Author’s response to reviews

Title: Effect of Gallic acid and Myricetin on ovarian cancer models: a possible alternative antitumoral treatment

Authors:

Luis Varela-Rodríguez (lvarela@cinvestav.mx)
Blanca Sánchez-Ramírez (bsanche@uach.mx)
Verónica Ivonne Hernández-Ramírez (arturomvi@hotmail.com)
Hugo Varela-Rodríguez (hugo.varela@cinvestav.mx)
Rodrigo Daniel Castellanos-Mijangos (rdcastellanos@intramed.net)
Carmen González-Horta (carmengonzalez@uach.mx)
Bibiana Chávez-Munguía (bchavez@cinvestav.mx)
Patricia Talamás-Rohana (ptr@cinvestav.mx)

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Author’s response to reviews:

REVIEWER ANSWERS

REVIEWER 1: Fedora Grande

We would like to thank Dr. Fedora Grande for her useful comments and observations.

Please find below our point by point responses, as well as a list of modifications done to the text according to her suggestions.

1. Keywords should not contain the same words present in the title (i.e Gallic acid, Myricetin and ovarian cancer).
We have added new key words and at the same time have left some of the originally selected words that we think will help people to find our manuscript one it is published.

Page 3, lines 33-34: Key words

New key words were added: Nu/Nu mice; toxicity; xenotransplanted mice

2. Where in the "methods" section well known procedures are described, authors should reference the original papers that the information have come from and then provide a summary of the methods rather than the whole text.

In the manuscript, the methods section is already referenced and summarized based on reported procedures and only important modifications that allow their reproducibility are described. However, 2.9 section: “Antineoplastic activity of GA and Myr” (Pages 8-9, line: 174-227) was described in detail and depth at the request of the BMC journal editor.

3. In the results section, for the biological activity of GA and MYR in cell lines, authors should also use a known drug (i.e. paclitaxel) as positive control.

Paclitaxel, a well-known drug used for ovarian cancer treatment, was indeed used both for in vitro and in vivo experiments as a positive control for cell damage. All figures except figure 2 include the results obtained with Paclitaxel. In the case of figure 2, although the experiments were also done with Paclitaxel, only the results obtained with GA and Myr are presented.

Please see below the changes done in the methods and results sections regarding the use of paclitaxel.

Methods:

Pag. 5, lines 108-110

….Vehicle (0.5 % DMSO in 1X PBS, v/v) was used as a negative control, and Paclitaxel, a drug used for the treatment of ovarian cancer, was used as a positive control (5 µg/mL in cells or 5 mg/kg body weight in mice) (Sigma®)…. 
Results:

Page 11, line 288-290

…same cell lines, compared to the treatment of negative control group (vehicle) (p ≤ 0.05, Dunnett) (Figure 2A and B). Positive control with paclitaxel administered at 5 μg/mL diminished viability to 50 % compared with the vehicle group (data not shown).

Pag. 12, line 294

…activity at 25 and 64 μg/mL respectively, compared to the vehicle treated group (p ≤ 0.05,

Lines 315-316

…necrosis (26.6 / 15.1 %) in SKOV-3 cells respectively; this effect, although of less intensity, was similar to that observed with paclitaxel (p < 0.05, ANOVA) (Figure 3A).

Pag. 13, lines 324-325:

…. These changes were also present in cells treated with paclitaxel, but absent in cells treated with the vehicle (Figure 3B).

Lines 329-330:

…or the increase observed in G2/M phase (15.3 %) in the cells treated with paclitaxel.

Line 343:

..treated with vehicle (p > 0.05, Tukey), or paclitaxel (Figure 4A). At the end of the

Pag. 14, line 360:

These results agree with those found with the larger diameter of the tumoral lesions in the
Line 362:

…with a relative increase in size

Line 363:

..was a 23.3 % reduction, in comparison

4. In the results section, for the in-silico studies, authors declare that "evaluation in silico studies demonstrated that GA and MYR interact with carbonic anhydrase and PIK3CG, respectively". It would be appropriate to carry out some molecular docking studies on the PDB structure of both proteins to obtain more detailed information regarding this point.

We agree with the reviewer that molecular docking and also experimental studies need to be performed to declare that our results “demonstrate”. Thus, we have modified the writing of this particular sentence in the results section of the abstract that is now as follows:

Pag. 2, lines 20-21

….. In silico studies suggest that GA and MYR could interact with carbonic anhydrase and PIK3CG, respectively….

5. The conclusions should be improved to provide a more comprehensive summary of the entire work and, above all, avoiding to repeat the same sentences used in the "conclusion" of the abstract.

We thank the reviewer suggestion to improve the conclusions section. Thus, a new complete paragraph has been written to provide a more comprehensive summary of the entire work.
Conclusions

GA and Myr presented biological activity against ovarian adenocarcinoma cells such as SKOV-3 (50 and 166 μg/mL) and OVCAR-3 (43 and 94 μg/mL) respectively, demonstrating differences of sensitivity in the effect of both compounds. Additionally, GA and Myr had cytotoxic activity in transformed/non-tumorigenic cell line as BEAS-2B (25 and 64 μg/mL), confirming low selectivity in their biological activity, possibly related to the cellular phenotype. Also, both polyphenol compounds induced morphological changes in SKOV-3 cells, mainly in the actin/tubulin cytoskeleton, cell cycle arrest and activation of cell death by apoptosis, through the generation of ROS. Finally, the peritumoral administration of GA and Myr (doses of 50 mg/kg) did not reveal behavioral changes or toxicity signs in rodents, but inhibited the development of ovarian tumor lesions, that allowed a stable progression of the disease. However, histological and paraclinical analysis of organs and blood extracted from mice during the toxicological study, revealed that GA induced hepatic necrosis, leukocyte infiltration, hypertransaminasemia, and hypoazotemia, which are related to hepatic failure due to chronic inflammation caused by loss of liver parenchyma; whereby additional studies are needed to find an adequate therapeutic dose for GA. In silico studies using the SEA approach allowed to suggest that carbonic anhydrase IX and PI3K proteins could be the most probable targets for GA and Myr respectively. Experimental and docking studies will allow to confirm this proposal. Therefore, GA and MYR could be considered as a starting point for the development of novel anticancer agents.

6. Furthermore, the authors say that "these compounds could be used in the chemotherapy of this pathology" I suggest to rewrite this sentence indicating that "GA and MYR could be considered as starting point for the development of novel anticancer agents".

Following the reviewer suggestion, the sentence has been changed both in the abstract conclusion Page 3, lines 31.32) and in the final conclusions section (Page 20, lines 533-534). We really appreciate your valuable contribution.

7. In table 4, please replace the stick and ball structure of the molecules with the linear ordinary chemical structure directly drawn by using ChemDraw or other similar software.

The chemical structures present in Table 4 were modified based on the reviewers’ suggestion. We appreciate your observation.

In the manuscript, the references list was updated with the references proposed by the reviewer and by other three additional references. All of them have been edited following the format provided by BMC journal guidelines, as suggested by the reviewer. In consequence, the references numbers have changes and are marked in green in the corrected version.

REVIEWER 2: Leticia Gonzalez-Maya

We would like to thank Dr. Leticia González-Maya for all her valuable comments on our work and for her suggestions for improving its quality.

1. In general, the resolution of the figures, 2C, 3B, 3C (immunofluorescence) are not adequate.

All figures have been modified to enhance their resolution up to 300 – 500 d.p.i. approximately, according to the BMC Journal requirements.

2. The size of the figures are too small (3B, 3C).

The size of the panels in Figure 3 was increased to improve their visualization.
3. Figures 5A and 5B are not clear enough, maybe changing the contrast.

The brightness and Contrast of A - B panels in Figure 5 were increased to improve their visualization.

4. Figures 4A and 5D, morphological changes should be indicated with arrows.

Figures 4D and 5D were modified by adding arrows that indicate the morphological changes observed during the histological studies.
Additional modifications to the text:

ABSTRACT
Page 2, lines 4-5
R1: with little effectiveness in later stages of the disease and severe toxicological effects.
R2: with little effectiveness in later stages and severe toxicological effects

Line 20
R1: In silico studies demonstrated that GA and MYR interact with carbonic anhydrase and PIK3CG, respectively.
R2: In silico studies suggest that GA and MYR could interact with carbonic anhydrase IX and PI3K, respectively.

Lines 28-31:
R1: GA and Myr (50 mg/kg) inhibit the development of ovarian tumor lesions in 29 rodents administered by peritumoral route, with little toxicological effects. However, 30 additional
studies will be necessary to find the appropriate therapeutic dose for GA. Therefore, these compounds could be used in the chemotherapy of this pathology.

R2: GA and Myr (50 mg/kg) administered by peritumoral route, inhibit ovarian tumor lesions development in rodents with some toxicological effects. Additional studies will be necessary to find the appropriate therapeutic dose for GA. Therefore, GA and Myr could be considered as a starting point for the development of novel anticancer agents.

1. Background:

R1: PI3K-PKB/Akt,
R2: PI3K-PKB/Akt/mTOR

2. Methods

Page 5, lines 87-89 Section 2.1

R1: Compounds evaluated in this study were GA (G7384) (50 mg/kg of body weight) and Myr (M6760) (50 mg/kg of body weight)

R2: Compounds evaluated in this study were GA (G7384) (50 µg/mL in cells or 50 mg/kg of body weight in mice) and Myr (M6760) (166 µg/mL in cells or 50 mg/kg of body weight in mice)

Lines 108-110, Section 2.3

New addition

R2: Vehicle (0.5 % DMSO in 1X PBS, v/v) was used as a negative control, and Paclitaxel (5 µg/mL, Sigma®), a drug used for the treatment of ovarian cancer, was used as a positive control
Page 16, lines 430, section 3.4

R1: ATM/chk2/p53 and the inhibition of the

R2: ATM/Chk2/p53 and the inhibition of the carbonic anhydrase IX, COX-2/NF-kB

Page 17, lines 459-473

R1: The in silico analysis to determine the molecular mechanism and therapeutic targets of GA and Myr, revealed that GA might induce ATM-Chk2 activation and the inhibition of COX-2/NF-kB [20], while Myr is a general inhibitor of protein kinases, such as PI3K [18]. Interestingly, GA could have an additional mechanism of action, which involves the enzymatic inhibition of carbonic anhydrase, which is a zinc-dependent metalloenzyme responsible for regulating the intracellular pH, through the conversion of CO2 and H2O into HCO3 by catalysis, while Myr can stabilize the microtubules in the cell cytoskeleton. None of the mechanism of these compounds has been studied in depth. Recent work has demonstrated the fundamental role of carbonic anhydrase in different cancer types and parasitic pathologies [35], while additional studies found that carbonic anhydrase is very abundant in ovarian cancer, unlike other types of cancer such as renal cancer [36].

R2: The in silico analysis to determine the molecular mechanism and therapeutic targets of GA and Myr, revealed that GA might induce the activation of ATM/Chk2/p53 and the inhibition of COX-2/NF-kB and GSH [25], while Myr is a general inhibitor of protein kinases, such as PI3K-PKB/Akt/mTOR, MEK1, Fyn, and JAK1-STAT3, among others [22, 24]. Interestingly, both compounds could have an additional molecular mechanism of action based on results obtained in SEA approach. GA possibly can inhibit the carbonic anhydrase IX protein, which is a zinc-dependent metalloenzyme responsible for regulating the intracellular pH, through the conversion of CO2 and H2O into HCO3 by catalysis [41]; while Myr possibly can bind to tubulin and stabilize the microtubules in the cell cytoskeleton. None of these molecular mechanisms has been studied in depth. Although in this study we have proposed some target molecules only using the SEA approach, this analytical tool has been used widely to successfully predict the targets, toxicity and mechanism of action in diverse marketed drugs [42]; in addition, SEA has been proposed for the virtual detection and construction of a pharmacological network in the study of
medicinal plants [43]. Whereby, the proposed interaction, GA-carbonic anhydrase IX or Myr-tubulin, most likely can take place in experimental and natural conditions, but additional studies will be required to confirm them.

Page 18, lines 477-482:

R2: New paragraph On the other hand, PI3K/Akt/mTOR signaling pathway is dysregulated in diverse cancer types as glioblastoma or ovarian cancer, and mTOR is a key mediator of cellular processes such as growth, proliferation, metabolism, and angiogenesis [24]. Thus, the development of new drugs to inhibit these target proteins in cancer is an interesting perspective to address in the treatment of the disease. Moreover,

Pag. 3, line 29

…with some toxicological effects.

Pag. 28, lines 811-813:

(C). The arrows and symbols indicate: fibrosis (black arrowhead), necrotic area (NEC), vascularization (*), leukocytic infiltrates (grey arrowhead), and apoptotic cells (white arrowhead) (D).

Lines 823-824

…and color arrows indicate loss of hepatic parenchyma (black arrowhead) or leukocyte infiltrates (grey arrowhead).

6. ABBREVIATIONS.

Page 20, new abbreviations were added in R2

line 550: recommended daily dose; mTOR: mammalian target of rapamycin; MTT: 3-(4,5-

line 555: Phosphatidylinositol 4,5-bisphosphate 3-kinase; PKB (Akt): protein kinase B; p.t.: