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Cathepsin B inhibitors: Further exploration of the nitroxoline core

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ABSTRACT

Human cathepsin B is a cysteine protease with many house-keeping functions, such as intracellular proteolysis within lysosomes. Its increased activity and expression have been strongly associated with many pathological processes, including cancers. We present here the design and synthesis of novel derivatives of nitroxoline as inhibitors of cathepsin B. These were prepared either by omitting the pyridine part, or by modifying positions 2, 7, and 8 of nitroxoline. All compounds were evaluated for their ability to inhibit endopeptidase and exopeptidase activities of cathepsin B. For the most promising inhibitors, the ability to reduce extracellular and intracellular collagen IV degradation was determined, followed by their evaluation in cell-based *in vitro* models of tumor invasion. The presented data show that we have further defined the structural requirements for cathepsin B inhibition by nitroxoline derivatives and provided additional knowledge that could lead to non-peptidic compounds with usefulness against tumor progression.

Human cathepsin B (catB, EC 3.4.22.1) is an intracellular lysosomal cysteine protease that is ubiquitously expressed in many tissues and is involved in a number of physiological processes.[1] The enzyme possesses an endopeptidase and a dipeptidyl carboxypeptidase activity,[2] a unique feature for cysteine cathepsins that is attributed to the presence of the occluding loop.[3] This is a flexible 20 amino acid insertion that in the so-called closed (i.e., exopeptidase) conformation spans from the left domain to cover the primed subsites S3′ and S2′ of the active site cleft and prevents the access of large endopeptidase substrates.[4,5] The occluding loop is held in this conformation via two salt bridges, His110-Asp22 and Arg116-Asp224. Additionally, two histidine residues (His110 and His111) are located at its tip providing positively charged anchors for the negatively charged C-terminal carboxyl group of the substrates.[4,6] Besides the well-known exopeptidase activity that has a pH optimum of around 5.0,[7] the endopeptidase function of catB greatly increases when the ionic contacts that bind the loop to the body of the enzyme are weakened.[6] It has been shown that the endopeptidase activity of catB increases with a rising pH value, reaching its maximum at neutral pH.[7]

CatB can actively participate in the majority of processes that significantly contribute to cancer progression. Particularly, it can modify the tumor microenvironment through the turnover and degradation of the extracellular matrix (ECM) either directly via proteolytic degradation of its components or indirectly via activation or amplification of other proteases in the proteolytic cascade.[8,9] This ECM breakdown is a crucial step that promotes tumor invasion, and enables angiogenesis and metastasis.[10,11] The degradation of metalloprotease inhibitors and the release of growth factors that are bound to the ECM components are two additional mechanisms through which catB contributes to angiogenesis.[12–14] CatB was also found to have a role in chemotherapy resistance, as it was shown that lysosomal leakage of catB, caused by chemotherapeutics 5-florouracil and gemcitabine, activates the Nlrp3 inflammasome and promotes tumor growth.[15] Importantly, high pharmacological relevance of catB has also been established in various tumor mouse models using catB-deficient mice[16–20] rendering catB as a validated and druggable target for the design of new anti-tumor drugs.

Abbreviations: Abz, 2-aminobenzoyl; AMC, 7-amido-4-methylcoumarin; catB, cathepsin B; DIPEA, *N,N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HOBt, hydroxybenzotriazole; K_i, inhibition constant; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; Z, benzyloxycarbonyl.

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Several types of exogenous inhibitors of catB have been identified and the majority of them have peptidyl backbones that contain an electrophilic functionality in the position of the scissile peptide bond. Various electrophilic warheads were explored in preclinical studies and these form either an irreversible (epoxysuccinyl, vinyl sulfone, acyloxymethyl ketone) or a reversible (ketone, nitrile) covalent bond with the catalytic cysteine in the active site.[21] Due to their low reactivities towards other cellular nucleophiles, the nitrile-containing inhibitors are receiving the most attention in the development of inhibitors of catB,[22] as well as other cysteine cathepsins.[23,24] The peptidic nature of currently available inhibitors can be a cause of low bioavailability and poor metabolic stability, which limit the use of a best part of such compounds to research only.[25] Given the fact that researchers involved in this field continue to appreciate the importance of cathepsins in disease management[26] and their involvement in cancer progression,[27] there is a substantial need for the advances towards new catB-inhibiting scaffolds that can bypass the limitations of peptidic catB inhibitors.

Our work in the field of catB inhibitors has been focused on nitroxoline and its derivatives and is summarized in Fig. 1. On the basis of these previous results, we herein report a complementary and focused set of compounds used to further explore the chemical space and the structure-activity relationships (SARs) of nitroxoline-based derivatives. Our efforts have resulted in several compounds that inhibit both catB activities in the low micromolar range, inhibit degradation of the ECM, and concurrently reduce invasiveness in cell-based *in vitro* models of tumor invasion.

The fragment-like characteristics of the 5-nitro-8-hydroxyquinoline core of nitroxoline enable numerous possibilities for structural elaboration. In our design, we pursued four different modifications to nitroxoline (Fig. 2).

First, several different 7-carboxamido substituted derivatives were prepared. Besides incorporating substituents that were used in our previous series of 7-aminomethylated derivatives,[28,30] we focused also on amidoacetonitriles. These nitrile-based substituents are very often used as mildly electrophilic warheads in inhibitors of different cathepsins.[22] Furthermore, some of the most recent and the most potent catB inhibitors known to date possess substituents of this exact nature.[23] Next, we synthesized a variety of compounds with nitrile-based substituents, i.e., either aminoacetonitriles or amidoacetonitriles at position 2 of the 8-hydroxy-5-nitroquinoline scaffold. The underlying reason for the preparation of these compounds was based on our recent molecular dynamics studies of the binding of nitroxoline and its derivatives into the active site of catB.[32] These results indicated that the quinoline ring can rotate around the axis, which is represented by the quinoline-NO_2 bond; a characteristic that is not evident from the crystal structure of the complex.[28] In addition, docking of a representative 2-amidoacetonitrile substituted 8-hydroxy-5-nitroquinoline **22** further corroborated this approach (Figs. S1 and S2 in the Supplementary data). Thus, interactions of the nitrile group of substituents at position 2 of the nitroxoline core with the catalytic cysteine are also possible. Third, a series of truncated compounds was prepared, in which the pyridine moiety was omitted. Within this group of derivatives, the same substituents as in the 7-carboxamido group were used and all were appended at position *ortho* with respect to the hydroxyl group. By preparing truncated derivatives we wanted to determine if the *ortho* substituted 4-nitrophenol represents a sufficient pharmacophore to be recognized by catB and to enable binding in its active site. Finally, we prepared a variety of compounds with diverse substituents attached directly to position 8 of the 5-nitroquinoline ring. Despite knowing the potential toxicity issues associated with NO_2-substituted aryl derivatives, this functionality was present in the synthesized molecules from all four classes. We established previously[28,30] that NO_2 group is a prerequisite for the inhibition of catB as it interacts with two histidines, His110 and His111, in the occluding loop of catB.

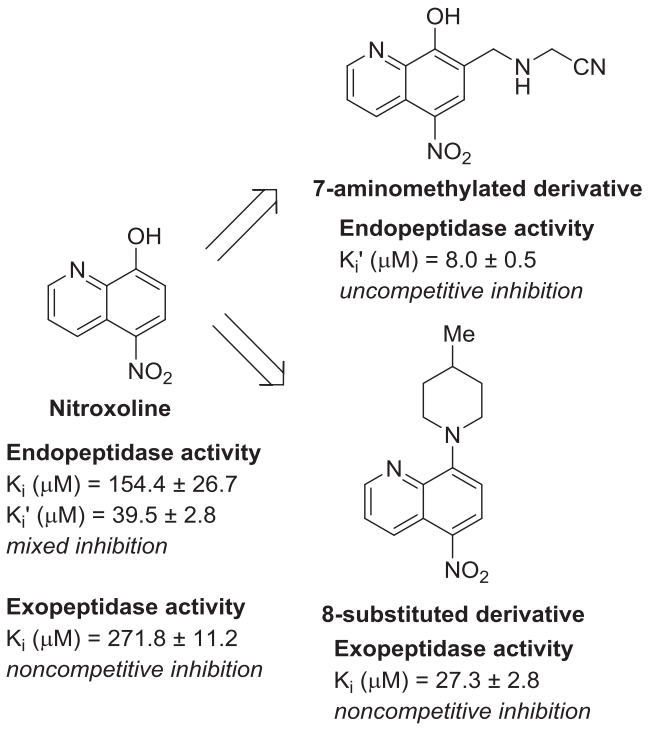

Fig. 1. Nitroxoline (5-nitro-8-hydroxyquinoline) and its endopeptidase and exopeptidase inhibition of catB.[28,29] Two representative examples of a first set of optimized derivatives[30,31] are also represented.

The nitroxoline carboxylic acid **1**[30,33] was used as substrate for the preparation of 7-carboxamido substituted nitroxoline derivatives **2**–**14** by modification of the carboxylic acid via an acid chloride (Scheme 1: rectangle A). Interestingly, all attempts to synthesize these derivatives using coupling reagents were unsuccessful. To prepare 2-substituted derivatives, five different commercially available quinolinol derivatives were used as starting material (Scheme 1: rectangle B). An efficient nitration procedure was applied in the first step using either a mixture of KNO_3/97% H_2SO_4 in acetic acid (for compounds **15** and **19**), 65% HNO_3 in acetic acid (for compounds **16** and **17**), or a mixture of 65% HNO_3/97% H_2SO_4 in acetic acid (for **18**). The regioselective introduction of the NO_2 group was confirmed by two-dimensional NMR experiments, such as homonuclear correlation spectroscopy and Nuclear Overhauser effect spectroscopy. The 2-aminoacetonitrile derivative **20** was synthesized from **18** by reductive amination procedure using $Na(OAc)_3BH$ as a reducing agent, whereas the amidoacetonitriles **21**–**24** were prepared by HATU-mediated coupling of 2-carboxy-8-hydroxy-5-nitroquinoline (**19**) and the corresponding amines (Scheme 1: rectangle B). Of note, several 2-substituted-5,7-dinitro derivatives were prepared (compounds **S1–S6**, Scheme S1 in the Supplementary data) and assayed for inhibition of catB.

For the 2-aminomethylated 4-nitrophenols **25**–**31**, either Mannich reaction conditions (**25**–**29**) or reductive amination (**30** and **31**) were used. The 2-carboxamide 4-nitrophenols **33**–**39** were synthesized via nitration of the salicylic acid to obtain intermediate **32**, followed by the reaction with $SOCl_2$ to generate the acid chloride *in situ*. Different amines were used subsequently for the formation of an amide bond in good overall yields (Scheme 1: rectangle C).