Bioorganic & Medicinal Chemistry 21 (2013) 2975–2987

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Development of cell-active non-peptidyl inhibitors of cysteine cathepsins

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ARTICLE INFO

Article history:
Received 16 January 2013
Revised 15 March 2013
Accepted 23 March 2013
Available online 2 April 2013

Keywords:
Cysteine protease inhibitor
Cathepsin inhibitor
Sulfonyloxiranes
Cancer metastasis
Cell migration

ABSTRACT

Cysteine cathepsins are an important class of enzymes that coordinate a variety of important cellular processes, and are implicated in various types of human diseases. However, small molecule inhibitors that are cell-permeable and non-peptidyl in nature are scarcely available. Herein the synthesis and development of sulfonyloxiranes as covalent inhibitors of cysteine cathepsins are reported. From a library of compounds, compound **5** is identified as a selective inhibitor of cysteine cathepsins. Live cell imaging and immunocytochemistry of metastatic human breast carcinoma MDA-MB-231 cells document the efficacy of compound **5** in inhibiting cysteine cathepsin activity in living cells. A cell-motility assay demonstrates that compound **5** is effective in mitigating the cell-migratory potential of highly metastatic breast carcinoma MDA-MB-231 cells.

1. Introduction

Precisely controlled proteolysis of cellular and extracellular proteins is a critically important cellular event that is elegantly orchestrated by a large family of 561 human proteases.[1] Among these are the fifteen members of cathepsin proteases that are primarily housed in membrane-bound organelles called lysosomes. Members of the cathepsin family, classified in terms of their catalytic nucleophilic residue, consist of 11 cysteine proteases (cathepsin B, C/J/dipeptidyl peptidase I, F, H, L, K, O, S, W, V and Z/X/P), two serine proteases (cathepsin A and G), and two aspartyl proteases (cathepsin D and E).[2] Recent decades of research strongly suggest that cysteine cathepsins play critically important and non-redundant roles in many physiological processes, such as cell death, the immune response, collagen degradation, and neurobiology.[3,4] Furthermore, either gain or loss of function as a result of their mis-regulation has been directly associated with a variety of human pathologies, such as cancer, osteoporosis, and autoimmune and metabolic disorders.[5–8] In addition to their importance in human biology, cysteine cathepsins are also implicated in many other types of diseases involving lower organisms such as parasites.[9,10] Consequently, they are important therapeutic targets for drug development.

It is now well established that over-expression and aberrantly regulated activity of cysteine cathepsins play a major role in promoting many hallmarks of cancer.[5,11] The expression of cysteine cathepsins (e.g., B, F, and L) has indeed been found to be significantly upregulated in solid tumors from various origins, such as breast, skin, colorectal, pancreatic, ovarian, brain, head and neck.[11,12] More importantly, a well-defined role for cysteine cathepsins in promoting cell invasion and cancer metastasis has recently emerged.[13,14] Indeed high level expression of cysteine cathepsin B was observed in invasive tumor cells derived from metastatic cancer patients of distinct origins.[15,16] Deletion of either cysteine cathepsins, B, L or S led to decreased tumor invasion with tumors progressively returning to a more benign form.[12] In a separate study, cysteine cathepsin X was able to compensate in part for the absence of cathepsin B in a mouse model of mammary cancer.[13] In this study, inhibition of cysteine cathepsin X with a neutralizing antibody resulted in a drastic loss of invasive behavior in cathepsin B null cultured cells. E-cadherin, an important adherens junction protein that maintains cell–cell adhesion in epithelial cells, was recently identified as a direct proteolytic target of cysteine cathepsins B, L and S.[12] Since it is well established that functional loss of E-cadherin promotes cell invasion and metastatic behavior of cancer cells, an effective inhibition of this family of enzymes is anticipated to provide significant therapeutic benefit in reducing metastatic potential of solid tumors.[17] These studies have led to an interesting hypothesis that promotes a

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http://dx.doi.org/10.1016/j.bmc.2013.03.062

poly-pharmacological approach to target pro-tumorigenic members of cysteine cathepsins collectively for the development of effective anti-cancer agents.

While numerous studies of cysteine cathepsins have clearly established them as important therapeutic targets for the development of anti-cancer agents, progress towards development of small molecule inhibitory agents that are non-peptidyl in nature remains slow. Currently, a majority of the reported inhibitory agents of cysteine cathepsins are peptidyl or peptidomimetic in nature, making them poorly cell permeable (reviewed by Turk et al.).[18] Small molecule inhibitory agents of cysteine cathepsins that are uncharged and non-peptidyl in nature are therefore desirable so that they can be further developed as suitable biological reagents, in addition to serving as lead chemotypes for drug development. Furthermore, if the mechanism of inactivation could be designed to produce a covalent and irreversible chemistry with the catalytic active site Cys residue, then the inhibitors could also be developed as activity-based probes (ABPs) for the functional analysis of cysteine proteases in complex proteomes. Activity-based probes have found extensive applications in functional annotation of enzymes in their native biological environments.[19]

2. Results and discussion

In this study, we synthesized a library of compounds containing sulfonyloxirane moiety in the context of a small non-peptidyl molecule and screened for their efficacy as time-dependent inhibitors of cysteine cathepsins. Compound **5** was identified as the lead inhibitory agent of cysteine cathepsin B. Enzymology and mass spectrometry-based experiments were performed to demonstrate that this class of molecules inhibited cysteine cathepsin B in a covalent and irreversible manner. Importantly, **5** was shown to be effective in inhibiting intracellular activity of cysteine cathepsin B, and the cell migratory behavior of highly metastatic human breast carcinoma MDA-MB-231 cells.

2.1. Design and synthesis of compounds 1–13 and time-dependent inhibitory evaluation

The advent of E-64 from *Aspergillus japonicus*, an epoxysuccinyl-derived inhibitory agent, was a critically important stepping stone for deciphering the undocumented function of cysteine cathepsins family of enzymes.[20] Although, E-64 is a generic non-selective inhibitor of cysteine cathepsins, structural investigation of the E-64-inhibited complex have subsequently led to the development of selective cysteine cathepsin B inhibitors (e.g., CA030 and CA074).[21,22] The marked success of E-64 and its subsequent analogues as effective inhibitors of cysteine cathepsins relies on the exclusive reactivity of an active site cysteine residue with the electrophilic 'warhead' epoxy group. The catalytic mechanism of cysteine cathepsins involves participation of this invariant and reactive cysteine residue.[23] A close visual inspection of the structures of CA030 and CA074 in complex with cysteine cathepsins revealed that there exist well-defined binding pockets, S2 and S1′, surrounding the S1 site (Schechter and Berger nomenclature)[24] that prefer to accommodate aromatic hydrophobic groups.[21,22] It was therefore hypothesized that an arylsulfonyl-substituted oxiranyl moiety could serve as a small molecule covalent inactivator of cysteine cathepsins, targeting the key residues of the core enzyme active site. This approach envisions that (a) an appropriate binding of inhibitory compounds within the active site of cysteine cathepsin will allow the reactive Cys residue to be entrapped by the activated sulfonyloxirane group, (b) the sulfonyl group will likely mimic the potential H-bonding interactions that the carbonyl group α to the epoxy moiety exhibits in the inhibited CA030-cathepsin B complex,[21] and (c) the sulfonyl group will further enhance the electrophilicity of the oxiranyl carbon, compared to a carbonyl group, thereby facilitating the entrapment of the active site Cys residue. Upon successful demonstration of the aforementioned hypotheses, this class of molecules could serve as a new type of chemical motif on which selective cysteine cathepsin inhibitors could be developed in future, exploiting the surrounding S3 and S′ pockets. Such a strategy has indeed been utilized to develop potent and selective inhibitors of cysteine cathepsins.[25–30]

A small library of sulfonyloxirane compounds was therefore synthesized. Cysteine cathepsin B, a promoter of cancer metastasis, was chosen as the initial inhibitory target to evaluate the efficacy by following a time-dependent loss of enzyme activity (Table 1). The initial screening was performed by incubating a fixed concentration of compounds **1**–**13** with cathepsin B under a pseudo-first order condition. A small aliquot of the incubation mixture was withdrawn at a fixed time interval and the remaining cathepsin B activity was measured in a large volume of assay mixture (17.5-fold dilution) containing cathepsin B substrate. The IC_{50} values were deliberately not estimated for this library, since they are not considered a good yardstick for measuring a time-dependent irreversible loss of enzyme activity due to a non-equilibrium binding mechanism.[31]

Compounds **1** and **2** which bear a non-polar 4-fluorophenyl and a propyl substituent respectively at the second carbon of the oxirane ring were non-inhibitory. This result indicated that a second substitution at the oxirane moiety in this class of molecules was perhaps detrimental to their inhibitory efficacies. Furthermore, compound **12**, which incorporates a methylene linker between the arylsulfonyl group and oxirane moiety, was also inert. We surmised that the lack of reactivity of **12** could presumably be due to lack of assistance from a general acid appropriately positioned in the cathepsin B active site to promote nucleophilic opening of oxirane ring by Cys29-S_γ residue. So, a thiirane analogue, **13**, that potentially contains a better leaving group thiolate (compared to alkoxide in **12**), was synthesized, and was also found to be ineffective. This again indicated that appropriate positioning of arylsulfonyl group with respect to the reactive oxirane group within the cysteine cathepsin active site was critically important for its inhibition. Efforts then became focused on synthesizing analogues of 2-(arylsulfonyl)oxiranes where functional variations of the aryl ring was further investigated.

Among the synthesized library, compound **5** was found to be most effective in inhibiting cysteine cathepsin B activity. The effectiveness of compound **5** was evident from a progressive loss of enzyme activity when it was incubated with active cathepsin B (Fig. 1A). No gain in cathepsin B activity was observed even after dilution of **5**–inactivated cathepsin B complex, thereby indicating that the mechanism of inhibition was perhaps covalent and irreversible in nature (data not shown). To assess the inhibitory efficacy, inactivation experiments were performed at appropriate concentrations of compound **5** under pseudo-first order conditions, and the experimental data thus obtained were analyzed using a simple two-step inhibition model (Fig. 1B):

$$E + I \overset{K_i}{\rightleftharpoons} E \cdot I \overset{k_i}{\rightarrow} E - I$$

where K_i represents the reversible equilibrium binding constant for the first step, and k_i is the first order inactivation rate constant for the second irreversible step. To obtain these two parameters, the following procedure was adopted. The pseudo-first order rate constants of inactivation (k_{obs}) were obtained at appropriate concentration of **5** ([I] ≫ [cathepsin B]), and a Kitz–Wilson analysis was performed to fit the experimentally obtained data (Fig. 1C).[32] This procedure yielded the equilibrium binding constant (K_i = 86 ± 3 μM) for the first equilibrium binding step, and the first order