Structural insights into isoform-selective regulators of mammalian Cryptochromes

The circadian clock is a biological timekeeper that regulates sleep-wake behavior, hormone secretion, body temperature, and metabolism with ~24-hour periodicity. The transcription factors CLOCK and BMAL1 activate transcription of their own inhibitors, encoded by *Period* (*Per*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. Cryptochromes (CRYs) are light-responsive flavoproteins related to DNA repair enzymes, photolyases. In mammals, however, CRYs typically do not bind a flavin cofactor, FAD, and function as light-independent transcriptional repressors of the circadian clock by forming complexes with PER proteins, and translocating to the nucleus where they inhibit CLOCK-BMAL1 to close the negative feedback loop. Dysfunction of CRY proteins affects circadian period, and has been associated with sleep disorders, metabolic disease, and cancer, thus making them attractive therapeutic targets. The first CRY-targeting compound KL001 was found to stabilize CRY proteins and lengthen the circadian period, but KL001 binds to both CRY1 and CRY2 without preference [1]. Since CRY1 and CRY2 have overlapping and distinct cellular functions, the development of isoform-selective modulators is important for the elucidation of unique regulatory mechanisms between CRYs. Crystal structures of the photolyase homology regions (PHRs) of CRY1 [2] and CRY2 [3] provided a foundation for the understanding of compound interactions. However, very high sequence identity in the FAD-binding pockets of CRY1 and CRY2 (where compounds bind) has hampered isoform-selective compound design.

By conducting phenotypic screens of circadian period modulators, we identified first-in-class compounds KL101, TH301, and KL201 that stabilize CRY1 and CRY2 in an isoform-selective manner (Fig. 1) [4,5]. To understand the mechanisms of action of these unique compounds, we determined X-ray crystal structures in complex with CRY1- and CRY2-PHR proteins. Crystals were initially screened at SPring-8 **BL32XU** using ZOO automated data collection, and the final diffraction data were subsequently collected manually at SPring-8 **BL44XU**. We determined the structures of CRY1-apo and CRY1-KL101 at a resolution of 2.0 Å, and CRY1-TH301 and CRY1-KL201 at 2.1 Å [4,5]. All structures formed canonical photolyase folds comprising N-terminal α/β and C-terminal α-helical domains, connected by an extended linker region (Fig. 2). The FAD-binding pocket is located within the α-helical domain and is composed of 17 residues, with only one variant residue between CRY1 and CRY2 (Fig. 3). We divided the FAD-binding pocket into three regions: Hydrophobic region 1, hydrophobic region 2, and the affinity region, due to interactions formed with various compound moieties (Fig. 3).

The compounds KL101 (CRY1-selective) and TH301 (CRY2-selective) consisted of a phenylpyrazole moiety (Fig. 1) which formed multiple hydrophobic interactions with residues in hydrophobic region 2, and an H-bond with R358 (Fig. 3). A methoxy group in TH301 engaged in an H-bond with W397. Amide groups in KL101 and TH301 formed an H-bond to S396, and TH301 formed a unique compound-induced interaction between the amide oxygen and Q289 (a typically disordered residue). A *meta*, *para*-dimethylphenyl (KL101) and a cyclopentyl (TH301) group bound at hydrophobic region 1, with a notable difference in the conformation of W399: "Out" in CRY1-KL101, and "in" in CRY1-TH301. The steric bulk of the *meta*, *para*-dimethylphenyl group (KL101) appeared to preclude an "in" conformation of W399, and revealed a potential CRY1-selective mechanism in KL101 binding. The smaller steric bulk of the cyclopentyl induced a stacking interaction with W399, which rotated to an "in" conformation that was significantly different to the "out" conformation in CRY1-apo. We analyzed structural activity relationships (SARs) of KL101 and TH301 derivatives to help characterize their interactions observed in the crystal structures. The *para*-methyl group of KL101 (Fig. 1, blue) and the *meta*-methyl (Fig. 1, red) were important for compound activity. Removal or substitution of these groups resulted in severely reduced activity. Regarding TH301 derivatives, removal or substitution of the methoxy group caused inactivity and reduced activity, respectively, and the cyclopentyl and chlorophenyl

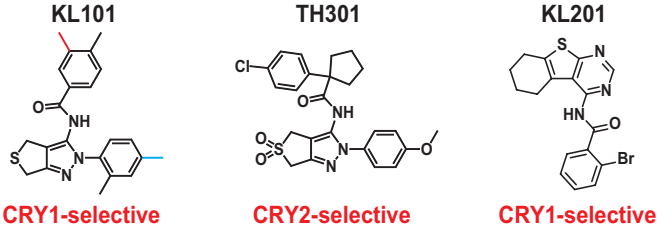

Fig. 1. CRY1 and CRY2 isoform-selective compounds. Important KL101 *para*- and *meta*-methyl groups are colored blue and red, respectively.

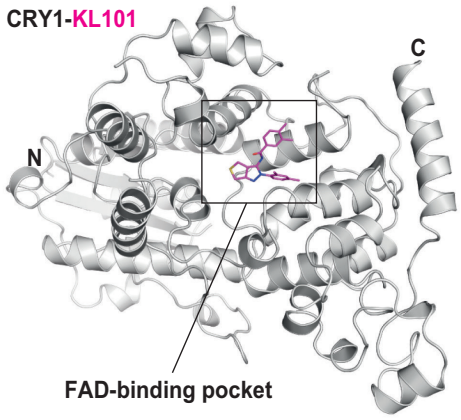

Fig. 2. Overall crystal structure of CRY1 in complex with KL101.

were essential for activity. The CRY1-selective compound KL201 contains a small bromophenyl group that occupied hydrophobic region 2, with the bromine atom facilitating a π-stacking interaction between the phenyl group and W397 (Fig. 3). A heterocyclic thienopyrimidine and cyclohexyl moiety occupied hydrophobic region 1, forming hydrophobic and stacking interactions with aromatic residues. W399 adopted an "out" conformation perhaps due to increased steric bulk compared to TH301. The KL201 amide formed a canonical H-bond with S396, as well as an H-bond with R358. SAR analyses of KL201 derivatives identified an essential role of the bromophenyl in compound activity, whereas the steric bulk of the cyclohexyl (connected to the thienopyrimidine) had a size-dependent effect.

The subsequent determination of the CRY2-TH301 structure (PDB: 6KX8) revealed very similar binding mechanisms to CRY1-TH301 [4]. Comparison of KL101, KL201 and TH301 structures, showed differential organization of two core FAD-binding pocket residues: H355 and W399 in CRY1 (corresponding to H373 and W417 in CRY2). Equivalent residues in *Drosophila* CRY have been implicated in the binding of a CRY C-terminal tail (CCT). We tested the response of compounds on mutant CRY1 and CRY2 proteins and found the CCT to play an important role in isoform selectivity, perhaps by forming interactions with differentially arranged FAD-binding pocket residues. The results of our studies provide a basis to understand how isoform-selective compounds interact with CRYs, and a rationale into the design of compounds with potentially improved selectivity.

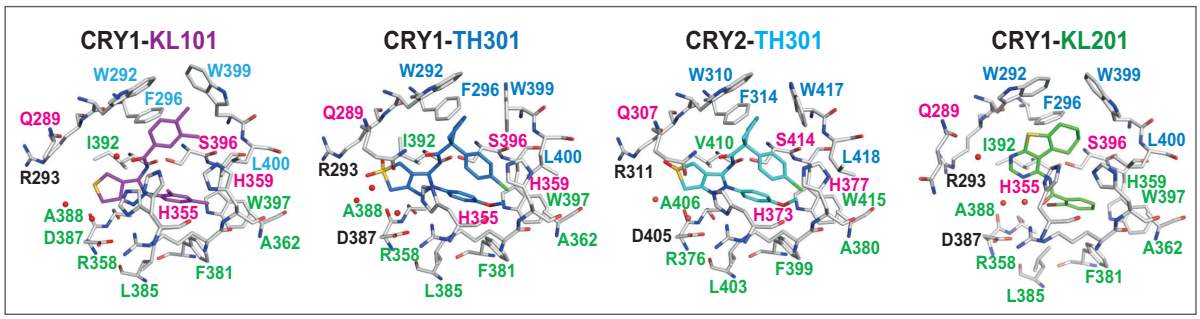

Fig. 3. Interactions of KL101, TH301, and KL201 with CRY1, and TH301 with CRY2. CRY1 H355 and W399 (corresponding to CRY2 H373 and W417) were differentially organized by compounds.

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