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Cryptochrome structures reveal insights into isoform selectivity and design of circadian clock-modulating compounds

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1 Introduction

The circadian clock is a biological timekeeper that drives daily rhythms of physiological functions in synchrony with environmental day–night cycles. The core circadian clock consists of the heterodimeric transcription factors CLOCK and BMAL1, which activates the transcription of its own repressor genes: *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1* and *Per2*). CRY and PER proteins associate into complexes and translocate to the nucleus where they bind to and suppress CLOCK–BMAL1 transcriptional activity. This suppression is alleviated by rhythmic proteasomal degradation of CRYs and PERs enabling a new circadian cycle (day) to begin. CLOCK and BMAL1 also activates the transcription of thousands of genes to regulate physiological rhythms. Disruption of circadian rhythms has been linked to multiple diseases including cancer, heart disease, diabetes, and obesity. Therefore, core circadian clock proteins are prime targets for modulation by small molecules in potential therapeutic applications.

We previously identified first-in-class isoform-selective compounds against CRY1 and CRY2. Our research has focused on molecular insights into the function and regulation of CRY isoforms. CRYs are composed of a structurally ordered photolyase homology region (PHR) with high sequence conservation, and a disordered CRY C-terminal tail (CCT). Located in the PHR is an FAD pocket (where ligands bind) and a lid loop. CRY1 and CRY2 FAD pockets and lid loops have very high sequence conservation. In contrast, the CCTs have low sequence conservation and have been shown to impart compound isoform selectivity[1].

We obtained 3-D structures of both apo and compound-bound forms[1–3]. Our crystal structures elucidated intrinsic structural isomerism in several core FAD pocket residues in CRY1 and CRY2. The most important of which is W399 in CRY1 and the corresponding W417 residue in CRY2. W399 and W417 intrinsically adopt different rotamer conformations that are stabilized by differential interactions with their corresponding lid loops[3]. This isomerism imparts selectivity in compound binding, hence W399/W417 were named as the gatekeeper residue. These structural insights have provided an important basis for new compound design.

Here, we report two key discoveries from crystal structures obtained from the Photon Factory: Project 1) The design of a first-in-class photo-switchable CRY1-selective compound[4]; and Project 2) The first structural insights into the anti-glioblastoma compound SHP656[5]. Glioblastoma is the most aggressive and deadly type of brain tumor.

2 Experiment

Project 1: X-ray diffraction data for CRY1(PHR)–TH129 and CRY1(PHR)–TH303 crystals were collected at 0.98 Å and 100 K at the Photon Factory (BL-17A). The datasets for CRY1(PHR)–TH129 (PDB: 7D19) and CRY1(PHR)–TH303 (PDB: 7D1C) were processed to 2.35 Å and 1.9 Å, respectively. The structures were solved by molecular replacement (MR) using CRY1-apo (PDB: 6KX4) as a search molecule.

Project 2: Datasets for CRY2(PHR)–SHP656 and CRY2(PHR)–SHP1703 were collected at 0.98 Å and 100 K at BL-17A. CRY2(PHR)–SHP656 (PDB: 7V8Z) and CRY2(PHR)–SHP1703 (PDB: 7V8Y) were processed to 1.95 Å and 1.9 Å, respectively. Both structures were solved using CRY2–TH301 (PDB: 6KX8) as a template for MR.

3 Results and Discussion

Project 1: Design of the the first-in-class photo-switchable CRY1-selective compound

We identified a new CRY1-selective compound TH129 from phenotypic screening of circadian clock modulators. TH129 is a derivative of the CRY1-selective compound KL101 with substitution of the dimethylphenyl group with a benzophenone group[4]. We obtained the crystal structure of CRY1(PHR) complexed with TH129, which revealed a unique binding mode that induced a conformational change in the gatekeeper tryptophan and rearranged the lid loop[4]. We noted that the benzophenone moiety in TH129 was structurally similar to the *cis* form of azobenzene (a moiety that can reversibly undergo rational azologization from a *cis* to *trans* conformation with different wavelengths of light). Substitution of the benzophenone (TH129) for an azobenzene (GO1323) facilitated the design of the first-in-class CRY1-selective compound that could be activated and deactivated via irradiation with different wavelengths of light (Fig. 2). This photo-switchable compound shows promise in the field of photopharmacology where protein modulators can be spatiotemporally turned on or off to potentially limit side effects.

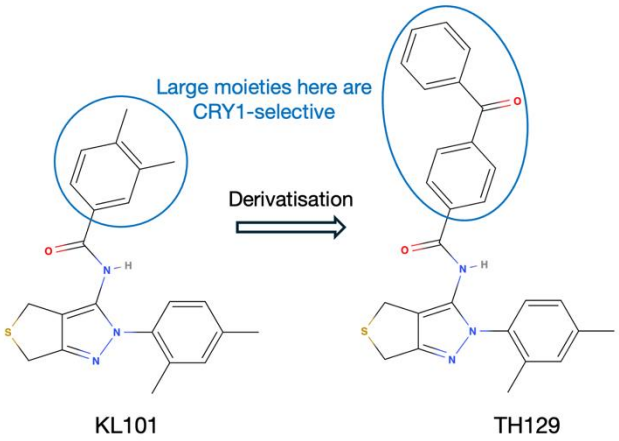

Fig. 1: Substitution of the dimethylphenyl in KL101 (left) for a benzophenone in TH129 (right).

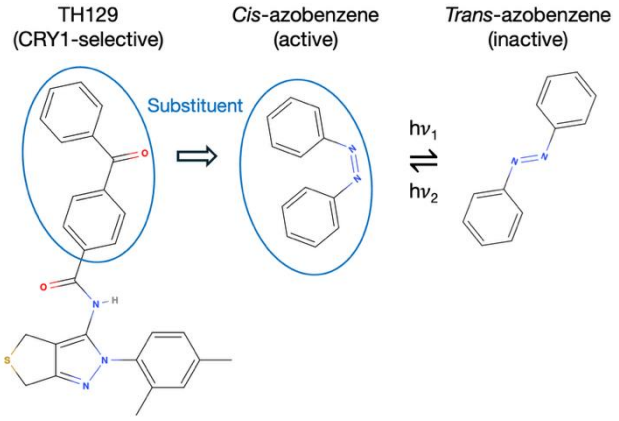

Fig. 2: The *cis*-form of azobenzene is structurally similar to benzophenone and active against CRY1.

Project 2: Structural insights on CRY2 selectivity of an anti-glioblastoma compound

SHP656 is a small-molecule compound that has previously been shown to exhibit anti-glioblastoma activity via potential interactions with CRY(s)[6,7]. Utilizing cell-based degradation assays, reporter gene assays, and in vitro thermal shifts, we determined that SHP656 preferentially targets CRY2. To obtain molecular insights into the mechanism of action, we determined the 3-D structure of SHP656 in complex with CRY2(PHR). SHP656 contains a small 2-imidazolidinone moiety that occupies hydrophobic region 1 in the FAD pocket. Notably, this moiety is similar in steric bulk to the cyclopentane moiety in the CRY2-selective compound TH301[1,5]. Furthermore, superimposition of SHP656 and TH301 showed a similar stacking interaction with the gatekeeper, W417 (Fig. 3). Moreover, we determined that only the *R*-form of SHP656 bound to CRY2 in crystal structures. Because SHP656 was a racemic mixture containing both *S*- and *R*-forms, we synthesised pure *R*-form (SHP1703) and determined its crystal structure in complex with CRY2[5]. The binding modes of SHP656 and SHP1703 were nearly identical. Together, these data showed that SHP656 was compatible with the intrinsic conformation of the FAD pocket in CRY2, namely the gatekeeper tryptophan that imparts a significant proportion of compound-binding selectivity.

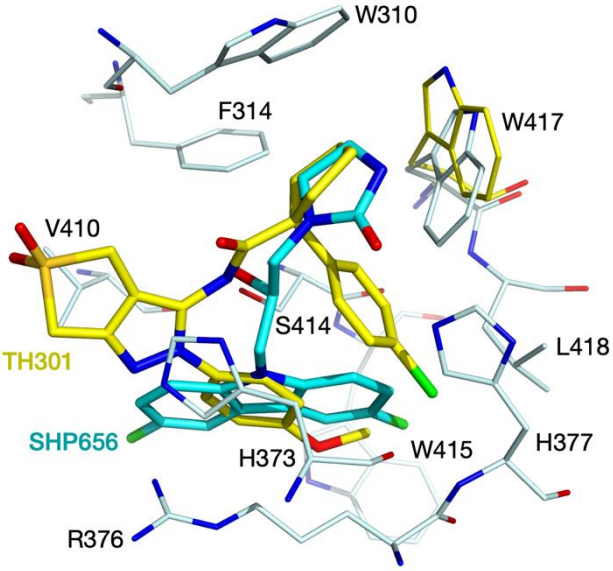

Fig. 3: CRY2–SHP656 (cyan) superimposed on CRY2–TH301 (yellow). The gatekeepers (W417) adopted similar rotamer conformations.

Next, we analyzed the effects of SHP1703 in cellular and in vitro assays. SHP1703 showed greater period lengthening than SHP656 on reporter genes in U2OS cells, and SHP1703 had a more potent thermal stabilising effect on recombinant CRY2(PHR) compared to CRY1(PHR)[5]. Moreover, in cell viability assays utilizing human lung fibroblasts (IMR-90), osteosarcoma cells (U2OS), and patient-derived glioblastoma stem cells (MGG 31), SHP1703 exhibited a more potent effect on MGG 31 cells with reduced viability at lower doses, compared to IMR-90 and U2OS cells[5]. In contrast, pure *S*-form SHP1704 showed reduced activity. Together, these data show that the *R*-form (SHP1703) is the active enantiomer, and that its activity is selective to patient-derived glioblastoma stem cells.

In summary, our structural, chemical, and cell biology insights into the binding modes of CRY isoform-selective compounds, have facilitated the rational design of more potent and selective compounds, and also provided a foundation for more targeted therapy with lower doses and reduced side effects.

Acknowledgement

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