

Reviewer 4:

Comment #1: One of the primary concerns with this paper is the lack of clarity in defining the scope and objectives of the study. The authors have not clearly outlined the specific research questions or goals they aimed to address. As a result, the paper appears to be a collection of loosely connected ideas rather than a cohesive discussion.

Reply: Thank you, we admit this criticism. In the revised version we outlined the study in *Introduction* (page 3). In *Discussion* we re-iterated the formulations of the goal and major results (page 15). Namely, the pyrido[3,4-g]quinazoline chemotype provides vast opportunities for the search of active compounds specifically as DYRK/CLK antagonists. We were interested in disclosing the mechanism whereby one single structural substitution can dramatically change target selection and therefore cellular effects. To analyze this problem, it is important to compare close derivatives. We selected **1** and **2** out of a series of original pyrido[3,4-g]quinazolines synthesized and published by our group. Compounds **1** and **2** differed only in one chemical function at the periphery of the heterocyclic core. Thus, we aimed at identifying the role of a particular structural unit, and this role appeared to be critical. Such an analysis is especially important for rational design of isoform-specific inhibitors within the enzyme families.

Comment #2: Another major issue is the lack of rigorous methodology. The paper does not provide any information regarding the systematic studies used to perform this computation.

Reply: Historically, the key experiments in the study were *in vitro* kinase assays that demonstrated the ‘reshaped’ inhibitory profiles of DYRK/CLK isoforms for structurally close compounds **1** and **2**. With this information in mind we turned to theoretical calculations to understand how one single substitution can influence the target selection. Alone, the physico-chemical and electrochemical characteristics of **1** and **2** did not explain the differential isoform preferences. We next explored the *in silico* interactions of **1** and **2** with DYRK3 and CLK4, respectively, the targets identified in *in vitro* kinase assays. We used PDB models of each enzyme in complexes with their reference ligands. Methodologically, we went from the initial docking to molecular dynamics simulations and finally to the clustering of target-ligand interactions. Our sequential analysis unveiled non-trivial peculiarities of complex formation by **1** and **2** with their respective targets. Once **2** was identified as a CLK4 binder, we asked whether the calculated peculiarities were translatable into biochemical characteristics, that is, RNA splicing regulation and cytotoxicity for patient-derived GBM neurospheres. Previously our group has validated these models for the pyrido[3,4-g]quinazoline chemotype (ref.22). The experimental analysis demonstrated numerous consequences of **2**-CLK4 interaction on the expression of individual genes; these alterations were causatively associated with glioblastoma cell death. Overall, we combined the *in silico* methodologies with cell based and molecular investigation, a strategy widely used in modern medicinal chemistry.

In the revised text we summarized these systematic approaches on pp. 3, 15, 16. Sections 2.5 and 2.6 (new) were edited. Also, as commented by other reviewers, we added more experimental and calculation evidence (new Figures 5B,C on pp.12,13 and Figure S5, RMSF).

Comment #3: The paper also suffers from a lack of critical analysis and evaluation of the reviewed technologies. Merely describing and summarizing the existing technologies without offering any meaningful insights or comparisons diminishes the value of the work. The paper should have critically examined the strengths, limitations, and potential areas of improvement for each approach.

We included a new section *Limitations* (page 16). Therein we state that, regardless of the detailed *in silico* analysis, the X-ray structures of drug-target complexes are worthy for an unambiguous judgment; also, **1** and **2** might have other splicing-related (and perhaps -unrelated) targets. Indeed, intracellular interactions of **2** are multifaceted: RNAseq studies identified genome-wide changes of expression of multiple genes with critical functions for tumor cell biology, namely, DNA damage response, p53 signaling, and transcription. In the present study these changes were translated into the cytotoxicity for patient-derived GBM neurospheres, demonstrating the practical applicability of the pyrido[3,4-g]quinazolines. It is the subject of future research to uncover how pharmacological targeting of splicing is multiplied into (or manifested as) a plethora of cytotoxic phenomena. On the one hand, such a manifold net effect is

therapeutically beneficial for intractable tumors including GBM. On the other hand, caution is needed when evaluating the clinical perspective: the fundamental role of RNA splicing may underlie potential side effects. From this viewpoint the differential potency of **1** and **2** due to single substitution indicates that a pipeline of derivatives with desired characteristics can be generated based on the pyrido[3,4-g]quinazoline scaffold.

Comment #4: The overall organization and structure of the paper are inadequate. The flow of ideas is unclear, and there is a lack of coherence between sections. The paper should have presented a clear introduction, outlined the main themes or categories of technologies, and provided a concise summary or conclusion to tie the information together.

Reply: Our explanations are given in the replies to **Comments 1-3**. The flow of ideas was the following.

1. *General goal:* to explore the pyrido[3,4-g]quinazoline scaffold as a source of RNA splicing inhibitors with therapeutic potential against GBM, an aggressive brain tumor.
2. *Methodology:* a detailed *in silico* and experimental investigation of the significance of individual chemical moiety in pyrido[3,4-g]quinazolines, an investigational chemotype for GBM treatment.
 - 2a. Selection of compounds **1** and **2** as the original pyrido[3,4-g]quinazoline derivatives with the published inhibitory activities against individual splicing kinases. The selection was based on the fact that **1** and **2** differed in one single function. This allows for identifying the role of the individual structural unit within the chemotype.
 - 2b. Investigation of the inhibitory profiles of **1** and **2** against all isoforms of DYRK and CLK families of splicing kinases. Identification of differential isoform preference for **1** and **2** (target selection).
3. *Mechanistic studies*
 - 3a. A detailed *in silico* analysis of interactions of **1** and **2** with their preferred targets DYRK3 and CLK4, respectively. Identification of structural reasons for differential drug-target complex formation. Validation of **2** as a CLK4 binder for RNA splicing deregulation.
 - 3b. Experimental proof of **2**-CLK4 complexation in splicing deregulation and death of patient-derived GBM neurospheres.

Comment #5: Spacing issues. Correct such issues throughout the manuscript.

Reply: Could be that the spacing was damaged during the submission process. We have double checked spacing in the revised text.

Comment #6: Abbreviations must be explained next to the first mention or wherever appropriate to avoid reader struggle understanding the terminology. We have identified technical jargons which must be avoided by the authors.

Comment #7: Unrequired abbreviations otherwise should be discarded.

Reply: We revised the text thoroughly and corrected a few awkward terms.