Faculty of Medicine
Department of Anatomy, Cell Biology and Physiological Sciences

Assaad Eid, DSc, HDR, EMBA
Professor & Interim Chair

Marwan El-Sabban, PhD
Professor & Vice Chair

Wassim Abou Kheir, PhD
Associate Professor

Elie D. Al-Chaer, PhD, JD
Professor

Kassem Barada, MD
Professor

Ali Bazarbachi, MD, PhD
Professor

Anwar Bikhazi, PhD
Professor Emeritus

Georges Daoud, PhD
Associate Professor

Abdo Jurjus, PhD
Professor

Nada B. Lawand, PhD
Assistant Professor

Hala Gali-Muhtasib
Professor

Fadi Mourad, MD
Professor

Rihab Nasr, PhD
Associate Professor

Dany Nassar, MD, PhD
Assistant Professor

Makram Obeid, MD
Assistant Professor

Raya Saab, MD
Associate Professor

Youssef Zeidan, MD, PhD
Associate Professor

Talar Terzian
Senior Office Assistant

June 2022

Lian-Sheng Ma,
President and Company Editor-in-Chief
Baishideng Publishing Group Co.
World Journal of Gastroenterology

Dear Doctor Lian-Sheng Ma,

On behalf of my colleagues, I would like to submit our revised manuscript entitled **"The novel therapeutic Diiminoquinone exhibits anticancer effects on human colorectal cancer cells in 2D and 3D in vitro models"**. We have provided a point-by-point response addressing reviewers' comments and those of the editorial office. We uploaded the manuscript with tracked-changes as a supplementary material. We also uploaded the original figures as a PowerPoint file along with the tables as a Word file. We believe that our paper is now in a form that is acceptable, and we look forward to your positive response.

Sincerely Yours,

Wassim Abou-Kheir, PhD
Associate Professor
Department of Anatomy, Cell Biology and Physiological Sciences
Faculty of Medicine
American University of Beirut
Bliss Street, DTS Bldg, Room 116-B
Beirut-Lebanon, 1107-2020
Tel: 961-1-350000, Ext. 4778
Mobile: 961-76994308
Fax: 961-1-744464
E-mail: wa12@aub.edu.lb

Point-by-point response

Reviewers' comments:

Reviewer #1:

Scientific Quality: Grade B (Very good)

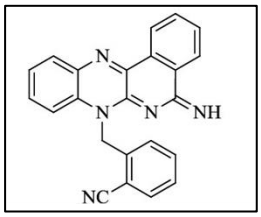
Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: Authors investigated the anticancer activity of DIQ against CRC cell lines and primary CRC stem cells. They showed that DIQ targets CSCs, reducing their tumorigenic potential by downregulating the ß-catenin, AKT and ERK oncogenic signaling pathways.

Q1 :The authors refer to their previous study on the characterization of the antitumor activity of DIQ (Monzer, 2019), but in that paper a number of compounds was investigated. The author should clearly specify in materials and methods or results which of those compounds they are investigating, showing structure and IUPAC name. The presence of such (partial) information in the abstract alone is not, in my opinion, sufficient.

Answer Q1: We thank the reviewer for this comment. In our previous paper, we investigated compound DIQ3 and its analogues (DIQ4-6). In this manuscript, we used the compound DIQ (previously referred to DIQ3) compound and not any of its analogues, since this compound caused sufficient inhibition of the self-renewal ability of the highly resistant cancer stem cells. So, DIQ and DIQ3 are the same compound. This has been mentioned in the MATERIALS AND METHODS section. Also, The following structure of DIQ has been added to the *DIQ preparation and treatment*-MATERIALS AND METHODS section on page 8:



Structure 1: Chemical structure of DIQ compound

Q2 Pag 17 .. Following 72 h, the inhibitory effect of DIQ was accompanied with considerable changes in cell morphology and confluency…. I expect that, since cells viability is reduced upon DIQ treatment, cell morphology and confluency change. I suggest to remove this sentence

Answer Q2: Thank you for this comment, and we totally agree with the reviewer. The comment was addressed, and we decided to remove the following sentence as suggested by the reviewer on page 17 in the revised paper:

American University of Beirut
Diana Tamari Sabbagh Bldg., 1st Floor, Room 130
P.O.Box 11-0236 / Anatomy, Cell Biology and Physiological Sciences
Riad El-Solh / Beirut 1107 2020, LEBANON

American University of Beirut
3 Dag Hammarskjold Plaza,
8th Floor,
New York, NY 10017-2303, USA

"Following 72 h, the inhibitory effect of DIQ was accompanied with considerable changes in cell morphology and confluency…. I expect that, since cells viability is reduced upon DIQ treatment, cell morphology and confluency change".

Q3 MTT allows to assess cell viability, as a surrogate for cell counts. Reduced viability could be ascribed to cell death and / or reduced cell proliferation. To assess cell proliferation a CFSE or BRdU assay should be performed. Please modify figures and text accordingly. Minor point Pag 17. …HCT116 and HT29 human CRC cells micromolar concentrations… è HCT116 and HT29 human CRC cells at micromolar concentrations

Answer Q3: Thank you for the comment. MTT assay is a classic assay that we use in our lab (Farah Ballout et al.,Oncotarget, 2020; Tarek H. Mouhieddine et al. Front. Neurosci, 2015; Rabih El-Merahbi et al, PLoS ONE, 2014). MTT assay is used to assess the cellular metabolic activity as an indicator of cell proliferation. The MTT results were further confirmed by trypan blue exclusion method that assess cell viability. Also, the effect of DIQ on the cell proliferation of the CRC cell lines was assessed by checking the expression of the proliferation-associated protein PCNA, which was markedly decreased by DIQ treatment in both cell lines, consistent with the results of MTT (measuring cell proliferation) assay and the Trypan blue exclusion (measuring cell viability) assay.

We agree with the reviewer regarding using a CFSE or BRdU assays to assess cell proliferation; however, unfortunately, these assays are not available in our laboratory.

The typo mistake was addressed and "at" was added to the following sentence in page 17:

"The MTT results revealed that DIQ significantly inhibited the proliferation of HCT116 and HT29 human CRC cells at micromolar concentrations in a time- and dose-dependent manner (Figure 1A)."

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: The authors should be commended on taking on a project which aims to advance our understanding of chemoresistance. The manuscript is however, far too long. The abstract is almost double the length of a standard abstract, and the methods section is hard to understand. My feeling is

that this manuscript would best be published in a journal with more focus on pre-clinical/laboratory findings. Further comments see below:

1 Title. Does the title reflect the main subject/hypothesis of the manuscript? Title is ok.

2 Abstract. Does the abstract summarize and reflect the work described in the manuscript? Abstract is too wordy – 460 words. Standard length is between 200-300 words. Moreover, I cannot really agree with claims such as "Chemotherapy for colorectal cancer (CRC), the second leading disease of cancer-related mortality, has so far revealed partial success." Firstly, it depends on which stage of CRC is in question. But it appears that the authors refer to stage 4 disease, that is patients with metastases. Although there are no studies so far that can show prolonged OS with oncological treatment, it shows increases PFS. Furthermore, with triple agents, up to 70% of patients will respond to treatment, and can be candidates for curative surgery. The conclusion "This study is the first documentation of the molecular mechanism of the novel anticancer therapeutic DIQ via targeting CSC, findings that will certainly have therapeutic implications for colon cancer patients. " is not warranted by the findings.

Answer: We thank the reviewer for the valuable comments .The comments were addressed. The abstract was revised and modified according to the reviewer's suggestions.

3 Key words. Do the key words reflect the focus of the manuscript? ok

4 Background. Does the manuscript adequately describe the background, present status and significance of the study? The authors should be commended for trying to advance our understanding of chemoresistance. Though the background fails to communicate the oncological treatment regimens that are available – they mention 5FU, which is one of the oldest treatment agents, which is often used in combination with other drugs. Moreover the treatment entails the use of several drugs, not just one. There are several strong statements such as the following, which lack proper underpinning: "The presence of chemoresistant CSCs has been determined to be one of the most significant causes of tumor recurrence." Moreover, the sentence reads in a peculiar way – how can chemoresistant CSC be the cause of rumor recurrence? If they are resistant, and not dead, if they appear in a manifest form (say CT) they have not properly recurred, but have been there all the time.

Answer: We thank the reviewer for her/his valuable comments. All comments were addressed and some sentences about cancer stem cells were modified . We added a couple of sentences regarding the oncological treatment regimens that are available for colorectal cancer.

"Current medical treatment of CRC includes a wide array of systemic therapies, which include chemotherapeutic (such as 5-fluorouracil (5FU)), targeted therapy (such as epidermal growth factor receptor (EGFR) inhibitors), in addition to immunotherapy, depending on the stages of CRC."

Although 5FU is an old treatment, we focused on this treatment in the manuscript since it is a standard of care, readily available for studies, and was used in many different studies in this system in the lab.

5 Methods. Does the manuscript describe methods (e.g., experiments, data analysis, surveys, and clinical trials, etc.) in adequate detail? The methods section is very extensive, and hard to follow. The authors need condense it, and seek out to describe the key methods. The remaining can be attached as supplementary.

Answer: We thank the reviewer once again for the valuable comments. We agree with the reviewer that the methods section is very extensive. We made some changes in the methods section, but it was hard to push some assays to supplementary. We left the majority of the assays as we believe that detailed assays are essential for the clarity of the experiments that we have used.

6 Results. Are the research objectives achieved by the experiments used in this study? What are the contributions that the study has made for research progress in this field? The authors need to be more specific about what there findings have contributed to the field, this is something which should be explained in the discussion. The results section reads as a combination of intro with sentences about the characteristics of cancer (say invasions), and results.

7 Discussion. Does the manuscript interpret the findings adequately and appropriately, highlighting the key points concisely, clearly and logically? Are the findings and their applicability/relevance to the literature stated in a clear and definite manner? Is the discussion accurate and does it discuss the paper's scientific significance and/or relevance to clinical practice sufficiently? The authors fail to put the findings in context – what has been shown before, and why this is study is unique.

Answer: According to the reviewer's comments, the discussion was rephrased, reorganized and some paragraphs were removed. The main findings of our study was DIQ targeting the CSC in 2D and 3D colonospheres and patient-derived colorectal organoids. Interestingly, DIQ effect and response on these organoids showed the heterogeneity of the corresponding tumor patient tissues.

8 Illustrations and tables. Are the figures, diagrams and tables sufficient, good quality and appropriately illustrative of the paper contents? Do figures require labeling with arrows, asterisks etc., better legends? The ilustrations are generally of good quality.

9 Biostatistics. Does the manuscript meet the requirements of biostatistics? The statistics section is extremely brief, and the quality of the English is poor. It is hard to understand exacly which test that used when.

Answer: Thank you for the comment. We have clarified the statistical analysis in the MATERIALS AND METHODS section as follows in the revised paper:

American University of Beirut
Diana Tamari Sabbagh Bldg., 1st Floor, Room 130
P.O.Box 11-0236 / Anatomy, Cell Biology and Physiological Sciences
Riad El-Solh / Beirut 1107 2020, LEBANON

American University of Beirut
3 Dag Hammarskjold Plaza,
8th Floor,
New York, NY 10017-2303, USA

"All statistical tests were performed using GraphPad Prism 7 (version 7.0, GraphPad Software Inc., La Jolla, CA, USA). Student's t-test, One-way or two-way ANOVA tests were used in this study. In all statistical tests, the mean of treated groups was compared to the mean of control groups. Statistical significance was reported at p-values of < 0.05. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. Experimental values are means ± standard error of the mean (SEM)."

10 Units. Does the manuscript meet the requirements of use of SI units? Yes

11 References. Does the manuscript cite appropriately the latest, important and authoritative references in the introduction and discussion sections? Does the author self-cite, omit, incorrectly cite and/or over-cite references? There are severaly strange references – specifically ref 2 and 3.

Answer: All references were revised and double-checked.
Here are the links of References 2 and 3:
Ref 2: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6791134/
Ref 3: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7478464/

12 Quality of manuscript organization and presentation. Is the manuscript well, concisely and coherently organized and presented? Is the style, language and grammar accurate and appropriate? The manuscript is on the whole far too wordy. It needs to be condensed and better organized. Language is ok, though there are several grammatical errors, and choice of words are at times a bit odd.

13 Research methods and reporting. Authors should have prepared their manuscripts according to manuscript type and the appropriate categories, as follows: (1) CARE Checklist (2013) - Case report; (2) CONSORT 2010 Statement - Clinical Trials study, Prospective study, Randomized Controlled trial, Randomized Clinical trial; (3) PRISMA 2009 Checklist - Evidence-Based Medicine, Systematic review, Meta-Analysis; (4) STROBE Statement - Case Control study, Observational study, Retrospective Cohort study; and (5) The ARRIVE Guidelines - Basic study. Did the author prepare the manuscript according to the appropriate research methods and reporting? Yes,

14 Ethics statements. For all manuscripts involving human studies and/or animal experiments, author(s) must submit the related formal ethics documents that were reviewed and approved by their local ethical review committee. Did the manuscript meet the requirements of ethics? Yes

---

American University of Beirut
Diana Tamari Sabbagh Bldg., 1st Floor, Room 130
P.O.Box 11-0236 / Anatomy, Cell Biology and Physiological Sciences
Riad El-Solh / Beirut 1107 2020, LEBANON

American University of Beirut
3 Dag Hammarskjold Plaza,
8th Floor,
New York, NY 10017-2303, USA

Answer to the Editorial Office's comment

EDITORIAL OFFICE'S COMMENTS

Authors must revise the manuscript according to the Editorial Office's comments and suggestions, which are listed below:

(1) Science editor:

The manuscript has been peer-reviewed, and it' s ready for the first decision.

Language Quality: Grade B (Minor language polishing)

Scientific Quality: Grade C (Good)

(2) Company editor-in-chief:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. Please authors are required to provide standard three-line tables, that is, only the top line, bottom line, and column line are displayed, while other table lines are hidden. The contents of each cell in the table should conform to the editing specifications, and the lines of each row or column of the table should be aligned. Do not use carriage returns or spaces to replace lines or vertical lines and do not segment cell content. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022. Before final acceptance, when revising the manuscript, the author must supplement and improve the highlights of the latest cutting-edge research results, thereby further improving the content of the manuscript. To this end, authors are advised to apply a new tool, the Reference Citation Analysis (RCA). RCA is an

artificial intelligence technology-based open multidisciplinary citation analysis database. In it, upon obtaining search results from the keywords entered by the author, "Impact Index Per Article" under "Ranked by" should be selected to find the latest highlight articles, which can then be used to further improve an article under preparation/peer-review/revision. Please visit our RCA database for more information at: https://www.referencecitationanalysis.com/.

Answer: We thank the EDITORIAL OFFICE for their valuable comments and suggestions: The manuscript was revised according to the EDITORIAL OFFICE'S comments and suggestions. We provided decomposable Figures (in which all components are movable and editable in a single PowerPoint file, and the tables were edited as suggested by the EDITORIAL OFFICE.

American University of Beirut
Diana Tamari Sabbagh Bldg., 1st Floor, Room 130
P.O.Box 11-0236 / Anatomy, Cell Biology and Physiological Sciences
Riad El-Solh / Beirut 1107 2020, LEBANON

American University of Beirut
3 Dag Hammarskjold Plaza,
8th Floor,
New York, NY 10017-2303, USA