Development of an indole-amide-based photoswitchable cannabinoid receptor subtype 1 (CB₁R) “cis-on” agonist

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ABSTRACT: Activation of the human cannabinoid receptor type 1 (hCB₁R) with high spatiotemporal control is useful to study processes involved in different pathologies related to nociception, metabolic alterations and neurological disorders. To synthesize new agonist ligands for hCB₁R, we have designed different classes of photoswitchable molecules based on an indole core. The modifications made to the central core have allowed us to understand the molecular characteristics necessary to design an agonist with optimal pharmacological properties. Compound 27a shows high affinity for CB₁R (Kᵣ(cis form) = 0.18 µM), with a marked difference in affinity with respect to its inactive “trans-off” form (CB₁R Kᵣ(trans/cis ratio = 5.4). The novel compounds were evaluated by radioligand binding studies, receptor internalization, sensor receptor activation (GRABeCB₂.0), and calcium mobilization assays. The data shows that the novel agonist 27a is a candidate for studying the optical modulation of CB₁Rs serving as a new molecular tool for investigating the involvement of hCB₁R in disorders associated with the endocannabinoid system.

INTRODUCTION

In 1964 Gaoni and Mechoulam first identified Δ⁹-3₄-tetrahydrocannabinol as an active component from Cannabis sativa L. which was the vital first step in understanding the endocannabinoid system.² From there, as a result of multiple investigations, it was possible to identify the two canonical cannabinoid receptors reported to date, named as human cannabinoid receptor type 1 (hCB₁R) and type 2 (hCB₂R).²,³ Although both receptors share characteristics at the structural level, the distribution of the tissues where they are expressed, as well as the differences in the transduction systems makes the clinical relevance between both receptors differ.²⁻⁵

Specifically, CB₁R agonist ligands have great clinical relevance in the treatment of various pathologies. In studies with knockout animals, it has been found that the administration of CB₁R agonists causes a decrease in depressive and anxiety-like behaviors, whose biological effect is abolished by the administration of CB₁R antagonists.⁶,⁷ Similarly, by activating CB₁R in murine epilepsy models, it has been possible to reduce the intensity of seizures, suggesting that these agonists may be beneficial for patients with treatment-resistant epilepsy.⁸ Another aspect of clinical relevance is weight control, where CB₁R agonists have been shown to be useful in treating of certain metabolic disorders. CB₁R activation induces an antiemetic and satiety-inhibiting effect, which generates a positive energy mainte-
nance effect by increasing food intake and, therefore, increasing body weight. These effects are beneficial for the treatment of metabolic disorders such as cachexia and anorexia generated in patients with underlying pathologies such as cancer, HIV/AIDS or Alzheimer’s disease.\textsuperscript{9,12} Additionally, the antinociceptive effects produced by CB\(_{1}\)R agonist ligands in both inflammatory and neuropathic pain models open the possibility of new types of treatments with fewer adverse effects for patients with chronic disabling diseases.\textsuperscript{13}

Despite having multiple therapeutic uses, some CB\(_{1}\)R agonists are sold on the black market for their recreational effects, being used to induce hallucinogenic and relaxing effects, as well as substances with the potential to "increase creativity".\textsuperscript{14,15} However, its misuse can lead to multiple adverse effects, especially when consumed in excess. It has been documented that intoxication can produce a variety of cardiotoxic, psychiatric and neurological effects, as well as develop a certain degree of dependence in some individuals.\textsuperscript{16}

All these biological effects have led to the need for a more detailed exploration of CB\(_{1}\)R, so that through new ligands designed as molecular tools, the receptor activation mechanism can be understood in detail at both central and peripheral levels. Molecular tool compounds are useful substances in the study of molecular biology and medicinal chemistry. These molecules allow analyzing the signaling networks, and the biological effect given by the interaction with the receptor in a specific tissue or organ in a spatiotemporal manner.\textsuperscript{17} To achieve this specificity, it is necessary to use various external stimuli to generate the release or activation of the ligand directly in the selected tissue.\textsuperscript{18} Many of the new molecular tools designed to study the nature of biological receptors have the disadvantage of generating irreversible type reactions (for example, controlled release systems through hydrolysis of molecular bonds). In this way, although it is possible to activate the receptor in a precise temporal-spatial way, it is not possible to return to the initial state spontaneously. To solve this, new ligands have been designed which can function as molecular switches in such a way that they can activate or deactivate the receptor through the use of external light sources.\textsuperscript{19} When irradiated at specific wavelengths, these photo-ligands can isomerize in such a way that they can go from an inactivated "off" form to an active "on" photo-isomer, generating precise time-control of the biological activity of the receptor.\textsuperscript{19-21}

The use of photo-pharmacological tools for the exploration of receptors framed within complex pathologies is a topic of growing interest.\textsuperscript{22,23} Specifically for the CB\(_{1}\)Rs, novel photoswitchable molecules designed for the study of the biological activity of the endocannabinoid system have been reported. By coupling \(\Delta^9\)-THC scaffold with an azobenzene moiety, Trauner, Carreira and coworkers designed two photoswitchable ligands with high affinity for CB\(_{1}\)Rs.\textsuperscript{24} These novel molecules allowed spatiotemporal control of CB\(_{1}\)R and its signaling pathways by isomerization with external light sources. Although the molecules derived from \(\Delta^9\)-THC are pioneers in the photopharmacological study of CB\(_{1}\)Rs, however, like its parent compound, they could be non-selective toward other biological targets.\textsuperscript{25} Such potentially pros-

miscuous ligands can give rise to undesirable adverse effects derived from the non-specific interaction with a variety of targets. To improve its specificity, in previous work by our research group, new photoligands derived from the benzimidazole core were synthesized. These partial agonist ligands showed a pronounced selectivity for CB\(_{2}\)R over CB\(_{1}\)R, having higher affinity in its cis photo-isomer.\textsuperscript{26} Subsequently, Carreira, Frank and coworkers described the synthesis of highly selective CB\(_{1}\)R agonists coupled to fluorescent and photoswitchable moieties. This new class of hybrid photo-ligands has the ability to monitor CB\(_{1}\)R signaling by controlling calcium release in real time.\textsuperscript{27,28} Recently, Tao et al. have described the design of azaesters derived from the pharmacophoric structure of the CB\(_{1}\)R antagonist AM10257.\textsuperscript{29} Using rational remodeling approach, they replaced the amadantyl group of the parent compound with an azobenzene moiety, thereby creating a new photoligand with an affinity for CB\(_{1}\)R in its cis form more than 40 times higher than its trans form. These results opened up new possibilities for photoligand design through a novel structure-guided approach.

In a recent work of our research group, we describe the synthesis of "photo-rimonabant" as a novel ligand designed from the rimonabant pharmacophore, substituting one of the pyrazole side arms for an azobenzene moiety by means of azo-extension techniques.\textsuperscript{30} As a result, we obtained an antagonist with high selectivity for CB\(_{1}\)R over CB\(_{2}\)R and 15-fold higher affinity in its cis photo-isomer compared to the trans isomer.

For the present work, we wanted to design novel photoswitchable compounds with agonist activity on CB\(_{1}\)R. Although derivatives of \(\Delta^9\)-THC were previously reported in the literature,\textsuperscript{24} in our group we wanted to focus on compounds derived on the indole core. The advantage of this core is that the target molecules are smaller and easier to synthesize. The synthesized compounds were photocharacterized and tested in different biological assays, validating the differences between their two isoforms of the most representative compounds with an amide bridge at C-3.

**RESULTS AND DISCUSSION**

**Chemistry.** For the design of the CB\(_{1}\)R agonist compounds, the structures of promising substances previously described in the literature were considered (Chart 1).\textsuperscript{14,31-34} Within these substances, both the indole and indazole are the most common aromatic systems used as central core, which are substituted at the C-3 and N-1 positions with hydrophobic groups. Regarding N-1 substitutions, short aliphatic chains with 3-5 carbon atoms are usually attached, which may contain a halogen atom in their distal position, however, other hydrophobic substituents - more voluminous - are equally accepted.\textsuperscript{14} The substitution at C-3 usually occurs with bulky moieties such as naphthyl, benzyl or adamantyl, which are attached to the indole by a carbonyl linker, usually a ketone or amide bridge. This is because the carbonyl group is essential, generating hydrophilic interactions in one domain of the ligand binding pocket of CB\(_{1}\)R.\textsuperscript{35}

In view of the highly hydrophobic nature of CB\(_{1}\)R, it was decided to functionalize the central indole ring by attaching a moiety capable of isomerization by light. The chosen photosomerizable group was azobenzene, due to its structural...
characteristics that simulate common bulky groups such as naphthyl. Additionally, azobenzenes are stable over a wide pH range and are usually easy to synthesize.\textsuperscript{26-30}

Although agonist CB\textsubscript{1} photo-ligands based on the structure of THC have been previously reported in the literature,\textsuperscript{24} in this project we decided to synthesize new cannabimimetic photoswitchable ligands core-based on the indole ring. These new photo-compounds are “small” and much easier to synthesize. Similarly, its relatively simple structure may enable higher selectivity for CB\textsubscript{1}.

Chart 1. Similarities found in CB\textsubscript{1} agonist molecules described in the literature.\textsuperscript{14, 31-34}

The first class of CB\textsubscript{1}R ligands was designed by introducing different hydrophobic substituents at positions N-1 and C-3 of the central indole ring. First, to preserve the naphthyl group in position C-3 starting from the pharmacophoric structure of reference compounds 1 and 2. We decided to modify only the lateral aliphatic chain, attaching the azobenzene moiety to N-1 (Scheme 1). For this purpose, acylation of unsubstituted indole (6) was carried out by addition of Grignard reagent and 1-naphthyl chloride, followed by a selective hydrolysis to yield intermediate 7. By alkylation of compound 7 in position N-1, through the reaction with NaH and 1-bromo-4-nitrobenzene, intermediate 8 was obtained. Then, the nitro group was subsequently reduced to its respective amine 9 by hydrogenation. In parallel, aromatic amines 10a-b were partially oxidized with MPS to generate the corresponding nitroso compounds 11a-b. The mixture of the nitroso derivatives and intermediate 9 in acid conditions yielded the target compounds 12a-b in moderate yields.

To compare the influence of the photoswitchable azobenzene at different positions of the central ring, the following classes of compounds were designed to incorporate azobenzenes at position C-3 of the indole. As a first approach (Scheme 2), the indole 6 was reacted with substituted nitrobenzoyl chloride - in para (13a), meta (13b) and ortho (13c) positions - in the presence of Grignard reagent at 78°C. The yield of this reaction was highly dependent on substitution of the nitro group: substitution in ortho-position did not generate the desired product, but mainly a by-product corresponding to the alkylation of N-1. Substitution in meta-position gave low yields, with an overall value of 10% for compound 15b after hydrolysis. On the other hand, substitution in para-position generated the best results, obtaining compound 15a with an overall yield of 53%. Later, by deprotonation of the nitrogen with NaH, the aliphatic chain was incorporated into the intermediates to yield molecules 16a-b. The nitro-compounds 16a-b were hydrogenated to obtain the respective anilines 17a-b. As a final step, the Baeyer-Mills reaction in acidic medium was performed to obtain the final compounds with substitution in para (18) and meta (19a-b) positions.

Scheme 1. Synthesis of photo-CB\textsubscript{1}R ligands with introduction of azobenzene in position N-1.\textsuperscript{a}

\textsuperscript{a}Reagents and conditions: (a) MeMgBr, 1-naphthoylchloride, THF, -78°C, 4h; (b) LiOH, MeOH, THF, rt, 2h, 56%; (c) 1-bromo-4-nitrobenzene, NaH, DMF, 65°C, 3h, 58%; (d) H\textsubscript{2}, Pd/C, EtOH, rt, 2h, 60%; (e) MPS, DCM/H\textsubscript{2}O, rt, overnight; (f) AcOH/TFA/toluene, rt, overnight, 19-26%.

The second approach to synthesize substituted indoles with the azobenzene moiety in position C-3, was planned by replacing the ketone bridge with an amide bridge, like the pharmacophores in reference molecules 4 and 5 (Figure 1, Scheme 3). We also considered our previous experience regarding the synthesis of both CB\textsubscript{1}R antagonists’ photo-ligands as well as CB\textsubscript{2}R photoswitchable agonists, where slight changes in aromatic substitution of the azobenzene moiety, could translate into large differences in affinity between the different structural isomers.\textsuperscript{26, 30} To evaluate the aromatic substitution, the synthesis proceeded by starting with amino-substituted aminobenzylamines at ortho (20a), meta (20b) and para (20c) positions.

The synthesis of intermediates 23a-c was carried out according to the protocol previously described by our group.\textsuperscript{30} Following that synthetic route, indole-3-carboxylic acid (24) was treated with oxalyl chloride and DMF to obtain the
corresponding acyl chloride 25. Coupling between intermediate 25 and amines 23a-c resulted in the formation of the amides 26a-c. Subsequently, deprotonation with NaH and addition of 1-bromo-pentane or 3-fluoropropyl 4-methylbenzenesulphonate yielded compounds 27a-c and 28a-c with a fluorinated chain of 5 and 3 carbon atoms, respectively.

Scheme 2. Synthesis of photo-CB1R ligands with introduction of azobenzene in position C-3 (ketone bridged).a

![Scheme 2](image)

Reagents and conditions: (a) MeMgBr, THF, -78°C, 4h; (b) LiOH, MeOH, THF, rt, 2h, 10-53%; (c) 1-bromo-5-fluoropentane, NaH, DMF, 65°C, 3h, 74-81%; (d) H2, Pd/C, EtOH, rt, 2h, quant; (e) AcOH/TFA/toluene, rt, overnight, 14-22%.

Scheme 3. Synthesis of photo-CB1R ligands with introduction of azobenzene in position C-3 (amide bridged).a

![Scheme 3](image)

Reagents and conditions: (a) TEA, Boc2O, DCM, 0°C, 2 h, quant; (b) nitrosobenzene, acetic acid, rt, overnight, 58-68%; (c) TFA, DCM, rt, overnight, quant; (d) (COCl)2, DMF, DCM, 0°C→rt, 2 h; (e) TEA, DCM, 0°C→rt, 30 min, 50%; (f) 1-bromo-5-fluoropentane, NaH, DMF, 65°C, 3h, 32-48%; (g) 3-fluoropropyl 4-methylbenzenesulphonate, NaH, DMF, 65°C, 3h, 19-29%.

Considering the ease in the synthesis of amide bridge photo-ligands (compared to the low yields obtained in the coupling with ketone bridges), and in view of the highly hydrophobic nature of CB1R, the following synthesis was planned by introducing small aliphatic substituents in the distal ring of azobenzene to analyze whether these changes translate into a greater difference between the cis and trans isomers. The new molecules are derivatives of the target compounds 27a and 28a, whose modifications allowed us to evaluate the effect of azobenzene substitution regarding the affinity for CB1R (Scheme 4).

Scheme 4. Derivatization of ortho-substituted amide bridged photo-CB1R ligands.a

![Scheme 4](image)

Reagents and conditions: (a) MeOH, H2SO4, 80°C, 94%; (b) Alkyl fluoride, NaH, DMF, 65°C, 3h, 92-98%; (c) NaOH, EtOH, 80°C, 2 h, 86-98%; (d) (COCl)2, DMF, DCM, 0°C→rt, 2 h; (e) 2-aminobenzylamine, TEA, DCM, 0°C→rt, 30 min, 97%; (f) MPS, DCM/H2O, rt, overnight; (g) AcOH, rt, overnight, 11-71%.

As in the previous synthetic route, the synthesis began with compound 24, whose carboxylic acid esterified by a reaction with MeOH and H2SO4, generating the ester 25. The indole was deprotonated at position N-1 with sodium hydride and subsequently by reaction with alkyl fluoride, yielding intermediates 30 and 31 with 3 and 5 carbon atoms in the side chain respectively. Subsequently, basic hydrolysis of the ester was carried out, generating intermediates 32 and 33. The carboxylic acid was activated with oxalyl chloride, and subsequently added to a solution of 2-aminobenzylamine, TEA, and DCM, 0°C→rt, 30 min, 97%; (f) MPS, DCM/H2O, rt, overnight; (g) AcOH, rt, overnight, 11-71%.

As in the previous synthetic route, the synthesis began with compound 24, whose carboxylic acid esterified by a reaction with MeOH and H2SO4, generating the ester 25. The indole was deprotonated at position N-1 with sodium hydride and subsequently by reaction with alkyl fluoride, yielding intermediates 30 and 31 with 3 and 5 carbon atoms in the side chain respectively. Subsequently, basic hydrolysis of the ester was carried out, generating intermediates 32 and 33. The carboxylic acid was activated with oxalyl chloride, and subsequently added to a solution of 2-aminobenzylamine and TEA to yield the ortho-substituted anilines 34 and 35. In parallel, the nitroso compounds 11a-e were obtained in situ from the partial oxidation of the anilines 10a-e with...
MPS. The coupling between nitroso compounds 11a-e and anilines 34 and 35 was carried out with the Baeyer-Mills reaction under acid conditions to generate the final compounds 36a-e and 37b-e, respectively with 3 and 5 carbon atoms in their side chain.

Classical azobenzenes are thermodynamically stable in their trans isomer, undergoing isomerization to their cis form by light irradiation with wavelengths in the UV region of the spectra, and relaxation to their stable photoisomers by irradiation with wavelengths $\lambda > 400$ nm. However, recent publications have detailed the characterization of cyclic azobenzenes, which - and in contrast to classical azobenzenes - are thermodynamically stable in their Z form. These diazocines are isomerized to their E isomer by irradiation $\lambda < 400$nm and converted back to their Z isomer by irradiation at wavelengths $\lambda > 500$nm. Given that only few publications to date have explored these novel azobenzenes for pharmacological applications, we carried out a synthetic route for incorporation of diazocines into the central ring of the indole through amide bridge formation (Scheme 5).

The synthetic route started with a Sonogashira coupling reaction between aryl-iodide 38 and TMS-acetylene, yielding compound 39. Subsequent elimination of TMS with K$_2$CO$_3$ in MeOH generated intermediate 40, which reacted in a second Sonogashira coupling reaction with methyl 4-iodo-3-nitrobenzoate to give rise to molecule 41. Simultaneous reduction of the alkyne and the two nitro groups by hydrogenation at high pressure yielded diamine 42. Subsequently, slow addition of a mCPBA solution to compound 42 allowed partial oxidation of one of the anilines to nitroso, which reacted intramolecularly to yield diazocine 44. However, the byproduct azoxyazobenzene 43 was also formed. To increase the overall yield, we adapted a procedure of the literature, treating byproduct 43 with MoO$_3$(dmf)$_2$ and PPh$_3$ to deoxygenate to the corresponding azobenzene 44. Carboxylic acid 45 was obtained by hydrolysis of 44 under basic conditions with NaOH and EtOH. The carboxylic acid was then activated with oxalyl chloride and further reacted with aqueous ammonia solution to give the primary amide 46. Subsequently, a Hofmann rearrangement with metallic sodium and NBS generated carbamate 47. Hydrolysis of the carbamate to the corresponding primary amine 48 was achieved by hydrolysis under basic conditions with NaOH. Finally, coupling of the amine 48 and the carboxylic acid 24 was achieved by Mukaiyama reagent and DIPEA, giving the target C-diazocine 49. In parallel, the azobenzene 50 was synthesized according to the protocol previously described in literature. This last intermediate 50 reacted with Mukaiyama reagent and DIPEA, yielding the target N-diazocine 51.

**Scheme 5. Synthesis of photo-C$_3$B$_3$R ligands as C-3-indole-coupled diazocines.**

- Reagents and conditions: (a) Cul, PdCl$_2$(PPh$_3$)$_2$, TMS-acetylene, TEA, THF, rt, overnight, quant; (b) K$_2$CO$_3$, MeOH, rt, 10 min, 75%; (c) methyl 4-iodo-3-nitrobenzoate, Cul, PdCl$_2$(PPh$_3$)$_2$, TMS-acetylene, TEA, THF, rt, 72 h, 72%; (d) H$_2$, Pd/C, MeOH, rt, 5 h, 97%; (e) mCPBA, AcOH, DCM, rt, slow addition over 12 h, 52-70%; (f) PPh$_3$, MoO$_3$(dmf)$_2$, THF, 70°C, 3 h; (g) NaOH, EtOH, 80°C, 2 h, 76%; (h) (COCl)$_2$, DMF, 0°C, 1 h; (i) DCM, NH$_2$OH, 65°C–rt, 30 min, 63%; (j) Na, NBS, MeOH, 65°C, 1.5 h, 64%; (k) NaOH, MeOH, 70°C, 12 h, quant; (l) 2-chloro-1-methylpyridinium iodide, DIPEA, DCM, rt, 4 h, 41-52%.

**Photophysical properties.** All the target compounds were photocharacterized by UV/vis spectroscopy. The isomerization capacity was tested by applying external light irradiation at different wavelengths to find the most suitable ones for achieving maximal conversion to their respective photo-
isomerized to their thermodynamically stable trans form (70 - 85%) isomer. When measured after irradiation with UV light (λ = 366 nm), the ratio of cis isomer increased considerably, becoming the predominant photo-form. Meanwhile, diazocines 49 and 51 were distributed almost entirely to their thermodynamically stable isomer Z (>90%). When measured after irradiation with light λ = 400 nm, the proportion of E-photo-isomer increased, however, in contrast to classical azobenzenes, it was only possible to obtain a mixture 1:1 of both photo-forms (c.f. Supporting Information, Figures S22-a and S23-a-b).

To evaluate a possible loss of photostationary behavior (photofatigue) after multiple cycles of trans→cis→trans isomerization, the target compounds were evaluated at the wavelength of maximum absorption for each case. The classical azobenzenes samples were prepared in a 50 µM DMSO solution for UV/Vis characterization, while the diazocines, due to their very low absorption, had to be prepared in higher concentrations (200 µM). The compound solutions were consecutively irradiated with wavelengths at which the maximum photoconversion was obtained for its two photoisomers (λ = 366 nm for cis and λ = 400 – 450 nm for trans). After 10 cycles, all target compounds were shown to be stable without significant changes in the absorption values (Figure 1b). Target compounds 49 and 51 did not show photofatigue either (Figure 2b), however, unlike the classic azobenzenes, both diazocines show a change in hue when they are photoconverted. In this way, in its thermodynamically stable isomer (Z), a methanolic solution of the final compound showed a pale-yellow color, however, when irradiated with UV light λ = 400 nm, the target compounds presented a pink or violet coloration in their E-photo-isomer. Additionally, when exposed to ambient light, or irradiated again with amber-red light, the diazocines switched back to their initial color. This process could be repeated for several cycles with no apparent loss of hue (Figure 2c).

As photoswitching and thermal relaxation is solvent dependent, we also evaluated the azobenzene based compounds in aqueous environment. Most of the compounds showed long thermal stability when a 50 µM (DMSO:buffer 4:1), pH = 7.4 solution photoisomerized to their thermodynamically less stable photo-form and stored into the dark (3h, 37°C) (Figure 1c and Supporting Information, Table S3), without significant photoconversion under the assay conditions. Additionally, the photostationary distribution of the compounds was quantified by HPLC. The analyses were performed at the isosbestic point of the UV spectrum for each substance, by testing a methanolic solution of the respective target compound. The final compounds containing a classical azobenzene moiety were distributed mainly towards their thermodynamically stable trans (70 - 85%) isomer. When measured after irradiation with UV light (λ = 366 nm), the ratio of cis isomer increased considerably, becoming the predominant photo-form. Meanwhile, diazocines 49 and 51 were distributed almost entirely to their thermodynamically stable isomer Z (>90%). When measured after irradiation with light λ = 400 nm, the proportion of E-photo-isomer increased, however, in contrast to classical azobenzenes, it was only possible to obtain a mixture 1:1 of both photo-forms (c.f. Supporting Information, Figures S22-a and S23-a-b).
Radioligand binding studies. After carrying out the photocharacterization of the target compounds, their affinity for CBRs was evaluated in radioligand binding assays. This assay was chosen as the first filter, because unlike optical assays in vitro, it can be performed without the need for any external light sources. This makes it easy to exclude possible false negatives due to back-isomerization of the ligands during the assay run or reading process. The radioligand binding studies are useful to check to which extent structural differences between each pair of photo-isomers translate into changes in the affinity to the receptor. A photoswitchable ligand with a marked difference in affinity can be switched from a low-activity state "switch-off" to a high-activity state "switch-on" or vice versa, by external light irradiation.

In the competition assays, most of the final compounds showed affinity for CB₁R in the 1-digit micromolar range (Table 1). Compounds with azobenzene attached to the indole by position N-1 showed low affinity for CB₁R. Specifically, compound 12b was unable to displace the reference CP55940 in the assays for both CB₁R and CB₂R. Similarly, compounds with a 3-carbon side chain attached to nitrogen showed a lower affinity for CB₁R compared to their 5-carbon chain counterparts. This goes along with the keto bridged compounds (3C-chain) 28b and 28c, who did not displace the radioligand in the assay. Additionally, when comparing the compounds of the 36b-e family with their 37b-e homologues, the compounds with a short side chain had lower affinity for the receptor, even without showing any binding in some cases (36c-trans, 36e-cis). This indicates that although short-chain agonist compounds with high affinity are found in the literature, nevertheless, for the compounds described in this project, the presence of a chain of at least 5 carbon atoms is preferable.

The best example of the above findings is observable when comparing analogs 27a (5 carbon atoms in the side chain) and 28a (3 carbon atoms in the side chain). Regarding the effect of aromatic substitution of the azobenzene-photoisomer, both ortho-substituted compounds showed higher affinity values for CB₁R compared to the respective meta (27b, 28b) and para (27c, 28c) substituted analogues. This could be explained based on data from the literature, where CB₁R indole-based agonists with unsubstituted benzyl position had lower potency and affinity for CB₁R compared to analogues with methyl or cumyl substitution. For this project, the distal azobenzene ring of the target 27a and 28a ortho-photo compounds could occupy a cavity in the vicinity of the carbonyl group when the compounds are in the cis form. However, meta- and para-analogs fall short of occupying this position upon isomerization. This theory is reinforced by analyzing the trans photoforms of 27a and 28a, which could not occupy this hydrophobic region necessary for activity, so they have a lower affinity for CB₁R compared to their 27a-cis and 28a-cis counterparts, respectively.

Similarly, both compounds 27a and 28a show higher affinity when compared to their derivatives with distally substituted azobenzene (37b-e and 36b-e, respectively), suggesting that the unsubstituted azobenzene ring might have less steric hindrance when binding to CB₁R. Despite these similarities between both analogs, compound 27a showed superior binding affinities for CB₁R in the sub-micromolar range (CB₁R Ki 27a-cis = 0.18 µM; CB₁R Ki 27a-trans = 0.97 µM) and the largest difference between both photo-isomers (trans/cis ratio = 5.4-fold) (Figure 3a), which reafirms the importance of a long side chain for this type of substances. Additionally, when performing binding studies in CB₁R, we found that photo-isomer 27a-cis has a 6.8-fold higher selectivity for CB₁R compared to CB₂R (CB₂R Ki 27a-cis = 1.22 µM).

When comparing the classic azobenzenes with the diazocines, it was found that this last class of compounds presents a moderate affinity in both isomers in the binding studies and fairly low differences between the photo-isomers. But in fact, the Z-photo-isomer, which is more stable in this case, was also the photo-form with the higher affinity, which demonstrates the feasibility of this approach.

Figure 2. Photochemical characterization of diazocines 49 (up) and 51 (down). a) Changes in the absorption spectrum upon irradiation with different wavelengths (Full Spectrum and Y-Axis Zoom). b) Absence of photofatigue after 10 switching cycles. c) Notorious color changes shown in their photostates (4 mM methanolic solution).
### Table 1. Affinity values at rCB1R and hCB2R determined in radioligand binding studies.

<table>
<thead>
<tr>
<th>Photoisomers</th>
<th>rCB1 pKi ± SEM(^a)</th>
<th>CB1 trans / cis ratio</th>
<th>hCB2 ([^{3}H] \text{CP55950}) displ. at 1 μM or hCB2 pKi ± SEM(^b)</th>
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<tbody>
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<td>CP55940</td>
<td>9.54 nM (8.02 ± 0.054)</td>
<td>-</td>
<td>13.7 nM (7.86 ± 0.071)</td>
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<tr>
<td>Rimonabant</td>
<td>62.9 nM (7.20 ± 0.07)</td>
<td>-</td>
<td>&lt; 10%</td>
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<tr>
<td>Reference compound</td>
<td><em>52</em></td>
<td>-</td>
<td>64.8 nM (7.19 ± 0.04)</td>
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<tr>
<td>12</td>
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<tr>
<td>12a R = H</td>
<td>cis 2.38 μM (5.62 ± 0.12)</td>
<td>3.8</td>
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<tr>
<td></td>
<td>trans 9.17 μM (5.04 ± 0.43)</td>
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<td>35%</td>
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<tr>
<td>12b R = CH(_3)</td>
<td>cis &lt;10%</td>
<td>-</td>
<td>12%</td>
</tr>
<tr>
<td></td>
<td>trans &lt;10%</td>
<td>-</td>
<td>23%</td>
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<td>cis 2.34 μM (5.63 ± 0.09)</td>
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<tr>
<td></td>
<td>trans 2.12 μM (5.67 ± 0.17)</td>
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<tr>
<td>19a R = H</td>
<td>cis 2.12 μM (5.67 ± 0.13)</td>
<td>1.6</td>
<td>64%</td>
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<tr>
<td></td>
<td>trans 3.32 μM (4.58 ±0.17)</td>
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<td>64%</td>
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<td>19c R = CH(_3)</td>
<td>cis 3.98 μM (5.40 ± 0.18)</td>
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<td>27</td>
<td>27a</td>
<td>27b</td>
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<td></td>
<td>ortho-substitution</td>
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<td>trans</td>
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<td>0.18 µM (6.75 ± 0.07)</td>
<td>9.7 µM (6.01 ± 0.08)</td>
<td>3.07 µM (5.51 ± 0.10)</td>
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<td>1.22 µM (5.99 ± 0.08)</td>
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<td>ortho-substitution</td>
<td>cis</td>
<td>trans</td>
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<td>4.97 µM (5.30 ± 0.25)</td>
<td>3.58 µM (5.45 ± 0.41)</td>
<td>11.20 µM (5.37 ± 0.25)</td>
</tr>
<tr>
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<td>14%</td>
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Cannabinoid receptor internalization. We next sought to determine efficacy using internalization of the receptors as a readout. The best compounds - according to the results of our radioligand binding assay - were evaluated regarding their effect on endocytosis of FLAG-epitope-tagged CB₁ and CB₂ receptors stably expressed in HEK293 cells, detected by fluorescently conjugated M1-anti-Flag antibody, and monitored by flow cytometry. Compounds were tested as agonists, expressed as percentage of internalization caused by CP55940, and as antagonists against 10 nM CP55940, in both the cis and trans forms. Irradiation with the respective wavelength was done for the dilution rows in DMSO before addition to the wells. Compound 27a behaved as an agonist for both photo-isomeric forms, with the cis-isomer showing 4.4-fold elevated potency and efficacy (PSS₃₆₅nm, EC₅₀ (CB₁))
This pronounced agonist profile of the cis isomer is consistent with the results previously obtained by radioligand binding assay. When analog 28a was tested, the compound did not show agonist or antagonist behavior in endocytosis at the CB₁R, whereas the parent compound 4 (EC₅₀ (CB₁) = 5.4 nM) proved to be an equally good agonist as CP55940 (EC₅₀ (CB₁) = 1.1 nM). Compound 27a was also tested against CB₂R to study selectivity. At 10 µM the amount of internalized CB₂ receptor was 53% of the maximum response caused by CP55940 (data not shown), proving that the compound acts as selective CB₁R agonist in the relevant concentration range.

Figure 3. Pharmacological evaluation of compound 27a. a) Radioligand binding assay: cis (purple, dotted line) and trans (blue, solid line) photo-isomers vs. rimonabant as control. Each data point and error bar represent the average of 3 independent experiments by triplicate ± standard error of the mean (SEM). b) Ligand-induced internalization in HEK293 cells. Data of compound 27a. Each data point and error bar represent the average of 3 independent experiments ± standard error of the mean (SEM).

GRABeCB₂.₀ sensor assay. To test the biological activity of the most active compound at the receptor level, we decided to directly measure the activity at CB₁R, using a genetically encoded fluorescent CB₁R sensor named GRABeCB₂.₀. For the biosensor design, a circularly-permuted green fluorescent protein (cpGFP) was inserted into a conformationally sensitive domain between transmembrane domains 5 and 6 of CB₁R. When testing an agonist substance capable of binding to the sensor protein, a strong increase in sensor fluorescence is generated, indicating receptor activation. For this project, an assay was designed in a 96-well plate using HEK293T cells with transient expression of GRABeCB₂.₀. Subsequently, the reference CB₁R agonist CP55940 was tested (Figure 4a), showing a concentration-dependent increase in fluorescence (EC₅₀: 64.8 nM), thus indicating the activation of GRABeCB₂.₀.

Figure 4. CB₁R-cpGFP sensor assay. a) Dose-dependent activation of GRABeCB₂.₀ by CP55940. b) Dose-dependent inhibition of 0.1 µM CP55940-induced GRABeCB₂.₀ response by photo-rimonabant: Fluorescence was measured at each well before and after addition of different concentrations of cis (purple, dotted line) or trans (blue, solid line) photo-rimonabant to calculate ΔF. c) Dose-dependent activation of GRABeCB₂.₀ by compound 27a: Fluorescence was measured at each well before and after addition of different concentrations of cis- or trans-27a to calculate ΔF. For all the curves each data point and error bar represent the average of 3 independent experiments (7 independent experiments for 4c) ± standard error of the mean (SEM).
Once the assay was validated with the reference agonist ligand, we wanted to study photoswitchable CB1/R ligands. To do so, we tested the previously described antagonist “photo-rimonabant” in this assay. Our group previously reported that photo-rimonabant is more active in its cis photo-form. For this assay, we observed that cis-photo-rimonabant inhibits the CP55940-mediated activation of GRABeCB2.0 with a concentration-dependent fashion. The cis-photo-isomer inhibited GRABeCB2.0 response with a 13-fold higher potency than trans-photo-rimonabant did (Figure 4b), exhibiting IC50 values in agreement with our last publication (IC50 cis: 47 nM, IC50 trans: 613 nM). With these data, we were able to verify the suitability of the GRABeCB2.0 assay for studying the pharmacology of photo-switchable CB1/R ligands.

Next, we studied the activity of 27a photo-isomers using the GRABeCB2.0 assay. In these experiments, both cis- and trans- photo-forms of 27a displayed a concentration-dependent activation of GRABeCB2.0, confirming their agonistic characters. The isomer 27a-cis displayed a higher maximal response (93% of response induced by 1µM CP55940) than 27a-trans (59.6%). Moreover, in its cis isomer, ligand 27a activated GRABeCB2.0 6-fold more potently than its trans isomer (EC50 cis: 2.1 µM, EC50 trans: 13.3 µM) (Figure 4c). The observed fold difference in EC50 was in accordance with the ones observed in the binding studies and internalization of the target compound. To validate the ligand-specific activation of the eCB2.0 sensor, we performed a negative control experiment in which we tested 27a on cells expressing an inactive mutant of the eCB2.0 sensor. In these experiments, neither the CB1/R agonist CP55940 nor the cis or trans isomers of 27a activated the sensor. These results indicate that the activation of eCB2.0 sensor by 27a is a ligand specific response, and not an artefact (data not shown).

hCB1/R calcium mobilization assay. To further characterize the target compounds, an intracellular fluorescent hCB1-activated Goq16-Coupled calcium mobilization assay was performed. This assay used CHO-K1 cells overexpressing hCB1/R as has been described in previous studies. The compounds were tested as both photo-isomers. For the photo-isomerization, a DMSO/buffer dilution (<1% DMSO) was irradiated with the respective wavelength prior to the experiment. CP55940 was used as CB1/R agonist reference. Although this technique in our laboratory has lower sensitivity for substances with affinity in the micromolar range, it was possible to evaluate compound 27a which was found to exert the desired agonism for hCB1. Although the determined half maximal effective concentration was lower than for the reference substance (CP55940, IC50 = 14.8 nM; 27a-cis, IC50 = 3.10 µM), it was confirmed that the photoswitchable compound has a higher efficacy in its cis-activated form (Figure 5). In its trans photo-isomer, compound 27a did only present an apparent effect at even higher concentrations. This can be explained by previous data from our radioligand binding assay, giving its affinity is in the micromolar range. All other synthesized compounds failed to show similar efficacies in calcium mobilization (data not shown).
Figure 6. ERK1/2 activation by CB1R ligands. Dose-dependent phosphorylation of ERK1/2 (pERK1/2) after CB1R stimulation by CP55940 and 27a both in the cis and trans photoform. The ERK1/2 phosphorylation was assessed after 15 minutes of stimulation by Western blot analyses using antibodies directed against the phosphorylated TEY motif (pERK1/2) and ERK1/2. Shown is a representative Western blot. The data represent the mean ± standard error of the mean (SEM) of 5 (cis and trans photoform of 27a) or 6 (CP55940) experiments. For statistical analysis ordinary one-way ANOVA and Šídák’s multiple comparison test as post hoc test was applied for the depicted stimulations with 27a in the cis and trans photoform and versus DMSO, and an unpaired two-sided Student's t-test was applied to the 1µM pERK1/2 signals to compare the efficacy of the target compound compared to the reference compound (1 µM 27a-cis vs. CP55940; of note the representative blot is cut, i.e. the 1mM band of 27a-cis is shown twice and the uncut Western blot is shown in Supporting Information, Figure S70).

NanoBiT® βarr2 recruitment assays. To further assess the CB1R activation potential of the photo-isomers of compound 27a, a NanoBiT® luciferase assay, based on the recruitment of the intracellular signaling protein βarr2 to the ligand-activated receptor, was performed. Prior to measurements, stock solutions were irradiated with the respective wavelengths and luminescence was monitored for 2 hours. Relative to the E_max of the reference standard CP55940, set at 100%, 27a-cis was found to have an efficacy of 363%, whereas the trans isomer had an E_max of 191% compared to the reference (Figure 7). Furthermore, 27a-cis had a 4-fold higher potency at CB1 than 27a-trans (EC_{50}cis: 221 nM, EC_{50}trans: 960 nM), confirming earlier findings obtained from the calcium assay, the GRABeCB2.0 sensor assay and the receptor internalization assay. At CB2R, both photo-isomers were found to be partial agonists compared to CP55940 with E_max values of 19.1% (27a-cis) and 26.0% (27a-trans) and potencies of 29.1 and 255 nM, respectively (c.f. Supporting Information, Figure S68).

To evaluate the reversibility of the photoswitching and the potential impact on the CB1R activity, stock solutions containing high concentrations of 27a were switched back and forth between cis and trans states and CB1R receptor activation by 27a in different phases of this cycle was measured (Figure 8).

In line with the previous experiments, the cis isomer (switched from the photostationary state to cis, switch 1) yielded the highest signals. Switching to trans (switch 2) resulted in a decrease in luminescence, which can be almost completely reversed by switching the compounds back to cis (switch 3), signifying that the higher activity of the cis isomer can be restored after earlier conversion to the trans isomer. After a fourth irradiation, switching 27a to trans, the luminescent signal is again reduced to a similar lower level, comparable to the signal after switch 2. Overall, this shows that the different photo-isomers retain their specific activity profile after being switched back and forth between the different cis and trans states (Figure 8).
Figure 8. Dynamic photomodulation of compound 27a at three different concentrations, demonstrated by β-arrestin2 recruitment to CB1R. Data (n=3) is given as the mean area under the curve (AUC) ± standard error of mean (SEM), normalized to the Emax of CP55940, set at 100%.

Molecular docking. To get insights into the possible binding modes of the two photo-isomers of 27a that could explain the differences in their binding behavior at the receptor, we used a docking routine previously optimized by us. Briefly, the ASP57 scoring function available within GOLD56 was found to consistently reproduce the binding pose of the CB1R agonist and delta-9-tetrahydrocannabinol (Δ9-THC) derivative, AM1154258 (PDB ID: 5XRA), with a top-ranked pose which differed only by 0.47 Å from the experimental pose (Figure 9a). Docking of the AM220131 derivative, reference 4, revealed a binding mode reminiscent of AM11542. The indole core and 5-fluoropentyl tail of compound 4 occupy the same hydrophobic pocket as the 8-bromo-2-methyloctan-2-yl tail of AM11542, which is defined by F170, F189, L193, V196 and F268 (Figures 9a and b). Moreover, the carbonyl group of reference 4 could form a hydrogen bond with S383, as experimentally observed for AM11542 (Figure 9b). Importantly, the cumyl group overlaps with the hydrophobic core of AM11542, adequately filling the pocket defined by F170, F174, F177 and F189 (Figure 9e). The overlapping interaction networks of compound 4 and AM11542 suggested by our docking experiments may account for the potent CB1R agonistic effect of cumyl derivatives reported in the literature.59 It must be noted that the docking pose of ligand 4 depicted in Figure 9b was ranked third best out of 50 poses, with an ASP score of 52.69. However, this pose was selected for analysis based on being in the same score range as the top ranked pose (53.84 ASP score) and its interaction network consistent with experimental data, as previously described. Additionally, rescoring with the DSX_CSD60 scoring function yielded a better score for the selected pose than for the top ranked ASP pose (c.f. Supporting Information, Table S1).

Docking of the two photo-isomers of compound 27a yielded ASP and DSX_CSD top scored poses where the indole core, carbonyl group and 5-fluoropentyl tail of the molecule occupy a similar space as in the docking result of reference 4 (Figure 9b, c and d). These features correspond to the common sub-structure shared by compounds 27a and 4 and may therefore establish a similar interaction network within the orthosteric site of the CB1R. However, our docking results suggest that the azobenzene photo-switchable group adopts a more compact conformation in the cis photo-isomer, occupying the same hydrophobic pocket as the cumyl group of reference 4 and the hydrophobic core of AM11542 (Figure 9e and f). In contrast, the distal phenyl ring of the photo-switchable group on the trans photo-isomer is projected beyond the experimentally observed orthosteric site of AM11542, where it may not be well packed (Figure 9g). We hypothesize that the more compact conformation of the cis photo-isomer has a better spatial arrangement in the hydrophobic cavity within the CB1R orthosteric site. Thus, this steric arrangement may contribute to the experimentally observed 5.4-fold affinity ratio of the cis photo-isomer over the trans photo-isomer. Although the trans conformation has a slightly better ASP score than the cis conformation (68.78 vs 67.78), the scores are still within the same range as the scoring function is likely not sensitive enough to discriminate between poses with only a 5.4-fold affinity difference.
A comparison of target compounds 27a and 28a was made to analyze the difference in affinity for CB1R in relation to the length of the aliphatic side chain. Docking of the cis form of the 3-fluoropropyl derivative 28a produced a top-scored docking pose where most structural features of the molecule (core, photoswitchable group and aliphatic chain) overlap with the top-scored docking pose of the cis form of the 5-fluoropentyl derivative 27a (Figure 10a). After normalizing the ASP scores by the cubic root of the number of heavy atoms on each molecule to compensate for the bigger size of molecule 27a, the cubic root scores (CRS) for the 5 best ranked poses of compound 27a were superior to the 5 best ranked poses of compound 28a (Supporting Information, Table S2). This is consistent with the better binding affinity experimentally observed for compound 27a over 28a.

Our docking results suggest that the longer aliphatic chain of target 27a more adequately fills the side hydrophobic pocket where the tail of AM11542 is experimentally bound (Figure 10b), which may help explain the superior affinity of compound 27a over 28a. This observation is consistent with the experimental behavior of phytocannabinoids reported in the literature, where longer aliphatic chains such as in THCP (heptyl chain) and THC (pentyl chain), lead to a higher affinity value towards CB1R than shorter aliphatic chains such as in THCB (butyl chain) and THCV (propyl chain).

CONCLUSIONS

By developing different synthetic pathways, we successfully synthesized 21 new target molecules as CB1R agonists. These molecules were designed from an indole core with substitutions at N-1 and C-3 by adding an azobenzene moiety. All obtained compounds could be easily photosomerized by external light sources. This photoconversion can be repeated for many cycles without changes in absorption spectra of the synthesized compounds. Additionally, most of the compounds show good stability of their thermodynam-
ically less stable photo-isomers after irradiation and environmental light exclusion, demonstrating good thermal stability for a period of 3 hours at 37°C.

The pharmacological characterization showed that the compounds substituted with the azobenzene moiety at C-3 have a higher affinity for CB1R compared to those substituted at N-1. Similarly, compounds with amide bridges, aromatic substitution of azobenzene in the ortho position, and without additional substituents on the distal benzene ring of azobenzene are preferred. The fluorinated side chain in N-1 should have a length of five or more carbon atoms to enhance the potency of the compound. When evaluating all target compounds by several in vitro assays, it was concluded that target compound 27a, which presents all the previously listed structural characteristics, is the one with the best pharmacological properties.

Compound 27a shows affinity for CB1R in the sub-micromolar range with a pronounced difference in affinity between both photo-isomers. The 27a-cis isomer has a 5.4-fold higher affinity (KICB1R = 0.18 μM) than the 27a-trans isomer (KICB1R = 0.97 μM). The agonistic activity of compound 27a was verified in five different biological assays: receptor internalization, calcium mobilization, GRABeCB2.0 sensor assay, Western Blots for analysis of ERK1/2 activation, and as well as in NanoBIT® Purr2 recruitment assays. In all biological assays, the pharmacological profile was similar to the one initially obtained in competition binding, showing that the 27a-cis isomer activates CB1R with higher potency and efficacy than the 27a-trans photoform. This high reproducibility throughout diverse biological assays thus demonstrates the robustness of the obtained results. Additionally, we verified that compound 27a retain its specific activity profile after several cycles of cis-trans-cis irradiation, allowing optical control of their pharmacological profile. This suggests that these novel compounds have a clear advantage over traditional non-switchable ligands. It should also be mentioned that the affinity difference at the receptor level seems quite low, but numerous photopharmacological tool compounds have shown much more pronounced effects in assays investigating subsequent steps in the activation cascade.59, 60, 61, 62, 63, 64, 65, 66, 67

The pharmacological properties mentioned above can be explained by molecular docking, whose results allow us to hypothesize that azobenzene substituted in ortho of the 27a-cis isomer adopts a more compact conformation that can better fill the hydrophobic cavity at the orthosteric site of CB1R. Meanwhile, when isomerized to the 27a-trans photoform, the photo-switchable group is projected to a region where it is not adequately packed, resulting in a lower affinity towards CB1R.

Our results show that agonist 27a shows a "cis-on" activation, with high affinity for hCB1R. These properties constitute this novel compound as a new molecular tool to obtain more profound insights in GPCR pharmacology through optical modulation of hCB1R, which could allow a better understanding of the disorders associated with the endocannabinoid system in the future.

METHODS

Chemistry. General Methods. We acquired both the solvents and commonly used reagents from different commercial suppliers and they were used without further purification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under an argon atmosphere. The monitoring of the reactions was performed with thin layer chromatography (TLC) on silica gel 60 on alumina foils with fluorescent indicator, and spots were detected with UV light (254 nm). All the melting points were determined with a Stuart melting point SMP3 apparatus (Bibby Sterlin Ltd, Staffordshire, UK). Preparative TLC purification (prep-TLC) was done in house by preparing glass surface chromatographic plates (20 x 20 cm) with silica gel 60G254 (Merck). Silica gel 60, 230–400 mesh (Merck) was used for purification by column chromatography, Nuclear magnetic resonance spectra were recorded with a Bruker AV-400 NMR instrument (Bruker, Karlsruhe, Germany) in deuterated solvents and chemical shifts were expressed in ppm (DMSO: 1H - 2.50 ppm, 13C - 39.52 ppm; CDCl3: 1H - 7.26 ppm, 13C - 77.16 ppm; MeOD: 1H - 4.87 ppm, 13C - 49.0 ppm; for acetone-D6: 1H - 2.05 ppm, 13C - 203.6 ppm). To monitor the purity of the products by HPLC, a Shimadzu kit, equipped with a DGU-20A3 degassing unit, a LC20AB liquid chromatograph and an SPD-20A UV / Vis detector, were used. Also, we used an LCMS 2020, mass spectra. The stationary phase was a Synergi 4 μm fusion-RP (150 x 4.6 mm) column, and a MeOH / water gradient with 0.1% formic acid was used as the mobile phase (parameters: A: water, B: MeOH, V(A)/(V(A) + V(B)) = from 5 → 90% over 10 min, V(B)/(V(A) + V(B)) = 90% for 5 min, V(B)/(V(A) + V(B)) = from 90 → 5% over 3 min. The method was performed with a flow rate of 1.0mL/min and scan range of 60-1000 m/z). All target compounds were accepted if a purity of ≥ 95% was achieved. The same purity criteria was applied for testing the compounds in vitro. UV / Vis spectra experiments were made on a Varian Cary 50 Bio UV / Vis spectrophotometer using Hellma (Type 100-QS) cuvettes (10 mm light path).

General procedure I for Azo-Coupling under Acidic Conditions (28a, 36b-c, 37b-e). The respective amine (1 eq) and the nitroso compound (1.4 to 6 eq) were dissolved in AcOH. The mixture was stirred at room temperature overnight. After reaction, the solvent was removed in vacuo. The raw material was dissolved in DCM and washed with sat. aq. NaHCO3. The organic layers were dried over Na2SO4 and concentrated. The residue was purified by flash chromatography with isocratic gradient (petroleum ether: AcOEt; 95:5→5:95) to afford the title compound.

3-(Naphthalene-1-carbonyl)-1-(4-[(1E)-2-phenylidiazol-1-yl]phenylmethyl)-1H-indole (12a). Compound 9 (30 mg, 0.08 mmol, 1 eq) was dissolved in a mixture of AcOH / TFA / toluene (6/1/6). To this mixture, nitrosoazobenzene (17 mg, 0.16mmol, 2 eq) was added. The reaction was stirred at room temperature overnight. The mixture was diluted with ethyl acetate and washed with water. The organic layer was separated, dried over Na2SO4 and concentrated. The residue was purified by column chromatography (DCM/MeOH/NH4 = 120/1/0.5) to achieve the title compound (12a) as an orange solid. (19%, 18 mg). m.p. 190°C. H NMR (400 MHz, CDCl3); δ = 8.30 (dd, J = 14.5, 7.9 Hz, 1H), 8.02 (d, J = 8.2 Hz, 1H), 7.75 (d, J = 8.2 Hz, 1H), 7.32 – 7.65
(m, 4H), 7.63 (d, J = 8.3 Hz, 1H), 7.48 (d, J = 6.7 Hz, 1H), 7.33 – 7.28 (m, 3H), 7.28 (s, 2H), 7.17 (ddd, J = 7.9, 5.4, 2.4 Hz, 2H), 7.12 – 7.09 (m, 2H), 7.07 – 6.98 (m, 2H), 6.55 (d, J = 8.4 Hz, 1H), 5.07 (d, J = 60.2 Hz, 2H). 13C NMR (101 MHz, CDCl3): δ = 192.1 (1C), 152.5 (1C), 152.4 (1C), 138.9 (1C), 138.4 (1C), 138.2 (1C), 137.2 (1C), 133.8 (1C), 131.3 (1C), 130.8 (1C), 130.2 (1C), 129.1 (2C), 128.2 (2C), 127.4 (1C), 127.2 (1C), 126.8 (1C), 126.4 (1C), 126.0 (1C), 125.9 (1C), 124.6 (1C), 124.0 (1C), 123.4 (2C), 123.2 (1C), 123.0 (1C), 122.9 (2C), 118.3 (1C), 110.4 (1C), 50.6 (1C) ppm. LC: tx (min) = 10.53 (cis) + 11.60 (trans), purity = 97.4%. MS: [M+H]+ calc. for [C13H12N2O]+: 466.56, found 466.20. High-Resolution Mass: m/z calcd for C32H26N2O2, 466.1914; found, 466.1911.

1-(4-[(1E)-2-(2-methylphenyl)diazene-1-yl]phenylmethyl)-3-(naphthalene-1-carbonyl)indole (12b). Compound 9 (40 mg, 0.11 mmol, 1 eq.) was dissolved in a mixture of AcOH/TFE / toluene (6/1/6). To this mixture, nitrosothiolue (78 mg, 0.64 mmol, 6 eq.) was added. The reaction was stirred at room temperature overnight. The mixture was diluted with ethyl acetate and washed with water. The organic layer was separated, dried over Na2SO4 and concentrated. The residue was purified by column chromatography (DCM/Methanol/NH4OH = 99/1/0.5) to achieve the title compound (12b), as an orange solid. (26%, 13.2 mg, m.p. 167°C. 1H NMR (400 MHz, CDCl3): δ = 8.58 – 8.48 (m, 1H), 8.03 (d, J = 8.4 Hz, 2H), 8.00 – 7.95 (m, 4H), 7.61 (s, 1H), 7.58 – 7.49 (m, 3H), 7.44 – 7.33 (m, 3H), 4.43 (dt, J = 47.2, 5.8 Hz, 2H), 2.40 (t, J = 7.1 Hz, 2H), 1.99 – 1.91 (m, 2H), 1.72 (ddt, J = 13.7, 11.8, 5.9 Hz, 2H), 1.53 – 1.44 (m, 2H). 13C NMR (101 MHz, CDCl3): δ = 190.5 (1C), 154.6 (1C), 153.1 (1C), 143.1 (1C), 137.3 (1C), 132.0 (1C), 129.6 (2C), 127.8 (1C), 124.2 (1C), 123.5 (2C), 123.4 (1C), 123.3 (1C), 123.2 (1C), 116.2 (1C), 110.3 (1C), 84.0 (1C), 74.6 (1C), 30.2 (1C), 30.0 (1C), 23.3 (1C). LC: tx (min) = 10.18 (cis) + 11.07 (trans), purity = 98.7%. MS: m/z [M+H]+ calc. for [C26H20F2N2O]+ = 414.20, found 414.15. High-Resolution Mass: m/z calcd for C32H26N2O2, 414.1976; found, 414.1976.

(E)-(1-(5-fluorophenyl)-1H-indol-3-yl)(3-(phenyl diazenyl)phenyl)methaneone (18). Compound 17a (89 mg, 0.27 mmol, 1eq) was dissolved in AcOH: TFA (6:1). Then, nitrosobenzene 11a (89 mg, 0.82 mmol, 3 eq.) was added. The reaction mixture was stirred at 60°C for 4h. After completing the reaction, the mixture was diluted with EtOAc and washed with water and brine. The organic phases were collected and dried over Na2SO4. Lately, the product was purified by column chromatography (petroleum ether: EtOAc / 5:1) to afford the target compound (19a), as an orange solid. (26%, 0.31 mmol, 6 eq.) was dissolved in AcOH: toluene : TFA (6:6:1). Then, nitrosobenzene 11c was added (184 mg, 1.8 mmol, eq.) to the reaction mixture at 60°C and monitored by TLC until the end of reagents. After the reaction was completed, the mixture was diluted with ethyl acetate and washed with water and brine. The organic phases were collected and dried over Na2SO4. Lately, the product was purified by column chromatography (petroleum ether: EtOAc / 5:1) to afford the target compound (19c), as orange solid (29 mg, 22%). 1H NMR (400 MHz, CDCl3): δ = 8.47 – 8.29 (m, 1H), 7.98 (q, J = 8.5 Hz, 4H), 7.88 (d, J = 8.2 Hz, 2H), 7.60 (s, 1H), 7.44 – 7.30 (m, 5H), 4.43 (dt, J = 47.3, 5.8 Hz, 2H), 4.22 – 4.16 (m, 2H), 2.46 (s, 3H), 1.95 (dt, J = 15.0, 7.3 Hz, 2H), 1.81 – 1.64 (m, 2H), 1.54 – 1.42 (m, 2H). 13C NMR (101 MHz, CDCl3): δ = 190.2 (1C), 154.3 (1C), 150.9 (1C), 142.5 (1C), 142.4 (1C), 137.0 (2C), 130.0 (2C), 129.8 (2C), 127.4 (1C), 123.9 (1C), 123.2 (2C), 123.0 (1C), 122.9 (1C), 122.7 (2C), 115.9 (1C), 110.0 (1C), 83.7 (1C), 47.2 (1C), 30.0 (1C), 29.7 (1C), 23.0 (1C), 21.7 (1C). LC: tx (min) = 10.37 (cis) + 11.23 (trans), purity = 98.9%. MS: m/z [M+H]+ calc. for [C27H26F2N2O]+ = 428.53, found 428.20. High-Resolution Mass: m/z calcd for C27H26F2N2O, 427.2054; found, 427.2057.

(E)-(1-(5-fluorophenyl)-N-(2-phenyl diazenyl)benzyl)-1H-indole-3-carboxamide (27a). To a suspension of NaH (10.54 mg, 0.25 mmol, 3eq) in DMF was added compound 26a (29 mg, 0.061 mmol, 1eq). After stirring at room temperature for 30 min, 1-bromo-5-fluoropentane (33 µL, 0.25 mmol, 3eq) was added dropwise. The resulting mixture was heated at 65°C for 3h. After reaction, the mixture was diluted with ethyl acetate and washed with water and brine.
The organic layer was dried over Na₂SO₄ and concentrated in vacuo. The crude product was purified by column chromatography using (petroleum ether: ethyl acetate, 3:1) to afford the target compound (27a), as orange solid. (15 mg, 41%). m.p.163 °C. 1H NMR (400 MHz, CDCl₃): δ = 7.99 – 7.95 (m, 2H), 7.80 – 7.72 (m, 2H), 7.67 (d, J = 6.2 Hz, 2H), 7.57 – 7.51 (m, 3H), 7.49 – 7.37 (m, 2H), 7.31 (dt, J = 8.3, 0.9 Hz, 1H), 7.21 – 7.13 (m, 1H), 7.00 – 6.89 (m, 1H), 6.69 (t, J = 6.2 Hz, 1H), 5.19 (d, J = 6.0 Hz, 2H), 4.44 (t, J = 6.0 Hz, 1H), 4.32 (t, J = 5.9 Hz, 1H), 4.09 (t, J = 7.1 Hz, 2H), 1.92 – 1.82 (m, 2H), 1.73 – 1.62 (m, 2H), 1.44 – 1.37 (m, 2H). 13C NMR (101 MHz, CDCl₃): δ = 165.3 (1C), 153.5 (1C), 150.8 (1C), 138.4 (1C), 136.9 (1C), 132.1 (1C), 132.1 (1C), 131.8 (1C), 130.8 (1C), 129.4 (1C), 128.7 (1C), 128.5 (1C), 125.3 (1C), 122.8 (1C), 121.7 (1C), 120.5 (1C), 116.7 (1C), 111.4 (1C), 110.6 (1C), 84.1 (1C), 47.1 (1C), 40.8 (1C), 30.4 (1C), 30.0 (1C), 23.2 (1C). LC tR (min) = 9.87 (cis) + 10.68 (trans), purity = 99.1%. MS: m/z [M+H]+ calc. for (C₁₇H₁₂FN₃O)⁺ = 443.22, found 443.20.

High-Resolution Mass: m/z calc for C₁₇H₁₂FN₃O₄ 443.2242; found 443.2237.

(E)-1-(3-fluoropropyl)-N-(2-(phenyl diazenyl)benzyl)-1H-indole-3-carboxamide (28a). Protocol I: To a suspension of NaH (11.37 mg, 0.27 mmol, 3eq) in DMF was added compound 26a (33 mg, 0.09 mmol, 1eq). After stirring at room temperature for 30 min, 3-fluoropropyl 4-methylbenzenesulfonate (63 mg, 0.27 mmol, 3eq) was added dropwise. The resulting mixture was heated at 65°C for 3h. After reaction, the mixture was diluted with ethyl acetate and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated in vacuo. Protocol II: The reaction was carried out according to general procedure 1, using compound 34 (50 mg, 0.16 mmol, 1eq) and nitrosobenzene (24 mg, 0.22 mmol, 1.4eq). The crude product was purified to afford the title product (28a) as an orange solid. (47 mg, 71%). m.p. 151 °C. 1H NMR (400 MHz, CDCl₃): δ = 7.99 – 7.93 (m, 2H), 7.80 – 7.74 (m, 2H), 7.71 (s, 1H), 7.66 (dd, J = 7.4, 1.5 Hz, 1H), 7.57 – 7.50 (m, 3H), 7.47 (td, J = 7.4, 1.4 Hz, 1H), 7.40 (td, J = 7.6, 1.6 Hz, 1H), 7.34 (d, J = 8.3 Hz, 1H), 7.20 (dd, J = 8.3, 7.1 Hz, 1H), 6.98 (td, J = 7.5, 1.0 Hz, 1H), 6.82 (s, 1H), 5.18 (s, 2H), 4.41 (t, J = 5.5 Hz, 1H), 4.27 (dt, J = 13.5, 6.1 Hz, 3H), 2.23 – 2.08 (m, 2H). 13C NMR (101 MHz, CDCl₃): δ = 164.9 (1C), 153.0 (1C), 150.5 (1C), 137.9 (1C), 136.7 (1C), 132.0 (1C), 131.8 (1C), 131.5 (1C), 130.6 (1C), 129.4 (2C), 128.5 (1C), 125.3 (1C), 123.2 (1C), 122.8 (1C), 121.6 (1C), 120.3 (1C), 116.4 (1C), 111.3 (1C), 110.2 (1C), 80.4 (1C), 42.5 (1C), 40.5 (1C), 30.9 (1C). LC tR (min) = 9.51 (cis) + 10.43 (trans), purity = 97.6%. MS: m/z [M+H]+ calc. for (C₂₃H₁₈FN₃O)⁺ = 415.19, found 415.20.

High-Resolution Mass: m/z calc for C₂₃H₁₈FN₃O 415.1748; found, 437.1749.

(E)-1-(3-fluoropropyl)-N-(3-(phenyl diazenyl)benzyl)-1H-indole-3-carboxamide (28b). To a suspension of NaH (52.7 mg, 1.25 mmol, 3eq) in DMF was added compound 26b (150mg, 0.42 mmol, 1eq). After stirring at room temperature for 30 min, 3-fluoropropyl 4-methylbenzenesulfonate (290 mg, 1.25 mmol, 3eq) was added dropwise. The resulting mixture was heated at 65°C for 3h. After reaction, the mixture was diluted with ethyl acetate and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (water: methanol, 95:5 → 95:5) to afford the title product (28b) as an orange solid. (33 mg, 19%), m.p. 141 °C. 1H NMR (400 MHz, CDCl₃): δ = 8.00 (d, J = 7.6 Hz, 1H), 7.92 (dd, J = 9.7, 8.5 Hz, 3H), 7.85 (d, J = 7.4 Hz, 1H), 7.75 (s, 1H), 7.57 – 7.44 (m, 5H), 7.40 (d, J = 8.1 Hz, 1H), 7.32 – 7.23 (m, 2H), 6.38 (s, 1H), 4.83 (d, J = 5.8 Hz, 2H), 4.48 (t, J = 5.4 Hz, 1H), 4.37 – 4.28 (m, 3H), 2.28 – 2.13 (m, 2H). 13CNMR (101 MHz, CDCl₃): δ = 165.2 (1C), 153.1 (1C), 152.8 (1C), 140.2 (1C), 136.7 (1C), 131.5 (1C), 131.2 (1C), 130.5 (1C).
The reaction was carried out according to general procedure I, using compound 34 (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq). The crude product was purified to afford the title product (28c) as an orange solid. (25 mg, 29%). m.p. 120 °C. 1H NMR (400 MHz, CDCl3): δ = 7.97 (d, J = 7.3 Hz, 1H), 7.91 (d, J = 8.1 Hz, 4H), 7.76 (s, 1H), 7.58 – 7.44 (m, 5H), 7.41 (d, J = 7.8 Hz, 1H), 7.33 – 7.23 (m, 2H), 6.33 (s, 1H), 4.79 (s, 1H), 4.47 (d, J = 5.5 Hz, 1H), 3.48 – 3.40 (m, 30H), 2.31 – 2.22 (m, 1H), 2.18 (dd, J = 11.9, 6.1 Hz, 1H). 13C NMR (101 MHz, CDCl3): δ = 165.2 (1C), 152.8 (1C), 152.2 (1C), 142.1 (1C), 136.7 (1C), 131.6 (1C), 131.2 (1C), 129.2 (2C), 128.6 (2C), 125.6 (1C), 123.4 (2C), 123.0 (3C). 121.9 (1C), 120.5 (1C), 111.2 (1C), 110.3 (1C), 80.4 (1C), 43.4 (1C), 42.7 (1C), 30.9 (1C). LC: ts (min) = 9.43 (cis) + 10.38 (trans), purity = 98.5%. MS: m/z [M+H]⁺ calc. for (C23H20F3NO)⁺ = 415.19, found 415.20. High-Resolution Mass: m/z calc'd for C23H19F2NO, 415.1929; found, 415.1936.

(E)-1-(3-fluoropropyl)-N-(2-(2-tolyldiazenyl)benzyl)-1H-indole-3-carboxamide (36b). The reaction was carried out according to general procedure I, using compound 34 (50 mg, 0.16 mmol, 1 eq) and 2-nitrosoaniline (24 mg, 0.22 mmol, 1.4 eq). The crude product was purified to afford the title product (36b) as an orange solid. (47 mg, 71%). m.p. 131 °C. 1H NMR (400 MHz, CDCl3): δ = 7.81 – 7.72 (m, 2H), 7.70 (s, 1H), 7.65 (dd, J = 7.5, 1.6 Hz, 1H), 7.47 – 7.37 (m, 2H), 7.34 (dd, J = 8.3, 3.4 Hz, 3H), 7.21 (ddd, J = 8.3, 7.0, 1.1 Hz, 1H), 7.02 – 6.97 (m, 1H), 6.79 (s, 1H), 5.16 (s, 2H), 4.41 (t, J = 5.5 Hz, 1H). LC: ts (min) = 7.74 (cis) + 10.67 (trans), purity = 98%. MS: m/z [M+H]⁺ calc. for (C23H22FN2O)⁺ = 429.21, found 429.15. High-Resolution Mass: m/z calc'd for C23H22FN2O, 429.1524; found, 429.1526.

(E)-1-(3-fluoropropyl)-N-(2-(2-(tolyldiazenyl)benzyl)-1H-indole-3-carboxamide (36e). The reaction was carried out according to general procedure I, using compound 34 (34 mg, 0.1 mmol, 1 eq) and nitroso compound 11e (0.6 mmol, 6 eq). The crude product was purified to afford the title product (36e) as an orange solid. (28 mg, 73%). m.p. 132 °C. 1H NMR (400 MHz, CDCl3): δ = 7.87 (d, J = 8.3 Hz, 2H), 7.81 – 7.72 (m, 2H), 7.70 (s, 1H), 7.65 (dd, J = 7.5, 1.6 Hz, 1H), 7.47 – 7.37 (m, 2H), 7.34 (dd, J = 8.3, 3.4 Hz, 3H), 7.21 (ddd, J = 8.3, 7.0, 1.1 Hz, 1H), 7.02 – 6.97 (m, 1H), 6.79 (s, 1H), 5.16 (s, 2H), 4.41 (t, J = 5.5 Hz, 1H). LC: ts (min) = 10.00 (cis) + 10.86 (trans), purity = 97.7%. MS: m/z [M+H]⁺ calc. for (C23H22FN2O)⁺ = 443.22, found 443.22. High-Resolution Mass: m/z calc'd for C23H22FN2O, 443.2242; found, 443.2239.

(E)-1-(5-fluoropropyl)-N-(2-(2-tolyldiazenyl)benzyl)-1H-indole-3-carboxamide (37b). The reaction was carried out according to general procedure I, using compound 35 (51 mg, 0.14 mmol, 1 eq) and 2-nitrosoaniline (24 mg, 0.22 mmol, 1.4 eq). The crude product was purified to afford the title product (37b) as an orange solid. (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq). The crude product was purified to afford the title product (37b) as an orange solid. (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq). The crude product was purified to afford the title product (37b) as an orange solid. (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq). The crude product was purified to afford the title product (37b) as an orange solid. (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq). The crude product was purified to afford the title product (37b) as an orange solid. (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq). The crude product was purified to afford the title product (37b) as an orange solid. (59 mg, 0.19 mmol, 1 eq) and nitroso compound 11d (1.12 mmol, 6 eq).
mmol, 1.4 eq). The crude product was purified to afford the title product (37b) as an orange solid. (7 mg, 11%). m.p 162 ºC. 1H NMR (400 MHz, CDCl3): δ = 7.77 – 7.71 (m, 2H), 7.69 – 7.63 (m, 2H), 7.46 (td, J = 7.4, 1.4 Hz, 1H), 7.42 – 7.38 (m, 2H), 7.33 – 7.28 (m, 1H), 7.22 – 7.14 (m, 1H), 6.95 (t, J = 7.6 Hz, 1H), 6.76 (s, 1H), 5.20 (d, J = 6.1 Hz, 2H), 4.44 (t, J = 5.8 Hz, 1H), 4.32 (t, J = 5.8 Hz, 1H), 4.16 – 4.05 (m, 2H), 2.76 (s, 3H), 1.86 (p, J = 7.6 Hz, 2H), 1.75 – 1.59 (m, 4H), 1.46 – 1.35 (m, 3H). 13C NMR (101 MHz, CDCl3): δ = 165.3 (1C), 151.4 (1C), 151.1 (1C), 139.1 (1C), 138.5 (1C), 136.9 (1C), 132.2 (1C), 132.0 (1C), 131.9 (1C), 131.8 (1C), 128.7 (1C), 127.0 (1C), 125.6 (1C), 122.8 (1C), 121.7 (1C), 120.5 (1C), 116.6 (1C), 116.0 (1C), 111.5 (1C), 110.6 (1C), 84.1 (1C), 47.1 (1C), 40.9 (1C), 30.3 (1C), 30.0 (1C), 23.2 (1C), 18.1 (1C). t9/min = 9.96 (cis) + 10.82 (trans), purity = 96.8%. MS: m/z [M+H]+ calc. for (C26H35N3O) = 457.24, found 457.20. High-Resolution Mass: m/z calcd for C26H35N3O = 479.2218; found, 479.2218.

(E)-1-(5-fluoropyrrol-N)-N-(2-p-tolyl diazenyl) benzyl)-1H-indole-3-carboxamide (37c). The reaction was carried out according to general procedure I, using compound 35 (34 mg, 0.11 mmol, 1 eq) and nitroso compound 11c (0.6 mmol, 6 eq). The crude product was purified to afford the title product (37c) as an orange solid. (20 mg, 44%). m.p. 132 ºC. 1H NMR (400 MHz, CDCl3): δ = 7.93 – 7.77 (m, 2H), 7.68 (dt, J = 8.1, 2.6 Hz, 2H), 7.61 – 7.46 (m, 2H), 7.40 – 7.28 (m, 2H), 7.25 (t, J = 7.9 Hz, 3H), 7.14 – 7.06 (m, 1H), 6.96 – 6.85 (m, 1H), 6.65 (s, 1H), 5.10 (d, J = 6.0 Hz, 2H), 4.36 (t, J = 5.9 Hz, 1H), 4.25 (t, J = 5.8 Hz, 1H), 4.10 – 3.97 (m, 2H), 2.38 (s, 3H), 1.77 (p, J = 7.4 Hz, 2H), 1.70 – 1.51 (m, 4H), 1.32 (dd, J = 12.8, 10.5, 6.2 Hz, 2H). 13C NMR (101 MHz, CDCl3): δ = 186.4 (1C), 151.0 (1C), 150.4 (1C), 142.0 (1C), 137.6 (1C), 136.5 (1C), 136.5 (1C), 131.3 (1C), 130.4 (1C), 129.9 (2C), 128.3 (1C), 124.2 (1C), 123.1 (1C), 122.3 (1C), 121.2 (1C), 120.0 (1C), 116.2 (1C), 110.7 (1C), 84.2 (1C), 46.1 (1C), 40.4 (1C), 29.9 (1C), 29.6 (1C), 22.8 (1C), 21.6 (1C). t9/min = 9.92 (cis)+ 10.75 (trans), purity = 95.0%. MS: m/z [M+H]+ calc. for (C26H35N3O) = 457.24, found 457.20. High-Resolution Mass: m/z calcd for C26H35N3O = 479.2398; found, 457.2392.

(Z)-N-(11,12-dihydroidbenzoc[fg][1,2]diazocin-3-yl)-1-(5-fluoropyrrol-N)-1H-indole-3-carboxamide (49). To a mixture of amine 48 (22 mg, 0.1 mmol, 1eq), carboxylic acid 24 (34 mg, 0.14 mmol, 1.4 eq) and 2-chloro-1-methylpyridinium iodide (38 mg, 0.14 mmol, 1.4 eq) in DCM at 0 ºC under argon atmosphere, was added DIPEA (54 µl, 0.3 mmol, 3 eq). The reaction mixture was allowed to warm to r.t. and stirred. After 4-24h (check by TLC), water was added, and the layers were separated. The aqueous layer was extracted with DCM. The combined organic layers were dried over Na2SO4 and concentrated in vacuo. The crude product was purified by flash chromatography with isocratic gradient (hexane: AcOEt = 100:0–100) to afford the title compound (49) as an orange solid. (18 mg, 41%). m.p. 186 ºC. 1H NMR (400 MHz, CDCl3): δ = 8.01 – 7.96 (m, 1H), 7.73 (s, 1H), 7.68 (s, 1H), 7.39 (dt, J = 8.3, 2.2 Hz, 2H), 7.33 – 7.27 (m, 2H), 7.17 – 7.11 (m, 2H), 7.06 – 6.99 (m, 2H), 6.97 (d, J = 8.3 Hz, 1H), 6.86 (dd, J = 7.7, 1.3 Hz, 1H), 4.47 (t, J = 5.8 Hz, 1H), 4.36 (t, J = 5.9 Hz, 1H), 4.17 (t, J = 7.1 Hz, 2H), 3.02 – 2.94 (m, 2H), 2.80 – 2.72 (m, 2H), 1.92 (p, J = 7.2 Hz, 2H), 1.79 – 1.62 (m, 2H), 1.49 – 1.40 (m, 2H). 13C NMR (101 MHz, CDCl3): δ = 163.1 (1C), 155.9 (1C), 155.6 (1C), 137.3 (1C), 136.8 (1C), 131.7 (1C), 130.5 (1C), 129.8 (1C), 128.7 (1C), 127.3 (1C), 126.9 (1C), 125.5 (1C), 123.6 (1C), 123.0 (1C), 122.0 (1C), 120.3 (1C), 119.0 (1C), 118.6 (1C), 111.1 (1C), 110.5 (1C), 110.0 (1C), 83.8 (1C), 46.9 (1C), 31.8 (1C), 31.4 (1C), 29.9 (1C), 29.7 (1C), 23.0 (1C). t9/min = 9.93 (Z) + 10.28 (E), purity = 99.2%. MS: m/z [M+H]+ calc. for (C26H35N3O) = 455.22, found 455.20. High-Resolution Mass: m/z calcd for C26H35N3O = 455.2242; found, 455.2254.
iodide (28 mg, 0.11 mmol, 1.2 eq) in DCM at 0 °C under argon atmosphere, was added DIPEA (52 µl, 0.29 mmol, 3 eq). The reaction mixture was allowed to warm to r.t. and stirred. After 4-24 h (check by TLC), water was added, and the layers were separate. The aqueous layer was extracted with DCM. The combined organic layers were dried over Na2SO4 and concentrated in vacuo. The crude was purified by flash chromatography with isocratic gradient (hexane: AcOEt; 100:0→0:100) to afford the title compound (49) as an orange solid. (22 mg, 52 %), m.p. 149 °C. 1H NMR (400 MHz, CDCl3): δ = 8.35 – 8.28 (m, 1H), 7.35 – 7.23 (m, 4H), 7.21 – 7.09 (m, 3H), 6.96 (dt, J = 7.9, 1.4 Hz, 2H), 6.94 – 6.87 (m, 2H), 6.56 (s, 1H), 5.13 – 4.70 (m, 2H), 4.50 – 4.29 (m, 2H), 3.94 (s, 2H), 1.73 – 1.54 (m, 4H), 1.21 (d, J = 15.2 Hz, 2H). 13C NMR (101 MHz, CDCl3): δ: 165.7 (1C), 155.7 (1C), 153.5 (1C), 135.6 (1C), 131.6 (1C), 130.6 (1C), 130.4 (1C), 130.1 (1C), 128.8 (1C), 128.4 (1C), 128.2 (1C), 128.1 (1C), 127.9 (1C), 124.2 (1C), 122.8 (1C), 122.4 (1C), 121.6 (1C), 119.2 (1C), 119.1 (1C), 109.5 (1C), 109.2 (1C), 83.7 (1C), 53.0 (1C), 46.6 (1C), 30.0 (1C), 29.4 (1C), 22.8 (1C), t½ (min) = 9.51 (Z) + 10.44 (E)); purity = 99.5%. MS: m/z [M+H]+ calc. for C27H25F2N-O2 = 441.21, found 441.20. High-Resolution Mass: m/z calc d for C27H25FN-O2NaO, 463.1905; found, 463.1908.

**Biological Assays. Radioligand Binding Assay.** For the experiments, hCB2-HEK cells were a gift from AbbVie Laboratories (Chicago, USA). Cells were grown in Dulbecco’s modified Eagle’s medium containing high glucose supplemented with 10% fetal calf serum (FCS), penicillin / streptomycin (100 U / mL / 100 µg / mL) and 25 µg/ml zeocin in a 37 °C incubator in the presence of 5% CO2 (100 U / mL / 100 µg / mL) to afford the title compound (26) given from an alternate project by Prof. Kristina Lorenz of the Institute of Pharmacology and Toxicology at the University of Würzburg. The respective rCB2R membranes were prepared as described in literature.26 The rCB2R membrane homogenate was prepared from brains of adult female rats frozen at -80 °C65, 66 given from an alternate project by Prof. Kristina Lorenz of the Institute of Pharmacology and Toxicology at the University of Würzburg. The respective rCB2R membranes were freshly prepared according to the protocol described by Catani and Gasperi for preparation of membrane homogenates.

CBr saturation and competition assays were performed according to protocols previously established in the M. Decker research group.26, 30. For the determination of Ks value of the membrane samples, saturation assays were done taking 8 concentrations of [3H] CP55940 (Hartmann Analytic GmbH) ranging from 0.1 nM to 5.0 nM. Reactions were started by adding membrane (12.5 µg/well for rCB2 or 8 µg/well for hCB2) of a 96 well Multiscreen filter plate (Millipore) containing the radioligand in assay buffer (50 mM Tris-HCl pH = 7.4; 5 mM MgCl2 x 6 H2O; 2.5 mM EDTA). After 3 h incubation at 37°C, the reaction was stopped by vacuum filtration and each well was washed 4 times with cold binding buffer (50 mM Tris-HCl, pH = 7.4; 5 mM MgCl2 x 6 H2O; 2.5 mM EDTA). The filter plate was dried at 45 °C. After cooling to ambient temperature, 20 µL IRGA Safe plus-scintillation cocktail (Perkin Elmer) were added to each well. The activity was counted in a MicroBeta TriLux-Counter (Wallac - PerkinElmer). Competition assays were done with 7 concentrations (0.10 nM – 0.32 mM) of target compound (previously isomerized to their cis or trans states with light at different wavelengths according to the nature of each compound) and 0.62 nM [3H] CP55940. The positive controls for the assays over rCB1 and hCB2 were the respective the selective ligands rimonabant and reference compound 52 (both synthesized in house).

The stock solutions of all tested compounds (5 mM) were prepared as solution in DMSO. The dilution rows of all compound stock solutions were prepared in binding buffer.

Statistical analyses and sigmoidal dose-response curve fittings were performed with GraphPad Prism 6 for Windows (v. 6.01, September 21, 2012). Ks values were determined according to the Cheng-Prusoff equation:

$$K_s = \frac{EC_{50}}{1 + \frac{L}{K_D}}$$

with [L+] as radioligand concentration. The Ks values were calculated of at least two individual experiments. Ks values were measured in at least two individual experiments for each new batch of prepared membrane.

**Internalization of CB1R by flow cytometry.** The cDNA of the hCB1R was obtained from the cDNA resource center (www.cdna.org). PCR was used to delete amino acids 1-25 (as previously described in the literature) and create homology sequence with the signal-sequence-Flag vector.69 The cDNA of the hCB1R was a generous gift from Dr. Guillermo Yudowski, University of Puerto Rico, and PCR was used to amplify the full receptor. NEBuilder was then used to create the final SS-Flag-CB1R and SS-Flag-CB2R. Stable cell lines were created by transfecting HEK293 cells and selecting with Zeocin. Resistant colonies were selected and screened for expression by fluorescent microscopy using anti-Flag M1 (Sigma) conjugated with AlexFluor -647 (ThermoFisher). Internalization was measured using a previously described assay.49 Briefly, Flag-tagged surface receptors were labelled with the calcium sensitive antibody anti-FlagM1 conjugated to AlexaFluor647 for 30 min at 37°C. Compound dilutions were prepared in DMSO and irradiated with the respective wavelength for 5 min before addition to the wells and were incubated for 45 min at 37°C (containing 0.2 % DMSO). For testing compounds in antagonist mode, receptors were stimulated by simultaneously adding the agonist CP55940 (2) in a fixed concentration of 10 nM with the respective test compound dilutions. Following stimulation, cells were rapidly washed with PBS/EDTA (0.04 %) to remove any surface antibody. Cells were suspended in PBS/EDTA (0.04 %), pelleted (1100 rpm, 4°C, 5 min) and resuspended in PBS (containing Ca2+) to measure endocytosis. The increase in fluorescence due to previous internalization of the fluorescent tagged receptors was analyzed using a FACSCalibur (BD Biosciences) with 5000 events being analyzed in all cases. The data was analyzed from at least three independent experiments using sigmoidal dose-response curve fitting in GraphPad Prism 6 for Windows (v. 6.01, September 21, 2012).

**Fluorescence-based measurement of GRABeCB2.0 activation.** HEK293T cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) (PAN Biotech) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Biochrom AG), 2 mM L-glutamine, and penicillin/streptomycin (100 U/mL / 100 µg/mL). Cells were maintained at 37°C and 5% CO2 in a humidified atmosphere. Cells were routinely passaged by aspirating the medium, washing with phosphate-buffered
saline (PBS) (Sigma-Aldrich), de-attaching with trypsin
0.05%/ethylene-dinitrilo)tetraacetic acid 0.02% in PBS and
resuspending in culture DMEM. To perform the assays, 2
million HEK293T cells were seeded in 10-cm cell culture
dishes. After 24 hours, cells were transfected with 2 µg
GRABεCB2.0-containing plasmid DNA using the jetPRIME
reagent according to the manufacturer’s protocol. Later, 24
hours after transfection, cells were resuspended in culture
DMEM and seeded into a black-walled, black-bottom 96-
well plate, at a density of 50,000 cells per well. Prior to per-
forming the assay, photoswitchable ligands were isomer-
ized under LED light (CoolLED pE-4000) at λ = 365 nm (cis)
or λ = 450 nm (trans) for 10 minutes. After isomerization,
ligand dilution series were prepared under dark conditions.
After 24 hours, cells were washed with PBS, and then 90 µL
of prewarmed PBS was added to each well. After 5 minutes
of incubation at 37 °C, fluorescence was measured at 37 °C
using a Synergy Neo2 Plate Reader (Biotek) using an excita-
tion wavelength of 479/20 nm and emission window of
520/20 nm (detector gain: 120V). Each well was measured
once before and after treatment with 10 µL volume of lig-
ands at different concentrations. Fluorescence change upon
ligand treatment was calculated using the following for-
mula:
\[
\Delta F = \frac{F_{post} - F_{pre}}{F_{pre}} \times 100
\]
where \(F_{pre}\) is the measured fluorescence value before ligand
stimulus and \(F_{post}\) is the fluorescence after stimulus. For
each ligand concentration 4 to 8 wells were measured per
plate and responses were averaged. For the statistical an-
alysis, each data point and error bar represent the average of 3-
7 independent experiments ± standard error of the mean
(SEM). Data was fit to Hill equation with variable slope to ob-
tain the EC50 values using GraphPad Prism Prism 6 for Win-
dows (v. 6.01, September 21, 2012).

**hCB Calcium Mobilization Assay.** Chinese hamster ovarian
(CHO-K1) cells stably expressing Gaq16 with either hCB1; or
hCB2 receptor were cultivated in Ham’s nutrient mixture
F12 (Merck) supplemented with 10 % FCS and penicil-
in/streptomycin (90 U/mL / 90 µg/mL). hCB1 cells were
selected using hygromycin (200 µg/mL) and genetin (400
µg/mL). Zeocin (200 µg/mL) and hygromycin (200 µg/mL)
were used for the selection of hCB2. Cells were plated onto
96-well plates (30,000 cells/well) to a final volume of 100
µL/well and incubated at 37 °C for 24 h. Cells were washed
with freshly prepared assay buffer made up from 10 x
Hanks’ balanced salt solution (HBSS, containing final con-
centrations of 137.0 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.4
mM MgSO4, 0.5 mM MgCl2, 0.3 mM Na2HPO4, 0.4 mM
KH2PO4, 4.2 mM NaHCO3, and 5.6 mM D-glucose), 1M HEPES
(final concentration 20 mM), bovine serum albumin (1%)
and Probened (dissolved in 1 M NaOH, final concentration
2.7 mM) adjusted to pH 7.4. Cells were loaded with 100 µL
Fluo-4 AM (4 µM) at 37 °C for 1 h. The dye was removed
and 60 µL of assay buffer (100 µL for controls) was added to the
dye-loaded cells. Calcium flux was monitored using an auto-
mated plate reader (FlexStation 3, Molecular devices) at an
excitation wavelength of 495 nm and an emission wave-
length of 525 nm. Test compounds were irradiated with the
wavelength of interest for 10 min directly before addition.
After measuring the baseline for 15 sec, test compounds in
assay buffer (< 0.5 % DMSO) with various concentrations,
compound CP55940 (positive control) or DMSO (negative
control) was added automatically, and fluorescence was
monitored for 5 min. All experiments were performed in du-
plicates in three independent experiments. EC50 values
were analyzed from the respective area under curve with
sigmoidal dose–response curve fitting using GraphPad
Prism 9 for Windows (v. 6.01, September 21, 2012).

**Analysis of ERK1/2 activation by Western Blot.** CHO cells
expressing Gaq16 and hCB1 were plated in 6 well plates
(1*106 cells/well) for 24 h and starved for another 24 h in
serum free media. Test compounds were irradiated with the
wavelength of interest for 5 min. Cells were stimulated for
15 min with the indicated compounds and lysed using an
ice-cold lysis buffer (1% (V/V) Triton-X-100, 5 mM EDTA,
300 mM NaCl, 50 mM Tris (pH 7.4), 20 µg/mL soybean tryp-
sin inhibitor, 0.4 mM benzamidine, 1 mM PMSE, 50 mM NaF,
5 mM Na3PO4, 1 mM NaVO4 and 1.5 mM NaN3). After 10
min (4 °C), sonification was performed and the lysates were
centrifuged (20,000 g, 15 min). The colorimetric BCA pro-
tein Assay Kit (ThermoScientific) was used to determine the
protein concentration. Equal amounts of protein per sample
were separated using SDS/PAGE. After the transfer to a
PVDF membrane (Amersham; Hybond-P), the membranes
were blocked with 5 % (w/v) fat-free milk dissolved in Tris-
buffered saline with 0.1 % (w/v) Tween-20 or 5 % (w/v) BSA
dissolved in Tris-buffered saline with 0.2 % (V/V) NP-40
for 1 h at room temperature. The membranes were incu-
bated with the indicated primary antibodies over night at 4
°C. Following primary antibodies were used: phosphoERK1/2(TY2) (1:1,000; no.9101L, Cell Signaling), ERK1/2 (1:1,000; no. 9102, Cell Signaling). A horseradish-
peroxidase-linked antibody was used as secondary anti-
body (1:10,000; no. 111–035–144). Membrane bound anti-
bodies were detected using a luminol solution (125 mM lu-
minal, 45 mM coumaric acid, TRIS 1 M (pH 8.3), 0.015 %
(V/V) H2O2). Data analysis was performed with ImageJ and
GraphPad Prism 9 for Windows (v. 9.3.1).

**NanoBiT® βarr2 recruitment assays.** CBR activation po-
tential was evaluated using live cell NanoBiT® assays, fol-
lowing the recruitment of βarr2 to the receptor upon acti-
vation. The NanoLuc Binary Technology, developed by
Promega, is based on functional complementation of a split
nanoluciferase. The establishment of the used assays has
been described before.79-72 HEK293T cells stably expressing
either the CB1-βarr2 or CB2-βarr2 reporter system were rou-
tinely maintained at 37 °C, 5% CO2 under humidified atmo-
sphere in Dulbecco’s modified Eagles medium (Glu-
MaxTM) (DMEM) supplemented with 10% heat-inactivated
bovine fetal serum (FBS), 100 IU/mL of penicillin, 100
µg/mL of streptomycin and 0.25 µg/mL of amphotericin B.
On the day prior to experiments, cells were seeded in white
96-well plates, coated with poly-D-lysine, and incubated
overnight. Stock solutions were made by dissolving com-
pound 27a in DMSO and working solutions were made by
serial dilution in Opti-Mem I Reduced Serum and MeOH
(50/50 % v/v), resulting in an in well DMSO concentration
of 0.09 %. The next day, cells were rinsed twice with 150
µL of Opti-MEM, after which 100 µL of this assay medium
was added to each well. Subsequently, 25 µL of the 20-fold
diluted Nano-Glo® Live Cell reagent, containing the cell-
permeable furinazime substrate was added and the plate
was placed into a TriStar® LB 942 Multimode Microplate Reader (Berthold Technologies GmbH & Co., Germany). Luminescence was measured for 10-15 min until the signal stabilized. To evaluate CB activity of the 2 photo-isomers of 27a, 13.5x concentrated stock solutions were either irradiated with $\lambda = 365$ nm (cis) or $\lambda = 450$ nm (trans) for 5 min and added to the cells. Luminescence was then monitored for 2 hours. A concentration range of reference standard CP55940, as well as appropriate solvent controls were present on each plate. To assess dynamic photomodulation of 27a, 3 high concentrations (10 µM, 1 µM and 100 nM) were prepared as described previously. Stock solutions then were subjected to 1, 2, 3 or 4 irradiations, switching between 27a-cis and 27a-trans. Stock solutions were added to the cells and the assay was performed as mentioned above. Raw data were initially processed using Microsoft Excel 2019. Luminescence values were baseline-corrected for inter-well variability and a blank correction was performed by subtracting the area under the curve (AUC) values of the solvent controls. Using the GraphPad Prism software (Version 9.3.0) (San Diego, CA, USA), concentration-response curves were obtained by normalizing to the Emax of the reference standard CP55940, set at 100%. Curve fitting of the concentration-response curves via nonlinear regression (three-parameter logistic fit) was performed to obtain EC50 (potency) and Emax values (efficacy).

**Molecular Docking.** The 2D structures of the compounds were drawn with ChemDraw (v. 18.0, PerkinElmer) and MarvinSketch for Windows (v. 19.10.0, ChemAxon Ltd.). Docking experiments were performed as previously described.$^{35}$ The crystal structure of the agonist 1hCB:R (PDB ID: 5XRA) was prepared using MOE$^{73}$ (version 2016.08). MOE’s protein structure preparation pipeline$^{74-79}$ was used to remove ligands and water molecules, followed by automatic assignment of tautomer and protonation states using Protonate3D$^{80}$ at pH 7.5. Geometry optimization of AM1542, reference compound 4 and target compounds 27a and 28a was performed using the MMFF94s$^{81, 82}$ force field (gradient convergence criterion: 0.001 RMS kcal·mol$^{-1}$Å$^{-1}$). Pose reproduction performance was assessed by RMSD between docked poses and the experimental binding pose of AM1542 (PDB ID: 5XRA). The GOLD program (v5.4.1) was used to dock the ligands under default settings. The knowledge-based scoring function ASP was found to robustly model experimentally observed intermolecular interactions of AM1542 and to yield the best crystal pose reproduction among all of GOLD’s scoring functions (RMSD of 0.47Å for the top ranked pose). The 50 best ASP poses of each ligand were rescoring using the knowledge-based scoring function DSX (v9.89), with CSD and PDB potentials (version 05/11). Compound 4 and the cis and trans isomers of compound 27a, and compound 28a-cis were manually prepared in MOE’s Builder tool and subsequently prepared and evaluated under the same conditions as for AM1542.

To reliably compare the docking scores of molecules with different molecular sizes, the original ASP scores were normalized by the cubic root of the heavy atom count of each ligand (cubic root score),$^{61}$ as defined by the expression:

$$CRS = \frac{\text{Score}}{\sqrt[3]{\text{Heavy atom number}}}$$

**ASSOCIATED CONTENT**

The supporting information is available free of charge via the Internet at http://pubs.acs.org.

The document contains: HPLC chromatogram of target compounds, molecular docking and scoring, photo stationary distribution (compounds 27a and 49), $^1$H-NMR and $^1$C-NMR spectra of target compounds, thermal stability graphics of target compounds, supplementary figures of biological assays, and process for the synthesis of selected intermediates.

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**Author Contributions**

M. Decker was responsible for the supervision and development of the whole project. D. A. Rodríguez-Soacha performed the chemical synthesis, testing of the physicochemical properties of the target compounds and performing the radioligand binding assays. Additional radioligand binding experiments were done by S. A. M. Steinmüller. Western Blots were obtained by J. Fender under the supervision of K. Lorenz. M. H. Deventer performed bar2 assays (NanoBit®) and dynamic photomodulation experiments under the supervision of C. P. Stove. The calcium mobilization assay was established with the help of J. Fender under supervision of K. Lorenz, and the experiments were performed by S. A. M. Steinmüller and A. Tutov. Computational docking studies were performed by Y. Ramírez with supervision by C. Sotriffer. A. Ishbilir with supervision by M. J. Lohse performed fluorescence biosensor-based receptor activation assays. The flow cytometry analysis of internalization was performed by S. A. M. Steinmüller under supervision of J. N. Hislop. All authors have given approval to the final version of the manuscript.

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**Notes**

The authors declare no competing financial interest.
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ABBREVIATIONS

hCB-R, human cannabinoid receptor 1; hCB2-R, human cannabinoid receptor 2; rCB1R, rat cannabinoid receptor 1; Boc-O, D-tart-butyl decarboxate; DCM, dichloromethane; DIPEA, N,N-Diisopropylethylamine; mCPBA, meta-chloroperbenzoic acid; MPS, potassium peroxymonosulfate; PPh3, triphenylphosphine; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TMS-acetylene, trimethylsilylacetylene.

REFERENCES


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