Reviewer’s report

Title:Carnosine inhibits the carbonic anhydrase IX-mediated extracellular acidosis and reduces the growth of HeLa tumor xenografts

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Reviewer:Herman H Yeger

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The studies presented by Ditte et al are based on polder observations of anti-cancer properties of a natural metabolite carnosine. The focus here is on the pH regulatory carbonic anhydrase IX which has been associated with aggressive tumor behavior especially metastasis. They used MDCK, HeLa, HT-29 and SiHa cell lines to examine CAIX expression in monolayers and derive spheroids relative to modulation of HIF-1alpha and its target genes. They show that carnosine raises extracellular pH and increases HIF-1alpha and targets expressions. External pH measurements confirmed a reduction in acidic pH although the mechanism appeared to be somewhat different than expected. This in my opinion makes the study provocative because by looking at protein associations it would seem that carnosine actually interferes with CAIX functional conformation and association with other proteins.

Discretionary: The question thus arises whether this is due to steric effects or direct interference with AE2 examined or the active site for metabolizing CO2. Using a FITC-CA labeled inhibitor for binding/interference supported the hit on CAIX. In vitro clonogenic and nude mouse xenograft studies demonstrated the potent tumor growth inhibitory effects. Measuring ATP showed depletion and suggested involvement at the mitochondrial level; it would be interesting to explore this aspect further given the chemical structure of carnosine. There are many examples in nature of highly bioactive and specific activity of dipeptides so what makes carnosine different with respect to cancer? I feel that the authors should place some discussion on such aspects and provide further insights into how the chemical structure of carnosine could perturb enzyme functions given that histidines are often found to be critical amino acids in active site and conformational bridging.

Major Compulsory:A number of other points need to be addressed:

1. Dose responses, Fig 2, show 20mM to be optimal; why since this is already a high does from a physiological point of view and in the pharmacological range.
2. Fig 3A, one would expect good induction of HIF-alpha in the ctrl extant of potentiation by carnosine. Please explain.
3. Fig 5C- significance not shown for effect of hypoxia?. In Fig5 a, pH shown but not given in the legend?
4. Fig 1- why does the culture start off at pH8 when media are near neutrality?
5. Fig6- is this retarded growth? Do the authors have a proliferation marker here? Certainly if vascularization is affected by carnosine both CAIX via HIF1alpha can be strongly upregulated. This can confuse what carnosine actually does to CAIX or perhaps it basically works at the mitochondrial level to reduce ATP which will limit growth severely, and other such diversions. How is carnosine itself metabolized? Do the spheroids just show a reduced growth rate or is there loss of cells at the slightly higher pH, which in fact is at the pH of media, ie, this is the normal growth environment? Rapidly growing cells acidify the growth medium. This makes the data in Fig5A a bit hard to explain.

Minor Essential Revisions: Minor points include spelling errors—check ‘microenvironment’, ‘trypsin’; poor sentence construction of “used carnosine dosing….,” In Discussion.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

No conflicts. I declare that I have no competing interests.