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COMMUNICATION

Identification of synthetic inhibitors for the DNA binding of intrinsically disordered circadian clock transcription factors

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Essential components of the human circadian clock, BMAL1 and CLOCK, which are intrinsically disordered transcription factors, were expressed and subjected to a fluorescent *in vitro* binding assay using an E-box DNA fragment. Screening of a chemical library identified 5,8-quinoxalinedione (1), which was found to inhibit binding of the heterodimer BMAL1/CLOCK to E-box at low micromolar concentrations.

Intrinsically disordered proteins and regions (IDPs and IDRs) are unfolded proteins that lack well-defined tertiary structures and are predicted to be present in about 40% of all human proteins.[1] Significant attention has been given to these disordered proteins due to their crucial roles in controlling cell signalling transduction through weak protein–protein interactions.[2-7] Binding to their partner proteins with dissociation constants (K_d) in the micromolar range and lifetimes of seconds triggers transient structural changes in IDPs and IDRs, allowing for dynamic assembling of weak and fast signalling transmissions.[8] Recent studies have shed light on their central role in liquid–liquid phase separation,[9,10] a process that generates non-membrane liquid-like cellular compartments. These include signalling clusters,[11] RNA granules,[12] and nucleoli,[13] demonstrating that reversible assembly of IDPs and IDRs mediates diverse biological processes.[10,14-15]

Because IDPs and IDRs have been implicated in many illnesses, including cancers and neurodegenerative diseases,[2] there is tremendous interest in developing therapeutic agents for this untapped class of drug targets.[1-7] However, their structural flexibility and complex regulatory mechanisms involving posttranslational modifications[16] complicate a rational approach to the development of IDP inhibitors. In addition, because of a tendency for aggregation, the experimental handling of recombinant disordered proteins remains challenging.[17] Recent efforts involving yeast two-hybrid assay,[18] surface plasmon resonance,[19] NMR,[20] thermal shift assay,[21] and virtual screening,[22] have successfully led to several low-molecular-weight inhibitors of c-Myc, EWS-FLI1, p27, NUPR1, and α-synuclein, respectively. In addition, a designed short helical peptide was reported to bind disordered Id proteins.[23] Although these studies have highlighted the promise of IDP-directed drug discovery, examples are still limited.

We envisioned that an *in vitro* fluorescence polarisation (FP) assay using recombinant IDPs might be useful for exploration and functional evaluation of inhibitors via high-throughput

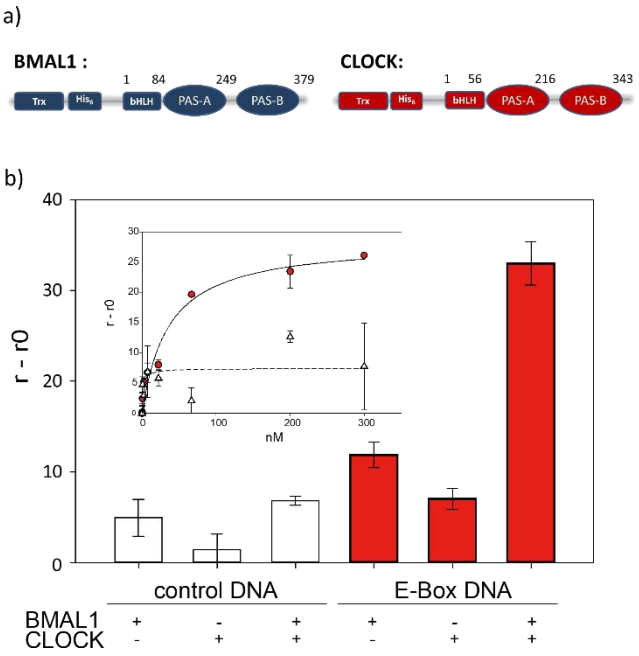

Fig. 1 a) Domain organization of truncated BMAL1 and CLOCK. b) Change in the fluorescence polarisation of FAM-labelled oligo dsDNA fragment (10 nM) upon addition of either BMAL1 or CLOCK protein (500 nM) or an equimolar mixture of BMAL1 and CLOCK; E-box DNA (5'-GCGCGGTCACGTTTTCCACT-3'), control DNA (5'-GCGCGGTGCTAGTTTCCACT-3'). FP was measured in 10 mM Tris HCl (pH 8.0), 150 mM NaCl, and PEG200 (10% v/v) and excited at 488 nm and detected at 526 nm. n=3. Inset: Titration curves of the equimolar mixture of BMAL1 and CLOCK (0–300 nM, solid line with red circles) to dsDNA (10 nM, dash line with white triangles) at 25°C. n = 3. The mean value ±SEM are presented.

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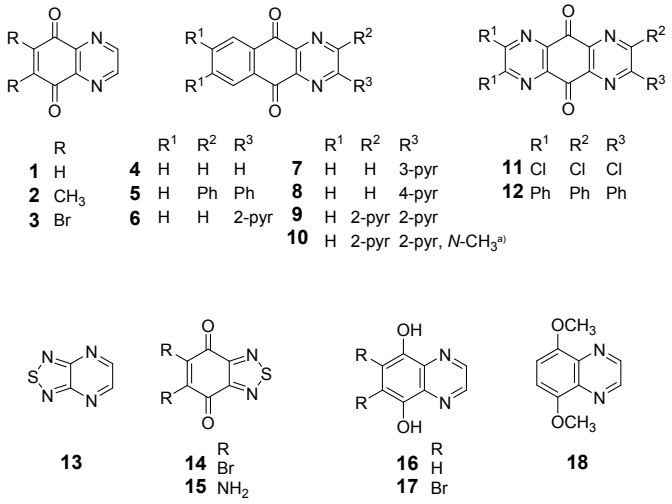

Fig. 2 Chemical structures of the compounds evaluated in this study. a) TfO^- (trifluoromethanesulfonate) salt.

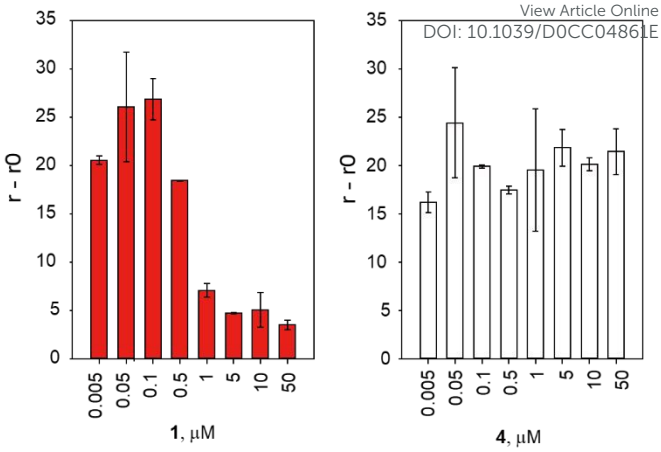

Fig. 3 Concentration-dependent inhibitory activity of **1** and **4** (0.005–50 μM) against binding of BMAL1/CLOCK (500 nM) to 5'-FAM-labelled E-box DNA fragment (10 nM). The reaction mixture was incubated at 25°C for 20 min prior to measurement of the r value. r0: the r value of the oligo DNA alone. n = 3. The mean value ±SEM are presented.

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screening (HTS) of chemical libraries. To this end, we focused on brain and muscle ARNT-like 1 (BMAL1)[24] and circadian locomotor output cycles kaput (CLOCK),[25] the highly disordered circadian transcription activators.[26] These proteins form a heterodimer and bind to E-box regulatory elements in *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes, activating their transcription during daytime. At night, the protein products PER and CRY accumulate, dimerise, translocate to the nucleus, and bind to BMAL1/CLOCK to reduce its transcriptional activity.[27,28] This transcriptional feedback loop is key to the 24-hour circadian clock of mammals. Recent studies have shown that disturbances in the circadian rhythm mechanism are linked to psychiatric disorders[29] and tumorigenesis.[30] Thus, synthetic molecules that can modulate the heterodimer formation and its binding to E-box DNA are desirable for the development of new therapeutic agents. Here, we report a new fluorescent *in vitro* assay platform, in which truncated BMAL1 and CLOCK proteins were prepared and their binding to an E-box DNA fragment evaluated. Screening of a chemical library led to identification of 5,8-quinoxialinedione (**1**), which significantly inhibited binding of BMAL1 and CLOCK to E-box DNA at low micromolar concentrations. The results of a structure–activity relationship study are also reported.

The 69-kDa BMAL1 and 95-kDa CLOCK proteins contain basic helix-loop-helix (bHLH) domains, Per-ARNT-Sim (PAS) domains A and B, and transactivation domains (TADs). The bHLH is an *N*-terminal DNA-binding domain,[31] whereas the PAS domains serve as a versatile sensor in signalling pathways.[32] Previous studies reported that more than 30% of BMAL1 and nearly 60% of CLOCK are predicted to be disordered[26] by computational simulation of disordered probability[33] and that the intrinsically flexible PAS domains adopt various conformations depending on bound ligands or interacting partners.[32,34] Recent X-ray structural analysis of the heterodimer of mouse BMAL1/CLOCK revealed that the dimerisation interface involves not only the leucine zippers in bHLH, but also both the PAS A and B of each protein, engaging contact areas of ~1,950 and 700 Å^2, respectively, through electrostatic and hydrophobic interactions including aromatic residues such as Trp427 of BMAL1 and Trp284 of CLOCK.[34] These structural features clearly indicate that the PAS domains play an essential role in dimer formation, thus in the DNA binding. Therefore, we prepared a model protein of BMAL1 and CLOCK comprising both bHLH and PAS domains, with $(His)_6$ and thioredoxin, a soluble tag, attached at each *N* terminus (Fig. 1a).[35]

Recombinant human BMAL1 (68 kDa) and CLOCK (62 kDa) were expressed in *Escherichia coli* according to a standard method (see ESI†). Each protein was obtained as an inclusion body, which was dissolved in urea, purified by an Ni-NTA column, and dialysed to refold. The protein electrophoresis analysis showed that the resulting BMAL1 was fairly pure, whereas CLOCK contained two unknown protein bands at around 30 kDa (Fig. S1). Further purification attempts were unsuccessful. Thus, we decided to proceed with evaluation of the DNA-binding properties of these proteins.

An *in vitro* FP-based binding assay was performed by incubating protein (500 nM) with a 5'-fluorescein (FAM)-labelled double-stranded oligo DNA fragment (10 nM) possessing either a *Per2* E-box sequence or a mutated sequence as a control.[34] To mimic the crowded environment of the nucleus, 10% v/v PEG200 was added (Fig. S2).[36] While BMAL1, CLCOK, or a 1:1 mixture of the proteins failed to show apparent binding to the mutated dsDNA (Fig. 1b, white bars), an equimolar mixture of the proteins showed a significantly increased FP value compared with each protein alone (Fig. 1b, red bars).[37] These results suggest that the recombinant proteins retain the ability to form the heterodimer, thereby binding to E-box DNA. Furthermore, the titration experiments confirmed that 1:1 binding of an equimolar mixture of BMAL1/CLOCK to E-box DNA had a K_d value of 36 ± 10 nM (Fig. 1b, inset: red circle), which is consistent with a previously reported value (59 ± 10 nM).[34] No binding was observed with the control DNA (Fig. 1b,

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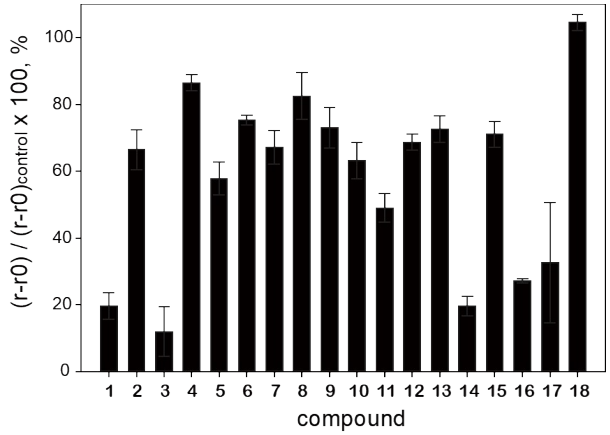

Fig. 4 Inhibitory activities of compounds **1–18** (50 μM) against binding of BMAL1/CLOCK (500 nM) to 5'-FAM-labelled E-box DNA fragment (10 nM). n = 3. The mean value ±SEM are presented.

inset: white triangle), clearly demonstrating the selective binding of the resulting heterodimer to the E-box fragment.

To explore synthetic compounds that are able to disrupt the binding of BMAL1/CLOCK to DNA, a chemical library of heterocycles (1,785 compounds) was screened in a 96-well format (ESI†). The results identified 5,8-quinoxalinedione **1** (Fig. 2) as a hit compound that significantly reduced the FP value (Fig. S3). Compound **1** demonstrated concentration-dependent inhibitory activity against the binding of BMAL1/CLOCK to E-box DNA with an IC_{50} value of about 1 μM (Fig. 3, red bars). Importantly, structurally similar and bulkier benzoquinoxaline-5,10-dione **4** was inactive (Fig. 3, white bars). These results validate the inhibitory activity of **1** for DNA binding.

Next, we wanted to assess the specificity of the inhibitory activity of **1**. To this end, we tested **1** for binding of specificity protein 1-zinc finger transcription factor (Sp1ZF) to a GC box DNA fragment by conducting an electrophoretic mobility-shift (EMSA) assay.[38] The results indicated that **1** did not interfere with the binding of Sp1ZF to the GC box DNA at the concentration range of 1–10 μM (Fig. S4). Thus, the activity of **1** is selective for BMAL1/CLOCK and E-box over Sp1ZF and GC box, suggesting that the activity is likely not caused by nonspecific binding of **1** to a protein or DNA fragment.

The distinct activities observed for **1** and **4** prompted us to perform structure–activity relationship studies of **2**–**15** (Fig. 2). Interestingly, the dimethyl derivative **2** was less active, whereas dibromo-substituted **3** was as potent as **1**, suggesting that the electrophilicity of the quinone moiety is important (Fig. 4). All benzo/pyrazino derivatives **4**–**12** were less active, indicating that addition of the fused ring to the enedione of **1** hampers inhibition. Furthermore, the results showing that the thiadiazolopyrazine derivative **13** was less active than **1** while the dibromo-quinone derivative **14** was equally potent as **3** clearly indicated that the quinone moiety is required for activity. As seen in **15**, replacement of the dibromo groups in **14** by amino groups reduced activity, which is consistent with the trend observed for **1**–**3**, in which electron-deficient compounds with addition/substitution reactivity at the enedione unit (R = H or Br) were more potent.

Because quinones undergo two-electron reduction to afford the corresponding hydroquinones under biological conditions,[39] we suspected that **1** would convert to **16** in solution. Evaluation of **16**–**18** demonstrated that hydroquinones **16** and **17** were as potent as **1**, whereas the dimethylether derivative **18** was inactive (Fig. 4). Notably, HPLC analysis showed that **16** was readily converted to **1** in an aqueous solution at room temperature (Fig. S5). These results indicate that **1** and **16** exist in equilibrium and suggest that either **1** or **16**, or both, may act as the active species.

The PAS A and B domains of BMAL1 and CLOCK are cysteine-rich domains (13 and 8 residues, respectively). Considerable effort has recently focused on the development of PAS-directed inhibitors for treating cancer[40] and neurological disorders,[41] and an allosteric inhibitor for hypoxia-inducible factors 2α (HIF 2α) is currently being tested in a clinical trial.[42] Studies have revealed that this aromatic compound binds to a hydrophobic cleft in the PAS B domain of HIF 2α, inducing a structural alteration and thereby disrupting dimerisation with ARNT protein.[43] The three-dimensional structures of the PAS B domains of HIF 2α and BMAL1 are highly preserved and BMAL1 notably possesses Cys373 near the hydrophobic cleft (Fig. S6). Because nature uses covalently bound quinone cofactors, such as cysteine tryptophylquinone,[44] and the covalent attachment of quinones to cysteine residues has been well studied,[43] it is reasonable to hypothesise that hydrophobic electrophiles may react with these cysteine residues.

We attempted MALDI-TOF analysis of the chemically treated proteins, and the spectra observed for BMAL1 were significantly broadened compared with a non-treated control and CLOCK, suggesting that BMAL1 may be more vulnerable to chemical reaction with **1** than CLOCK (Fig. S7). We then tested whether masking of cysteine residues affects the inhibitory activity of **1**. When BMAL1 and CLOCK were separately treated with iodoacetamide, dialysed, and then combined, the same level of DNA binding as the control was observed, but the inhibitory activity of **1** was significantly reduced (Fig. S8). Furthermore, electrophoresis analysis demonstrated that treatment of **1** causes smearing in the case of BMAL1, but this was less evident for CLOCK (Fig. S9a). This smearing clearly disappeared when proteins were pre-treated with iodoacetamide (Fig. S9b). These results indicate that **1** preferably reacts with nucleophilic residues such as cysteines in BMAL1. Cell-based evaluation of **1** by luciferase reporter assay[45] did not show significant activity at 10 μM against BMAL1/CLOCK interaction, presumably due in part to its low stability in the cellular environment.

In conclusion, a fluorescent-based chemical library screening using recombinant BMAL1 and CLOCK permitted identification of **1** as a potent inhibitor of binding to E-box DNA at low micromolar concentrations. Our results suggest that **1** covalently reacts with protein(s) and may modulate the dimer formation. Although further study is required in order to elucidate the details of the mechanism of action and improve cellular activity, to our knowledge, this is the first example of a synthetic compound that disrupts binding of BMAL1/CLOCK to an E-box DNA fragment. This simple low-molecular-weight heterocycle may serve as a useful scaffold for the development of BMAL1/CLCOK inhibitors.

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Conflicts of interest

There are no conflicts to declare.

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Identification of synthetic inhibitors for the DNA binding of intrinsically disordered circadian clock transcription factors

Table of Contents

Fluorescent-based library screening for the DNA-binding of disordered BMAL1 and CLOCK identified heterocyclic inhibitors which possess low micromolar activity.