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Development of Photoswitchable Tethered Ligands that Target the μ-Opioid Receptor

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Converting known ligands into photoswitchable derivatives offers the opportunity to modulate compound structure with light and hence, biological activity. In doing so, these probes provide unique control when evaluating G-protein-coupled receptor (GPCR) mechanism and function. Further conversion of such compounds into covalent probes, known as photoswitchable tethered ligands (PTLs), offers additional advantages. These include localization of the PTLs to the receptor binding pocket. Covalent localization increases local ligand concentration, improves site selectivity and may improve the biological differences between the respective isomers. This work describes chemical, photophysical and biochemical characterizations of a variety of PTLs designed to target the μ-opioid receptor (μOR). These PTLs were modeled on fentanyl, with the lead disulfide-containing agonist found to covalently interact with a cysteine-enriched mutant of this medically-relevant receptor.

Introduction

Throughout the past decade, extensive research has been conducted in expanding the repertoire of photoswitchable ligands that interact with a range of biological targets.[1] The attractiveness of photoswitchable probes stems from the ability to use light to reversibly change their chemical structure and/or properties. Importantly, this change may also alter the inherent biological activity of these compounds, resulting in photocontrol of ligand activity.[1] Such spatial, temporal and non-invasive control may be beneficial in enhancing kinetic and dynamic investigations.[2] This includes overcoming limitations of non-uniform start times in kinetic studies, as well as exploring receptor-ligand interactions and associated conformational changes in dynamic studies.[2] These investigations are particularly important for understanding the mechanisms and interactions of medically-relevant receptors, including G-protein-coupled receptors (GPCRs).[3]

Approximately 34% of clinically approved drugs exert their mechanism of action by modulating GPCR signaling.[4] Despite the successful application into clinics of drugs that target this class of receptors, there is still a knowledge gap in the research surrounding GPCR mechanisms and interactions.[5] This knowledge gap is particularly evident for the μ-opioid receptor (μOR). The μOR is notably targeted for pain relief, with drugs such as fentanyl and morphine available on the medical market. However, despite its importance, this GPCR plays a leading role in the current opioid epidemic.[6] In order to better understand the μOR, a wide range of valuable research has been accomplished over decades.[7] In the field of photopharmacology, fentanyl was developed by *Trauner et al.*[8] and later by our group[9] into photoswitchable ligands that enabled photocontrol of a μOR ionotropic and metabotropic response, respectively (Figure 1).

For ionotropic photocontrol, the *trans*-isomer (blue light irradiation) of photofentanyl 2 (**PF2**) was found to trigger μOR-mediated potassium influx through G-protein-coupled inward-rectifying potassium (GIRK) channels, while its respective *cis*-isomer (360 nm irradiation) retracted this μOR activation.[8] For metabotropic photocontrol, a photoswitchable ligand that is named here as fentanyl azopyrazole 1 (**FAPz 1**) displayed

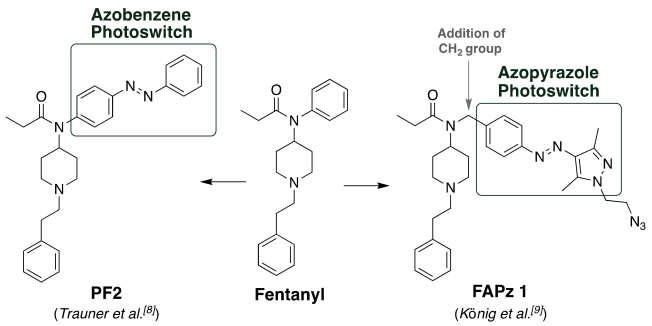

Figure 1. Structures of previously reported photoswitch-containing ligands that target the μOR (left and right), modeled on the fentanyl pharmacophore (middle). Previous work by *Trauner et al.*[8] attached an azobenzene photoswitch to the core structure of fentanyl, named photofentanyl 2 (**PF2**), while previous work by our group (*König et al.*[9]) attached an azopyrazole photoswitch unit (**FAPz 1**). When compared to fentanyl, **FAPz 1** contains a methylene insertion (+CH_2), which was reported to improve the photophysical properties of **FAPz 1**.[9]

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significant receptor efficacy differences between its respective *trans*- (528 nm irradiation) and *cis*-isomers (365 nm irradiation) in a G-protein activation assay (IP-One®).[9] In order to further exploit the possibilities of **FAPz 1** as a biochemical tool, this photoresponsive ligand was modified in this work into a range of tethered fentanyl azopyrazole (**tFAPz**) derivatives. These compounds were designed to contain a reactive group that could covalently interact with the μOR (Figure 2). More specifically, these fentanyl derivatives were designed in accordance with a class of covalent photoswitchable molecules known as photoswitchable tethered ligands (PTLs), which offer several advantages that are discussed in this paper. To covalently bind to a functionalized fentanyl derivative, we intended to use the μOR mutant N127C, which was shown to form a covalent receptor-ligand complex with a disulfide-functionalized PZM21 derivative.[7d] To date, there have been no PTLs that target μOR, and as a result, the work herein describes findings in the pursuit of these compounds.

Results and Discussion

PTLs have been successfully applied in targeting GPCRs, ion channels and enzymes.[10] These tool compounds are composed of a pharmacophore that is attached to a photoswitch unit, which in turn, is attached to a reactive unit.[11] The two segments that link these 3 components may vary in length. Longer segments result in a greater separation between these components, which may be beneficial in maintaining pharmacophore affinity. A greater separation between these components may also allow for more dramatic displacement of the pharmacophore from the binding pocket upon photoisomerization; however, too much flexibility could oppose this effect. Once the PTL is drawn by affinity to its respective binding site, the reactive unit is able to form a covalent bond via a cross-linking reaction, localizing the ligand to its biological target.[12] In doing so, PTLs overcome several limitations of freely diffusible photoswitchable ligands.[12] Covalent localization to the target receptor may improve site-selectivity, minimize off-target interactions, and is resistant towards sample washing or dilution. One of the most established extracellular bioconjugation techniques using PTLs has been the cysteine-maleimide system.[13] In this system, a sulfhydryl group of a cysteine residue near the receptor binding pocket reacts with an electrophilic maleimide moiety installed on the PTL, forming a covalent bond. Since reduced and solvent-exposed cysteine residues have a low natural abundance in proteins, mutation of amino acids near the binding site to a cysteine residue has commonly been required to allow for bioconjugation.[12] Despite the possibility for introduced cysteines to undergo oxidation reactions that may affect protein folding,[14] the disadvantages can be considered minor when compared to other biorthogonal approaches that require fusion proteins or other larger modifications.[10e] For example, the azide group of **FAPz 1** could be directly used in a bioorthogonal approach to covalently attach this fentanyl-based probe to the binding pocket of μOR,[15] however, this may require more complex genetic engineering of μOR. Furthermore, due to the low natural abundance and good reactivity of 'free' sulfhydryl groups, cysteine residues are considered to be one of the most convenient targets for selective bioconjugation.[16]

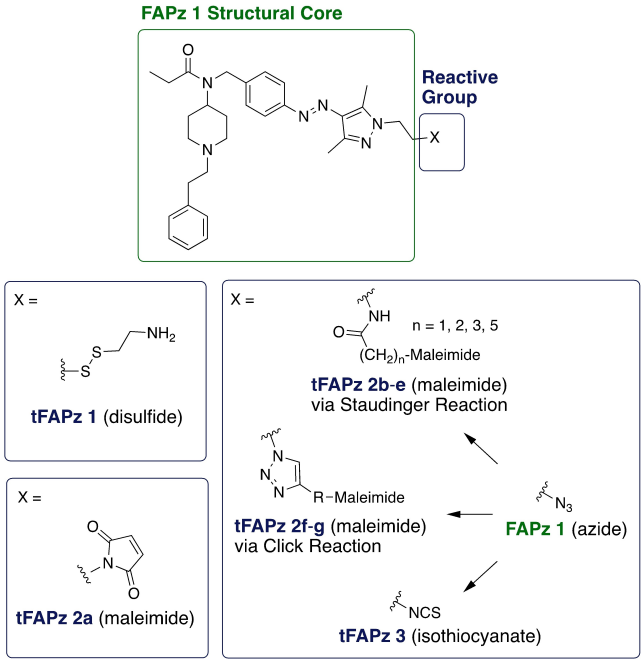

Figure 2. Structures of **tFAPz** that target the μOR, modeled on **FAPz 1**. The **tFAPz** explored in this work contain disulfide, maleimide or isothiocyanate reactive groups. Furthermore, maleimides **tFAPz 2 b**–**g** and isothiocyanate **tFAPz 3** were synthetically accessed directly from **FAPz 1**.

Overall, PTLs offer significant advantages as biochemical tools. As a result, it became of interest to expand **FAPz 1** that already possesses desirable biochemical and photophysical properties into PTLs.[9] Since **FAPz 1** already contains the fentanyl pharmacophore and an azopyrazole photoswitch unit, a reactive group was attached to the photoswitch unit via various linker lengths, resulting in an array of **tFAPz** derivatives (Figure 2). Various lengths of this linking segment were explored in order to achieve covalent interaction in or near the μOR binding pocket. The longest segment designed in this series was the PEG-4 linker in **tFAPz 2 g**. This derivative was hypothesized to form covalent interactions more distal to the ligand-receptor binding pocket in accordance with the design approach of photoswitchable orthogonal remotely tethered ligands (PORTLs).[11] Following both PTL and PORTL strategies, a maleimide series was developed (**tFAPz 2 a**–**g**) using different synthesis techniques for maleimide attachment to **FAPz 1**, including the well-known 'click' and 'Staudinger' reaction methods (Figure 2).[17] Even though maleimides have been effectively used in bioconjugation, hydrolysis and other drawbacks provided incentive to synthesize additional PTLs with other reactive groups.[16] The disulfide reactive group is highly selective for cysteine residues via disulfide exchange with free thiol groups. Despite susceptibility to reduction, disulfides have been proven valuable in bioconjugation for targeting GPCRs,[18] and as a result, a disulfide-containing PTL was designed (**tFAPz 1**).

Similarly, isothiocyanate **tFAPz 3** was developed due to its promising ability to engage in covalent interactions. Isothiocya-

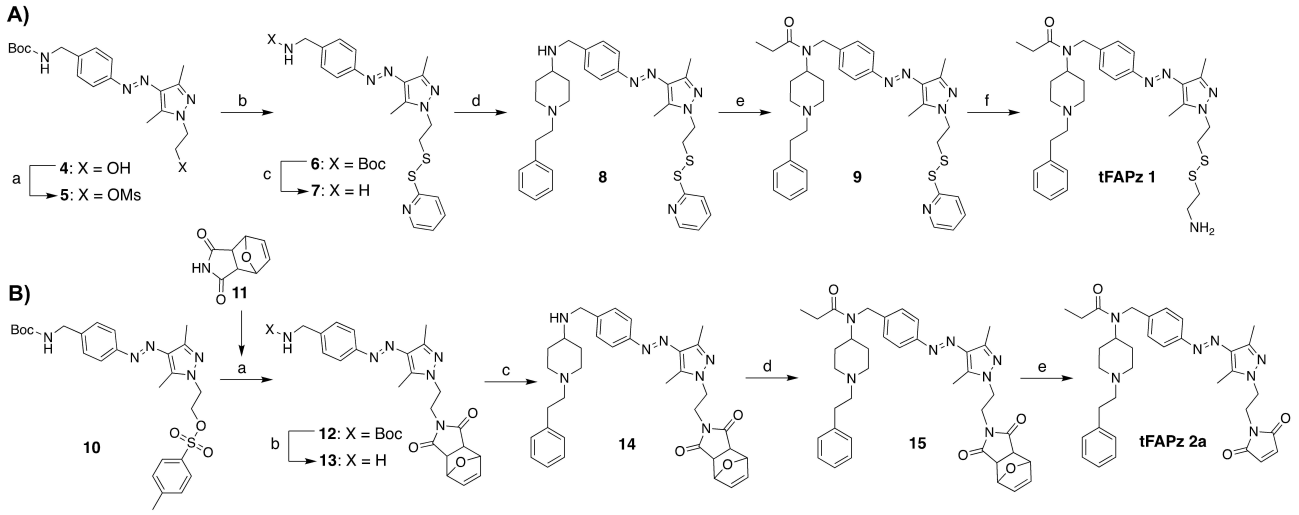

Figure 3. Synthesis of **tFAPz 1** and **2a**. A) Synthesis of **tFAPz 1**. (a) MsCl, Et_3N, DCM, 0 °C → rt, 1 h, 97%; (b) KSAc, acetone, reflux, 3 h; and then, Aldrithiol™-2, NaOMe, MeOH, rt, 16 h, 67% (overall yield); (c) TFA, DCM, 0 °C → rt, 1 h, 95%; (d) 1-Phenethyl-4-piperidone, $NaBH(OAc)_3$, AcOH, DCE, Ar, rt, 20 h, 88%; (e) Propionyl chloride, Et_3N, DCM, 10 min, rt, 95%; (f) Cysteamine hydrochloride, MeOH, Ar, rt, 0.5 h, 95%. B) Synthesis of **tFAPz 2a**. (a) K_2CO_3, DMF, 50 °C, 3 h, 56%; (b) TFA, DCM, 0 °C → rt, 1 h, 99%; (c) 1-Phenethyl-4-piperidone, $NaBH(OAc)_3$, AcOH, DCE, rt, 16 h, 61%; (d) Propionyl chloride, Et_3N, DCM, N_2, 24 h, rt, 34%; (e) DMSO (dried), 110 °C, 3 h, 51%.

nates have been shown to react with both sulfhydryl and amine nucleophiles, depending on pH, with a resistance towards water and alcohol-mediated hydrolysis.[18] It should be mentioned that in previous literature, an isothiocyanate group was attached to the terminus of the phenethyl moiety of fentanyl to form a covalent ligand (FIT or further modifications to SUPERFIT).[19] Despite possessing covalent properties, these ligands resulted in selectivity for δOR instead of μOR. Further modifications were explored in other literature work in attempts to obtain a fentanyl-based ligand that covalently targets μOR.[20] This included the incorporation of a reactive acryloyl group to the benzeneacetamide unit of fentanyl, however, a covalent ligand did not result.[20a] In 1990, an azido-containing photoaffinity derivative of carfentanil was developed that was successfully able to irreversibly label μOR.[20b] A drawback of this probe was the prolonged periods of 315 nm exposure for azide activation, which may not be compatible with several biological assays.[1a,b, 21] As a result, the desire to expand the repertoire of fentanyl-based covalent probes provided further incentive for the development of the proposed **tFAPz** ligands.

The synthesis of **tFAPz** either required the development of individual synthetic routes (**tFAPz 1** and **tFAPz 2a**, Figure 3) or could be easily accessed from **FAPz 1** via a one-pot synthesis reaction (**tFAPz 2b–g** and **3**, Figure 4, 5 and 6). To obtain **tFAPz 1** (Figure 3A), arylazopyrazole **4** that was synthesized according to literature procedures[9] was activated using methanesulfonyl chloride to form **5** in 97% yield. Nucleophilic substitution with potassium thioacetate resulted in a thioester, which was directly transformed to pyridyl disulfide **6** using Aldrithiol™-2 in an overall yield of 67%. Boc-deprotection with trifluoroacetic acid then afforded amine **7** in high yield. Subsequent reductive amination with 1-phenethyl-4-piperidone, afforded intermediate **8** in 88% yield, which was then acylated to obtain intermediate **9**

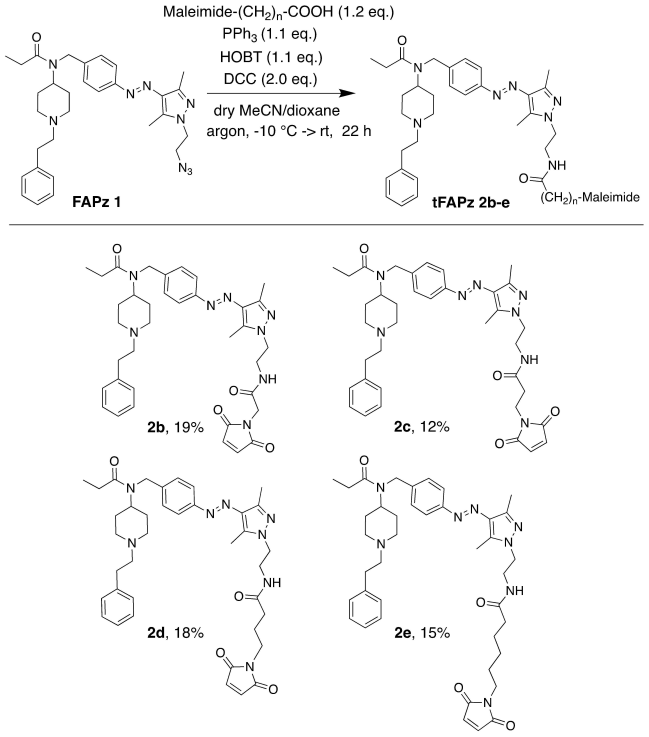

Figure 4. Synthesis of **tFAPz 2b–2e** via an adapted Staudinger reaction. Yields of isolated products are shown. Please refer to Supporting Information for detailed synthesis methods.

in 95% yield. Treatment with cysteamine, following previous literature,[22] afforded **tFAPz 1** in 95% yield.

The synthesis of **tFAPz 2a** (Figure 3B) involved preparing precursors **10** and **11** via previously reported procedures.[9,23] Using these materials, nucleophilic substitution yielded intermediate **12** in 56% yield. Boc-deprotection with trifluoroacetic acid afforded amine **13** in quantitative yield. Reductive amination

with 1-phenethyl-4-piperidone resulted in intermediate **14** in 61% yield. An acylation reaction then afforded intermediate **15** in 34% yield. The 2,5-dimethylfuran-protected maleimide was deprotected at 110 °C to afford **tFAPz 2a** in 51% yield. For the synthesis of **tFAPz 2b**–**e**, an adapted Staudinger reaction was employed, using a previously reported procedure (Figure 4).[24] In this adapted procedure, the carboxylic acid was first activated by HOBT. Activated esters were then reacted under dry conditions with the iminophosphorane intermediate of **FAPz 1**, using DCC as a coupling reagent, to form **tFAPz 2b**, **2c**, **2d** and **2e** in 19%, 12%, 18% and 15% yield, respectively.

In order to improve synthesis yields, Cu(II)-assisted click chemistry was employed to generate additional maleimide-containing PTLs (Figure 5). This was achieved using a similar method to that previously reported.[25] Using this approach, the azide of **FAPz 1** was reacted with alkyne derivatives that contained a maleimide group to obtain **tFAPz 2f** and **2g**. The alkyne used to obtain **tFAPz 2f** was synthesized via the reaction of activated 2-maleimidoacetic acid with N-propargylamine (see Supporting Information), while the alkyne used to obtain **tFAPz 2g** was commercially obtained. In this assisted cycloaddition reaction, sodium ascorbate was used as an initiating reagent and Cu(II) was chelated with TBTA, which yielded **tFAPz 2f** and **2g** in higher yields of 47% and 46%, respectively. To obtain isothiocyanate **tFAPz 3** (Figure 6), previously synthesized **FAPz 1** was directly subjected to a one-pot reaction that has been previously reported.[26] This involved generating an iminophosphorane from the azide of **FAPz 1**, followed by a condensation reaction with carbon disulfide to afford **tFAPz 3** in 42% yield.

Once synthesized, it was important to determine whether the desirable photophysical properties of **FAPz 1** were maintained despite ligand extension into PTLs. Since chemical modifications were conducted at distal sites to the photoswitch unit to obtain the **tFAPz** derivatives, similar photophysical properties to **FAPz 1** were anticipated and obtained (Figure 7 and Supporting Information).[9] Similar to **FAPz 1**, irradiation wavelengths of 365 nm could be used to obtain the *cis*-isomers of the **tFAPz** ligands, while a desirable red-shifted wavelength (528 nm) could be used to obtain the respective *trans*-isomers. In addition, the **tFAPz** derivatives displayed resistance towards cycle fatigue for at least 5 isomerization cycles, exhibited comparable photostationary states (PSS) to **FAPz 1** and possessed *cis*-isomer thermal half-lives of at least 3 days in both DMSO and buffer solution. In particular, the long thermal half-lives allow for good photocontrol, as the abundance of either the *cis*- or *trans*-isomer can be spatially and temporally modulated by irradiation with either 365 nm or 528 nm, respectively.

Since the PTLs displayed desirable photophysical properties, it was next important to determine whether **tFAPz 1**, **2a**–**g** and **3** displayed a potency towards wild-type μOR (μOR_{wt}). These PTLs were subjected to a ligand-mediated μOR activation screen at

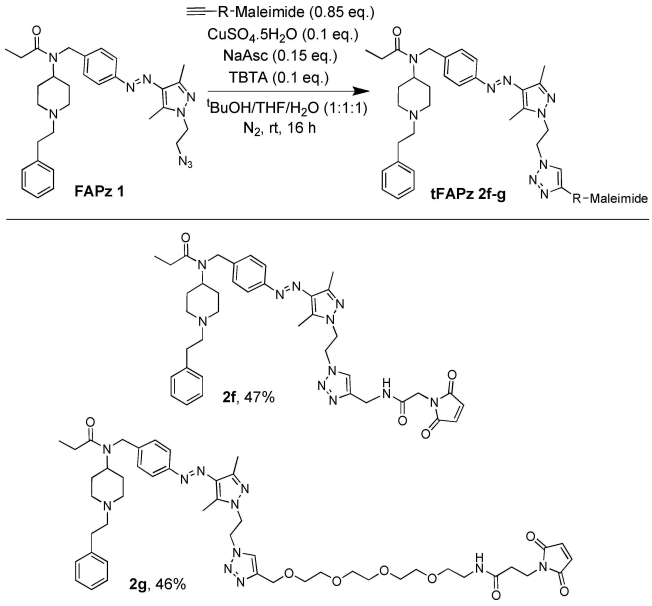

Figure 5. Synthesis of **tFAPz 2f**–**2g** via Cu(II)-mediated click reaction. Yields of isolated products are shown. Please refer to Supporting Information for detailed synthesis methods.

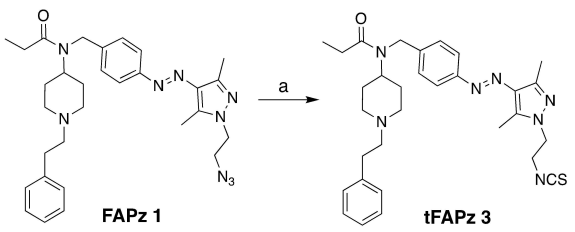

Figure 6. Synthesis of **tFAPz 3**. (a) PPh_3, CS_2, dry THF, N_2, reflux, 16 h, 42%.

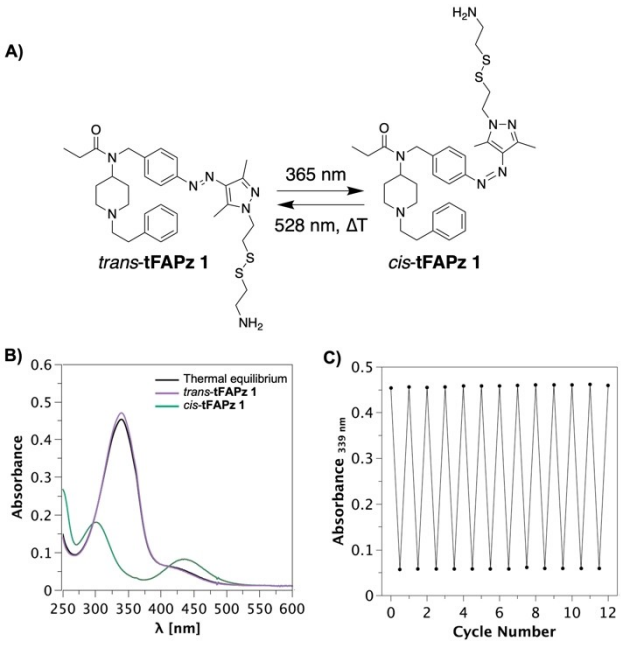

Figure 7. Photoinduced isomerization and cycle performance of **tFAPz 1**. A) Depiction of geometrical changes that occur upon photoinduced isomerization of **tFAPz 1**. B) UV/Vis absorption spectra of thermal equilibrium, *trans*-isomer and *cis*-isomer. C) Cycle performance (12 cycles) of **tFAPz 1** upon alternating irradiation of 365 nm and 528 nm, recorded at the absorbance maximum of the *trans*-isomer (339 nm). Results are shown of **tFAPz 1** (20 μM) in buffer solution (Tris·HCl buffer, pH 7.5) + 0.2% DMSO at 25 °C.

10 μM ligand concentration, using a G-protein activation assay (IP-One®).[27] This assay measures agonist-stimulated accumulation of IP in HEK293T cells, which were transiently co-transfected with the receptor and the hybrid G-protein $G\alpha_{qi5HA}$.[27] Each PTL was irradiated prior to biological analysis with the appropriate wavelength in order to obtain either the respective *trans*- or *cis*-isomer, and were compared to the full agonist reference DAMGO. In this screen, **tFAPz 2a–g** and **3** displayed weak receptor activation (SI Table 2). After an IP accumulation period of 3 h, both *cis*- and *trans*-isomers of **tFAPz 2b**, **2d** and **2f** displayed less than 31% μOR_{wt} maximum receptor response, while both respective isomers of **tFAPz 2a**, **2c**, **2e**, **2g** and **3** displayed no significant μOR activation. These poor activation profiles, especially when compared to fentanyl (100% response), may be due to suboptimal modifications to the pharmacophore or off-target interactions of the reactive tethering groups; however, compound degradation under the described conditions cannot be entirely excluded. In contrast to these derivatives, the disulfide-containing **tFAPz 1** displayed full agonist activity (E_{max} = 100%) similar to the efficacy stimulated by fentanyl. As a result, **tFAPz 1** was identified as a lead compound in this work.

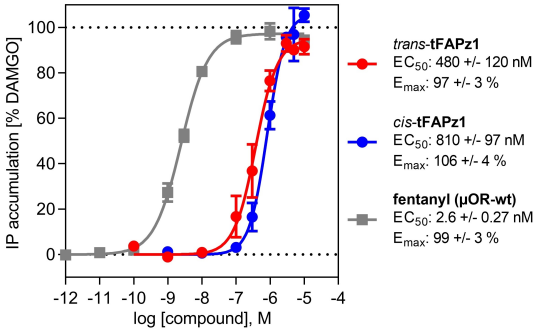

Figure 8. Activation of μOR by **tFAPz 1** and fentanyl. G-protein-mediated receptor activation by *trans*-**tFAPz 1**, *cis*-**tFAPz 1** and fentanyl was measured by applying the IP-One® accumulation assay in HEK293T cells transiently co-transfected with μOR_{M1} or the wild-type μOR (for fentanyl) and the hybrid G-protein $G\alpha_{qi5HA}$ after an incubation time of 180 min. Graphs show mean curves (±S.E.M.) of 4 (*cis*-**tFAPz 1**), 6 (*trans*-**tFAPz 1**) or 7 (fentanyl) individual experiments, each performed in duplicate.

Table 1. Ligand-mediated activation and radioligand binding with μOR_{M1}.

Ligand-mediated activation[a]				
Compound	EC_{50} [nM ± S.E.M.]	EC_{50} ratio[b]	E_{max} [% ± S.E.M.][c]	(*n*)[d]
trans-**tFAPz 1**	480 ± 120	1.7 (*trans*)	97 ± 3	6
cis-**tFAPz 1**	810 ± 97		106 ± 4	4
Fentanyl[e]	2.6 ± 0.27		99 ± 3	7
Radioligand binding studies[f]				
Compound	K_i [nM ± S.E.M.]		K_i ratio[b]	(*n*)[g]
trans-**tFAPz 1**	42 ± 10		1.9 (*trans*)	6
cis-**tFAPz 1**	80 ± 28			
Fentanyl[e]	11 ± 1.8			7
trans-**tFAPz 1**[e]	85 ± 32		1.3 (*trans*)	4
cis-**tFAPz 1**[e]	110 ± 27			

[a] IP-One accumulation assay (Cisbio). [b] The isomer shown in brackets has a greater potency than its respective isomer. [c] Maximum receptor activation in % ± S.E.M. relative to the full effect of DAMGO. [d] Number of individual experiments, each performed in duplicate. [e] Data is derived from experiments conducted with the μOR wild-type receptor. [f] Binding data to μOR_{M1} was determined by competition binding with [^{3}H]diprenorphine. [g] Number of individual experiments, each performed in triplicate.

The use of disulfide-containing compounds to covalently bind to GPCRs have been proven successful in generating stable and functional GPCR-ligand complexes.[18] Once such ligands diffuse via intrinsic affinity into the receptor binding pocket, a chemoselective disulfide exchange proceeds between the disulfide unit of the ligand and a nearby sulfhydryl group of a free cysteine residue.[28] This highly selective reaction reduces the risk of off-target interactions with other nucleophilic amino acids and ultimately, would result in covalent binding to the target site. In order to ensure that **tFAPz 1** binds to an appropriate cysteine residue, a site-specific μOR mutant ($N127^{2.63}C$, abbreviated as μOR_{M1}) was established that contains a free cysteine residue in the μOR binding pocket.[7d,29]

Using this μOR mutant, ligand-mediated activation and binding affinity of **tFAPz 1** to μOR_{M1} were evaluated (Figure 8, Table 1). In order to determine ligand-mediated activation, full-dose response curves of both *trans*- and *cis*-isomers of **tFAPz 1** were generated using the IP-One® assay. In this study, both isomers of **tFAPz 1** behaved as full agonists with potencies of 480 nM (*trans*-isomer) and 810 nM (*cis*-isomer). Despite the reduction in potency when compared to fentanyl, both *trans*- and *cis*-isomers were still able to activate the receptor in a nM range, with approximately 2-fold higher activity found for *trans*-**tFAPz 1** than *cis*-**tFAPz 1**. Importantly, the previously published efficacy ligand **FAPz 1** possessed a lower potency than **tFAPz 1** in the same activation assay with wild-type μOR (*trans*-**FAPz 1**: EC_{50} = 4,700 nM; *cis*-**FAPz 1**: EC_{50} = 2,300 nM; compare with fentanyl: EC_{50} = 2.6 nM).[9] These results suggest that modification of **FAPz 1** to **tFAPz 1** by replacing the azide moiety with a disulfide unit may be better tolerated for receptor activation. Radioligand binding studies revealed competitive binding of **tFAPz 1** to μOR_{M1}, with K_i values of 42 nM and 80 nM obtained for its *trans*- and *cis*-isomers, respectively (Table 1). Similar to activation studies, *trans*-**tFAPz 1** displayed approximately 2-fold higher affinity for μOR_{M1} than *cis*-**tFAPz 1**. Furthermore, the former isomer displayed only a 3.8-fold attenuation in binding affinity when compared to fentanyl, which shows that the ligand specifically recognizes μOR_{M1}.

Since **tFAPz 1** displayed significant affinity and activation profiles towards μOR_{M1}, it was crucial to determine whether this lead PTL was able to covalently bind to μOR_{M1}. Specific binding of [^{3}H]diprenorphine was determined, according to previous procedures,[28,30] for membranes pre-treated with *trans*- or *cis*-**tFAPz 1**. Corresponding findings were compared to a control homogenate that was incubated with the reversible ligand naloxone or fentanyl. For membranes that were treated with **tFAPz 1**, the determined specific binding indicates the amount of blocked receptor binding sites by covalently bound ligand after washing and exposure to excess radioligand. In comparison

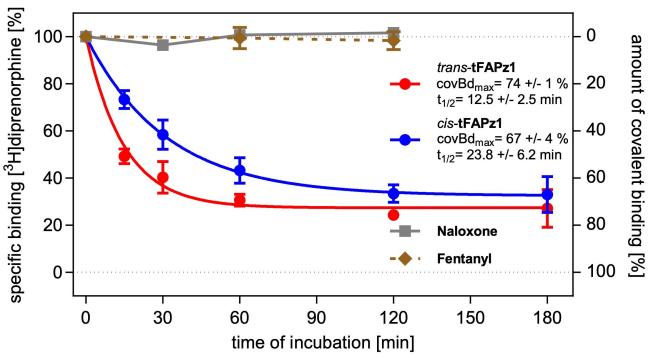

Figure 9. Covalent binding of **tFAPz 1**. Covalent binding was determined in a radioligand depletion assay, with membranes from μOR_{M1} expressing HEK293T cells and the radioligand [^{3}H]diprenorphine. The PTL **tFAPz 1** displayed significant covalent binding, with a covalent binding maximum ($covBd_{max}$) of 74 ± 1% by *trans*-**tFAPz 1** (1 μM) and 67 ± 4% by *cis*-**tFAPz 1** (1 μM), while the reversible control ligands naloxone (0.1 μM) and fentanyl (0.1 μM) displayed no covalent binding properties. Kinetic analysis of this covalent process revealed 2-fold faster interaction of *trans*-**tFAPz 1** ($t_{1/2}$ = 12.5 min) with the orthosteric binding site, compared to *cis*-**tFAPz 1** ($t_{1/2}$ = 23.8 min). Graphs show mean curves (± S.E.M.) of 3 (for naloxone) or 6 (for **tFAPz 1** and for fentanyl) individual experiments, each performed in quadruplicate.

to the control, both isomers of this newly synthesized PTL were able to covalently bind to μOR_{M1} (Figure 9). After an incubation time of 45 min, *trans*-**tFAPz 1** was able to covalently bind to 74 ± 1% of μOR_{M1} binding sites, as indicated by the curve shown in Figure 9. In contrast, *cis*-**tFAPz 1** was only able to covalently bind to 67 ± 4% of μOR_{M1} binding sites after 120 min.

The superiority of the *trans*-isomer over the *cis*-isomer was also reflected by the kinetics of covalent binding. While *cis*-**tFAPz 1** displayed covalent binding to the receptor with a half-life ($t_{1/2}$) of 23.8 min, a $t_{1/2}$ of 12.5 min was obtained for *trans*-**tFAPz 1**. This result indicates 2-fold faster kinetics for the latter isomer (Figure 9, SI Table 3). These findings were further validated by the observation that *trans*-**tFAPz 1** decreased specific binding by 51% after 15 min, while *cis*-**tFAPz 1** only decreased specific binding by 27% in the same amount of time. Since differences in covalent binding properties were observed between the isomers of **tFAPz 1**, it may be inferred that the differing geometry of each isomer was maintained upon diffusion into the receptor binding pocket and during the process of ligand-receptor complexing. Overall, these findings establish **tFAPz 1** as an attractive probe that can localize and covalently bind to μOR_{M1}.

Conclusions

The work described herein focuses on the development of PTLs, modeled on the potent μOR agonist fentanyl. The newly synthesized ligands displayed ideal photophysical properties that were similar to that of the previously reported **FAPz 1**, including long thermal half-lives (of at least 3 days) and cycle performance resistance (for at least 5 cycles) in both buffer and DMSO systems.[9] Furthermore, respective *cis*-isomers could be accessed with 365 nm irradiation and respective *trans*-isomers could be accessed using a desirable red-shifted wavelength of 528 nm. The lead disulfide-containing PTL **tFAPz 1** displayed full agonist properties in a metabotropic functional assay, with significant potencies towards μOR_{M1} (*trans*-**tFAPz 1**: EC_{50} = 480 nM; *cis*-**FAPz 1**: EC_{50} = 810 nM). High binding affinities were further validated in a radioligand binding assay (*trans*-**tFAPz 1**: K_i = 42 nM; *cis*-**FAPz 1**: K_i = 80 nM). Notably, the *trans*-**tFAPz 1** displayed 2-fold greater potency than it's respective *cis*-isomer in both assays. Importantly, **tFAPz 1** was found to form covalent ligand-receptor complexes with μOR_{M1}, with differences observed between isomers. Covalent binding of *trans*- **tFAPz 1** proceeded with a $t_{1/2}$ of 12.5 min and a maximum of 74%. In contrast, covalent binding of *cis*-**tFAPz 1** proceeded at approximately half the rate ($t_{1/2}$ = 23.8 min), with a maximum of 67%.

These findings not only establish **tFAPz 1** as a photoswitchable agonist that can form covalent ligand-receptor complexes in this system but demonstrate that the structural differences between isomers were maintained upon covalent interaction with μOR_{M1}. Future work may consist of minor structural modifications to **tFAPz 1** in order to further enhance biological differences between isomers. For example, lower affinity PTLs may allow for more significant compound placement/displacement in/out of the receptor binding pocket upon photoisomerization.[10e] Due to the high binding affinity of *trans*-**tFAPz 1** (K_i = 42 nM) and *cis*-**tFAPz 1** (K_i = 80 nM), structural modifications to slightly reduce affinity may be beneficial. Further work may also consist of exploring isomer activity differences of the ligand once in complex with the receptor, which could be best explored using different systems, including both metabotropic and ionotropic systems.[8–9] As a result, the development and confirmation of **tFAPz 1** as a covalent and photoswitchable μOR probe provides a foundation to further expand and improve the repertoire of **tFAPz** ligands. Furthermore, this covalent probe may be beneficial in future biochemical investigations surrounding μOR and fentanyl-based structure relations, with the added advantage of utilizing the photoswitchable properties of **tFAPz 1** when desired.

Supporting Information

The authors have cited additional references within the Supporting Information.[31]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: covalent · fentanyl · μ-opioid receptor · G-protein-coupled receptor · photopharmacology

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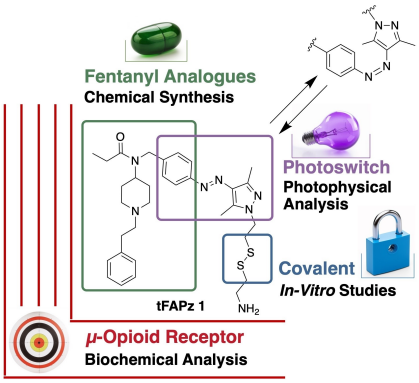
RESEARCH ARTICLE

Fentanyl-based photoswitchable tethered ligands (PTLs) were developed, targeting the μ-opioid receptor (μOR). Such ligands allow for spatial, temporal and non-invasive regulation of this medically-relevant receptor, using light. Covalent interaction via a tethering group offers several advantages, including ligand localization to μOR. This interaction was achieved in this work by the disulfide-containing PTL **tFAPz 1**.



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Development of Photoswitchable Tethered Ligands that Target the μ-Opioid Receptor