Inhibitors

Diarylethene-Based Photoswitchable Inhibitors of Serine Proteases
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Abstract: A bicyclic peptide scaffold was chemically adapted to generate diarylethene-based photoswitchable inhibitors of serine protease Bos taurus trypsin 1 (T1). Starting from a prototype molecule—sunflower trypsin inhibitor-1 (SFTI-1)—we obtained light-controllable inhibitors of T1 with Kᵢ in the low nanomolar range, whose activity could be modulated over 20-fold by irradiation. The inhibitory potency as well as resistance to proteolytic degradation were systematically studied on a series of 17 SFTI-1 analogues. The hydrogen bond network that stabilizes the structure of inhibitors and possibly the enzyme–inhibitor binding dynamics were affected by isomerization of the photoswitch. The feasibility of manipulating enzyme activity in time and space was demonstrated by controlled digestion of gelatin-based hydrogel and manipulating enzyme activity in time and space was demonstrated by controlled digestion of gelatin-based hydrogel and an antimicrobial peptide BP100-RW. Finally, our design principles of diarylethene photoswitches are shown to apply also for the development of other serine protease inhibitors.

The use of light to control functions of biomacromolecules has become an active field of research over the last decades. Reversibly photoisomerizable (i.e. photoswitchable) compounds have already demonstrated great promise in medicinal chemistry[1] and materials science.[2] Particular attention has been devoted to control the activity of enzymes by light.[3–6] as enzymes are important drug targets[6] and often are components of “smart” soft materials.[7a,b] Most of the known photocontrollable enzyme inhibitors have been designed using two general strategies, illustrated in Figure 1. One (Figure 1A) is based on the incorporation of a photoisomerizable fragment (photoswitch) into a known inhibitor as part of the enzyme-binding moiety. This strategy is well-suited for the design of small-molecule constructs; many photocontrollable enzyme inhibitors have been designed using this second approach. Here, we introduce yet another design—grafting the target-binding and photoregulating units joined through a bridge in the bicycle.

We were inspired by success in using bicyclic scaffolds for design of non-photocontrollable multifunctional peptides. For example, Pei et al. reported recently on bicyclic cell-permeable PIN-1 inhibitors,[8] in which the enzyme-binding and cell-penetrating units were joined through a bicyclic peptide core.
Although the photoswitch moiety is distant to the binding unit in our constructs, we demonstrate here that both units can “communicate” through the bicyclic scaffold, resulting in efficient photomodulation.

With the suggested strategy, we designed and optimized DAE-based peptidic inhibitors of chymotrypsin-like (S1) serine proteases, the largest family of all peptidases. Serine proteases have a broad biological significance and immense potential as drug targets, as their malfunction is linked to numerous pathologies. These enzymes have been in the focus of intensive research directed towards the discovery of new inhibitors, including photocontrollable ones. The latter were based exclusively on azobenzenes as the photoswitching moiety; no DAE-derived inhibitors have been reported to date for this class of enzymes.

*Bos taurus* trypsin 1 (T1) represents the S1 family by its catalytic mechanism, inhibitor susceptibility profile and substrate specificity, and was thus selected as the model target. As the reference compound and as the starting template, we used the sunflower trypsin inhibitor-1 (SFTI-1). It is bicyclic, well-studied and is one of the strongest natural inhibitors of trypsin-like endopeptidases ($K_i$ 0.1–3.4 nM for T1). The SFTI-1 molecule contains two antiparallel β-strands stabilized by a disulfide bridge (Figure 2).

Figure 2. Sequence, nomenclature and structure of SFTI-1. A) Schematic representation of the SFTI-1 peptide and its structural/functional parts. B) Molecular structure and hydrogen bonding network (PDB: 1BLJ).

When designing the analogues of SFTI-1, we relied on the known mechanism of inhibition and the relationship between structure and activity of SFTI-1. We also considered the changes in molecular dynamics that the diarylethene moiety causes upon photoisomerization. SFTI-1 inhibits trypsin by the Laskovski mechanism, which is, it binds tightly to the protease (Figure 2A) but resists hydrolysis, thereby blocking the enzyme. The resistance to hydrolysis has been attributed to a rigid bicyclic structure and a tight network of hydrogen bonds (Figure 2B).

Modifications of the reactive loop are known to drastically deteriorate the inhibitory activity, while the structural loop can be modified without substantial activity drop. Therefore, we incorporated the photoswitch within the structural loop of SFTI-1, using building block 1 (Figure 3) developed previously by us. We aimed at the compounds in which the ring-open DAE fragment would cause minor changes in the enzyme inhibition, while the ring-closed DAE photoform would minimize this activity.

The first series of DAE-containing SFTI-1 analogues ($S1n$, $S2–S4$, Table 1) was prepared to explore the impact of the structural loop size upon the inhibitory activity and photoswitching efficiency, and to check the role of the bridge on the functionality of the entire construct. As can be seen from the data in Table 1, compound $S1n$ proved to be a strong T1 inhibitor, comparable to SFTI-1. However, this peptide could not be converted to the ring-closed form, presumably because of severe constraints in the structural loop due to its small size. The analogue $S2$, lacking the bridge, was well photoisomerizable. (See the data about the photostationary state under UV irradiation for all the compounds studied here in the SI. Conversion from ring-closed forms to the ring-open forms under visible light irradiation (at 570 nm) was in all cases practically quantitative.) However, we observed a significant drop in its activity compared to the native inhibitor. The smaller bridge-free analogue $S3$ also showed a reduced activity. Besides, it was obtained with a very low yield at the cyclization step. The smallest bridge-free analogue $S4$ (ring-open), although being a potent inhibitor, also did not photoisomerize into the ring-closed form. These results from the first series suggest that incorporation of the DAE into the structural loop of SFTI-1 may indeed result in potent T1 inhibitors. However, more conformational freedom should be provided in the structural loop to enable effective photoisomerization, and the bridge should be kept in place.

We hypothesized that an extension of the bridge might provide the required freedom. Replacement of the disulfide bond in SFTI-1 with surrogates has been described in the literature. The majority of such substitutions were functionally tolerated, providing a significant increase in the redox-stability. Hence, we designed the second series of analogues with elongated bridges ($S5n–S7n$, Table 1).

The proteolytic stability of the peptide inhibitors is the second major factor, besides $K_i$, that is of importance for any practical utility. The hydrolysis rate does not strictly correlate with $K_i$ but depends on the activation barrier of the hydrolysis reaction. It is known that T1 may cleave SFTI-1 at Arg2 in the structural loop. Since neither Arg2 nor Phe12 have been reported as critical in binding to trypsin, we supplemented the second series of SFTI-1 analogues by “inverted” compounds. Here, Arg2 and Phe12 were swapped in order to evaluate the effect of Arg2 on the proteolytic stability ($S5i$, $S5–S7i$, Table 1). As anticipated, all analogues with elongated bridges ($S5n–S7n$, $S5i–S7i$) were photoisomerizable. Generally, upon extending the bridge, the potency and stability of the peptides decreased. At the same time, we...
| Name       | Sequence | K_\text{[nM]} (open) | K_\text{[nM]} (closed) | K_\text{Ki (closed)} /K_\text{Ki (open)} | k_\text{H [M^{-1} s^{-1}]} (open) | k_\text{H [M^{-1} s^{-1}]} (closed) | k_\text{H (closed)} /k_\text{H (open)} |
|------------|----------|-----------------------|------------------------|------------------------------------------|----------------------------------|----------------------------------|-------------------------------------|
| Reference compounds |
| SFTI-1     | ![Structure](image1) | 3.4 ± 0.2             | –                      | 2.0 ± 0.1 × 10^{-5}                      | –                                | –                                 | –                                   |
| SFTI-1i    | ![Structure](image2) | 12.5 ± 0.8            | –                      | 2.0 ± 0.2 × 10^{-5}                      | –                                | –                                 | –                                   |
| First series of inhibitors |
| S1n        | ![Structure](image3) | 4.9 ± 0.3             | –                      | 7.8 ± 0.2 × 10^{-5}                      | –                                | –                                 | –                                   |
| S2         | ![Structure](image4) | 203 ± 6               | 659 ± 33               | 3.2                                       | –                                | –                                 | –                                   |
| S3         | ![Structure](image5) | 101 ± 9               | –                      | –                                         | –                                | –                                 | –                                   |
| S4         | ![Structure](image6) | 22 ± 3                | –                      | –                                         | –                                | –                                 | –                                   |
| Second series |
| S1i        | ![Structure](image7) | 13.4 ± 1.4            | –                      | 8.3 ± 0.3 × 10^{-5}                      | –                                | –                                 | –                                   |
| S5n        | ![Structure](image8) | 95 ± 6                | 231 ± 14               | 2.4                                       | 2.5 ± 0.1 × 10^{-3}              | 3.4 ± 0.5 × 10^{-2}               | 13.6                                |
| S5i        | ![Structure](image9) | 102 ± 14              | 182 ± 16               | 1.8                                       | 2.9 ± 0.1 × 10^{-3}              | 5.0 ± 0.8 × 10^{-2}               | 1.7                                 |
| S6n        | ![Structure](image10) | 135 ± 8               | 1120 ± 109             | 8.3                                       | 6.7 ± 0.9 × 10^{-3}              | 2.5 ± 0.8 × 10^{-2}               | 3.7                                 |
| S6i        | ![Structure](image11) | 441 ± 43              | 7700 ± 600             | 17.5                                      | 1.9 ± 0.1 × 10^{-2}              | 1.1 ± 0.2 × 10^{-1}               | 5.8                                 |
| S7n        | ![Structure](image12) | 63.4 ± 4.4            | 2200 ± 214             | 34.7                                      | 2.0 ± 0.2 × 10^{-3}              | 6.1 ± 1.2 × 10^{-2}               | 30.5                                |
| S7i        | ![Structure](image13) | 263 ± 30              | 7220 ± 360             | 27.5                                      | 7.5 ± 0.2 × 10^{-3}              | 5.5 ± 1 × 10^{-1}                 | 73                                  |
| Third series |
| S8n        | ![Structure](image14) | 5.2 ± 0.4             | 125 ± 8                | 24                                        | 1.3 ± 0.7 × 10^{-4}              | 5.3 ± 1.4 × 10^{-4}               | 4.1                                 |
| S9n        | ![Structure](image15) | 8.3 ± 0.6             | 55 ± 3.8               | 6.6                                        | 1.5 ± 0.3 × 10^{-4}              | 1.3 ± 0.1 × 10^{-3}               | 8.7                                 |
| S10n       | ![Structure](image16) | 17.5 ± 0.7            | 181 ± 7                | 10.3                                       | 2.3 ± 0.2 × 10^{-4}              | 3.6 ± 0.3 × 10^{-2}               | 15.7                                |
| S10i       | ![Structure](image17) | 5.1 ± 0.4             | 44.8 ± 2.2             | 8.8                                        | 5.3 ± 0.4 × 10^{-5}              | 2.2 ± 0.3 × 10^{-4}               | 4.2                                 |
| S11i       | ![Structure](image18) | 1720 ± 130            | 5370 ± 420             | 3.1                                        | 2.8 ± 0.3 × 10^{-2}              | 9.1 ± 1 × 10^{-2}                 | 3.3                                 |
Table 1: (Continued)

| Name | Sequence | $K_i$ [nM] (open) | $K_i$ [nM] (closed) | $K_i$ (closed) / $K_i$ (open) | $k_i$ [M$^{-1}$ s$^{-1}$] (open) | $k_i$ [M$^{-1}$ s$^{-1}$] (closed) | $k_i$ (closed) / $k_i$ (open) |
|------|----------|------------------|-------------------|-----------------------------|-------------------------------|--------------------------------|----------------------------|
| S12i | ![image](image) | 2440 ± 215 | 12 400 ± 900 | 5.0 | 1.4 ± 0.3 × 10$^{-2}$ | 4.2 ± 0.4 × 10$^{-2}$ | 3.0 |

[a] T1 inhibitory activities ($K_i$), T1-induced hydrolysis rate constants ($k_i$), and their ratio for the ring-closed to the ring-open isomers. The residue of T1 is highlighted by red color and underlined.

observed a systematic increase in the activity difference between the ring-open and ring-closed forms. We also found that the Arg2/Phe12 inversion improved the proteolytic stability of some analogues.

In the third series of photoswitchable SFTI-1 analogues, we explored the extension of the peptide cycles. One or two additional aliphatic residues (Val and Leu) were introduced at the flanks of the photoswitch (S8b–S10n, S10i, Table 1). Two triazole-linked analogues (S11i, S12i) bearing elongated bridges were also prepared. This design yielded the best photoswitchable analogues, which not only retained a high inhibitory potency in the low nanomolar range, but also showed efficient photoswitching of their activity. Compound S8b, which is the best performing molecule in terms of inhibitory potency and photoswitching efficiency of the inhibiting activity, was found to change its inhibitory potency and photoswitching efficiency of the controlling photoisomerization ($K_i$ from 5.2 to 125 nM upon photoisomerization), that is, by a factor of 24. The hydrolysis rates for the disulfide-bearing inhibitors of this series were close to that for native SFTI-1. The triazole-bridged S11i and S12i, however, showed a drastic loss of activity as well as proteolytic stability.

Structural preorganization—resulting in a low entropic penalty upon enzyme binding—was postulated to be the key factor determining the high potency of SFTI-1.[13a] To understand the mechanism by which the DAE photoswitch in our compounds modulates the $K_i$ and hydrolysis rate, we measured hydrogen/deuterium exchange rates for the compounds in D$_2$O using MALDI mass spectrometry. From these data, the number of hydrogen bonds could be quantified and compared with the values obtained for $K_i$ and $k_i$.[25] Figure 4 illustrates the relationship between conformational stability in terms of the number of protons in slow exchange, proteolytic stability, and the potency of each inhibitor/photoswitchable state. The prototype SFTI-1 and its “inverted” mutant SFTI-2i possessed 7.0 slow-exchanging protons ($r_{i>2} > 40$ s). The best-performing analogues S8b, S9n, S10n, and S10i had a comparable number of slow-exchanging protons in their ring-open forms (5.5 to 6.5), but far less upon photoswitching, which correlated well with their $K_i$ and $k_i$. This correlation provides evidence that the ring-open DAE facilitates preorganization of the reactive loop through hydrogen bonds, which stabilize the conformation and enhance the potency.

The structure–activity relationship of the entire library is not as straightforward, however. Compound S6i, for example, has approximately the same number of exchanging protons for its two photoforms (approx. 3.4), yet its $K_i$ and $k_i$ changed significantly upon photoisomerization (Figure 4). Although an influence of the DAE moiety on the structural preorganization of the S6i molecule cannot be excluded, it appears more probable that the DAE photoswitching modulates the dynamics of the enzyme–inhibitor complex. The DAE is expected to significantly influence the dynamics, because this photoswitch possesses a very different flexibility in its two photoisomers. The more flexible ring-open peptides can thus have more favorable conformational and vibrational entropy changes upon binding to the target protein than the corresponding ring-closed photoisomers.[30]

SFTI-1 serves as a lead in developing inhibitors of other serine proteases.[25c,25b,26] To demonstrate that our photoswitchable T1 inhibitors can be modified to inhibit other members of the chymotrypsin-like family, we synthesized SSF, an analogue of S10n in which Lys5 at the P1 position was changed to Phe. Such modification in the native SFTI-1 had been reported to result in a potent inhibitor of $\alpha$-chymotrypsin. A non-photoswitchable mutant SFTI-5F was taken as a control, whose $K_i$ value was measured to be 2.8 nM. As expected, SSF inhibited $\alpha$-chymotrypsin (from bovine pancreas Type II) very well. The $K_i$ values of the two photoisomers differed by 21-fold (5.8 nM for the ring-open form, 122 nM for the ring-closed form), proving that the present scaffold can be adapted to inhibit other serine proteases in the
same photocontrollable manner. We also wondered how selective our inhibitors were and characterized SSF against trypsin and its prototype S10n (one of the best-performing trypsin inhibitors) against α-chymotrypsin. As expected, only weak inhibition with IC₅₀ values at 50–100 µM (>3 orders of magnitude lower compared to their parent proteases’ inhibition) was observed in each case, proving sufficient enzyme selectivity of our compounds.

Finally, photo-stability in repeated cycles of reversible photoisomerization was studied on three selected compounds (linear Ac-Ala-I-Ala-NH₂, peptide S2 lacking the bridge, and S10n). All three compounds demonstrated moderate photofatigue resistance in these tests (see the SI) declining less than 30% in 15 cycles. We consider this acceptable for most biomedical applications, where only one or a few cycles of the photoconversion are usually needed.[1-3]

To demonstrate the manipulation of the enzyme activity in space and time by light, we set up two experiments. The first one was based on digestion of a gelatin-based hydrogel with trypsin. A water solution of gelatin (20 mg/mL⁻¹) was mixed with S10n (ring-closed form) and trypsin (10 µM and 100 nM final concentrations, respectively). Bromophenol blue was added for better visualization of the gel, which was formed in a Petri dish at 0°C in the dark. The light (approx. 10 mW/cm²) was then applied for 3 min to the gel trough a mask to convert S10n to the ring-open form, an active trypsin inhibitor. Incubation of the Petri dish at 20°C after the irradiation resulted in liquefying of the gel only in places where the light did not reach the mixture. The liquid was removed, leaving the intact areas where trypsin was inhibited by the photoactivated S10n (Figure 5; an image of the whole Petri dish after the experiment can be seen in the Table of Contents picture).

An analogous experiment was designed to show the photoregulation of BP100-RW peptide activity by digestion with trypsin. BP100-RW (sequence RRLFRRILRWL-NH₂) is known as a potent antimicrobial peptide (AMP).[27] Growth of E. coli DSM 498 was monitored in a media containing the peptide (64 µg/mL⁻¹, twice as high as the minimal inhibitory concentration), trypsin (3 nM), and S10n ring-closed (300 nM). Irradiation of the mixture prevented the bacterial growth, while the bacteria grew almost as fast as without the BP100-RW in a control experiment in dark (Figure S9).

In summary, we have evaluated a strategy for the design of efficiently photocontrollable bicyclic peptide-based enzyme inhibitors. Our compounds possess the enzyme-binding fragment in one cyclic unit, and the photoswitchable fragment in the opposite cyclic unit of the macro-bicycle. We used the diarylethene photoswitch for effective regulation of inhibiting activity of serine proteases, which has never been used before for this class of enzymes and demonstrated the utility of the obtained enzyme inhibitors for photoregulation of hydrogel digestion and antibacterial activity of an AMP. These results pave the way for the development of new macro-bicyclic inhibitors of other chymotrypsin-like family proteases, in particular, taking natural serine protease inhibitors as the templates.

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Conflict of Interest

The authors declare no conflict of interest.

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Bicyclic diarylethene-containing analogues of the sunflower trypsin inhibitor-1 (SFTI-1) can modulate activity of serine proteases in the nanomolar–micromolar range in highly efficient photocontrollable manner.