Improved Selectivity for DYRK Kinases

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Supporting Information

ABSTRACT: DYRK kinases are involved in alternative pre-mRNA splicing as well as in neurodegenerative disorders such as Alzheimer’s disease and Down Syndrome. In this study, we present the design, synthesis, and biological evaluation of indirubins as DYRK inhibitors with enhanced selectivity. Modifications of the bis-indole included polar or acidic functionalities at positions 5′ and 6′ and a bromine or a trifluoromethyl group at position 7, affording analogues that possess high activity and pronounced specificity. Compound 6i carrying a 5′-carboxylate moiety demonstrated the best inhibitory profile. A novel inverse binding mode, which forms the basis for the improved selectivity, was suggested by molecular modeling and confirmed by determining the crystal structure of DYRK2 in complex with 6i. Structure–activity relationships were further established, including a thermodynamic analysis of binding site water molecules, offering a structural explanation for the selective DYRK inhibition.

KEYWORDS: DYRK inhibitors, indirubins, kinase selectivity, pre-mRNA splicing, docking calculations, WaterMap

The dual-specificity tyrosine phosphorylation-regulated kinase (DYRK)/minibrain family of protein kinases represents a relatively unexplored portion of the human kinome in terms of inhibitor development. The DYRK1a gene is localized in the Down syndrome (DS) critical region of chromosome 21, and its overexpression has been related to the development of DS features.1 DYRK2 regulates p53 to induce apoptosis in response to DNA damage and serves as a scaffold for an E3 ubiquitin ligase complex.2,3 It is mainly expressed during development, but it has been found to be overexpressed in adenocarcinomas of the esophagus and lung.4 DYRK2 has been recently identified as the priming phosphorylation enzyme for oncogenes c-Jun and c-Myc, although the precise role of DYRK2 in cancer needs further investigation.5 As such, DYRKs can be regarded as emerging targets, and selective inhibitors could assist in elucidating the various biochemical mechanisms in which they are implicated.

A limited number of compounds demonstrate selective inhibition toward DYRK kinases, although binding with cdc2-like kinases (CLKs) is frequently observed.6–10 The bis-indole indirubin has been utilized as an interesting lead for the development of potent kinase inhibitors.11–17 Regardless of the notable selectivity of the various indirubin analogues, they all share a common interaction pattern with their target enzyme active site (for example, see PDB structures 1E9H, 2BHE, 1UV5, and 1UNH). Recently, the structural basis of selective inhibition of CLK3, a closely related kinase, by leucettamine-B derivatives was reported.8 Interestingly, the cocrystal structure revealed a significantly different binding mode from the typical ATP-competitive interaction pattern. A similar binding orientation was subsequently reported as the structural basis for selective DYRK1a inhibition by harmine.9 Those studies prompted us to investigate whether selective indirubin analogues targeting the closely related DYRK2 could be designed.

The initial series of inhibitors had minor modifications on the indirubin core that were designed with the objective of generating a noncanonical binding mode and subsequently an...
improved selectivity profile toward DYRKs. A bulky modification such as bromine or trifluoromethyl was introduced at position 7 based on previous studies of the analogue 7BIO (7-bromoindirubin-3′-oxime; Table 1) that showed a minor negative influence on affinity due to a putative steric clash between the inhibitor and the kinase hinge.15 In addition, the removal of a hydrogen bond donor from the indirubin core by methylating the lactam nitrogen at position 1 was explored as a more drastic way to perturb the interactions. Such methylated analogues are totally inactive against CDKs and glycogen synthase kinase 3β (GSK3β) and serve as negative control compounds.13 However, the low water solubility of the bisindole system necessitated the introduction of polar or ionizable functionalities such as carbomethoxy, methylene hydroxy, aldehyde, carboxy, tetrazole, cyano, and oxime groups at positions 5′ and 6′. Moreover, positions 5′ and 6′ represent the least explored sites of this scaffold. Thus, results would contribute toward a systematic exploration of the indirubin SAR landscape. The routes employed for derivative synthesis are summarized in Schemes S1 and S2 in the Supporting Information. All compounds were evaluated against a panel of five kinases (Table 1).

Table 1. IC_{50} Values (μM) of Indirubin Analogues Binding to Five Kinases

| compd | R1   | R2 | R3 | R4 | R5 | R6 | CDK5 | GSK3 | CK1 | DYRK1α | DYRK3 |
|-------|------|----|----|----|----|----|------|------|-----|--------|-------|
| 6BIO  | NOH  | H  | H  | Br | H  | H  | 0.083| 0.005| 1.2 | 1.7    | 2.1   |
| 7BIO  | NOH  | H  | H  | Br | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 5a    | O    | H  | H  | Br | CH3| H  | >10  | >10  | 0.58| >10    | >10   |
| 5b    | O    | COOCH3| H | H  | H  | H  | 3.40 | 1.10 | 1.40| 2.00   | 1.80  |
| 5c    | O    | COOH | H  | H  | H  | H  | 10   | 25   | >10 | >10    | >10   |
| 5d    | O    | COOCH3| H | H  | Br | H  | >10  | >10  | >10 | >10    | >10   |
| 5e    | O    | COOCH3| H | H  | CF3| H  | >10  | >10  | >10 | >10    | >10   |
| 5f    | O    | CN  | H  | H  | CF3| H  | >10  | >10  | >10 | >10    | >10   |
| 5g    | O    | tetrazole| H | H  | CF3| H  | >10  | >10  | >10 | >10    | >10   |
| 5h    | O    | COOCH3| H | H  | Br | CH3| >10  | >10  | >10 | >10    | >10   |
| 5i    | O    | H   | COOH | H  | H  | H  | >10  | >10  | 1.70| 2.60   | 1.10  |
| 5j    | O    | H   | COOCH3| H | Br | H  | >10  | >10  | >10 | >10    | >10   |
| 5k    | O    | H   | COOCH3| H | CF3| H  | >10  | >10  | >10 | >10    | >10   |
| 5l    | O    | H   | COOH | H  | Br | H  | >10  | >10  | 9.30| 1.30   | 1.30  |
| 5m    | O    | H   | COOH | H  | CF3| H  | >10  | >10  | >10 | >10    | >10   |
| 5n    | O    | CH2OH| H  | H  | Br | H  | >10  | >10  | >10 | >10    | >10   |
| 5o    | O    | CH==NOH| H | H  | Br | CH3| 0.20 | 0.20 | 0.80| 1.10   | ND    |
| 6a    | NOH  | H  | H  | H  | CF3| H  | >10  | >10  | >10 | >10    | >10   |
| 6b    | NOH  | H  | H  | Br | H  | CH3| >10  | >10  | >10 | >10    | >10   |
| 6c    | NOH  | H  | H  | H  | H  | H  | 0.52 | 0.40 | 0.30| >10    | >1 |
| 6d    | NOH  | H  | H  | H  | H  | H  | 0.53 | 0.07 | 0.42| 0.31   | 0.35  |
| 6e    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6f    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6g    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6h    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6i    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6j    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6k    | NOH  | H  | H  | H  | H  | H  | >10  | >10  | >10 | >10    | >10   |
| 6l    | NOH  | H  | H  | H  | H  | H  | 0.2 | >10  | >10  | 0.13  | 0.22  |
| 6m    | NOH  | H  | H  | H  | H  | H  | 3.30 | 6.10 | 0.80| 0.63   | 1.90  |
| 6n    | NOH  | H  | H  | COOCH3| H | H  | >10  | >10  | >10 | >10    | >10   |
| 6o    | NOH  | H  | H  | COOCH3| H | CF3| H  | 1.7  | 0.1 | 5.0   | 0.11  |
| 6p    | NOH  | H  | H  | COOH | H  | H  | >10  | >10  | 7.40| 0.60   | 1.70  |
| 6q    | NOH  | H  | H  | COOH | H  | CF3| H  | 3.1  | >10  | >10  | 7.0   | >10   |
| 6r    | NOH  | CH2OH| H | H  | Br | H  | 0.40 | 0.40 | >10 | 1.60   | ND    |
| 6s    | NOH  | CH==NOH| H | H  | Br | H  | 0.60 | 0.60 | 7.40| 2.10   | ND    |

*aAll measurements were determined in triplicate, and mean values are reported. The standard error of determination in all cases does not exceed 10%. The K_{m} for ATP with DYRK2 is 7.7 μM."
for an acidic substitution to maintain DYRK inhibition. The presence of a 3’-oxime moiety generally improved potency with minor effects on selectivity. Finally, the influence of methyl-capping N1 was unfavorable for DYRK activity (7BIO as compared to 6b). Inspection of the SAR landscape suggested that the combination of the 7-bromo, 3’-oxime, and S’-carboxy substitutions would be optimal for DYRK selective inhibition. The corresponding 7-bromo-S’-carboxyindirubin-3’ oxime (6i) proved to be a potent inhibitor of DYRK1a and DYRK2 (IC_{50} values 210 and 130 nM, respectively). DYRK activity was accompanied by a marked selectivity, as 6i was inactive toward cyclin-dependent kinase 5 (CDK5), GSK3β, and casein kinase 1 (CK1). Interestingly, the introduction of the bulky bromine in position 7 enhanced selective DYRK inhibition only when combined with the carboxylate substitution, which was evident comparing 6d and 6i. Quite unexpectedly, the carboxylate bioisostere tetrazole compounds (5g and 6h) were inactive. Moreover, analogues where the carboxylate moiety was replaced by a polar but neutral oxime group (may adopt a binding mode that is significantly different from the experimentally established indirubin-kinase binding mode). Two distinct modes were observed in the docking studies, distinguished by a 180° flip of the indirubin core with respect to either its primary or secondary axis (Figures S1A and S1B in the Supporting Information, respectively). In both cases, the usually observed hydrogen bond triplet between the indirubin pharmaphore and the kinase hinge was either disrupted (mode I) or inverted (mode II). In both cases, the S’-carboxyamide formed a salt bridge with either the Lys165 (mode I) or the catalytic Lys178 (mode II).

To confirm the inverse binding mode, we determined the crystal structure of 6i bound to DYRK2 at a resolution of 2.28 Å (PDB ID 3KVW). The structure (Figures 1A and S2 in the Supporting Information) revealed that the binding mode was indeed inverted with respect to the geometry of all previously reported indirubin-kinase complexes and consistent with the predicted binding mode II. The predicted salt bridge anchoring the anionic S’-carboxylate to the side chain of Lys178 was observed in the crystal structure. An additional hydrogen bond was present between the S’-carboxylate and the NH backbone of Asp295. Furthermore, three water molecules participated in the hydrogen bond network by bridging the inhibitor carboxylate and Lys178 ammonium groups to the side chains of Asp295 and Glu193. At the hinge, two hydrogen bonds were formed between the lactam carbonyl of 6i and the NH backbone of Leu231 (n + 3 from the gatekeeper) and between the lactam nitrogen of the inhibitor and the backbone carbonyl of the same residue. The solvent-accessible surface area buried upon binding was 417 Å² (229.4 Å² nonpolar) for the ligand and 254.8 Å² (222.8 Å² nonpolar) for the protein, underscoring the hydrophobic nature of this binding site. The bromine at position 7 was almost half-exposed to the solvent. Encouragingly, docking calculations using Glide with the crystal structure of DYRK2 predicted mode II as the best pose of 6i. Interestingly, docking in the crystal structure of DYRK2 showed that 7BIO would also adopt the inverted mode, while 6BIO was predicted to bind in the usual indirubin-kinase orientation, further supporting the putative role of the flipped binding mode in determining selectivity.

A superposition of the inhibitor cocrystal structure of DYRK2 with that of the apoenzyme (PDB ID 3K2L, manuscript in preparation) revealed that the S’-carboxylate moiety of 6i overlaps with a crystallographic water molecule appearing in the active site of the apoenzyme and interacting with Lys178 (Figure 1A). To understand the thermodynamic characteristics of the system and that particular water molecule, calculations were run with the WaterMap program, which combines molecular dynamics, solvent clustering, and statistical thermodynamics to assess the enthalpy, entropy, and free energy. The 7BIO-water displacement site is colored by free energy, with red being the most unfavorable. The hydration site adjacent to Lys178 (ΔG = 1.6 kcal/mol) is shown as a large semitransparent blue sphere, and the crystallographic water is shown as a small opaque blue sphere. Compound 6i is superimposed to show the displacement of the hydration sites. The view has been rotated to improve visibility of the key water.
energy of water “hydration sites”.

WaterMap has been successfully applied to study selectivity in kinases and PDZ domains, as well as several studies of understanding binding affinity and SAR series.

Figure 1B shows the predicted WaterMap hydration sites in the apoenzyme. The site near Lys178 is in near-perfect accordance with the crystallographic water. This hydration site has a thermodynamic profile making the total free energy slightly worse than bulk water (+1.5 kcal/mol). While the site is highly unfavorable entropically (+3.4 kcal/mol) due to the localization around Lys178, it is enthalpically favorable (−1.9 kcal/mol) due to the interactions with Lys178. Displacement of this hydration site by a ligand functional group that also replaces the water interactions is predicted to improve potency, in agreement with the experimental 10-fold affinity difference between 7BIO and its carboxylated analogue 6i.

This shows the importance of including water molecules in the analysis and assessment of binding energies.

The selectivity of 6i and 6l toward DYRKs prompted us to investigate their inhibition profile over a broader panel of protein kinases. Compounds 6i and 6l along with 6BIO and 7BIO were assayed in vitro against a panel of 42 kinases (Table S1 in the Supporting Information). Compounds 6i, 6l, and 7BIO were inactive against all assayed kinases. In contrast, 6BIO showed a broad inhibitory profile. Apart from its well-established target, GSK-3β, 6BIO was weakly active toward the receptor tyrosine kinases fibroblast growth factor receptor 3 (FGFR3) and platelet-derived growth factor receptor (PDGFR) and showed a notable inhibition of proto-oncogene tyrosine-protein kinase receptor (RET). Although the crystal structure of 6l-DYRK2 was not determined, the similar specificity profile of 6i and 6l suggests that the presence of the N1-methyl does not induce an alternative binding mode.

To conclude, the combined presence of a bromine substitution at position 7 and an acidic functionality at position 5′ of the indirubin scaffold turns the nonselective bis-indole indirubin into a potent and selective DYRK inhibitor. Structural insights offered by docking, crystallographic studies, and solvent thermodynamic calculations suggest that selective DYRK inhibition can be attributed to a nonstandard kinase binding mode where the indirubin core adopts an inverted pose. Data indicate that the driving force for the inverted binding orientation is the occurrence of a steric clash between the bulky halogen of position 7 and the kinase hinge. The acidic substitution at position 5′ further enhances activity by displacing an unstable water and establishing a salt bridge between the 5′-carboxylate and the Lys178. The need for the simultaneous substitutions at position 5′ and 7 was evident by the fact that neither of the two substitutions alone resulted in the desired activity-selectivity profile. As a result, the desired selectivity profile was achieved with an ATP-competitive but not ATP-mimetic inhibitor using a variety of rational design strategies.

## ASSOCIATED CONTENT

### Supporting Information

Detailed information about compound synthesis, computational methodology, protein production, crystalization, data collection, refinement statistics, and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

## Accession Codes

The 6l-DYRK2 structure has been deposited to the PDB with accession code 3KVW.

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These authors contributed equally.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

FGFR3, fibroblast growth factor receptor 3; PDGFR, platelet-derived growth factor receptor; RET, proto-oncogene tyrosine-protein kinase receptor; CDK5, cyclin-dependent kinase 5; GSK3β, glycogen synthase kinase 3β; CK1, casein kinase 1

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