**) were weaker inhibitors (Ki in the micromolar range). Molecular modeling studies support the fact that the azaGly surrogate is more favorable than the triazole to sustain both a suitable selectivity and an effective affinity between substrate-derived inhibitors and their targets.

2. Results and discussion

2.1. Substrate-derived inhibitors

Triazolopeptides **1a** and **2a** and azapeptides **1b** and **2b** (Fig. 1) were synthesized by Fmoc-based solid phase peptide synthesis (SPPS), by incorporating the pseudopeptide bonds during the solid phase process. For triazolopeptides, the P1′ residue was coupled as the α-azide derivative of the parent amino acid (respectively N_3Phe-OH and N_3Ala-OH), followed by copper-catalyzed azide/alkyne cycloaddition (CuAAC) [30,31] with *N*-Fmoc-propargylglycine using an optimized protocol we recently described [24]. Azapeptides **1b** and **2b** were obtained through coupling of the P1 azaGly residue using Fmoc-hydrazide preactivated with carbonyldiimidazole [32]. All four peptides were purified up to >99% purity by RP-HPLC.

2.2. Inhibition of cathepsin S

First, we tested *in vitro* a series of proteases, including human cysteine proteases (cathepsins B, H, L, K and S), a matrix metalloproteinase (MMP-2), an aspartyl protease (CatD), and serine proteases (i.e. trypsin, chymotrypsin and neutrophil elastase). The substrate-derived triazolopeptide **1a**, whose peptide sequence (Gly-Arg-Trp-His-Pro-Met-Gly-Ala-Pro-Trp-Glu-D-Ala-D-Arg) is derived from a specific substrate of CatS [27], did not inhibit trypsin, chymotrypsin, HNE, MMP-2, CatD, CatH, and CatB. **1a** inhibited CatS, but also both CatK and CatL. It has to be noticed that the parent peptide substrate was not tested against CatK and CatH in the initial study [27]. Similarly, the substrate-derived azapeptide **1b** did not inhibit trypsin, chymotrypsin, HNE, MMP-2, CatD and CatB, but impaired the peptidase activity of CatS, CatK, and CatL. **1b** also inhibited CatH to a weaker extent. Analysis by RP-HPLC of the different incubation mixtures established the absence of proteolytic degradation for **1a** and **1b**. Moreover we postulated that transforming a CatS peptidyl substrate into a triazolopeptide or an azapeptide would result in a competitive substrate-derived inhibitor of CatS. We assessed the reversibility of the inhibitory mechanism by incubating CatS with **1a** or **1b** (concentration in excess to ensure a complete inhibition over the time of experiment) before adding an irreversible synthetic inhibitor Biot-$(PEG)_2$-Ahx-Leu-ValGly-DMK (Supplementary file S1). Labelling of CatS by the biotinylated activity-based probe showed that both **1a** and **1b** were removed from the active site of CatS, thus confirming the reversibility of the interaction as previously observed [33]. Also the peptidase activity was restored following addition of an excess of Z-LR-AMC (not shown). We then determined the kinetic constants of the two inhibitors. **1a** inhibited reversibly and competitively cathepsins S, K, and L with K_i values in the micromolar range (Table 1) at pH 5.5. Unlike most of lysosomal cysteine cathepsins that are optimally active in acidic environments and are rapidly inactivated at neutral pH, CatS remains stable and active at pH 7.4 [4]. This therefore suggests **1a** may gain a greater selectivity for CatS at a pH value close to the extracellular medium. Indeed **1a** inhibited CatS, but not CatK and CatL, in a comparable manner at pH 5.5 and pH 7.4. Otherwise **1b** powerfully inhibited CatK (K_i = 3 nM), CatL (K_i = 5 nM) and CatS (K_i = 26 nM) at pH 5.5. As observed for **1a**, **1b** hampered CatS, but not CatK and CatL, with a comparable Ki at pH 7.4 (K_i = 17 nM), substantiating that the selectivity of both **1a** and **1b** is partly pH-driven. Also this initial result suggests that the introduction of the azaGly residue (**1b**) resulted in a much more powerful and specific substrate-derived inhibitor of cysteine cathepsins than the introduction of a 1,2,3-triazole (**1a**). Interestingly this result corroborates previous data based on a similar approach: a potent reversible inhibitor of human neutrophil proteinase 3