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Photoswitches

A High-Quality Photoswitchable Probe that Selectively and Potently Regulates the Transcription Factor RORγ

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Abstract: Retinoic acid receptor-related orphan receptor γ (RORγ) is a nuclear hormone receptor with multiple biological functions in circadian clock regulation, inflammation, and immunity. Its cyclic temporal role in circadian rhythms, and cell-specific activity in the immune system, make it an intriguing target for spatially and temporally localised pharmacology. To create tools that can study RORγ biology with appropriate spatiotemporal resolution, we designed light-dependent inverse RORγ agonists by building azobenzene photoswitches into ligand consensus structures. Optimizations gave photoswitchable RORγ inhibitors combining a large degree of potency photocontrol, with remarkable on-target potency, and excellent selectivity over related off-target receptors. This still rare combination of performance features distinguishes them as high quality photopharmaceutical probes, which can now serve as high precision tools to study the spatial and dynamic intricacies of RORγ action in signaling and in inflammatory disorders.

Retinoic acid receptor-related orphan receptors (RORs, NR1F1-3) are a family of three ligand-activated transcription factors, RORα, RORβ, and RORγ, binding oxysterols as natural ligands.[1–3] Together with the two related rev-ERBs (NR1D1-2), RORs are integral parts of the circadian clock. Both receptor families have oscillating expression and compete for the same DNA response elements (RORE), with RORs as constitutively active transcriptional inducers and rev-ERBs as repressors, for genomic control of circadian rhythm and metabolic homeostasis (Figure 1a).[2, 4] The RORγ splice variant RORγt is also involved in immune system regulation and has become an experimental drug target for treating autoimmune diseases,[2, 5] since inhibition of the constitutive RORγt activity in naive T cells using inverse agonists can prevent their differentiation to T_H17 cells and reduce chronic inflammation.[2, 6–9] However, no drug targeting RORγ has yet been approved. Despite high levels in lymphocytes, RORγ is widely expressed in multiple tissues. Even when applied topically with low-efficacy dosing,[6] experimental agents suffered systemic adverse effects, leading to attrition of inverse RORγ agonists in clinical trials.[2, 4, 9] Probes allowing temporally resolved and spatially localised control of RORγ would thus be highly desirable: in basic research, they could reveal unique aspects of its roles in health and disease; and in translational settings, they could orient therapeutic development e.g. by testing whether localized RORγ inhibition might improve efficacy and safety.[10]

Photoswitches are ideally suited to make small molecules reversibly light-responsive, enabling spatiotemporal control

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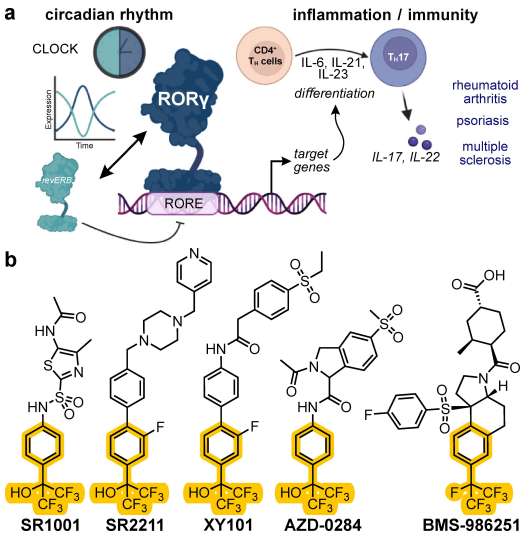
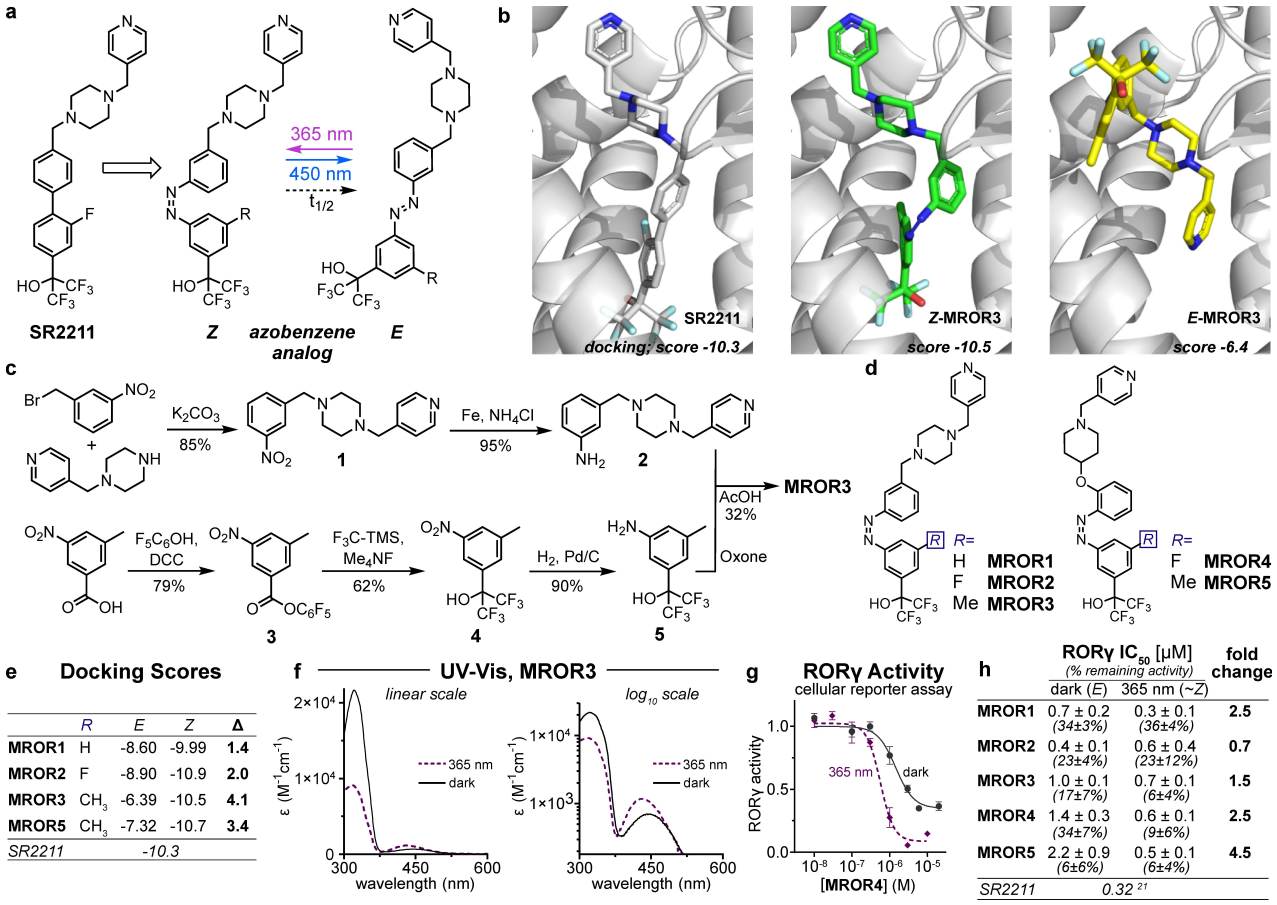


Figure 1. a) RORγ function and biological roles. b) Inverse RORγ agonists featuring a terminal (hexafluoroisopropyl) phenyl motif near a central aryl ring.

over drug bioactivity across various target classes by simple localised light application.[11–13] Azobenzene photoswitches have been applied in light-controlled probes of nuclear hormone receptors,[14] notably including agonists for peroxisome proliferator-activated receptors (PPARs),[15] estrogen receptor,[16] and liver X receptor (LXR).[17] Photopharmaceutical approaches to control circadian rhythms have also emerged, including light-responsive ligands for casein kinase I or CRY1,[18–20] showing a natural fit of the temporal reversibility of photoswitch chemistry to clock genes. However, despite high target potential both for spatially localised studies in inflammation and for temporally specific studies of circadian rhythms, RORs remain unexplored by photopharmacology.

Here we report the design and structural optimization of first-in-class photoswitchable inverse RORγ agonists which can be reversibly activated and deactivated by light. The most active compound **MROR6** has single-digit nanomolar potency in its more active photoisomer, remarkable >99% efficacy in blocking RORγ activity, and outstanding selectivity for RORγ over many related nuclear hormone receptors. Its photoswitchability opens new opportunities to study the role of RORγ in circadian rhythm, and its potential in immunotherapy, with spatiotemporal resolution.

The structure–activity relationships of RORγ ligands have been explored in detail over many pharmaceutical programs,[4, 21] and several co-crystal structures of the RORγ ligand binding domain (LBD) with bound ligands have been published. Our initial design for a photoswitchable RORγ tool was inspired by a structurally similar subset of inverse agonists, that feature a hexafluoroisopropylphenyl ring held rigidly near a "central" aryl ring which tolerates broader substituent scope and usually has a polar tail (Figure 1b). We envisioned that bridging the two aryl rings with a photoisomerisable diazene could drive substantial activity differences between *E* and *Z* photoisomers, by changing the separation and relative orientation of the aryls. We chose the biaryl SR2211, a selective inverse agonist of RORγ, as a starting point.[22] Aiming at a tool that is a RORγ inverse agonist only when illuminated (i.e., as the *Z*-isomer formed under UV/violet illumination),[23] we designed the diazene in *ortho* to the original biaryl bond (Figure 2a) to mimic the



	R	E	Z	Δ
MROR1	H	-8.60	-9.99	**1.4**
MROR2	F	-8.90	-10.9	**2.0**
MROR3	CH_3	-6.39	-10.5	**4.1**
MROR5	CH_3	-7.32	-10.7	**3.4**
SR2211			*-10.3*	

	RORγ IC_{50} [μM] (% remaining activity) dark (*E*)	365 nm (~*Z*)	fold change
MROR1	0.7 ± 0.2 (34±3%)	0.3 ± 0.1 (36±4%)	**2.5**
MROR2	0.4 ± 0.1 (23±4%)	0.6 ± 0.4 (23±12%)	**0.7**
MROR3	1.0 ± 0.1 (17±7%)	0.7 ± 0.1 (6±4%)	**1.5**
MROR4	1.4 ± 0.3 (34±7%)	0.6 ± 0.1 (9±6%)	**2.5**
MROR5	2.2 ± 0.9 (6±6%)	0.5 ± 0.1 (6±4%)	**4.5**
SR2211	*0.32* [21]		

Figure 2. SR2211-based azologues **MROR1–5** as photoswitchable RORγ ligands. a) A SR2211 azologue. b) Docking of SR2211 and *E*/*Z*-**MROR3** into the RORγ LBD (PDB: 6NWT;[29] the low score for *E*-**MROR3**, and its unrealistic pose, are coherent with a steric clash). c) Synthesis of **MROR3**. d) Structures of **MROR1–5**. e) Docking scores of *E*/*Z*-azologues. f) Representative UV/Vis spectra, as all-*E* (dark) or mostly-*Z* (PSS 365 nm) (Figure S2a). g–h) Mostly-*Z*-**MROR1–5** are submicromolar inverse RORγ agonists, similar to SR2211;[22] but their *E*-isomers are not weaker enough to qualify as *usefully switchable* ligands (Figure S3a), data shown as mean ± S. E. M. from at least three independent experiments each with two technical replicates.

biaryl as the *Z* but not *E* isomer.[24–27] Structural evaluation indicated that the RORγ ligand pocket can accommodate the *Z*-azobenzene similarly to the biaryl, since rather larger molecules like BMS-986251 are also tolerated (Figure 1b). Molecular docking supported the feasibility of this hypothesis for *Z*-active compounds (Figure 2b,e, Figure S1).

Based on this, we prepared a set of azobenzene analogues ("azologues") of SR2211 to vary a substituent near the diazene (R group in Figure 2a as H, F, Me in **MROR 1–3**), and reorient the polar tail (**MROR 4–5**), scanning for structure-dependent effects (Figure 2c–d). We synthesised **MROR 1–5** by oxidatively coupling their two aniline halves, that were each assembled rapidly in good yield, relying on Handlon's procedure[28] to install the hexafluoro-2-hydroxyisopropyl group from the 3-nitrobenzoic acid (Figure 2c and Supporting Information). **MROR 1–5** displayed good photoswitching, with efficient *E*-to-*Z* isomerization by 365 nm light (Figure 2f, Figure S2a), and were evaluated for RORγ inhibition in a Gal4 hybrid reporter gene assay in HEK293T cells comparing dark (*E*) to UV (mostly-*Z*) potencies (Figure 2g).

In cells, the *Z* isomers of **MROR 1–3** achieved similar submicromolar RORγ inhibition as SR2211, but so did their *E* isomers, making them not useful as photoswitchable tool compounds (Figure 2h). Reorienting the polar pyridin-4-ylmethyl group relative to the hexafluoroisopropanol anchor in **MROR 4–5** gave larger *E/Z* activity differentials (2.3 to 4.4-fold; Figure S3a), but still below the 10-fold activity switching minimum we advocate for robust photoswitchable cellular tools.[23]

Aiming to increase the activity switch from *E* to *Z* isomers, we tried another polar group, *para*-(ethylsulfonyl) phenylacetamide, found in several potent RORγ ligands (Figure 3a). We oriented it in *meta* or *ortho* to the diazene in **MROR 6–9** (Figure 3b,c). Their photoswitching was typical (Figure 3d, Figure S2b). However, *meta* **MROR 6–7** excelled as photopharmaceuticals in cells: with *Z*-isomer potencies in the low nanomolar range paired with *E/Z*-activity differentials of up to 20-fold (Figure 3e,f, Figure S3b).

MROR 6–7 undergo spontaneous *Z*→*E* relaxation far slower than bioassay timescales (half-life ~10 h, Figure S2d), making them bistable in practice. It was also of interest to explore faster-*Z*→*E*-relaxing ligands, since in assays where ligands can diffuse rapidly after photopatterning,[30–31] suppressing *Z*-bioactivity by relaxation outside photoactivation zones can give higher spatiotemporal resolution.[32] We chose to use *para*-hydroxylation or -amination to increase relaxation speed, as these also allow shifting the optimal *E*→*Z* photoisomerisation wavelengths from ca. 360 nm (classical azobenzenes, e.g. **MROR 6**) to a broader range ca. 400–500 nm.[33] We thus prepared **MROR 10–13**, which have sub-second *Z*→*E* relaxation rates in water as well as higher *E*→*Z* photoisomerisation response to standard microscope lasers than **MROR 6–7** (e.g. 405 nm; Figure 3g–i, Figure S3c). Despite lower absolute potency and *Z/E* bioactivity differential of **MROR 10–13** compared to **MROR 6–7** (Figure 3j,k), **MROR 12** still offers favourable tool characteristics for short-timescale assays where its 6-fold *Z/E* activity ratio can be supported by its rapid *Z*→*E* switch-off (further discussion at Figure S3).

With their high *Z*-potency and *Z/E* activity difference, **MROR 6/7** remained our leads for spatiotemporally precise cell biology. The final key feature we required of them to be useful as chemical tools was selectivity for targeting RORγ over related NHRs. Outstandingly, **MROR 6/7** exhibited no significant off-target activity at 1 μM (200×/50× their RORγ IC_{50}), and even confirmed selectivity versus LXR which binds structurally similar natural and synthetic ligands (Figure 4a).[17] As proof-of-concept before phenotypic biology applications, we monitored how in situ *E*→*Z* photoswitching of **MROR 6/7** affects cellular RORγ-regulated gene expression over time. For this we tracked RORγ-dependent expression of mCherry by fluorescence, in HEK293T cells kept in the dark (*E*) or under 365 nm pulsing (~*Z*), during 36 h. As expected, **MROR 6/7** at concentrations within their 12-to-20-fold working windows (Figure S3c) gave good photocontrol: with the *E*-isomers having little to no effect compared to untreated cells, but strong inhibition with *Z*-**MROR 6/7**.

Having thus established their chemical probe quality, we applied **MROR 6** to control cell fate, in a phenotypic experiment. Adipocytes (fat cells) are non-dividing terminally differentiated cells formed from replicative preadipocyte cells by a complex differentiation sequence that is precisely temporally orchestrated. Understanding the organisation and temporal dynamics of this sequence is crucial for tackling major metabolic pathologies, including obesity-associated insulin resistance and chronic inflammation. PPARγ as well as C/EBP proteins are central actors in preadipocyte differentiation; however, at least 100 other transcription factors are expressed in adipocytes, and the native sequence of events (as well as both therapeutic intervention opportunities, and risks of unwanted side-effects) remains unclear.[34] RORγ is expressed in adipose tissue,[35–36] as well as during differentiation of preadipocytes,[37] and a role of this circadian regulator in adipogenesis can also be speculated based on rhythmic expression of adipokines in mice.[38] We examined adipocyte-derived mesenchymal stem cells (ASCs), a model for preadipocytes that can be differentiated into mature adipocytes by treatment with a mixture of insulin, IBMX, dexamethasone, and pioglitazone. 300 nM of **MROR 6** powerfully counteracted this induced adipogenesis with full light- and dose-dependency: reducing adipogenesis under 365 nm pulsing by 40 %, with high statistical confidence, yet being inactive in the dark (Figure 4d–f): showing the value of this photohormone as high-potency, high-precision tool to reveal and study RORγ-dependent biology.

In conclusion, the photoswitchable inverse RORγ agonists **MROR 6–7** will enable for the first time a spatiotemporally resolved control over the key circadian clock and immunity regulator RORγ which is attracting major attention as promising therapeutic target. The

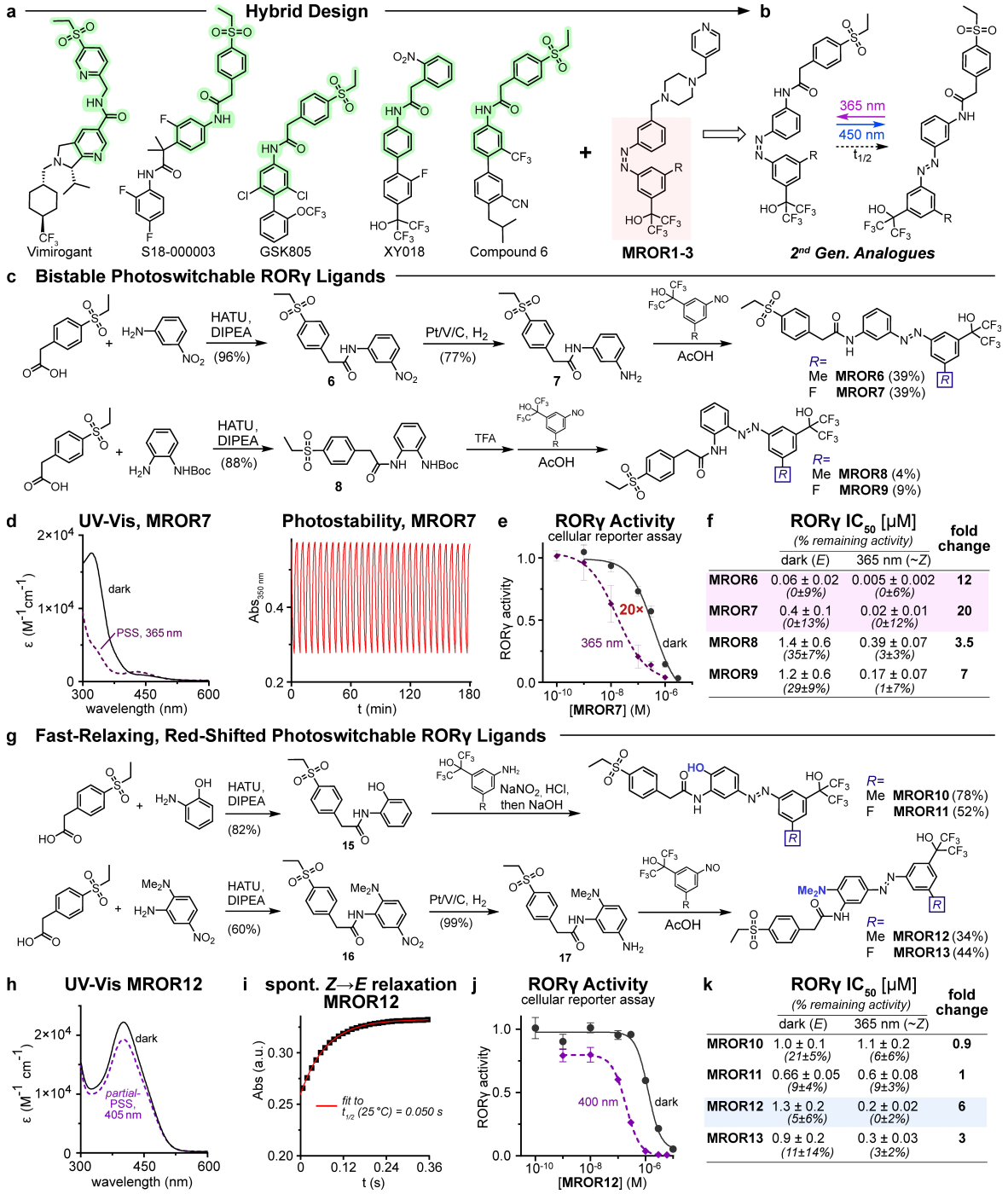


Figure 3. Design of hybrid photoswitchable RORγ ligands using *para*-(ethylsulfonyl) phenylacetamide, a common polar motif for inverse RORγ agonists. a–f) Bistable hybrids **MROR6–9**: a–b) design; c) syntheses; d) representative UV/Vis spectra and reversible $E \rightleftarrows Z$ photoswitching (365/450 nm) (Figure S2bd); e–f) **MROR6–9** have good light-dependency of cellular RORγ inhibition and high *Z*-potency (Figure S3bc). g–k) Fast-relaxing red-shifted **MROR10–13** (based on **MROR6–7**): g) synthesis; h–i) representative UV/Vis spectra and thermal $Z \rightarrow E$ isomerisation (Figure S2ce); j–k) light-dependent cellular RORγ inhibition (Figure S3d). (e, f, j, k: data are mean ± S.E.M. from at least 3 independent experiments each with 2 technical replicates). Higher-resolution Figure given as Figure S8.

remarkable potency of the light-activated *Z*-isomers (down to 5 nM), their large *E*/*Z* activity differences (up to 20-fold), and their outstanding selectivity against related NHRs support the value of **MROR6–7** as next-generation tools to study the biology and therapeutic potential of RORγ with local and temporal precision. Future work may also focus on more druglike compounds for more complex biological models, e.g. by swapping for an isosteric but metabolically resistant photoswitch such as C=C-based heterostilbenes.[39–40] We foresee many

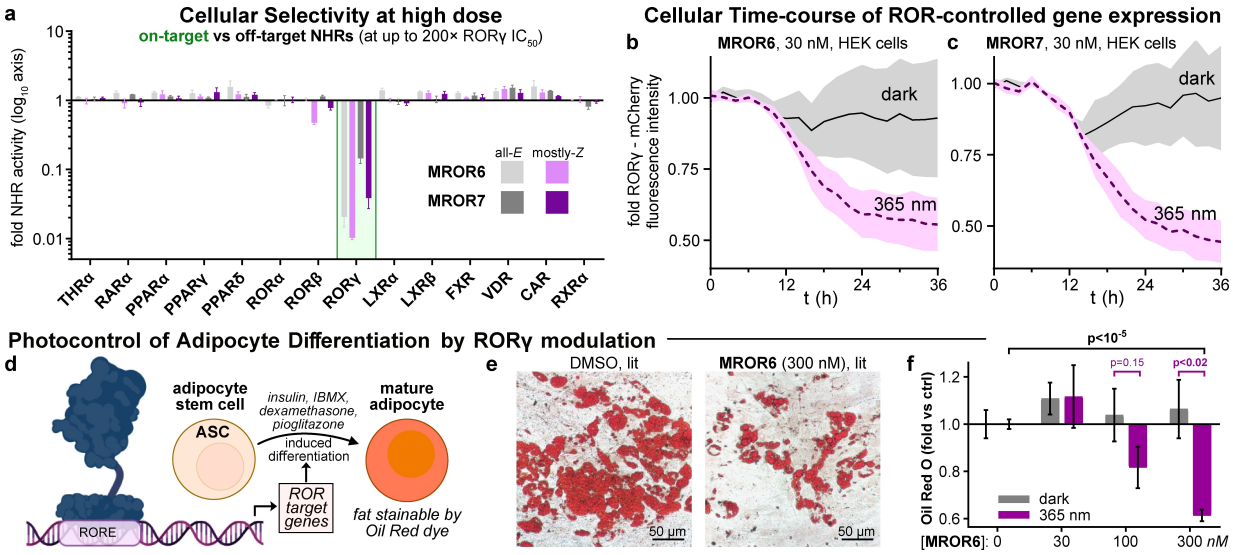


Figure 4. Cellular utility of **MROR6–7** as photopharmaceutical tools. a) Cellular NHR selectivity profiles of *E/Z*-**MROR6–7** at 1 µM (see also Figure S4), data shown as mean fold NHR activity remaining ± S.E.M. from at least 2 independent experiments each with 2 technical replicates. b–c) Time-course of RORγ-controlled protein expression depending on *E/Z*-**MROR6–7** (interpret: *Z* blocks RORγ-dependent transcription, so first target mRNA, and then target protein, levels drop relative to control; target here: mCherry; c. f. Figure S5); data are mean ± S.E.M. from 4 independent experiments each with 2 technical replicates. d–f) *Z*-**MROR6** counteracts ASC differentiation, showing a role for RORγ in adipogenesis (*ASC52telo,hTERT* cells; 21 days' differentiation; e: representative micrographs, lipid stain: Oil Red O, scale bar 50 µm; f: data as mean ± SEM; $n = 5$; statistical significance determined using a Welch Two Sample t-test). Full legend at Figure S9.

applications ranging from time-resolved studies on the clock regulator, through to testing the therapeutic potential of locally targeted RORγ modulation in models of metabolic and autoimmune diseases, and to studies where interventions in time-regulated networks of gene transcription must be performed with previously hard-to-obtain spatiotemporal precision.

Supporting Information

Compound design, synthesis, analysis, and biological applications are given in the Supporting Information (PDF). The authors have cited additional references within the Supporting Information.[41–52]

Abbreviations

C/EBP	CCAAT/enhancer binding protein
PPAR	peroxisome proliferator-activated receptor
PSS	photostationary state [$E:Z$ photoequilibrium]
ROR	Retinoic acid receptor-related orphan receptor
RORE	ROR response element.

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Conflict of Interest

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: azobenzenes · nuclear hormone receptors · Photopharmacology · spatiotemporal resolution · transcription factor

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