Reviewer’s report

**Title:** Cobalt Chloride Compromises Transepithelial Barrier Properties of CaCo-2 BBe Human Gastrointestinal Epithelial Cell Layers

**Version:** 0  **Date:** 09 Apr 2017

**Reviewer:** Susanne M. Krug

**Reviewer's report:**

Although the article of DiGuilio et al. deals with a very interesting topic, there are unfortunately many major concerns that I have with this study.

Besides being a pure descriptive and somehow only preliminary study, an adequate discussion of the result is missing. Also, the style of presentation and writing in general is not appropriate. I have doubts that a very well-established researcher like James Mullin was the one preparing the manuscript.

Often unusual descriptions are used and sometimes these may lead to confusion.

To give a few examples, "barrier friendly" and "unfriendly", or "both cell surfaces" when meaning apical and basolateral side. What is "physical scraping", or what would be non-physical scraping? Rt is less common than Rt or TER (or TEER). "mw" is no common unit, use Da.

The order of presentation is also a bit confusing. Before doing the experiments with one dose, the dose-finding data should be shown. Also the data finding the application-side should be one of the first data.

The descriptions of the methods is very detailed one on hand, but misses important information on the other.

- The cell line used is not named correctly. It is CaCo-2 BBe, not CACO-2. The addition BBe as mentioned in the methods section is essential, as CaCo-2 and CaCo-2 BBe cells are not identical.
- How were the electrophysiological measurements performed? Were Ussing chambers used? Which kind or brand? Which solutions were used, Ringer solutions? Please also add the composition.

- What do the authors mean with "various nutraneuticals … at the concentration that provided maximal barrier enhancement"?

- Although analyzing some tight junction proteins, there is no adequate discussion of the results. What do the respective changes mean for the paracellular barrier? Is each analyzed tight junction protein of similar impact? Why were those tight junction proteins chosen and other not? In addition, a short introduction into the tight junction field would be helpful for readers being not a specialist in this.

Furthermore, there are several tight junction proteins mentioned to be analyzed in the methods, but no data are shown. By the way, it very untypical to incubate with antibodies for only two minutes - might this explain why there were no data shown?

- For analysis of the tight junction expression the authors treated cells cultured in flasks. As they show that cobalt only is effective from the basolateral side, how can they expect any effects in flask-culture? The cells grow as monolayer on the plastic bottom, so that there is no realistic chance to be reached from basolateral side. The HIF-beta expression should be shown as control, too.

- How did the authors ensure that same protein concentrations were analyzed on the Western blots? There are no loading controls shown, e.g. beta-actin. Densitometric results should be corrected for that and images of blots should be shown, too.

- The authors mention the Cytotox96 cell viability assay, but do not show the data. "Data not shown" is a bad habit. If not very important for the whole study, one could always add such data as supplementary information.

- Does "seven-day post-confluent" mean that cells were grown until being confluent (when are they confluent?) and were analyzed 7 days later? Why do the authors not use the usual description of days after seeding?

As CaCo-2 cells in general (not only BBc) are very variable in their differentiation there needs to be an explanation how the authors assured similar quality and properties in their experiments.
Some studies use two-week old cells other three-weeks etc. A good choice would be the regular control of the TER by chopstick-electrode systems to check the differentiation easily.

- Most of the figures could be reduced or even combined. They also need huge improvement. I suggest the authors to have a look on different other publications. Bars in the diagrams are too broad and labeling is too small if one takes in account that in a paper the figures do not cover a whole page. Also labeling of the axes should be improved.

Fig. 1: "Percent of normalized control resistance" would be "Resistance (or TER) in % of controls". In this context, I also would like the authors to unify the type of presentation. Sometimes they show normalization, sometimes concrete values in other figures. Why did they chose one or the other? The "inset" is a sub-figure and should be properly labeled. What is shown here should be obvious without having to read the complete figure legend first.

Fig. 2: "Normalized band density": If normalized than one should normalized to percentage of the controls again. Please also show blots - and the loading control (as explained above).

Fig. 3: It is common to present fluxes. But these are not suitable when comparing different markers (as the authors do in Fig. 7), as they may vary a lot depending on the respective concentrations used. It is better to use permeability, which is a concentration-independent value.

Fig. 4: The used presentation of the time-curves is very unusual. The curve of the controls should be also shown as these also develop with time. So instead matching to the controls one should match to the initial start t=0.

How is HIF1-beta developing during the time-courses? And the tight junction proteins?

Fig. 7: Please use permeability as fluxes are concentration-dependent and it is not clear if for all markers same concentrations and experimental conditions were used (please add in methods).

Fig. 8: The abbreviation for days is not capitalized. Use "d". Do CaCo-2 BBe already form confluent monolayers three days after seeding? How are the non-normalized TER values? The reasons for the different reaction of different ages/differentiation states should be discussed.

Fig. 9: Again, the "inset" is a sub-figure and should be properly presented and labeled. There is no loading control.
Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

No

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.

Unable to assess

Are the conclusions drawn adequately supported by the data shown?
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