Bioconjugate Chemistry

Communication

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Firefly Luciferase Inhibitor-Conjugated Peptide Quenches Bioluminescence: A Versatile Tool for Real Time Monitoring Cellular Uptake of Biomolecules

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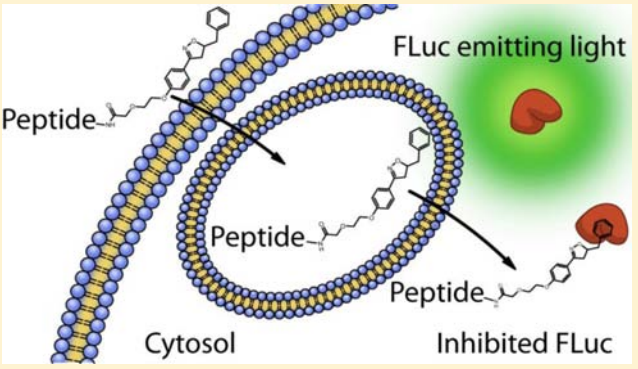
Supporting Information

ABSTRACT: In this paper, novel firefly luciferase-specific inhibitor compounds (FLICs) are evaluated as potential tools for cellular trafficking of transporter conjugates. As a proof-of-concept, we designed FLICs that were suitable for solid phase peptide synthesis and could be covalently conjugated to peptides via an amide bond. The spacer between inhibitor and peptide was optimized to gain efficient inhibition of recombinant firefly luciferase (FLuc) without compromising the activity of the model peptides. The hypothesis of using FLICs as tools for cellular trafficking studies was ensured with U87Fluc glioblastoma cells expressing firefly luciferase. Results show that cell penetrating peptide (penetratin) FLIC conjugate 9 inhibited FLuc penetrated cells efficiently (IC_{50} = 1.6 μM) and inhibited bioluminescence, without affecting the viability of the cells. Based on these results, peptide−FLIC conjugates can be used for the analysis of cellular uptake of biomolecules in a new way that can at the same time overcome some downsides seen with other methods. Thus, FLICs can be considered as versatile tools that broaden the plethora of methods that take advantage of the bioluminescence phenomena.



INTRODUCTION

Problems with formulations and bioavailability are one of the main reasons promising drugs fail in clinical trials. Conjugation of small molecules to, e.g., cell penetrating peptides can be used to make lipophilic drugs more water-soluble and more importantly transported to target tissue. [1] Most of the transporter molecules, such as peptides and nucleotides, are using mainly endocytotic pathways to penetrate the cell membrane. However, during the endocytosis, therapeutic macromolecules may be trapped in the endosomal/lysosomal compartment which reduces their bioavailability.[2−4] Several methods have been reported for elucidation of cellular uptake mechanisms and for tracking of the location of macromolecules inside the cell. One of the most widely used methods is the conjugation of macromolecules with a suitable fluorophore.[2,5] The main drawback of this method is, however, the difficulty discriminating between truly cytosol-internalized peptides from those bound to the membrane or trapped in the endosomes. Thus, the real time experiments in vitro and in vivo are not possible.[2] Fluorescence quenching methods have been used to investigate the delivery of therapeutic molecules to the cytosol by cell penetrating peptides (CPPs), but these assays represent end point studies.[6] One widely used method is to inhibit the potential route of the uptake pathway by using small molecule uptake inhibitors.[7] The main challenge with the uptake of inhibitors is the existence of parallel uptake mechanisms, which can substitute the inhibited delivery route.[8] Overall, novel techniques are needed to ensure the cytosol entry of the biomolecules to verify that the cargo is transported and released inside the cell.

One of the most studied imaging and transporter technique is the so-called controlled release technique, which takes advantage of the bioluminescence.[9] In this method the conjugate is actively cleaved from the transporter inside the cell to produce easily detectable light but it has also some down sides.[10,11] Briefly, the natural substrate of luciferase, D-luciferin, has been conjugated to peptides[9] or other macromolecules[12] using cleavable linkers that release the substrate after penetration into the cytosol, where the light producing enzyme, luciferase, is situated. However, the linker may already be cleaved inside the endosome, thus releasing only the easy membrane penetrating substrate D-luciferin, while the delivery vector remains entrapped inside the vesicle which could lead to

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situation were, e.g., "real" drug cargo is not released in cytosol of the cell.[10,11]

A number of potent inhibitors of firefly luciferase (FLuc, *Photinus pyralis*) have been recently reported.[13−26] However, with a few exceptions, including the dual luciferase reporter assays,[27] luciferase inhibitor compounds have not been previously brought forward as research tools. In this work, we modified our recently described[26] high affinity (subnanomolar IC_{50}) FLuc inhibitor compounds (FLICs, Figure 1) to allow covalent conjugation of FLIC with an earlier characterized trypsin inhibitor peptide (TIP)[28] or cell-penetrating peptide (CPP), penetratin.[29] The FLIC−peptide conjugates were evaluated with in vitro inhibition studies using recombinant firefly luciferase enzyme and for probing cellular uptake and endosomal escape of the conjugate (Figure 2, Figure S-1, Supporting Information). Based on these results, the optimized molecular scaffold (FLIC 3) was proven to be a versatile tool for detecting cellular uptake of CPP conjugated peptide. The main advantage of this method over the previous procedures is that the active quenching compound does not have to be cleaved from the peptide to be bioactive and vice versa. This enables direct measurement of release of the FLIC−peptide conjugate to the cytosol in an active form. The real time endosomal release of FLIC−transporter conjugate can be used to verify the penetration of the cargo into cytosol. Furthermore, our method can easily be applied to screening of cell internalization of large series of molecules, because the quantification of bioluminescence can be easily automated.

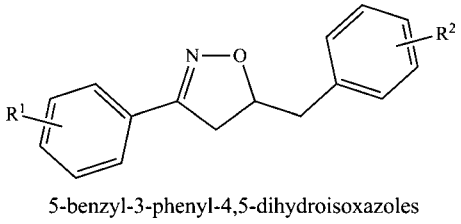

Figure 1. General structure of the FLICs.[26]

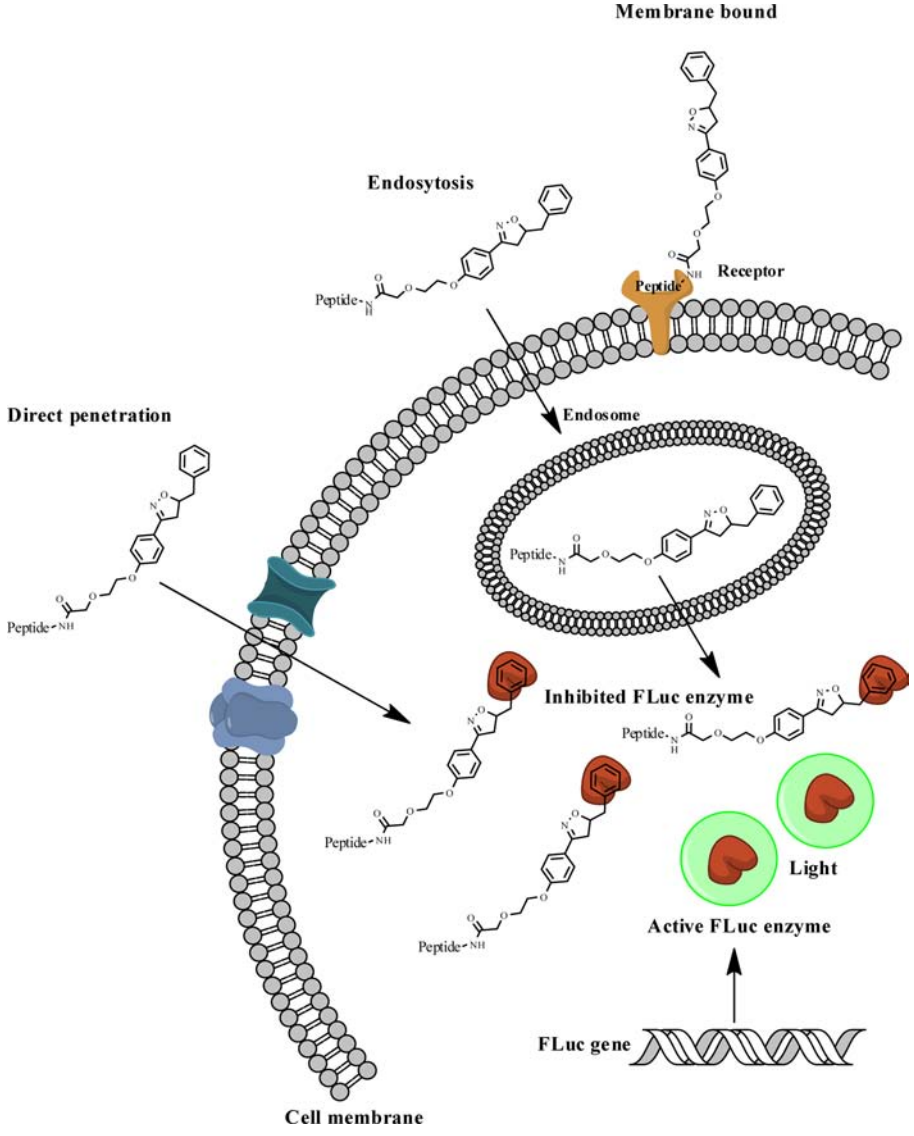

Figure 2. Principle of using FLICs to investigate the cellular accumulation of peptides. Peptide function is categorized into three classes: membrane-bound, endocytosis, and direct penetration.

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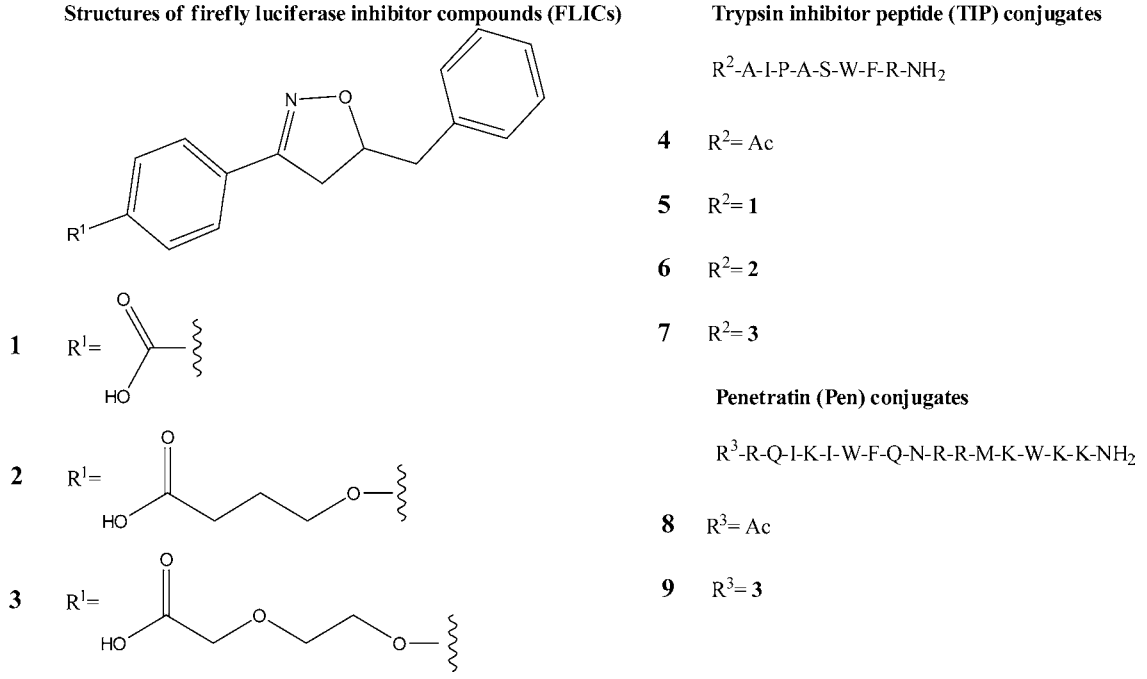
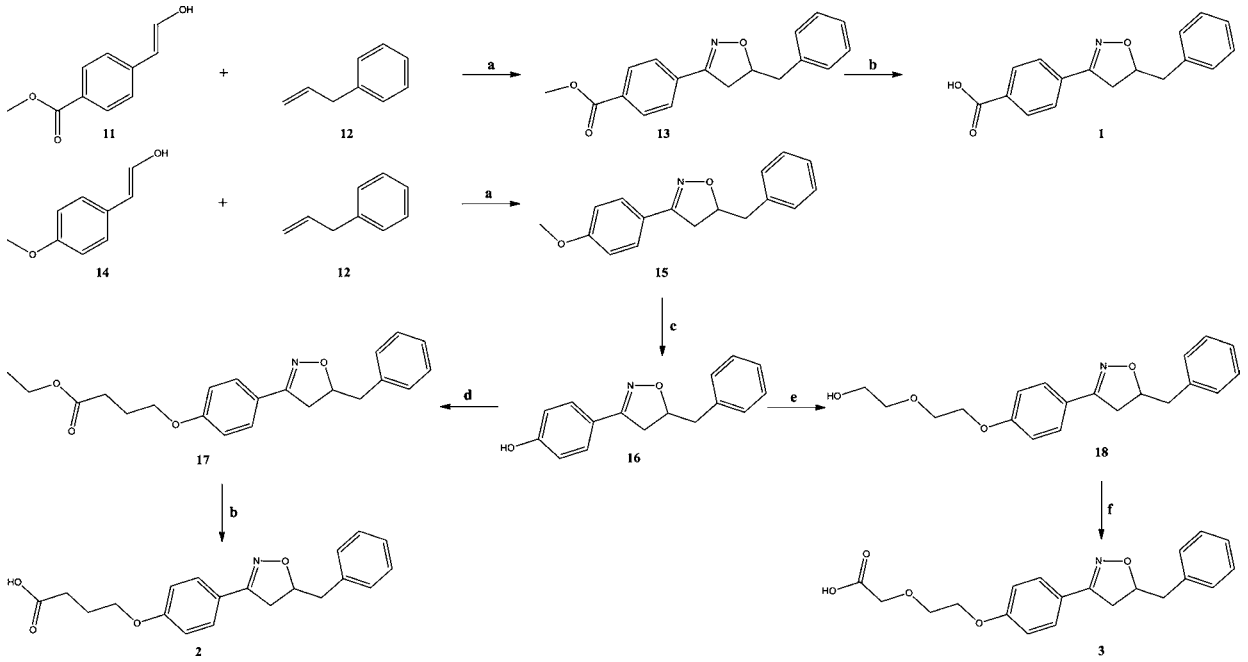


Figure 3. Structures of FLICs **1**–**3** and peptides **4**–**9**. FLICs were conjugated to the N-terminus of the peptide which was acetylated in the control peptide to keep the charge of the peptide conjugates the same.

Scheme 1. Synthesis of the 4,5-Dihydroisoxazoles[a]



[a]Reagents and conditions: (a) NaOCl, pyridine, CH_2Cl_2, 0 °C, 2 h; (b) LiOH, H_2O, MeOH, 55 °C, overnight; (c) BBr_3, CH_2Cl_2, RT, overnight; (d) 4-bromobutanoate, K_2CO_3, anhydrous acetone, reflux, overnight; (e) 2-(2-iodoethoxy)ethanol, K_2CO_3, anhydrous acetone, reflux, overnight; (f) CrO_3, H_2SO_4, acetone, 0 °C, 4 h.

RESULTS AND DISCUSSION

Molecular Design and Synthesis. In our previous study we have demonstrated that while R^1 of the FLICs (Figure 1) can be substituted with a variety of groups without losing the FLuc inhibitory activity of the ligands, almost any substitution on group R^2 is seriously hampering the inhibitory effect.[26] The basic objective of the present study was to design a FLuc inhibitor compound which could be linked via a covalent amide bond between R^1 of FLIC and the amino terminus of peptides in order to maintain the FLuc inhibition activity of FLIC. This was accomplished by synthesizing compounds with either a carboxylic group or a side chain possessing a terminal carboxylic group as R^1, like in FLICs **1**–**3**, that are readily usable in the solid phase peptide synthesis (SPPS). We did initial structural optimization of the FLICs by synthesizing several structural analogues possessing aliphatic or ethereal side

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chains of various lengths and made inhibition studies with the recombinant FLuc in order to find the compounds with the highest FLuc inhibition efficacy (data not shown). This screening revealed two potent spacer moieties presented in FLICs **2** and **3**.

The 4,5-dihydro-isoxazoles **13** and **15**[26] (Scheme 1) were chosen as precursors for the side chain attachments and they were prepared via a dipolar cycloaddition reaction of the nitrile oxide derivatives of appropriately substituted benzaldoximes **11** or **14** with allyl benzene (**12**). Synthesis of FLIC **1** with an aromatic carboxyl group was accomplished by hydrolyzing the corresponding methyl ester **10** with LiOH. Compound **16**[26]occupying a phenol group was synthesized by demethylating the aromatic methoxy group of isoxazole **15** by using BBr_3 and the desired side chains were attached to the phenolic hydroxyl group with potassium carbonate and ethyl 4-bromobutanoate or 2-(2-iodoethoxy)ethanol (prepared from commercially available 2-(2-chloroethoxy)ethanol by method described elsewhere[30]), resulting in compound **17** or **18**, respectively. Finally, ester **17** was hydrolyzed with LiOH to give carboxylic acid **2**, and alcohol **18** yielded via Jones oxidation with CrO_3 and H_2SO_4 carboxylic acid **3**.[31] After preparation, all the compounds were purified by semipreparative HPLC system.[32] Conjugation of the FLICs **1**–**3** to the N-terminus of the peptides was accomplished by following standard Fmoc chemistry as described in the experimental procedure. The purification of FLIC–peptide conjugates **5**–**7** and **9** with semipreparative HPLC was easy because of a significant difference in the retention times between unconjugated peptide and peptide conjugates.

Biological Evaluation. FLuc inhibition potential of the novel FLICs was tested using increasing concentrations to determine the IC_{50} value and to generate the concentration response curves (CRCs, Figure 4). The buffer and substrate reagent were designed to mimic the cell-based end point assay conditions by using commercial (Promega) products.

As shown in Table 1, the TIP **4** or penetratin **8** alone did not show any FLuc inhibition even at the highest concentration

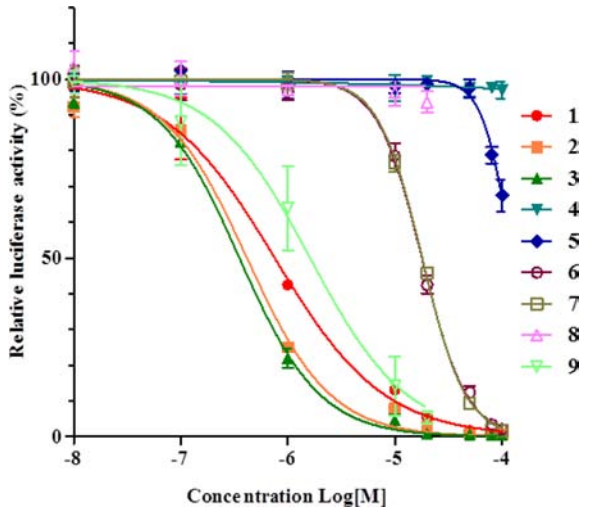

Figure 4. Recombinant FLuc inhibition potency of the FLICs and FLIC-conjugated peptides. The relative luciferase activity was measured after exposure to vehicle or 10 nM, 100 nM, 1 μM, 10 μM, 20 μM, 50 μM, 80 μM, and 100 μM concentrations of tested compounds. CRCs were analyzed using GraphPad Prism[33] software to evaluate IC_{50} values.

Table 1

compound	log IC_{50}[a]	log SE	inhibition % (20 μM)[b]
1	−6.12	0.03	94.5%
2	−6.38	0.03	97.2%
3	−6.45	0.02	99.0%
4	N.D.[c]		0.5%
5	>−4		1.5%
6	−4.75	0.01	57.5%
7	−4.75	0.01	54.2%
8	N.D.		
9	−5.79	0.07	94.6%

[a]FLuc inhibition is given as log IC_{50} analyzed at various concentrations in three replicates and presented together with standard error (log SE). [b]Relative inhibition power at 20 μM concentration is presented as percent (mean from three replicates, 100% refers to complete inhibition). [c]No significant inhibition was detected.

(100 μM) and the studied FLICs did not inhibit trypsin alone even at 50 μM concentration (Figure S2, Supporting Information). FLIC **1** was directly conjugated to TIP (FLIC-TIP **5**), but only weak inhibition of luciferase was detected. Also, the trypsin inhibition of the FLIC-TIP **5** was significantly decreased in comparison to TIP **4** (Figure 5.) and almost

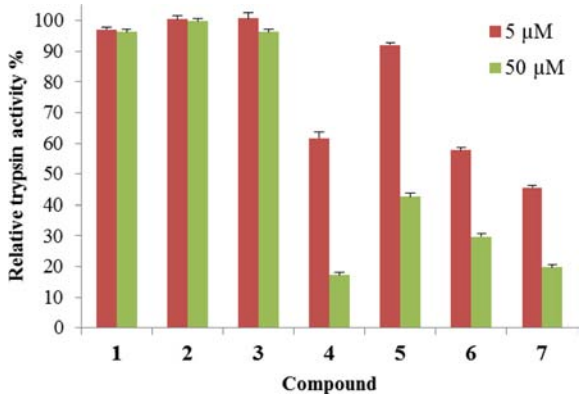

Figure 5. Trypsin activities after exposure to FLICs **1**–**3**, TIP **4** alone, or FLIC-TIPs **5**–**7**. The values are presented as relative percentages where trypsin is set as 100%. The slope is obtained from regression analysis from the linear section (0–15 min) of absorbance indicating trypsin reaction rate. Results are given as slope (absorbance unit/time) together with standard error analyzed from three different time points in three replicates. The activity was evaluated from linear area (Figure S2, Supporting Information, 0–15 min) of absorbance curves using regression analysis (GraphPad Prism).[33] The absorbance values were converted to inhibition percent using the formula: 100% × (Δ absorbance with inhibitor/Δ absorbance without inhibitor).

totally absent at 5 μM concentration, indicating that the peptide model was sensitive to the changes caused by the FLIC as expected. In addition, the new FLICs were designed to avoid the loss of activity and to increase the peptide–conjugate solubility. Optimized FLICs **2** and **3** were synthesized and conjugated directly to the amino terminus of the TIP on-resin. FLICs **2** and **3** showed a significantly better inhibition of the recombinant FLuc activity than FLIC **1**, and par excellence, both of them showed a similar, over 10 times higher inhibition when conjugated to the TIP. The IC_{50} values of FLIC-TIPs **6** and **7** were at 20 μM levels and the FLuc inhibition with the highest concentration tested (100 μM) was almost 100%. The ability of FLIC-TIP **6** to inhibit trypsin at 50 μM concentration

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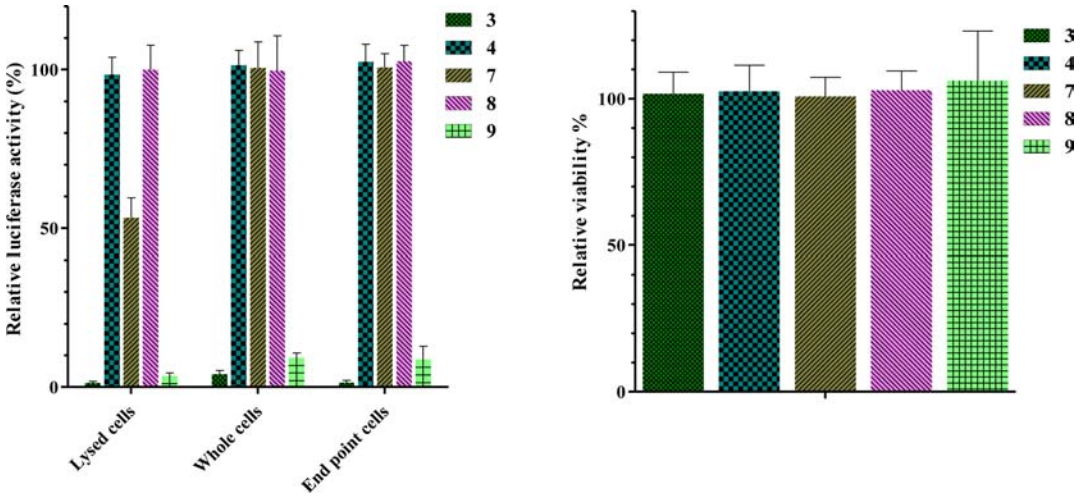

Figure 6. U87Fluc cells or their lysates were exposed to 20 μM of FLIC **3**, peptides **4** and **8**, or FLIC−peptide conjugates **7** and **9** for 2 h, and luciferase activity was measured. The untreated cells were set as 100 (three parallels). The lysed cells are lysed before exposure to test compounds to study how tested compounds inhibit luciferase in cell environment; and the end point assay possibly also reveals uptake, trapped, or membrane bound inhibitors, since the cells are washed and lysed after exposure to test compounds. Testing with living cells shows only the uptake and endosomal escaped transporter FLIC conjugates, since luciferase enzyme is found in the cytosol of the U87Fluc cells. Cell viability was analyzed using the propidium iodide method. The results are given relative to the ethanol vehicle (100%) and as a mean together with standard deviation (n = 3).

was significantly better than that of FLIC-TIP **5** but not as good as TIP **4** alone. The FLIC-TIP **7** did not suffer from loss of trypsin inhibition activity and it retained its significant FLuc inhibitory potential. These results indicate that, with optimal spacer, like in FLIC **3**, it is possible to link FLuc inhibitors directly to a bioactive peptide without significant interference or loss of the biological activity of inhibitor or macromolecule.

To further evaluate the potential of the peptide conjugated FLICs as tools for studies of cellular accumulation and distribution, we exposed intact living U87Fluc firefly luciferase-expressing human glioma cells or their lysates to the FLIC **3**−peptide conjugate (Figure 6). It was assumed that TIP is not penetrating into the cells, and thus, a well-known cell penetrating peptide, penetratin **8**, was conjugated to FLIC **3** to study the potential of FLICs with a real transporter peptide. The recombinant luciferase inhibition test showed that the FLIC−penetratin conjugate **9** had IC_{50} value in micromolar level, which is more promising than was seen with TIP conjugate **7**. In addition, the conjugation of FLIC **3** to penetratin decreased the inhibition efficacy of the free inhibitor only minimally. These results can be considered promising, since the IC_{50} value of **9** is relevant to cell based assays. In the actual in vitro cell based tests, the FLIC-TIP **7** showed similar inhibition with cell lysate as seen with the recombinant enzyme, but had no effect on the FLuc activity when tested with intact living cells. In conclusion, these results indicate that the FLIC-TIP was unable to penetrate into the cells. In order to confirm this finding, we used an end point study where the cells were treated with the FLIC-TIP **7** and washed twice with phosphate-buffered saline before lysing. However, the inhibition of FLuc was still undetectable, strongly indicating that the conjugate did not accumulate in the cells, nor was it bound to the cell membrane. In contrast and importantly, the FLIC **3** alone inhibited FLuc also in the living cell-based assays, demonstrating that the unconjugated FLIC compounds penetrate into the cells. These findings indicate that the FLIC−peptide conjugate is stable in the cell cultural media and do not show significant degradation in cell based assays. In addition, when TIP peptide was replaced with **8**, FLIC−penetratin conjugate **9** accumulated to living cells and efficiently inhibited the cytosolar luciferase. This finding verifies our hypothesis that the FLIC based method is a potent tool for the analysis of the cellular uptake of transporter conjugates. To confirm that FLICs or their conjugates are not cytotoxic, we measured cell viability after 2 h exposure to the compounds, but did not detect any significant alterations in cell viability (Figure 6).

The uptake kinetics of FLIC−penetratin **9** in varied concentrations was studied and the results are shown in Figure 7. Results show that conjugate **9** is a slow penetrating peptide, and the transport is mainly occurred before the one hour time point. In addition, results show how the kinetics for **9** can be detected with 5 μM concentration, which is consistent with the IC_{50} value measured with recombinant enzyme. Overall, these results show that FLICs are potent tools for recombinant, end point, and living cell assays, and could be further developed to be used in transporter uptake and release studies.

CONCLUSIONS

In this paper, we describe for the first time the use of FLICs as tools in biological assays to measure distribution of bioactive molecules at cellular level. We modified our FLICs so that they are directly suitable for SPPS to achieve simple conjugation to synthetic peptides on resin. The most promising compound FLIC **3** inhibited FLuc at micromolar IC_{50} level, and when conjugated to model trypsin inhibitor peptide (FLIC-TIP **7**), the luciferase inhibition activity was maintained without significantly altering the trypsin inhibitory activity of the TIP. Thus, the approach was assumed to be applicable, e.g., for measuring release of biomolecules into cell cytosol and to distinguish between possible endosome- or membrane-trapped and truly internalized biomolecules, which could be used, e.g., for transporting drug molecules to target tissues. Moreover, this hyphothesis was verified with well-known cell penetrating peptide, penetratin, which was conjugated to FLIC **3**. Based on the results, FLIC-penetratin **9** inhibited recombinant firefly luciferase efficiently (IC_{50} = 1.6 μM) and more importantly also

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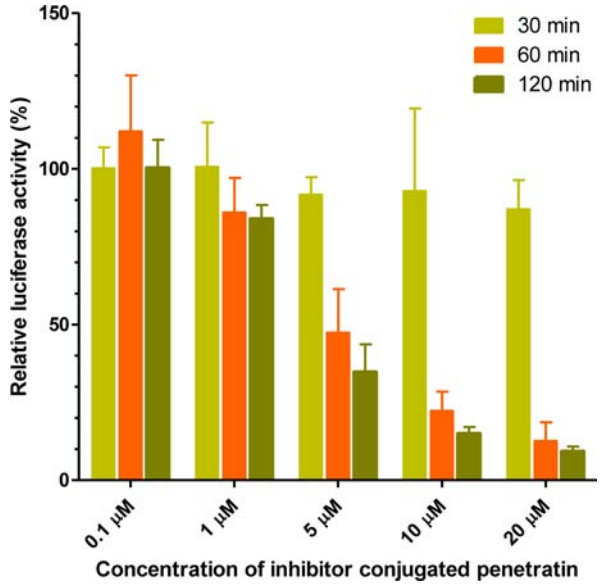

Figure 7. U87Fluc cells were exposed to varied concentrations of FLIC-penetratin (**9**), and luciferase activity was measured at indicated time points. Before luciferase inhibition can occur, the transporter–FLIC conjugate must be released to cytosol where the luciferase enzyme exists in U87Fluc cells. Results are given as mean together with standard deviation from three parallel measurements. Cells that were exposed to vehicle are set as 100% in each time point. The uptake kinetics indicates that **9** accumulates into the cell cytosol mainly within less than an hour at +37 °C.

in cell based assays with both lysed and living cells. In addition, none of the compounds (**3**, **4**, or **8**, or the conjugates **7** or **9**) caused significant effect on the viability of U87Fluc cells. Our proof-of-concept, using firefly luciferase-specific inhibitor compounds (FLICs) as tools for cellular internalization studies, provides a unique solution for the real time detection of the accumulation of a peptide into the cytosol and thus can be used to solve some technical problems seen especially with endosome trapped transporters. Moreover, this approach can be further used in an automated and quantitative manner, for example, to resolve the dilemma whether macromolecules can actually penetrate the cell membrane and carry cargo to the cytosol without being entrapped into an endosome, and thus lose the activity of the carried drug. In addition, the FLIC approach can be optimized in a case specific manner, e.g., using other types of quenching molecules, or luciferase can be expressed cell organ specifically to introduce novel methods to support intracellular transporter conjugate studies.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures and characterization data of the synthesized compounds and peptides, protocols of *in vitro* assays, IVIS images, results of trypsin activity measurements, and presentation of HPLC traces of the synthesized peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CRC, concentration response curve; DIPEA, *N,N*-diisopropylethylamine; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; FLIC, firefly luciferase-specific inhibitor compound; FLuc, *Photinus pyralis* (firefly) luciferase; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; SPPS, solid phase peptide synthesis; TIP, trypsin inhibitor peptide

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