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# Substrate-guided optimization of the syringolins yields potent proteasome inhibitors with activity against leukemia cell lines

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ABSTRACT

Natural products that inhibit the proteasome have been fruitful starting points for the development of drug candidates. Those of the syringolin family have been underexploited in this context. Using the published model for substrate mimicry by the syringolins and knowledge about the substrate preferences of the proteolytic subunits of the human proteasome, we have designed, synthesized, and evaluated syringolin analogs. As some of our analogs inhibit the activity of the proteasome with second-order rate constants 5-fold greater than that of the methyl ester of syringolin B, we conclude that the substrate mimicry model for the syringolins is valid. The improvements in in vitro potency and the activities of particular analogs against leukemia cell lines are strong bases for further development of the syringolins as anti-cancer drugs.

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## 1. Introduction

The 26S proteasome is a multi-subunit complex that effects targeted protein degradation in eukaryotic organisms. It has emerged as one of the highest value targets in drug discovery and development programs focused on cancer treatments.[1] Efforts to develop anti-cancer drugs that target the proteasome are motivated by the success of Bortezomib (Velcade), a frontline drug for the treatment of multiple myeloma and mantle cell lymphoma.[2,3] This drug is a peptide boronate that reversibly inhibits the proteasome via substrate imitation and labile bonding between its boronic acid moiety and active site threonine residues of the proteasome's proteolytic β subunits. Interestingly, Bortezomib's substrate mimicry and its active site reactive "warhead" are features that it shares with virtually all naturally occurring and designed inhibitors of the proteasome that have been reported to date. Among these molecules are peptidyl aldehydes, peptidyl epoxyketones, and β-lactones.[4,5] It is remarkable that, with the exception of the peptidyl aldehydes, molecules from each of these structural classes are currently in or have completed clinical trials. Notably, an analog of a peptidyl epoxyketone natural product, Carfilzomib (Kyprolis), has recently been approved for the treatment of multiple myeloma. Clearly, optimization of the reactive substrate mimics is a viable strategy for the development of proteasome inhibitors with potential in medicine.[4–12]

Although natural products in the β-lactone and peptidyl epoxyketone classes of proteasome inhibitors have been thoroughly optimized in medicinal chemistry programs, those in the syringolin family have received much less attention. Syringolins were first isolated in 1998 from *Pseudomonas syringae* pv. *syringae* (Fig. 1)[13] and are characterized by a 12-membered macrocyclic lactam and an exocyclic dipeptide urea.[14] Irreversible proteasome inhibition by these molecules is a consequence of reaction of the α,β-unsaturated carbonyl moiety (i.e., the vinylogous amino acid) in their macrolactams with the catalytic threonine residues of the proteolytic subunits.[13,15] Syringolin congeners mostly differ with respect to their dipeptide urea moiety, but syringolins B and E are distinguished from the others by the absence of a unit of unsaturation in the macrolactam. The presence of the alkene likely strains the macrolactam such that its α,β-unsaturated carbonyl moiety is more reactive, as evidenced by the fact that syringolin A is the most potent congener. In the context of anticancer drug development, only a few syringolin analogs have been studied.[14,16–19] For instance, analogs with esters rather than carboxylic acids in the dipeptide urea moiety are reportedly more active.[16] The compounds with the most potent anti-cancer activities are the lipophilic variants of syringolin A (i.e., SylA LIP[16] and TIR-203[19]) (Fig. 1).

Curiously, the medicinal chemistry efforts on the syringolins described in the literature were not fully driven by considerations of the means by which the molecules bind to the proteasome. From

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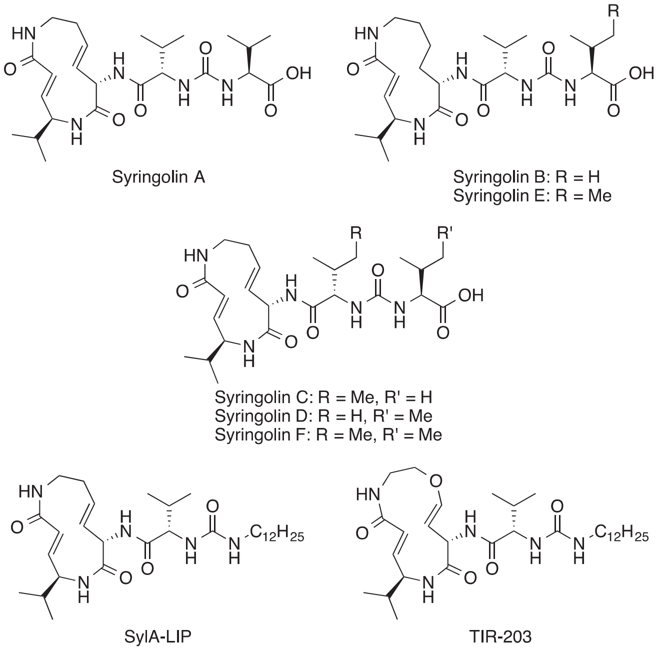
Syringolin A
Syringolin B: R = H
Syringolin E: R = Me
Syringolin C: R = Me, R' = H
Syringolin D: R = H, R' = Me
Syringolin F: R = Me, R' = Me
SylA-LIP
TIR-203

**Figure 1.** Syringolin natural products (syringolins A–F) and synthetic analogs thereof (SylA-Lip and TIR-203).

the published crystal structure of syringolin A bound to the yeast 20S proteasome,[15] it is evident that the side chains of the macrolactam's vinylogous amino acid and the amino acid residue appended to the macrocycle mimic those of amino acids at the P1 and P3 positions of a proteasome substrate, respectively (Fig. 2).[14] (A substrate's residue at the P1 position has the scissile bond and is separated from the one at the upstream P3 position by a single residue.) This model for substrate mimicry by the syringolins is consistent with their preferential (but not exclusive) reactivity with the β5 subunit of eukaryotic 20S proteasomes.[16] This subunit has a substrate specificity reminiscent of chymotrypsin, a protease that prefers substrates with aromatic amino acid residues (e.g., phenylalanine, tyrosine, and tryptophan) at the P1 position. In contrast, the β1 and β2 subunits have substrate preferences similar to caspase and trypsin, respectively. The former protease prefers acidic residues at the scissile bond, while the latter prefers those that are basic. Accordingly, the biased reactivity of the syringolins toward the β5 subunit is likely a consequence of their macrolactams having a hydrophobic, vinylogous amino acid (derived from valine) rather than one that is polar. In principle, the potency and selectivity of β5 subunit inhibition by the syringolins could be enhanced by replacing this mimic of valine with aromatic moieties, like those preferred at the P1 position of chymotrypsin substrates. Such design considerations could be coupled with recently reported findings of Chiba and co-workers that a syringolin A analog with a phenylalanine in the dipeptide urea (mimicking a substrate's P3 residue) was much more potent than the parent compound.[20] Herein, we report the synthesis and evaluation of syringolin analogs designed to closely mimic the preferred substrates of the proteasome subunit having specificity like chymotrypsin.

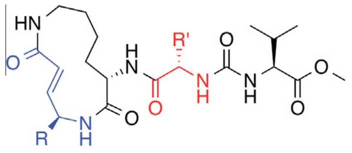

**Figure 2.** Model for substrate mimicry by the syringolins. R and R′ mimic the side chains of P1 and P3 residues of the proteasome substrate, respectively.

## 2. Results and discussion

We sought to test the prediction that the capacity of the syringolins to inhibit the proteasome could be improved by rendering their structures more like those of the preferred substrates of the β5 subunits of the proteasome. Our attention was focused on the vinylogous amino acid of the macrolactam (mimic of P1 residue) and the amino acid appended to the macrocycle (mimic of P3 residue). Although several syntheses have been presented in the literature,[16,20–24] we used a convergent synthetic approach developed by Pirrung and co-workers for the syntheses of syringolin B and analogs thereof (Schemes 1 and 2).[24] It is modular and thus amenable to diversity-oriented synthesis. For example, the linear precursor of the macrolactam is prepared from a Cbz-protected lysine residue having an acetyl phosphonate moiety on the ε-amino group and commercially available or easily synthesized 1,2-amino alcohols (Scheme 1).[25] Our design strategy dictated the selection of phenylalaninol, tryptophanol, or close analogs of these 1,2-amino alcohols as building blocks because their substituents mimic the aromatic side chains of the P1 residues of the preferred substrates of chymotrypsin substrates (see R groups in Scheme 1, Table 1). For the purposes of comparing analogs with aromatic substituents to those having aliphatic substituents at R, we used valinol and leucinol to prepare linear precursors of syringolin B macrolactam and the closely related compound having an isobutyl group at R, respectively (Table 1, entries 1 and 2). After coupling the amino alcohols to the protected lysine and oxidation of the products' primary alcohols, the reactive functionality mimicking the P1 residue of proteasome substrates was formed via an intramolecular Horner–Wadsworth–Emmons reaction. A modular route was also used for syntheses of the dipeptide urea side chain fragments whose constituents mimic the P3 residue of a proteasome substrate. Indeed, the selection of phenylalanine was informed by the report that a syringolin A analog with this amino acid at the same position was much more potent than those with glycine, alanine, leucine, or isoleucine.[20] In total, we synthesized 16 syringolin B analogs having esterified side chains and varying degrees of similarity to the substrates preferred by the β5 subunit of the human proteasome (Table 1).

Using purified human 20S proteasome (hs20S) and a fluorogenic substrate (Suc-LLVY-AMC), we performed in vitro assays to systematically assess proteasome inhibition by the syringolin analogs. Our choice of substrate was based on the fact that it is preferentially acted upon by the chymotrypsin-like β5-subunits of the proteasome due to the aromatic tyrosine residue at the scissile bond (i.e., P1 position).[26] From measurements of the rates of hydrolysis of the fluorogenic substrate by the proteasome in the presence of various concentrations of each inhibitor, we determined second-order rate constants, $k_{in}/K_i$ ($M^{-1}\,s^{-1}$) (Table 1), which reflect both the affinity of the non-covalent binding ($K_i$) and the rate of the chemical reaction with the enzyme ($k_{in}$).[27] All of the syringolin analogs were capable of inhibiting the chymotrypsin-like activity of hs20S. As is consistent with our design predictions, the most significant contributor to the apparent second-order rate constants of the compounds was their binding affinity ($K_i$) rather than the rates of inhibition (see Supplementary data). Accordingly, we found that an analog of syringolin B with an isobutyl substituent at R (Table 1, entry 2) rather than the isopropyl substituent of the parent (Table 1, entry 1) exhibited a diminished capacity to inhibit the proteasome. In contrast, most compounds with aromatic substituents (Table 1, entries 3–13 with exceptions of 4, 8, and 11) at the same position were

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