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Acridone alkaloids as potent inhibitors of cathepsin V

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ABSTRACT

Cathepsin V is a lysosomal cysteine peptidase highly expressed in thymus, testis and corneal epithelium. Eleven acridone alkaloids were isolated from *Swinglea glutinosa* (Bl.) Merr. (Rutaceae), with eight of them being identified as potent and reversible inhibitors of cathepsin V (IC_{50} values ranging from 1.2 to 3.9 μM). Detailed mechanistic characterization of the effects of these compounds on the cathepsin V-catalyzed reaction showed clear competitive inhibition with respect to substrate, with dissociation constants (K_i) in the low micromolar range (**2**, K_i = 1.2 μM; **6**, K_i = 1.0 μM; **7**, K_i = 0.2 μM; and **11**, K_i = 1.7 μM). Molecular modeling studies provided important insight into the structural basis for binding affinity and enzyme inhibition. Experimental and computational approaches, including biological evaluation, mode of action assessment and modeling studies were successfully employed in the discovery of a small series of acridone alkaloid derivatives as competitive inhibitors of catV. The most potent inhibitor (**7**) has a K_i value of 200 nM.

1. Introduction

Cathepsins, also known as lysosomal cysteine peptidases, are members of the papain-like peptidase family, which are implicated in many pathological conditions. These enzymes have been intensively studied as valuable targets for drug discovery and development.[1,2] Although the major role of cathepsins is related to the terminal protein degradation in lysosomes, it has been shown that these enzymes are also involved in other relevant biochemical pathways, acting at selective and controlled processes with specific functions associated to their restricted tissue localization.[3] In addition, evidence has indicated that cysteine cathepsins have specific intra- and extracellular functions, being involved in a number of diseases including cancer, osteoarthritis, osteoporosis, autoimmune disorders, and viral infections.[4]

Cathepsin V (CatV, EC 3.4.22.43) was identified as a lysosomal cysteine protease specifically expressed in thymus, testis and corneal epithelium.[5–7] The in vivo role of cathepsin V has been associated with the major histocompatibility complex (MHC) class II presentation pathway in humans[8] and, in pathological conditions, the enzyme has been considered as a potential diagnostic marker for colon tumors.[6] The enzyme is also expressed in colorectal and breast carcinomas but not in normal colon or mammary tissue. It is believed that cathepsin V plays a role in cancer progression, thus becoming a valuable drug target for oncology.[9] Moreover, cathepsin V has been identified as having potent elastolytic activity expressed in activated macrophages and, together with the lysosomal cathepsins L, K, and S, has been implicated in atherosclerosis.[10,11] Significant progress has been made to elucidate the relationship between cysteine cathepsins and several pathological states. For instance, cathepsins K and S have been validated as drug targets for the treatment of osteoporosis and immune disorders, respectively.[4]

Natural products have provided considerable value to the pharmaceutical industry over the past century. Natural products represent a rich source of structural diversity and biological activities, providing many drugs and lead compounds for the treatment of a variety of human diseases successfully developed for the treat of human diseases in many therapeutic areas.[12]

Several natural products have been investigated for their inhibitory effects on the catalytic activity of cathepsins.[4,13–15] Acridone alkaloids are a group of naturally occurring nitrogen heterocyclic compounds that have shown a large spectrum of biological activities, including antiparasitic,[16–18] antiviral,[19] and cytotoxic.[18,20–22] In the present work we described the biological evaluation and type of inhibition studies for a series of acridone alkaloids as potent inhibitors of cathepsin V. Molecular modeling and preliminary structure–activity relationship (SAR) studies were also performed to investigate the molecular basis underlying the binding affinity and inhibitory potency of this series of naturally occurring compounds. To the best of our knowledge, this is the first example

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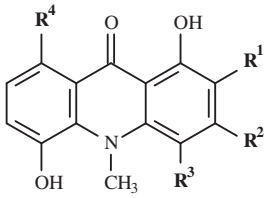
of the use of natural products as reversible and competitive inhibitors of CatV.

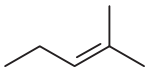
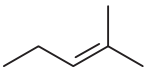
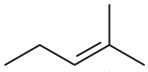
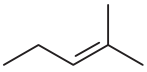
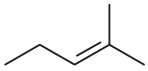
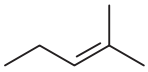
2. Experimental

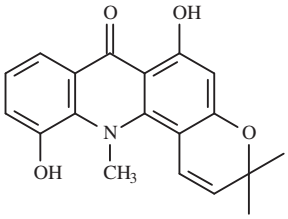
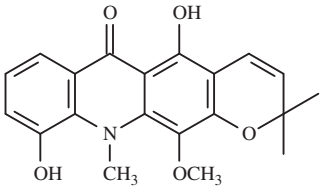
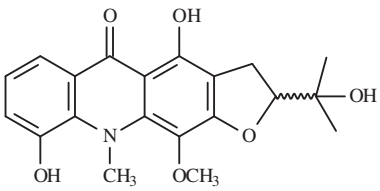
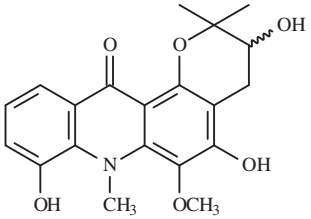
2.1. Chemistry

The acridone alkaloids 1,3,5-trihydroxy-4-methoxy-10-methyl-2,8-*bis*(3-methylbut-2-enyl)acridin-9(10*H*)-one (**1**), 1,3,5-trihydroxy-2,8-*bis*(3-methylbut-2-enyl)-10-methyl-9-acridone (**2**), glycocitrine-IV (**3**), glycocitrine-I (**4**), citrusinine-II (**5**), citrusinine-I (**6**), citibrasine (**7**), 5-hydroxynoracronycine (**8**), pyranofoline (**9**), 2,3-dihydro-4,9-dihydroxy-2-(2-hydroxy-propan-2-yl)-11-methoxy-10-methylfuro[3,2-*b*]acridin-5(10*H*)-one (**10**), and 3,4-dihydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-2*H*-pyrano[2,3-*a*]acridin-12(7*H*)-one (**11**) were isolated and identified from the methanol extract of the stem bark of *Swinglea glutinosa* (Rutaceae) by chromatographic and spectral methods (Table 1).

Table 1
Chemical structures and corresponding values of IC_{50} and K_i for a series of acridone alkaloids as inhibitors of cathepsin V



Compound	R^1	R^2	R^3	R^4	IC_{50}* (μM)	K_i (μM)
1		OH	OCH_3		2.5 ± 0.2	0.5
2		OH	H		3.9 ± 0.9	1.2
3		OH	OCH_3	H	2.2 ± 0.2	1.1
4	H	OH		H	25 ± 5	10.0
5	H	OH	OCH_3	H	10 ± 1	4.2
6	H	OCH_3	OCH_3	H	2.2 ± 0.6	1.0
7	OCH_3	OCH_3	OCH_3	H	1.2 ± 0.1	0.2

Compound	Structure	IC_{50}* (μM)	K_i (μM)
8		48 ± 5	–
9		44 ± 3	–
10		5.2 ± 0.2	4.4
11		2.8 ± 0.7	1.7

* The values represent means of at three individual experiments ± SD.

Detailed information on the isolation and characterization of the alkaloids can be obtained elsewhere.[18]

2.2. Expression and purification of cathepsin V

Recombinant human cathepsin V was produced using the *Pichia pastoris* expression system as previously described.[7] The molar concentration of the enzyme was determined by active site titration with E-64 following the conditions previously described.[23]

2.3. Kinetic measurements

All commercially available chemicals and reagents were purchased from Aldrich Chemical Co. and Sigma and used without further purification. Stock solutions of the compounds were prepared at a concentration of 1 mM in DMSO and diluted to the appropriate concentration prior to the assays. The inhibitors were screened against cathepsin V at an initial concentration of 25 μM. The in vitro enzyme inhibition experiments were carried out in triplicate (in 96-well black plates) as previously described.[24] The final volume of the reaction mixture was 200 μL, kept under stirring. Each well contained 191 μL of a 100 mM sodium acetate buffer (pH 5.5) containing 5 mM EDTA and 5 mM dithioerythritol (DTE), 2 μL of 1 mM Z-Phe-Arg-MCA dissolved in DMSO, 5 μL of sample, and 32 nM of cathepsin V. The enzyme was activated during 5 min with DTE at 27 °C, and then the reaction mixture was incubated during 5 min with the sample. The reactions were started by the addition of the fluorogenic substrate 4-methylcoumaryl-7-amide (MCA) and measurements were conduct using the Molecular Devices Spectra MAX GEMINI XS (excitation 355 nm, and emission 460 nm). Control assays were performed without inhibitor (negative control) and in the presence of the irreversible inhibitor for cysteine peptidase, E-64 (positive control). The IC_{50} values were independently determined by making rate measurements for at least five inhibitor concentrations. The type of inhibition and K_i values were determined under the same experimental conditions for three different inhibitor concentrations at five varying substrate concentrations (6.4, 9.6, 13, 19, and 38 μM). All kinetic parameters were determined from the collected data by nonlinear regression employing the SigmaPlot enzyme kinetics module. The values represent means ± SD of at least three individual experiments.

2.4. Molecular modeling

The X-ray crystallographic data for catV in complex with APC-3316 (an irreversible vinyl sulfone inhibitor) used in docking simulations were retrieved from PDB (PDB ID, 1FH0). For the calculations, hydrogen atoms were added in standard geometry, using the Biopolymer module as implemented in the SYBYL 8.0 package (Tripos Inc., St. Louis, MO) running on Red Hat Enterprise Linux workstations. The only exception was the catalytic cysteine residue (Cys25), which was deprotonated during docking simulations. The histidine, glutamine, and asparagine residues in the active binding site were manually checked for possible flipped orientation, protonation, and tautomeric states with the Pymol 0.99 (DeLano Scientific, San Carlos, CA) side-chain wizard script. The active site was defined incorporating all amino acid residues within a radius sphere of 6.0 Å centered on the bound ligand. Molecular modeling studies were carried out using the default parameters of FlexX (BioSolveIT, Sankt Augustin, Germany).[25] The FlexX scoring function was employed to select the representative pose for each compound of this series. Subsequently, the top-ranked conformations for each compound were submitted to the web-based interface DrugScore^{ONLINE} to rescore the proposed binding mode.[26,27] Finally, the individual ranks obtained from the consensus scoring were considered to establish a new rank order list where the top-ranked inhibitor were selected for visual inspection.

3. Results and discussion

3.1. Biochemical evaluation and preliminary SAR studies

As part of our ongoing research program aimed at discovering natural products as new potent inhibitors of cathepsins K, V, L, and S, we have screened a collection of 270 compounds, from which 111 were natural products isolated from Brazilian plants. The major classes evaluated in this study included cumarins, triterpenes, cinnamic acids, amides, lignans, flavonoids, alkaloids, and limonoids.

The percentage of inhibition was calculated according to the equation:

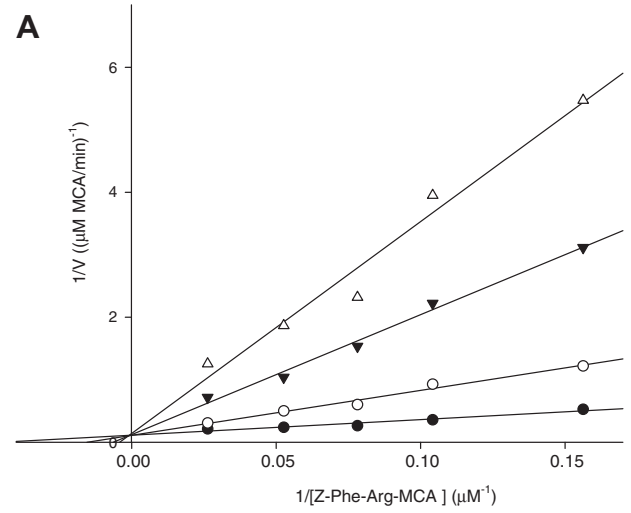
$$\% \text{ inhibition} = 100 \times (1 - V_i/V_0)$$

where V_i and V_0 are initial velocities (enzyme activities) determinate in presence and in the absence of inhibitor, respectively.

Among the compounds tested, the most promising results against the cathepsins were obtained for the alkaloid and flavonoid derivatives, including a series of acridone alkaloids (**1**–**11**), which showed potent inhibitory activity against cathepsin V. The chemical structures and corresponding IC_{50} values (concentration of compound required for 50% inhibition of cathepsin V) for the set of compounds (**1**–**11**) are shown in Table 1. As can be seen, compound **7** is the most potent inhibitor, with an IC_{50} value of 1.2 μM. This is a valuable result since acridone alkaloids as catV inhibitors have not been described in the literature. In addition to compound **7**, compounds **1**–**3**, **6**, **10**, and **11** also showed substantial inhibition with IC_{50} values in the range of 2.2–5.2 μM. Compounds **4** and **5** showed only moderate inhibition, with IC_{50} values of 25 and 10 μM, respectively. The isomeric tetracyclic system (compounds **8** and **9**) also showed low inhibitory potency, with IC_{50} values of 48 and 44 μM, respectively. The most potent inhibitors of the series (**2**, **4**–**7**, **10**, and **11**) were then selected for further kinetic studies for determination of the type of inhibition and dissociation constants (K_i).

3.2. Mechanism of inhibition

In order to explore the mechanism of inhibition in more detail, we have determined K_i values (Table 1) and the type of inhibition with respect to the substrate Z-Phe-Arg-MCA employing compounds **2**, **4**–**7**, **10**, and **11**. The results indicate that the inhibition of catV was found to be competitive with respect to the substrate Z-Phe-Arg-MCA (Fig. 1). The Lineweaver–Burk double-reciprocal plots show intercepts of all lines (obtained at three different inhibitor concentrations) converging at the *y*-axis ($1/V_{max}$), whereas the slope (K_M/V_{max}) and *x*-axis intercepts ($-1/K_M$) vary with inhibitor concentration. Consequently, the V_{max} values remain constant, whereas the apparent values of K_M (K_M^{app}, defined as $K_M(1 + [I]/K_i)$) increase with increasing inhibitor concentrations. This behavior is consistent with a mutually exclusive binding mode between inhibitor and substrate, therefore, these inhibitors (**2**, **4**–**7**, **10**, and **11**) compete with Z-Phe-Arg-MCA for the free enzyme active site. Another factor that must be considered in inhibitor drug design is the comparisons on the rational basis of the enzyme dissociation constant (K_i), since IC_{50} values can vary with substrate concentration for competitive inhibitors. The same data of Figure 1 was employed to determine the K_i values of the competitive inhibitors using Dixon plots, which consist in plotting the reciprocal of the initial velocity ($1/V_0$) versus a series of inhibitor concentrations at constant substrate concentrations, where the



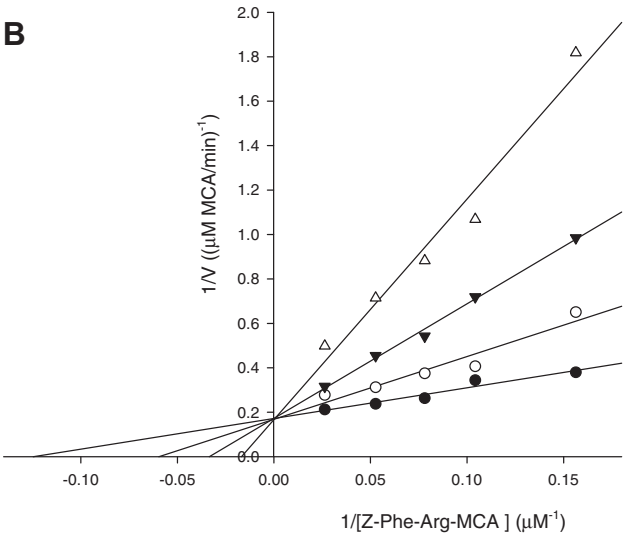


Figure 1. Competitive inhibitory profile of the CatV inhibitors **7** (**A**, K_i = 0.2 μM) and **11** (**B**, K_i = 1.7 μM). Kinetic assays were conducted in the presence of increasing concentrations of inhibitors. Panel A: 5 μM (△), 3 μM (▼) and 1 μM (○). Panel B: 7 μM (△), 3 μM (▼) and 1 μM (○). The absence of inhibitor is depicted by ● in panels A and B.

lines converge above the *x*-axis, indicating the inhibition constant $-K_i$. The K_i values for inhibitors **2**, **4**–**7**, **10**, and **11** are listed in Table 1. Accordingly, the results confirm the acridone alkaloid derivatives as a new class of competitive inhibitors of catV, with affinity values in the low micromolar range.

3.3. Molecular modeling studies

The integration of experimental and computational approaches has become a vital component of structure- and ligand-based approaches.[28–31] Despite the strong evidence that the acridone alkaloids bind to the active site of the enzyme, the kinetic data does not provide insights into the binding profile of the inhibitors (in the substrate pocket of catV). Therefore, molecular modeling studies were carried out to complement the kinetic findings and to investigate the mode of interaction of these inhibitors.[30,31] To achieve this, the compounds of this series were docked into the active site of catV. Although a quantitative agreement between the model and

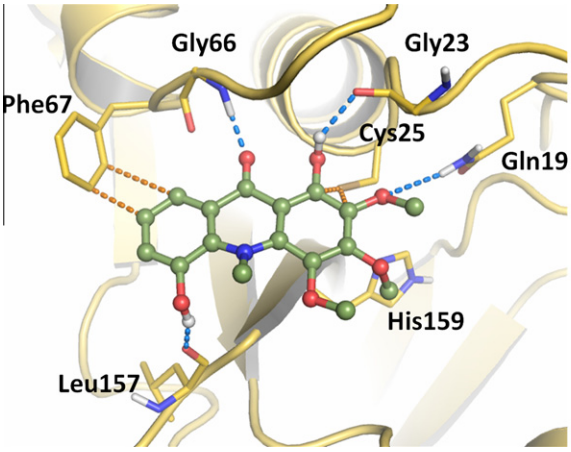


Figure 2. Predicted conformation of the representative compound **7** (green) within the catV binding pocket. Key residues involved in ligand binding are indicated as stick models and hydrogen bonds and van der Waals interactions as blue and orange dashed lines, respectively.

experimental SAR data was not expected, the model provided relevant insights into the inhibitory activity of the acridone alkaloid derivatives. The best predicted orientation of the representative inhibitor of this series, compound **7** (K_i = 200 nM), within the catV binding pocket is depicted in Figure 2. It can be seen that the inhibitor binds to the central region of the catV substrate binding site, close to the catalytic residues Cys25 and His159. The inhibitor interacts to the enzyme through a set of four hydrogen bonds: (i) the 1-hydroxyl substituent is positioned at the S1 pocket as a hydrogen bond donor to the main-chain carbonyl group of Gly23; (ii) the oxygen atom of the 2-methoxy substituent accepts a hydrogen bond from the NH_2 side-chain of Gln19, which is part of the oxyanion hole in catV;[10] (iii) the 5-hydroxyl substituent binds to the S2 pocket acting as a hydrogen bond donor to the main-chain carbonyl group of Leu157; and (iv) the 9-carbonyl group binds to the S3 pocket by accepting a hydrogen bond from the NH main-chain of Gly66. In addition to the polar contacts, non-polar interactions contribute to the orientation of **7** in the catV binding site. The 1-hydroxyl and 2,3,4-trimethoxy substituted rings form van der Waals contacts with the side-chains of Phe67 and Cys25, respectively. These findings are in agreement with our SAR data, which indicated that the presence of polar substituents at R^1 (2-position) and less bulky groups at R^4 (8-position) are favorable for enhanced inhibitory activity. Moreover, this conformation also suggests a structural feature that might be involved in the preferential binding of the 2-methoxy derivatives at R^1 (**7**) compared with the corresponding isoprenoid (**1**–**3**) or unsubstituted derivatives (**4**–**6**; **8**–**10**). According to the model, the Gln19 residue would represent a hydrogen-bonding site to interact with the inhibitor in order to incorporate selected molecules inside the binding cavity of the enzyme, thereby contributing to the improved affinity of the inhibitor **7**. This observation is supported by the finding that the S1′ would play a major role in the design of inhibitors having improved potency and affinity.[10]

4. Conclusion

In this work, we described a new series of acridone alkaloid derivatives as potent inhibitors of the enzyme catV. The kinetic and type of inhibition studies revealed that the compounds of this class are reversible competitive inhibitors of the target enzyme with affinity in the low micromolar range. The SAR and molecular modeling investigations indicated important molecular and structural features underlying the inhibitory activity of this series. In

sum, compounds **7** (K_i = 200 nM) is a promising lead candidate for future medicinal chemistry efforts designed to discover new competitive inhibitors of catV with enhanced affinity and potency.

Acknowledgments

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