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# Synthesis and proteasome inhibition of lithocholic acid derivatives

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ABSTRACT

A new class of proteasome inhibitors was synthesized using lithocholic acid as a scaffold. Modification at the C-3 position of lithocholic acid with a series of acid acyl groups yielded compounds with a range of potency on proteasome inhibition. Among them, the phenylene diacetic acid hemiester derivative (**13**) displayed the most potent proteasome inhibition with $IC_{50}$ = 1.9 μM. Enzyme kinetic analysis indicates that these lithocholic acid derivatives are noncompetitive inhibitors of the proteasome.



The proteasome is an important protein complex that plays a critical role in maintaining cellular homeostasis.[1–3] The main function of the proteasome is intracellular degradation of damaged, unwanted, and misfolded proteins. The 20S proteasome has a cylindrical structure containing four rings stacked on top of each other. The two outer rings each contain seven structural α-subunits that do not have enzymatic activity. The two inner rings, each with seven β-subunits, contain three major proteolytic activities: a chymotrypsin-like (β5), a trypsin-like (β2), and a caspase-like (β1) activity. These proteolytic activities allow the proteasome to cleave unwanted proteins into 8–12 amino acid peptides. The chymotrypsin-like activity is believed to be the most important activity in protein degradation and is thus the primary target of most proteasome inhibitors.[2,3]

By regulating cellular protein levels, the proteasome is critical for maintaining many important cellular functions, such as the cell cycle, apoptosis, and immune response. Targeting proteasomal proteolysis may lead to new treatments for a variety of clinical conditions, such as cancers, inflammation, and neurodegenerative diseases. One successful example is the proteasome inhibitor peptide boronate, PS341 (Bortezomib), which was developed into an anti-cancer drug for the treatment of multiple myeloma.[4]

In addition to bortezomib, a number of other proteasome inhibitors have been developed either as experimental tools or as potential drug candidates for clinical usage, especially for anti-cancer therapy. Originating from both chemical synthesis and natural sources, the majority of these proteasome inhibitors have peptide-related structures, interacting with the proteasome at the catalytic site to competitively inhibit the proteolysis of substrate. Examples of these compounds include MG132, CEP1612, PS341, lactacystin, TMC-89A, and argyrin A.[5] On the other hand, non-peptide proteasome inhibitors are less common than the peptide-related proteasome inhibitors. Two triterpene derivatives, celastrol and withaferin A, and some green tea polyphenols, have been reported with proteasome-inhibitory effects.[6–9] We recently reported that a series of triterpene 18β-glycyrrhetinic acid derivatives had potent inhibitory activity on the 20S proteasome.[10] Although the competitive proteasome inhibitors targeting the catalytic sites are well documented, noncompetitive inhibitors are less common and have generally not been well characterized[11] though some quinolines were reported to be noncompetitive inhibitors.[12,13] In this paper, we report a class of novel proteasome inhibitors that inhibit the proteasome in a noncompetitive manner.

Our previous study indicated that glycyrrhetinic acid can be used as a scaffold to synthesize proteasome inhibitors through esterification of its C-3 hydroxyl group.[10] In an effort to search for new scaffolds for the synthesis of proteasome inhibitors, several natural products, including moronic acid, ursolic acid, oleanolic acid, and lithocholic acid (LA), were evaluated as potential scaffolds.[10,14] Among these natural products, only LA inhibited the chymotrypsin-like activity of the 20S proteasome with an $IC_{50}$ of 18.1 μM (Table 1). Therefore, LA was used as a new scaffold to further increase the potency of the proteasome inhibition. The usage of LA as the scaffold is advantageous in that it is readily available and less expensive than other triterpene natural products. The molecular size of LA is also smaller than other triterpenes.

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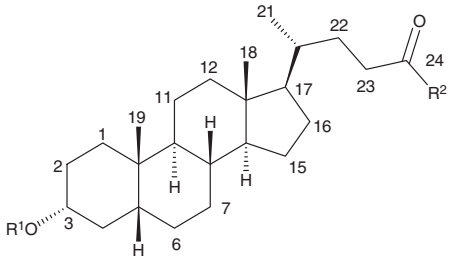
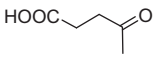
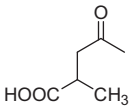
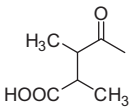
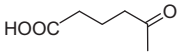
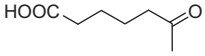
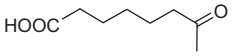
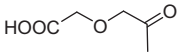
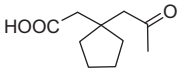
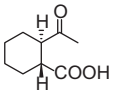
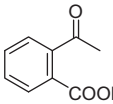
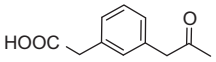
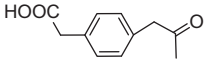
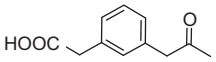
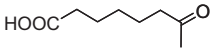
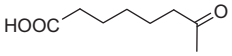
**Table 1**
Inhibition of proteasome activities by lithocholic acid and its derivatives



Lithocholic acid and its derivatives

| Compound | $R^1$ | $R^2$ | $IC_{50}$[a] (μM) |
|---|---|---|---|
| Lithocholic acid | H | OH | 18.1 ± 3.3 |
| **1** | H | $OCH_3$ | NA[b] |
| **2** | H | $OC_2H_5$ | NA[b] |
| **3** |  | OH | 10.3 ± 2.1 |
| **4** |  | OH | 11.5 ± 2.3 |
| **5** |  | OH | 5.8 ± 0.9 |
| **6** |  | OH | 10.8 ± 1.7 |
| **7** |  | OH | 3.5 ± 0.6 |
| **8** |  | OH | NA[b] |
| **9** |  | OH | 6.9 ± 1.5 |
| **10** |  | OH | 2.2 ± 0.3 |
| **11** |  | OH | 8.5 ± 1.4 |
| **12** |  | OH | 8.6 ± 1.3 |
| **13** |  | OH | 1.9 ± 0.3 (6.5)[d] (9.1)[e] |
| **14** |  | OH | 3.8 ± 0.5 |
| **15** |  | $OCH_3$ | NA[b] |
| **16** |  | $OCH_3$ | NA[b] |
| **17** |  | $OC_2H_5$ | NA[b] |
| LLM-F[c] | | | 5.2 ± 0.8 |
| Lactacystin[c] | | | 5.6 ± 0.9 |

[a] The inhibition of chymotrypsin-like (ChT-L) activities of the 20S proteasome was determined in the presence of various concentrations of the compounds as previously described.[10] $IC_{50}$ is the concentration that inhibits the proteasomal activity by 50%. The value of $IC_{50}$ is expressed as mean ± standard deviation from three independent assays.
[b] No inhibition.
[c] LLM-F and lactacystin are known proteasome inhibitors.
[d] $IC_{50}$ against caspase-like activity of the proteasome.
[e] $IC_{50}$ against trypsin-like activity of the proteasome.

The synthesis of LA C-3 ester derivatives was accomplished by treating LA or a LA methyl ester with the corresponding dicarboxylic acids or anhydrides under general esterification conditions (Scheme 1). Compounds **3**, **4**, **6**, **9**, **11**, and **12** were obtained by treatment of LA with corresponding anhydrides in the presence of 4-(dimethylamino)pyridine (DMAP), using microwave-assisted heating. Treatment of LA, LA methyl ester, or LA ethyl ester with corresponding dicarboxylic acid under microwave heating in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC)/DMAP yielded the compounds **5**, **7**, **8**, **10**, **13**–**17**.

The synthesized compounds were evaluated at various concentrations to determine their potencies against the chymotrypsin-like activity of the proteasome by using a previously described method.[10] Two known proteasome inhibitors Ac-Leu-Leu-Met-CHO (LLM-F) and lactacystin were included in the assay as controls (Table 1).

Results of the proteasome assay indicated that with the exception of compound **8**, C-3 esterifications of LA in general increased the potency of inhibition of chymotrypsin-like proteasome activities by 2- to 10-fold when compared to unmodified LA. In addition, compounds with C-3 aromatic or cyclic acetic acid moiety, such as **10**, **13**, and **14**, were in general more potent ($IC_{50}$ = 1.9–3.8 μM) than compounds without any ring system on their C-3 side chains. However, compounds **11** and **12** with a cyclic carboxylic acid moiety at C-3 were not as potent as other compounds with a ring system on their C-3 side chains. Compound **8,** with an unbranched heptanoic acid C-3 side chain, did not inhibit the chymotrypsin-like proteasome activity.

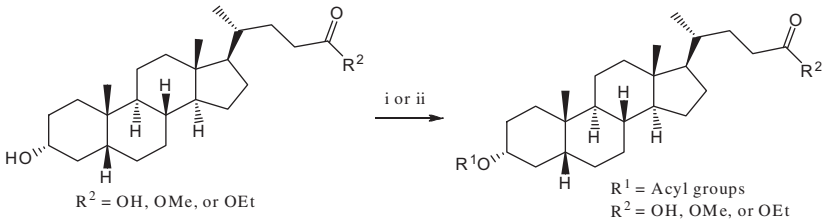
LA esterified at the C-24 position with methyl or ethyl groups resulted in a loss of activity in proteasome inhibition, as seen in compounds **1** and **2**, suggesting that the carboxylic acid moiety in the C-24 position is critical for inhibition of the chymotrypsin-like proteasome activities. The negative impact on proteasome inhibition by modifying the C-24 carboxylic acid was also evidenced by comparing compound **13** and its methylester derivative **15**, which lost inhibitory activity after esterification at the C-24.

To determine whether the lithocholic acid derivatives could also inhibit the other two main proteasomal activities, the most potent lithocholic acid derivative compound **13** was tested against the caspase-like and the trypsin-like activities of the proteasome. Compound **13** inhibited the caspase-like and the trypsin-like activities of the proteasome at 6.5 and 9.1 μM, respectively (Table 1).

Most proteasome inhibitors, such as the anti-cancer drug bortezomib, are competitive inhibitors that compete with substrates for the catalytic site binding. Since the lithocholic acid derivatives are not analogs of peptide substrates, it is possible that these compounds are not competitive inhibitors of the proteasome. To test this possibility, compound **10** was used in enzyme kinetic studies to determine its mode of action.

The chymotrypsin-like activity of the proteasome was determined using various concentrations of the substrate Suc-Leu-Leu-Val-Tyr-AMC in the presence or absence of 2 and 4 μM compound **10** (Fig. 1). The effects of compound **10** on the reaction rates of the chymotrypsin-like activity of the proteasome were analyzed using the Lineweaver–Burk plot (Fig. 1). Compound **10** at 2 and 4 μM reduced the maximum reaction rate ($V_{max}$) of the proteasomal activity without affecting the Michaelis–Menten constant ($K_m$) of the substrate, suggesting that compound **10** inhibited the proteasome in a noncompetitive manner. This data suggested that lithocholic acid derivatives are allosteric inhibitors of the proteasome.

In order to determine whether the lithocholic acid derivatives can inhibit the proteasome in living cells, HeLa $Ub^{G76V}$-GFP cells were treated with compound **10** at 7.5 μM for 24 h.[15] In the presence of proteasome inhibitors, HeLa $Ub^{G76V}$-GFP cells accumulate $Ub^{G76V}$-GFP which emits green fluorescence under fluorescent




Reaction conditions: (i) dicarboxylic acid anhydride, DMAP, pyridine, microwave heating, 120 °C, 2 hr; (ii) dicarboxylic acid, DCC, DMAP, pyridine, microwave heating, 120 - 160 °C, 10 min - 2 hr.

**Scheme 1.** Synthesis of lithocholic acid derivatives. Reagents and conditions.

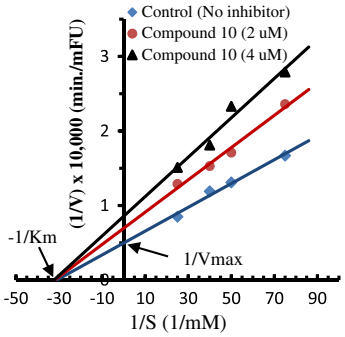



**Figure 1.** Compound **10** is a noncompetitive inhibitor of the proteasome. $K_m$ is the concentration of substrate (S) when the reaction rate ($V$) equals to 1/2 of the maximum reaction rate ($V_{max}$). The mean fluorescence units (mFU) are proportional to the amount of cleaved substrates. Each data point in the figure represents the average of two independent experiments.

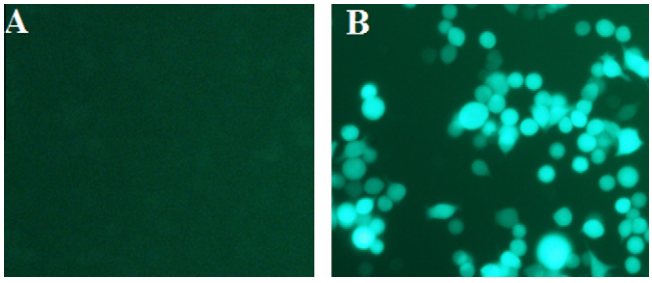



**Figure 2.** Compound **10** inhibited the proteasome in HeLa $Ub^{G76V}$-GFP cells. The cells were cultured in the presence (A) or absence (B) of compound 10 at 7.5 μM for 24 h. Emission of green fluorescence from $Ub^{G76V}$-GFP was documented with a Nikon fluorescence microscope system.

microscope.[16] Treatment of the HeLa $Ub^{G76V}$-GFP cells with compound **10** resulted in accumulation of $Ub^{G76V}$-GFP (Fig. 2, panel B). This result suggests that the lithocholic acid derivatives are able to get into the cells and inhibit the proteasome.

In summary, we have identified LA derivatives modified with a variety of acidic acyl substituents at the C-3 position exhibiting a range of inhibitory activity against the proteasome. Compound **13**, the most potent inhibitor with C-3 phenyl diacetic acid hemiester side chain, inhibited the chymotrypsin-like activity of the proteasome with an $IC_{50}$ of 1.9 μM. Based on the data shown in Table 1, the C-3 cyclic acetic acid moiety is critical for potent proteasome inhibition. Compounds **10**, **13**, and **14**, which possess a C-3 cyclic acetic acid moiety, were all more potent than the known proteasome inhibitors, LLM-F and lactacystin. In addition, a C-24 free carboxylic acid was found to be important for the proteasomal inhibitory activity. Esterification of the carboxylic acid at C-24 resulted in the loss of inhibitory activity, shown by compounds **1**, **2**, **15**, **16**, and **17**.

Bortezomib and most of the known proteasome inhibitors are competitive inhibitors that interact at the catalytic sites. To our knowledge, drug-like small molecule noncompetitive proteasome inhibitors are relatively rare. Our data indicate that compound **10** is a noncompetitive proteasome inhibitor, which suggests that the compound interacts with an allosteric site to inhibit the proteasomal activity. With further structural optimizations, this class of compounds might have potential therapeutic applications and could become a useful tool in dissecting the function of the proteasome.

## Acknowledgments

HeLa $Ub^{G76V}$-GFP cells were kindly provided by Dr. Nico P. Dantuma, Karolinska Institutet, Stockholm, Sweden. This work was supported by the National Institutes of Health grant GM084337 to CHC.

## Supplementary data

Supplementary data (the method of proteasome assay, general experimental procedures for preparation of compounds (Table 1 and Scheme 1), $^1H$ and $^{13}C$ NMR data, and high resolution mass spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.041.

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14. Huang, L.; Ho, P.; Chen, C.-H. *FEBS Lett.* **2007**, *581*, 4955.
15. *Cell based proteasome assay*: HeLa $Ub^{G76V}$-GFP cells were grown in DMEM with 10% (v/v) heat-inactivated fetal bovine serum (Sigma) at 37 °C with humidified 5% $CO_2$. HeLa $Ub^{G76V}$-GFP cells (10,000 cells each well) in 100 μl of growth medium were cultured in the presence of proteasome inhibitors in 96-well plates (Corning-Costar) for 24 h. Accumulation of $Ub^{G76V}$-GFP in the cells was documented by using a Nikon fluorescence microscope system.
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