Author’s response to reviews

Title: Selenized milk casein in the diet of BALB/c nude mice reduces growth of intramammary MCF-7 tumors.

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Author’s response to reviews: see over
Reviewer: 1
Comments to the Author

Authors need to include and discuss at least one literature reference by G.N.Schrauzer such as e.g. the one which appeared in Critical Reviews in Biotechnology, 2009, 29(1): 10-17, entitled 'Selenium and selenium-antagonistic elements in nutritional cancer prevention'.

AU: The literature reference by G.N.Schrauzer has been included and discussed on page 15.

Reviewer: 2
Abstract
1. Please mention in “Methods” that apoptotic cells and Bcl-2, Bax, and Cyclin D1 protein levels in tumors were determined.

AU: Included on page 2.

2. In “Results” it should be mentioned that final tumor mass, BCL-2, Bax, and Cyclin D1 protein levels in tumors were not significantly affected by different Se levels in the diet.

AU: Included on page 2.

3. The sentence “there was a significantly higher number of apoptotic cells in high-Se tumors as compared to low-Se tumors” should be moved from Conclusions to Results.

AU: Done. Page 2/3.

Introduction
4. Selenium is an essential trace element, but also toxic at high concentration. Please indicate which dietary level is considered adequate (or too high or too low).

AU: “Se becomes toxic at levels greater than 400 ug /d in the diet and deficient at levels lower than 40 ug/d.” added to Introduction. Page 4.

5. 2nd paragraph, 1st and 2nd sentence: both statements need a citation


6. p.5 first paragraph: “each increment in Se intake between 0.16 and 1.15 ppm of dry matter caused a decrease in tumor volume.” This statement suggests a strong concentration dependency which seems to be an overstatement considering the large variability in tumor size and growth pattern and the very small effect of the 0.51ppm Se diet.
AU: This was meant to convey the observation that tumor volume did decrease with increasing Se supplementation. I believe it is acceptable to keep because I did not state that there was a significant decrease with each increment, but merely that volume did decrease with each diet.

Materials and Methods
7. p.5 Casein isolation paragraph, 1st sentence: please specify what you mean by “basal diet”.
AU: Was left as is as a basal diet is well known to be a diet complete and adequate except for a single constituent.

8. p.6, Casein isolation and diet formulation: “AIN-76 diets (Research Diets Inc)” is confusing. I assume you mean the purified rodent diet AIN-76A, and that you prepared it yourself, since you had to add different caseins, or did the company prepare it with the casein provided from you? Please explain why you chose these dietary Se levels!
AU: “These low- and high-Se caseins were then sent to Research Diets Incorporated to be mixed with a standard rodent to produce four diets with varying Se levels.” added to page 6.

9. p.6 Animal trials: please indicate whether or not food and water was provided at libitum. Please indicate whether you measured food consumption. Did you determine the body weight during the experiment and at the end? How many animals did you have in each dietary group at the start of the experiment and did any animals die during the experiment.
AU: “Water and food were offered ad libitum. Food consumption was not measured, however body weights were measured biweekly throughout the trial.” Page 6.
“Only mice with tumors that reached a palpable volume within 3 weeks after implantation were included in the trial. The animal trial involved 72 mice; 18 mice per diet. 7, 8, 4, and 5 mice from treatments 1, 2, 3, and 4, respectively, were euthanized during this trial. These mice were prematurely euthanized for exhibiting criteria for euthanasia, either clinical signs or because tumor size exceeded 10% of body weight.” Page 7.

10. p.6 Animal trials cont.: “Once tumor volume reached …”. This sentence raises some concern about differences in tumor growth as strong modulating factor in the experiment. Please add information about the time period needed from implantation to inclusion in the dietary study for the animals. Although you randomly assigned the animals to dietary groups, did you follow the development of tumor growth in individual animals and analyzed whether there is a correlation between tumor growth before and after start on the diets?
AU: Added the fact that only mice with tumors that reached a palpable volume within 3 weeks after implantation were included in the trial. Individual tumor growth was only analyzed after beginning the mouse on a diet.

11. Statistical Analysis paragraph: did you try to compare the lowest Se diet group (Low adequate Se level) individually with the other groups? You could indicate significance between this group and others with an asterix in the figures in addition of giving the p value for the linear trend.
AU: This was considered, however we believed that analyzing linear trends was a stronger test and more statistically relevant.

12. p.9 Statistical Analysis cont: You list several parameters that resulted in an exclusion of animals from the data set, “mice put on treatment 3 weeks after inoculation or later”, “tumors with a grad 0”, “if their final volume was below 30 mm²”. Were these animals/tumors only not considered for the data in Table 2 or for all figures as well?
AU: These mice were excluded for all figures.

13. p.10 Statistical Analysis cont: “Because of the large variation around mean tumor characteristics within treatment”. You could add “(see Figure 1)” to explain what you mean with this statement.
AU: “(see Figure 4) added.

Results
14. p.10 Tumor growth, line 1f: “exhibited exponential growth to a plateau (Figure 1)”. I have a hard time to see a plateau for most of the tumors in Fig.1. Please explain better.
AU: This may only be seen with some individual growth curves, which is common in tumor growth studies as most tumors are not given enough time to reach their maximum volume before the animal must be euthanized.

15. p.10 Tumor growth, line 3f: “a tendency for final tumor mass to decrease (p=0.09; Fig 2b).” I see a downward trend (decrease) in volume for some tumors in Fig 1. Figure 2 shows a non-significant lower final tumor mass with the two higher Se diets.

16. p.11 line 3: “one mouse on 0.51ppm Se was excluded”. In addition the Vmax in this group is very high (Table 2). Please address this.
AU: It was stated this mouse was excluded due to a physiologically implausible best fit b value, implying these tumor growth dynamics were not modeled properly by Gompertz equation, explaining why the Vmax was also high.

17. p.11 Tissue analysis line 3: ”the DNA nick-end labeling also increased 2.4-fold with increasing Se level (Fig 6e).” This statement suggests a strict linear increase with concentration, but the 0.85ppm Se concentration is probably not significantly different from the 0.16ppm level.
AU: I feel this statement it valid because it does not claim there was a strict linear increase.

Discussion
18. p.12 line 1: “Our findings show, for the first time, that dietary Se is effective in reducing growth of human mammary tumors in vivo.” This statement seems to be incorrect, since Chen and coworkers just published a similar study (Chen et al., 2013).
AU: This statement was included to indicate that while similar work has been done by chen and coworkers with other forms of cancers, notable colorectal and colon, this is the first (as far as I know) with breast cancer. I am not sure if the Chen et al., 2013 you referred to was the one I found involving colorectal cancer, however if a study involving breast cancer has been published I will remove this statement.

19. p.12 line 5: “Number of large tumors after 8 weeks” please add “(tumors with Vfinal > 500 mm³)”
AU: This was added.

20. p.12 line 7-9: “Although … growth rate was reduced, the maximum volume that tumors were predicted to reach (Vmax)…” This is very confusing and needs to be clarified. Please add that you are here talking about the Gompertz fits, the estimated Vmax which was modeled using the growth data. It may help to use other abbreviations for the modeled data, like Vmax-G or something similar to distinguish this Vmax from the measured data.
AU: Added “Maximum volume and growth rate were generated using Gompertz fits of the observed growth data

21. p.13 end of page: “In vitro, the IC50 of SeMet against MCF-7 was 45 uM…we found in mammary tumors the equivalent of 23 uM SeMet…slightly less than the IC50.” It would be interesting to know the Se concentration in normal tissues? Please discuss why cancer cells are more vulnerable, i.e. is there any evidence that cancer cells accumulate SeMet more than normal cells or that cancer cell metabolize it differently, preferably to the postulated active form methylselenol (Zheng et al ..) or that they are more vulnerable due to specific changes?
AU: Added “Redman and coworkers [25] investigated the effects of SeMet on four cell lines in vitro: MCF-7 breast carcinoma, UACC-375 melanoma, DU-145 prostate cancer, as well as normal diploid fibroblasts. This study investigated the IC₅₀ of SeMet for for each cell line. SeMet concentrations ranged from 100-10000 µM. SeMet inhibited growth in all cell lines in a dose-dependent manner. In MCF-7 cells, cell viability was not affected by 0.01-10 µM, while 100-1000 µM significantly inhibited cell growth. In UACC-375 melanoma cells, concentrations greater than 1 µM were required to significantly inhibit cell growth. In prostate cancer cells DU-145, concentrations beyond 10 µM showed a marked decline in cell growth. In contrast to the micromolar concentrations of SeMet shown to inhibit cancer cell lines, inhibition of growth in diploid fibroblasts required millimolar concentrations. These results indicated that DU-145 prostate cancer cells are the most sensitive to SeMet treatment with a IC₅₀ of 40 µM, followed by MCF-7 and UACC-375 with 45 µM and 50 µM, respectively. Fibroblasts required 1 mM SeMet to induce 50% inhibition. According to these results, cancer cells may be more sensitive to selenium treatment than normal cells [25 ]. It was postulated that these discrepancies may be due to differences in uptake and metabolism of SeMet to anticarcinogenic metabolites, as SeMet may be metabolized to methylselenol or SeCys, which in turn is hydrolyzed to hydrogen selenide [25].”

22. p.14 end of 2nd paragraph: “…suggesting an alternative apoptotic pathway”. You may want to mention glutathione peroxidase 4, which is a Se-dependent
enzyme and was shown to influence apoptosis (Brigelius-Flohe and Maiorino, 2013).
AU: GPx was not included in discussion as GPx levels tend to plateau at lower Se levels than those required to reduce carcinogenesis.

23. p.14 end of 3rd paragraph: “...cyclin D1-mediated cell cycle progression does not appear to be responsible for the inhibitory effects of Se-casein on tumor growth.” You may want to mention another option here. A recent study showed an involvement of selenium-binding protein 1 in reduced proliferation of MCF-7 cells in the presence of MetSe (Zhang et al., 2013). Also, the alternative mechanisms mentioned, i.e. caspase activation and inactivation of Akt and ERK1/2, could be analyzed.
AU: “Selenium, however, has been shown to downregulate several genes controlling the expression of cell cycle proteins, including cyclin A, CDC25A, CDK4, PCNA and E2F (El-Bayoumy and Sinha, 2005).”

24. p.15 line 2: define SeMSC
AU: defined “Se-methylselenocysteine”

25. p.15 first or second paragraph: please provide information about possible toxic effects at high Se levels, like type 2 diabetes (Rayman and Stranges, 2013), or of wrong levels or wrong form possibly enhancing tumor growth and metastasis (Cassidy et al., 2013; Chen et al., 2013).

26. p.15 2nd paragraph: “Modeling tumor dynamics suggest that a diet high in Se-casein decreases the maximum growth rate.” The modeling data do not show linear concentration dependency and overall seem to be based on a lot of assumption. More convincing data to back up this statement is needed.
AU: This statement was removed.

27. p.16 1st sentence: “This study showed Se-casein to be an effective treatment of breast cancer, suggesting its potential role in adjuvant therapy.” This is an overstatement considering the limited amount of data in this study and needs to be modified. The authors could add a sentence about further experiment that are needed to shine light on the mechanism for the observed effects and eliminate safety concerns.
AU: Modified to “This study showed the potential for Se-casein to be an effective treatment of breast cancer, suggesting its potential role in adjuvant therapy.”
Added “Further study is required to elucidate the precise mechanism through which supranutritional Se-casein levels reduce carcinogenesis. The effects of high-Se casein on normal cells in addition to cancerous cells should also be well-characterized before it may be approved as an effective dietary supplement for chemoprevention in order to eliminate safety concerns.”

28. Figure 1: use the same scale for all figures, add the Se level in the left upper corner and provide the number of tumors (n) in each treatment.
AU: Modified Figure 1.