Steve Levine

Manuscript Number: RTP-09-41R1
Title: Dig1 protects against cell death provoked by glyphosate-based herbicides in human liver cell lines
Corresponding Author: Prof. Gilles-Eric Seralini
Authors: Celine Gasnier; Nora Benachour; Emilie Clair; Carine Travert; Frederic Langlois; Claire Laurant; Cecile Decroix-Laporte.

General Comments:

Overall Conclusions: It appears that the majority of the numerous grammatical/sentence syntax problems, poor definition of abbreviations, inadequate figure legends, etc. that were present in the original manuscript have been dealt with in the revised manuscript. However, the revised manuscript still disjointed, it is difficult to understand what was performed, has significant scientific flaws, contains speculative conclusions and inaccurately characterizes risk~~has major scientific flaws which make it unsuitable for publication~~. In addition, the work contained in the revised manuscript remains repetitious of that contained in previous publications from this group, and as such, it is not 'new' and does not represent information that would be useful to the Journal's readers. Further, the conclusions and model presented at the end of the paper is not supported by the data developed in the paper. For example, there are large gaps in mechanistic information that are required support the explanation for modulation of cytochrome P450 expression. Therefore, it is again recommended that this revised manuscript should not be accepted for publication in *Regulatory Toxicology and Pharmacology*.

The authors study design makes it impossible to separate the contributions of glyphosate, the surfactant or the lack of serum to the toxicity. The experiment that must be conducted to make this paper acceptable is one that will provide independent data of all variables. Test materials must include a glyphosate-based formulation, a formulation blank (all components except glyphosate), glyphosate, the surfactant in the formulation as well as additional surfactants and these experiments must be conducted in serum and serum-free conditions. Including additional surface acting agents is critical to put the observed effects in in vitro experiments into perspective - as demonstrated by other authors even those found in personal and home care products can produce similar effects without causing unacceptable toxicity to the consumers who use them.

Furthermore, one must question the authors' objectivity given the tone and ~~quality~~ content of their responses to comments. Indeed the authors' lack of objectivity appears to have ~~clouded~~ influenced their interpretation of ~~our~~ my original review. For example, the observed toxicity of the formulations in the in vitro studies was not questioned; what was questioned ~~was~~ is the interpretation of the results based on the study design. In addition, several references were provided in my previous review that had similar types of investigations for the authors to consider in reflection of their data, their interpretation of results and as a point of departure for their response. Yet the authors did not respond to the comments ~~in~~ regarding these references or include them in the current draft. This selective use of the literature is ~~most~~ troublesome.

Taken in sum this study displays neither the scientific rigor nor objectivity necessary for publication by the Journal.

Specific Comments

Abstract

Page 2, lines 40-41. It is stated that "CYP3A4 is specifically enhanced by R at doses 400 times less than used in agriculture (2%)". A similar statement appears page 13, lines 308-310 ("We tested R at sub-agricultural levels...below the maximum level of residues authorized in some feed ..."). These statements are apparent attempts to denote 'risk'to the liver cells of humans, but they actually reflect meaningless 'apples-and-oranges' comparisons. In the statement made here in the Abstract, the authors compare the *in vitro* concentrations used in their study to what they believe is the concentration of glyphosate in spray solutions typically used in agricultural operations. On page 13, the authors compare the *in vitro* concentrations used in their study to the highest existing tolerance for animal feed/hay uses. Both risk assessment comparisons are scientifically invalid. If the authors wish to relate their findings at the *in vitro* concentrations used in their experiments to actual/anticipated human exposures, then the appropriate exposure assessment must be conducted. In any case, the two referenced statements are misleading and especially inappropriate in a journal with the stated "Aims & Scope" of *Regulatory Toxicology and Pharmacology*.

Dig 1 was added to these cultures at a concentration of 2%, which equates to 20,000 ppm. Observing mitigation of cytotoxicity effect after adding 20,000 ppm of an organic extract is not an unexpected result. The authors have not justified why such a high concentration of the extract was added nor have they put into context what a 2% concentration translates to in an *in vivo* model. The extract most likely decreases bioavailability in much the same way that addition of BSA or serum would. The authors did not make these comparisons or discuss how serum free conditions do not represent physiological conditions.

Figure 2. This is not new information and was taken directly from Gasiner et al. 2009. Insert David's text

Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Séralini GE. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 2009 Aug 21;262(3):184-91.

Figure 5. The effect on caspase activity after 48 h of exposure to the formulation is uninterruptable. No where in the paper do the authors show the level of cytotoxicity for the R450 formulation after 48 hours of exposure. The caspase 3/7 activity noted in this figure reflects nothing more than a measure of cytotoxicity after exposure to a supra-physiological concentration of 60 ppm R450.

Figure 6. Same comment discussed for figure 5 applies to figure 6 for DAPI staining.

Figure 7. The authors still did not evaluate CYP1A1, CY2C9, CYP3A4 expression at the transcriptional and/or translational level. This is conventionally investigated to verify and further characterize modulation observed at the catalytic level. Without this assessment, it is neither possible to conclude a direct effect on cytochrome P450 activity nor conclude on the mode of action. This is a significant weakness in the manuscript and points to the lack of depth in this investigation.

Figure 8. In this experiment, it is stated cells were treated at the LC_{50} level for cytotoxicity of 25 ppm for the R400 formulation. Therefore, ~50% inhibition of GST activity is not unexpected and represents a measure of cytoxicity.

Figure 9. This model is not supported by the data developed in the paper. There is no direct evidence presented in this paper of induction of CYP3A4 and CYP1A2, nor the stimulation of metabolite production. Inhibition of GST is simply the result of cytotoxicity. Clearly, the decrease in succinate-dehydrogenase activity is the result of mitochondrial membrane disruption that consequently led to the induction of apoptosis (i.e., caspase induction and the consequently DNA condensation). However, the authors do not preface that this occurred only at high supra-physiological concentrations and these effects have been routinely observed by other researchers working with high concentrations of surfactants in cell culture.