We appreciate the feedback received from the Reviewers, plus to the work of the editor. Here we present our answers to comments and suggestions provided, colored in blue; insertions with new text are marked in italics.

Reviewer 1:

We are grateful for the time invested by the Reviewer in the corrections and suggestions made in the manuscript. Please note that the page citations correspond to the tracked version of the manuscript.

Apart from the suggested corrections applied to the manuscript, we also added modifications based on the proposed changes by the other reviewers:

- We added 2D structures of the compounds shown on the western blot results on Figure 4, so we do not miss any structural information.
- We modified the last paragraph from section 2.4 (line 285), in which we hypothesize the reason why Drug73 has a higher affinity than Drug120 and DEL-22379.

This study integrates scaffold hopping, virtual screening, and in vitro assays to identify new ERK2 dimerization inhibitors, highlighting Drug73 and Drug120 as promising analogs of DEL-22379 with better predicted pharmacokinetic and toxicity profiles. The work is original and relevant, presenting a coherent in silico-experimental workflow. However, the study would benefit from more rigorous docking validation, quantitative analysis of electrophoresis data, and additional biological assays to confirm functional relevance. Overall, it is a well-structured and valuable contribution that requires moderate methodological strengthening before publication.

Major suggestions:

Comment 1: The manuscript would benefit from a clearer explanation of the scaffold hopping strategy, including chemical rationale, diversity metrics, and how analogs were prioritized beyond H176 distance filtering.

Response 1: We appreciate the comment. We have modified the 2.1 section to provide a better and clearer explanation of the scaffold hopping procedure. The strategy we followed in this work was to obtain new structures by replacing, in some cases, the indoline-2-one group, the piperidine group, and/or the indole groups. This way, we could explore how these changes on the structure could affect the binding affinity with ERK2. Additionally, we have also added a Tanimoto-based similarity analysis, therefore have checked how similar the new compounds are to DEL-22379.

The modified paragraph would be the following one (Section 2.1. Scaffold hopping, lines 147-156):

"Scaffold hopping technique is used to generate new chemical compounds from a known effective molecule by changing its chemical scaffolds but maintaining its function [18, 19]. This way, hit compounds can be optimized by replacing scaffolds that can improve properties such as ADMET and bioactivity, but maintaining essential functional groups for interaction with the target. In this case, DEL-22379 already presented inhibitory effects of ERK2 dimerization, thus we applied this technique in order to optimize its structure. As a result, we obtained 536 derivative compounds, in which we can differentiate compounds that substitute the indoline-2-one group, others that change the piperidine group, and some that replace the indole core (Figure 2)."

Note that we have also reordered the section "2.2 Virtual screening", including a new figure S1, to improve clarity of the results and include the information regarding molecular similarity (new information added in lines 207-218).

"The similarity of these compounds with respect to DEL-22379 was measured using the Tanimoto index. The similarity scores of most of these new structures are above 0.5, which denote a high similarity with respect to the original structure

(Figure S1). This high similarity is potentially produced due to the preservation of the main structural cores of DEL-22379 and just modifying some regions of the molecule. "

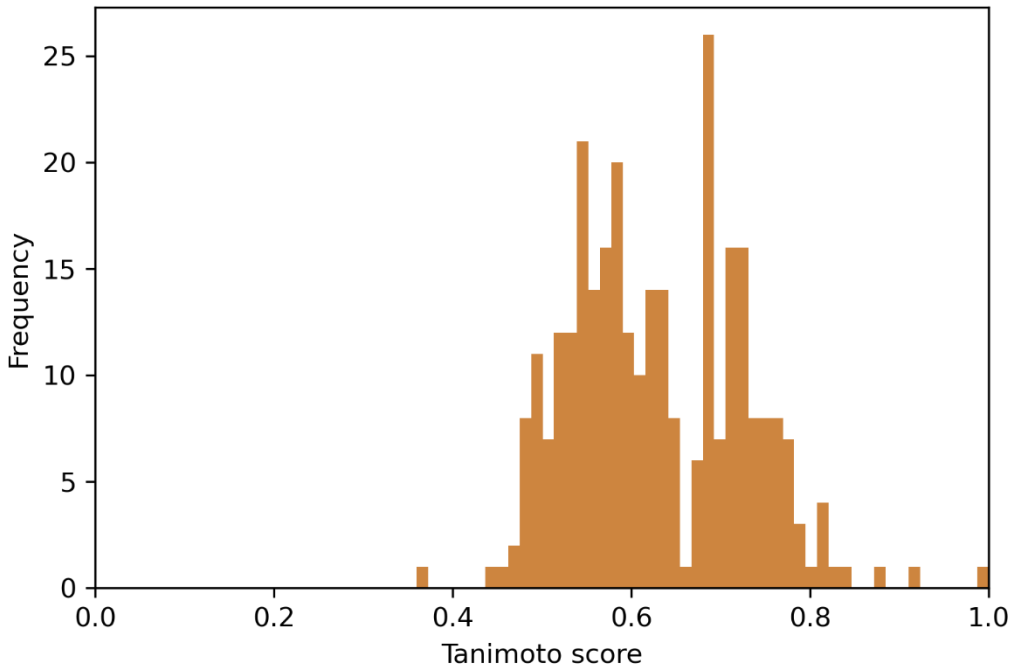


Figure S1. Similarity score distribution along the compounds generated by scaffold hopping.

We also added to Material and methods section (section 4.1. Scaffold hopping and virtual screening; lines 542-543) how we measured the similarity among the generated structures.

"For scaffold hopping, we used a web tool belonging to Mcule (https://mcule.com/, *accessed on May 2021*) *[35]*. The scaffold hopping technique is used to obtain derivative chemical compounds of a primary structure by changing functional groups... Virtual screening was carried out with YASARA software (v.21.6.17) [*36-38*]. Once the distance was obtained, the dataset was filtered. All compounds that had a distance higher than 5Å were removed. *We calculated MACCS fingerprint to evaluate the Tanimoto similarity scores using RDKit python package (v.2022.03.5) [39]*."

We also want to add that we did not use other requirements beyond H176 distance and docking scores for filtering our dataset before experimental validation. As not all the compounds were commercially available, we tested the ones that could be acquired, which is mentioned in line 222.

Comment 2: The molecular docking protocol requires more methodological rigor details such as grid dimensions, scoring function validation, and reproducibility assessment (e.g., redocking of DEL-22379 or RMSD comparison) should be included.

Response 2: We appreciate the reviewer's suggestion. In the revised manuscript, we tried to convey the molecular docking settings information on the material and methods section, including more information such as cell simulation dimensions. We have modified this to add coordinates information to make it clearer (lines 563-565).

"... We directed the docking simulation to the dimerization interface residues (H176, F181, F329, E332, L333, D335, L336, P337, E339, K340, and E343), *with a cubic simulation cell of 5Å around the dimerization residues (grid size of 38.97 Å in each axis) and setting the dimerization interface residues as flexible. Fifty* poses were generated..."

Regarding reproducibility, we acknowledge the reviewer's point and have added the grid box information to the Materials and Methods section. As of our understanding, with this information it is possible to reproduce our calculation. Moreover, as the ERK2–DEL-22379 complex has not been experimentally resolved, a direct redocking validation is not feasible. Nevertheless, our docking workflow is based on AutoDock Vina, a well-established and extensively validated tool, which uses its predefined scoring function, and which has demonstrated robust reproducibility across numerous protein–ligand systems, as reported by Vieira et al*. In our case, reproducibility was assessed by analyzing the RMSD and binding affinity consistency among the 50 generated poses. Molecules were clustered at an RMSD threshold of ≤ 3 Å, and the variation in mean binding affinity between clusters remained within ~1 kcal/mol. Despite minor differences in the spatial orientation of binding poses, they exhibit overall coherence, indicating stable and reproducible docking results.

*Vieira, T.F.; Sousa, S.F. Comparing AutoDock and Vina in Ligand/Decoy Discrimination for Virtual Screening. Appl. Sci. 2019, 9, 4538. https://doi.org/10.3390/app9214538

Comment 3: The experimental validation (native PAGE) lacks quantitative analysis.

Response 3: We thank the reviewer for this appreciation. We have modified the Figure 4, so the western blot intensity analysis is also included. We show the normalized monomer/dimer and normalized pERK/ERK ratios regarding intensity of control results. We have also modified this figure to add some changes suggested by other reviewers, such as the 2D structure of the shown compounds from the western blot results. Figure caption has been adapted, accordingly (lines 258-263).

"Figure 4. Identification of compounds that effectively inhibit ERK2 dimer formation without affecting ERK2 phosphorylation. *(a).* Native PAGE and SDS-PAGE of different tested compounds. HEK293 cells were stimulated with EGF (100 ng/ml for 10 min) and treated with the different compounds or DEL-22379 as the positive control (10 μM for 30 min). *Protein levels were quantified from western blots, and ratios are shown in the figures (b). 2D structure of shown compounds."*

We have also modified the 4.2 section (materials and methods), in which we added the software used for measuring band intensity (lines 549-550).

"... while SDS-PAGE was used to evaluate ERK phosphorylation status. *For the quantitative analysis of band intensity, we used FIJI software (v.17.02.0) [41], using mean gray value.*"

Moreover, we have also modified the Results section 2.3. Experimental evaluation (lines 264-273) to present these results:

"In cells treated with DEL-22379 only the lower mobility band *was evident*, indicating that only *ERK2* monomers are *present (monomer/dimer ratio of 5.36)*. The same happens *in the case of cells treated with* Drug21, Drug73 and Drug 120, *with a monomer/dimer ratio of 5.42, 4.6 and 4.25, respectively*. However, in the case of Drug21, phosphorylation *was impaired, as can be seen with the pERK/ERK ratio of 0.25, which is half of the value of DEL-22379 ratio*, indicating that its inhibitory effect on ERK dimerization is a *potential* consequence of preventing *ERK2* phosphorylation. Contrarily, Drug73 and Drug120 preserve ERK2 phosphorylation, *with ratios of 0.7 and 0.8,*

respectively, which were higher than DEL-22379, demonstrating that their inhibitory effect on dimerization is exerted by other means."

Comment 4: The functional significance of inhibition should be further validated, for example by testing cell migration or viability assays, to connect biochemical inhibition with phenotypic outcomes.

Response 4: We appreciate this comment and fully agree that assessing the functional consequences of inhibition would provide further insight into the biological relevance of the candidate compounds. However, the main goal of the present study was to elucidate the molecular mechanism of action of DEL-22379 derivatives and to characterize their biochemical interactions with ERK2 and related targets. The evaluation of downstream phenotypic responses, such as changes in cell migration, proliferation, or viability, would require dedicated in-vitro and in-vivo experiments that fall beyond the current scope and resources of this work. This study has allowed us to identify several promising derivatives that will be further investigated in future experimental assays, which are currently at first stages of development. We considered it important to first publish the computational and mechanistic aspects to establish a solid basis before expanding into the biological characterization of the most suitable candidates.

Nevertheless, we fully recognize this as a limitation of the present study. We have therefore added a sentence in the Discussion section (lines 518-524) to explicitly acknowledge that, although our results demonstrate strong mechanistic inhibition at the molecular level, further validation through phenotypic assays will be necessary to confirm the functional significance of these findings in a cellular context. We plan to address this aspect in future studies focused on the phenotypic and therapeutic implications of these compounds.

"*This study focuses primarily on the molecular and biochemical characterization of ERK2 dimerization inhibitors and does not assess the downstream phenotypic effects resulting from ERK2 inhibition. Evaluating such functional consequences, for example, on cell migration, proliferation, or viability, would help to better guide the design and optimization of future ERK2 dimerization inhibitors. Future studies will address these aspects to confirm the biological and therapeutic significance of the candidate compounds.*"

Comment 5: The ADMET predictions, while informative, should be cautiously interpreted and, if possible, supported by at least one experimental cytotoxicity or solubility assay.

Response 5: Thanks to the reviewer for this suggestion. For the ADMET prediction, we used different tools. For ProtoPRED tool, concretely, we used models from the ProtoADME module (i.e., bioavailability 20%, bioavailability 30%, Caco-2, HIA) and from the ProtoTOX module (i.e., neurotoxicity, acute oral toxicity). For Deeppk, we have employed two QSAR models, namely Acute toxicity in rat [LD50] and Chronic toxicity in rat (LOAEL). All these models have been developed following the OECD principles and guidelines to ensure regulatory validity (GUIDANCE DOCUMENT ON THE VALIDATION OF (QUANTITATIVE)STRUCTURE-ACTIVITY RELATIONSHIPS [(Q)SAR] MODELS[1]; (Q)SAR Assessment Framework: Guidance for the regulatory assessment of (Quantitative) Structure Activity Relationship models, predictions, and results based on multiple predictions). Moreover, regarding the metrics of the employed models, these presented values above 0.6 in accuracy, sensitivity and specificity in the validation set for classification models as bioavailability 20 and 30%, HIA, and acute oral toxicity (300 mg/kg); and above 0.7 for regression models such as Caco-2 and neurotoxicity, a common value used as threshold to consider acceptable.

To mark this better in the manuscript, we have modified the text as follows and include an additional Table S2, which contains the links with relevant information of the used models (lines 395-400):

"... and Deep-PK for acute and chronic rat toxicity. *We used these tools based on quantitative structure-acivity relationship (QSAR) (models implemented in ProtoPRED have been developed following the OECD principles) that present values above 0.6 in accuracy, sensitivity and specificity in the validation set for classification models; and above 0.7 for regression models. The metric of the models can be found in the corresponding QSAR Model Reporting Format (QMRF) documents or available information at Table S2*. As can be seen in Table 1, all candidate compounds satisfied Lipinski's rule of five, indicating no violations..."

We agree that the lack of characterization of the physchem properties and the toxicological profile of the compounds is a limitation of the study, but we are working in further characterization of the compounds and these properties will be considered in further investigations, as the scope of the present manuscript was to emphasize the usefulness of computational tools to identify potential candidates an accelerate the drug discovery process.

[1]https://one.oecd.org/document/ENV/JM/MONO(2007)2/en/pdf
[2]https://www.oecd.org/content/dam/oecd/en/publications/reports/2023/11/q-sar-assessment-framework-guidance-for-the-regulatory-assessment-of-quantitative-structure-activity-relationship-models-and-predictions_9b064821/d96118f6-en.pdf

Comment 6: Figures 5-7 could be enhanced with clearer residue labels and consistent color coding of ligands and binding sites to improve interpretability.
Response 6: We have considered the reviewer's advice and we have changed the color coding of the Figures. We have improved the images by unifying the ligands' color coding, maintaining the same colors in Figure 5 and Figure 7 (DEL-22379: cyan; Drug73: yellow; Drug120: red). We also made labels more visual and tried to make clearer which are the dimerization interface residues. (Figure 7; located after last paragraph of section 2.4). We hope this figure is now more comprehensible. We also modified figure caption (lines 383-387):

"Figure 7. In silico representation of the best binding mode for each ligand in complex with ERK2. Dimerization interface represented in orange; *DEL-22379 represented in cyan; Drug73 in yellow; Drug120 represented in red.* (a) All inhibitors in complex with ERK2; (b). DEL-22379; (c). Drug73; (d). Drug120. Visualization on ChimeraX.

Comment 7: Minor suggestions:

1. Several typographical and formatting inconsistencies should be corrected
2. Abbreviations such as HIA, LOAEL, and P-gp should be defined on first use in the main text.
3. The Results section could be shortened by merging overlapping descriptions of docking and ADMET predictions.

We thank the Reviewer for these remarks. We have revised the manuscript to check any of the inconsistencies that could be present on the text, and we have fixed them. We have also checked all the abbreviations that appear in the manuscript, so we reduce confusions related to their meaning.