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Title: Dig1 protects against cell death provoked by glyphosate-based herbicides in human liver cell lines
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General Comments:

Overall Conclusions: This manuscript has numerous grammatical/sentence syntax problems, poor definition of abbreviations, inadequate figure legends and misuse of terms, all of which result in very poor readability. Major revisions would be needed to make this manuscript acceptable for publication in any journal. More importantly, the manuscript has several major scientific flaws which make it unsuitable for publication. It does not contain any biologically relevant scientific information that would be useful to the Journal's readers. Therefore, it is recommended that this manuscript should not be accepted for publication in *Regulatory Toxicology and Pharmacology*.

The model presented at the end of the paper is not supported by the data developed in the paper. The effects on the measured endpoints are artifacts and can be attributed to the nonspecific action of the high concentrations of surfactants added to the cells in culture. Test materials were applied to cell cultures without consideration of relevant exposure levels at subcellular sites, which resulted in the exaggerated non-specific effects. For example, the decrease in succinate-dehydrogenase activity is clearly the result of mitochondrial membrane disruption that consequently led to the induction of apoptosis (i.e., caspase induction and the consequently DNA condensation).

The effects of the test material on cytochrome P450 activity is very mild and is likely the result from the non-specific effect on cellular membranes. The cytochrome P450 enzymes that were evaluated are anchored in the smooth endoplasmic reticulum where they are associated with reductases that supply them with reducing equivalents that allow them to catalyze monoxygentation reaction (hydroxylations). It is not uncommon to observe some modulation of cytochrome P450 enzymatic activity in the presence of surfactants that can either increase or decrease activity depending on the surfactant concentration and the extent of membrane disruption. This effect is related to a change in the arrangement of lipoprotein complexes in the membrane. Activation of enzyme activity following treatment with surfactants has been previously reported, particularly with microsomal proteins. For example, mammalian liver UDP glucuronyl transferase (decreased activity observed see figure 8) is firmly bound to microtonal membranes, and its activity has been shown to be strongly dependent on the presence of compounds that perturb membranes.

It is speculated by the authors that the observed effects are attributed to glyphosate and that glyphosate toxicity results from the addition of the adjuvant. However, the authors fail to

provide any evidence to support this conclusion. The authors did not separate the contributions to toxicity of glyphosate and the surfactant.

Further to the aforementioned points, an important inconsistency in the results is a reported induction of phase I metabolism but inhibition of phase II metabolism. This simply makes no sense since phase I and phase II metabolism have evolved as a coordinated system to promote rapid elimination of hydrophobic toxicants. A finding of induced phase I metabolism along with inhibition of phase II metabolism supports the conclusion that what is observed with the formulations is a non-specific effect.

Further to the comments on perturbation of cytochrome P450 catalytic activity discussed above, the authors did not evaluate CYP1A1, CY2C9, CYP3A4 expression at the transcriptional or translational level. This is conventionally investigated to verify and further characterize modulation observed at the catalytic level. This is a significant weakness in the manuscript and points to the lack of depth in this investigation.

The conclusions on how the plant extract Dig 1 is mitigating toxicity is based on unsupported speculation. The authors state on page 15 "*Our results show that D seems to penetrate the cells and does not only simply forms a shield of anti-penetrating agent for R that would have a fast and nonspecific action, but it implies particular levels of cell metabolism.*" There is no evidence presented in this paper showing how Dig-1 mitigates toxicity. The authors present information that cytochrome P450 activity is not affected by Dig-1. The affect of Dig-1 may very likely be one non-specific action countering another nonspecific action.

It is important to state that use of immortal cell lines derived from liver tissue were not an appropriate model to investigate hepatotoxicity in this paper. The authors are simply looking at the ability of a poorly characterized plant extract to potentially protect cells in culture. The authors neglected to test a known hepatotoxin or model adjuvants/surfactants to evaluate in parallel the effect of these compounds on these Hep cell lines. The authors misstate that these cell lines are commonly used to understand hepatotoxicity. These cell lines are primarily used to study cytochrome P450 regulation and activity since they constitutively express the receptors that regulate the CYP genes and express the CYPs proteins at relatively high levels.

Additionally, the authors provide a serious misrepresentation of citations. Many of these issues are related to the authors misunderstanding of the materials they are testing and the very nonspecific mode of surfactants particular when exposing cells in culture. It is evident to the reviewer that all of the specific responses observed in this study result from nonspecific membrane disruption of high concentrations of surfactants in cell cultures. Each of these major points is addressed below.

Specific Comments

Making specific comments on this manuscript was complicated by the fact that the authors did not number the lines in the manuscript, which is a standard practice for submitted manuscripts.

Introduction

Page 3. Many of the references cited by the authors were inappropriately or incorrectly used. Several instances of this are given below.

The authors references only one (Williams *et al.*, 2000) of many readily available documents and reviews of glyphosate highlighting low toxicity, including the US EPA Registration Eligibility Document (1993), European Commission Annex I Report and the Joint FAO/WHO Meeting on Pesticide Residues (2004).

Richard *et al.* (2005) was the first *in vitro* study by this author's group. The effects seen then and in their other publication including this one are secondary to the effect of surface acting substances, not a primary effect. This statement is not supported by the findings of that study.

Benachour *et al.* (2007b) is an inappropriate reference to earlier work by the same author.

The list of pesticidally inert ingredient from Cox (2004) was created from material safety data sheets on numerous pesticide products. The list is largely irrelevant, since only one of these is recognizably linked with glyphosate based formulations.

There is no basis for the conclusion extracted from Peluso *et al.* (1998). The authors states "some of these compounds may be genotoxic of form adducts to DNA" – but do not identify which compounds are they referring. The Peluso paper was an intraperotoneal (IP) injection of a Roundup branded formulation only sold in Italy. An IP injection is an irrelevant route of exposure for an herbicide. Heydens *et al.* (2008) demonstated that no effects were seen when the test material was dosed orally, that the results were related to the kidney and liver damage as a result of IP administration and test material adhering to the organs.

The Williams *et al.* (2000) citation is improperly used, since the paper did not address environmental fate, but rather it was a toxicology review and risk assessment for humans.

Takahashi *et al* (2001) does not provide evidence that "they also enter the food chain". This publication by Takahashi et al. is a description of an analytical method for residues of glyphosate AMPA in agricultural products. Residues of glyphosate and AMPA are legally allowed in agricultural products and food. The residues are approved and regulated by regulatory agencies around the world and scientific bodies such as CODEX.

The appearance of glyphosate and AMPA in surface water is inappropriately characterized. Glyphosate herbicides are approved by regulatory agencies for application to water bodies for control of aquatic plants. Therefore it is not surprising that glyphosate and its environmental degradate could be detected in surface waters. The World Health Organization determined that because of the their (glyphosate and AMPA) low toxicity, the health-based value derived for AMPA alone or in combination with glyphosate is orders of magnitude higher than concentrations of glyphosate or AMPA normally found in drinking-water. Under usual conditions, therefore, the presence of glyphosate and AMPA in drinking-water does not represent a hazard to human health. For this reason, the establishment of a guideline value for glyphosate and AMPA was not deemed necessary. Furthermore, they noted that most AMPA, the major metabolite of glyphosate found in water, comes from sources other than glyphosate degradation.

Acquavella *et al.* (2004) was a biomonitoring study with urine as the matrix. Forty percent of the applicators had no detectable levels of glyphosate in their urine (< 1 ppb), and those with detections were orders of magnitude below any regulatory standard. The authors did not provide the appropriate context of the study findings.

Clive (2007) is mis-referenced an inappropriately used. Clive James (the reference should be James, 2007) notes "biotech crops [are]..... associated with fewer insecticide and herbicide sprays". While Roundup use may increase, this is a substitution for pesticides with less desirable toxicological profiles which is generally considered to be a good trade-off for both human health and the environment. The author should acknowledge this.

Benachour *et al.* (2007b) worked with the human embryonic kidney 293 cell line. The effects noted were secondary due to surface active agents causing cell membrane disruption.

Page 4. The authors' hypothesis apparently attempts to link previous work conducted by the authors' group (Benachour *et al.*, 1007b) to the weak statistical associations noted in Savitz *et al.* (2007). However, in the Savitz *et al.* study, there were no statistically significant findings for glyphosate or Roundup related products.

The authors' inclusion of race and age for the source of immortal liver cell lines is unusual and not appropriate. However, this information could be considered in the Materials and Methods section if it is experimentally relevant.

The reference Knasmüller et al., (2004) could not be located.

Dig1 appears to be an uncharacterized plant extract from three different species. Neither explanation of the source of plant matter nor any extraction methods for Dig1 are provided. What is the purity of Dig 1, and were any impurities identified? In what way is Dig1 new – is it newly discovered for herbal remedies or recently isolated and identified, or perhaps it is new to the nutraceuticals market?

Statistical Analysis

Page 10. Significant descriptions of the statistical procedures are missing from the paper. LC/EC estimates are presented in the paper but there is no description of the procedure used to derive these estimates. Additionally, it is discussed that assays were repeated three times. This is taken to understand that this replication occurred across days (known as blocking by time). However, the stats were only done with a simple t-test and did not use a statistical model that considered the effect of day. Even more importantly, the authors failed to perform a statistical test that was adjusted for multiple comparisons. In other words, there were multiple treatments in each experiment, and performing multiple t-tests artificially inflates the power greatly, thereby increasing the Type I error rate (false positives). Therefore, the statistical analysis for this study was not done correctly and is consequently not suitable for publication.

Results

Page 11. The authors fail to acknowledge that exposures in the experiments shown in figure 1 are orders of magnitude above an environmentally realistic exposure. Exposing cells directly in culture to concentrations of glyphosate formulations at 0.5% is excessive. This exposure concentration equates to 5,000 ppm formulation. Surfactants are known to be toxic to cells in culture over this duration of exposure at concentrations in the low ppm range. A vast amount of literature has been developed in this area.

There are inconsistencies for duration of exposure and statements regarding growth rates (24 h vs 32 h) between the text, the legend for figure 1, and the Materials and Methods section.

It is incorrectly stated that cell death resulted from inhibition of succintate-dehydrogenase. Rather, the information presented only demonstrates decreased or inactivated succintate-dehydrogenase. The term inhibition is used to describe a specific type of interaction. The interaction described in this paper is evidently a nonspecific effect (membrane disruption) that leads to release of succinate-dehydrogenase from the mitochondrial membrane. This effect has been reported in the research for all classes of surfactants for several decades.

Page 12. As discussed in the general comments, the observed effects on P450 activity are of minimal magnitude. The effects of the test material on cytochrome P450 likely result from disruption of their anchoring in the endoplasmic reticulum where they are associated with reductases that supply them with reducing equivalents that allow them to catalyze monoxygentation reaction (hydroxylations). It is not uncommon to observe modulation of cytochrome P450 enzymatic activity in the presence of surfactants that can either increase or decrease activity depending on the surfactant concentration and the extent of membrane disruption. This effect is related to a change in the arrangement of lipoprotein complexes in the membrane. Activation of enzyme activity following treatment with surfactants has been previously reported, particularly with microsomal proteins (which is what is being evaluated in an S9 fraction). For example, mammalian liver UDP glucuronyl transferase (decreased activity observed see figure 8) is firmly bound to microsomal membranes, and its activity has been shown to be strongly dependent on the presence of compounds that perturb membranes (Graham AB, Wood GC. Factors affecting the response of microsomal UDP-glucuronyltransferase to membrane perturbants. *Biochim Biophys Acta.* 1973;7:45-50).

Discussion

Page 13. The use of LD50 is not appropriate and should be replaced with EC50.

The authors' reference to the residues in food or feed is not clear. The 400 ppm value cited appears to be a high tolerance for either alfalfa hay for livestock feed. There are no food use tolerances (what the authors refer to as "maximum level of residues authorized") for glyphosate at 400 ppm. Therefore, whatever the authors are attempting to infer about the relationship of the exposures used in their study to actual human dietary exposure, it is inaccurate.

Majer *et al.* (2004) – The aim of the study was to investigate the usefulness of two human derived hepatoma cell lines (HepG2 and Hep3B) for the detection of dietary and lifestyle related DNA-reactive carcinogens. Glyphosate is not DNA-reactive. The authors' use of this paper is an inappropriate attempt to link the Peluso *et al.* (1998) paper mentioned in the Introduction (an *in vivo* study using intraperatoneal injection, a non-relevant route of administration) to their *in vitro* methods and establish biological plausibility.

Page 14. It is speculated by the authors that the observed effects are attributed to glyphosate and that the glyphosate toxicity results from the addition of the adjuvant. However, the authors fail to provide any evidence to support this conclusion. The authors did not separate the contributions to toxicity of glyphosate and the surfactant. However, several studies have evaluated the effect of direct treatment of herbicide formulations on mitochondrial function (Oakes DJ, Pollack JK. Effects of a herbicide formulation, Tordon 75D, and its individual components on the oxidative functions of mitochondria. *Toxicology*. 1999;13;136:41-5 and Oakes DJ, Pollak JK. The *in vitro* evaluation of the toxicities of three related herbicide formulations containing ester derivatives of 2,4,5-T and 2,4-D using sub-mitochondrial particles. *Toxicology*. 2000;26;151:1-9.). Oakes and Pollak (1999) investigated the effect of another commercially available herbicide formulation on mitochondrial function using sub-mitochondrial particles. The herbicide formulation Tordon 75D® was found to inhibit electron transport with an IC50 value in the low micromolar range. By testing individual components of the herbicide formulation, it was shown that the proprietary surfactant polyglycol 26-2, when tested alone, uncoupled mitochondrial respiration to an equal level as the commercial formulation. None of the other components of the herbicide formulation had an inhibitory effect. This is consistent with the findings in another publication where a Roundup branded formulation and a formulation blank containing the surfactant produced an equivalent effect on mitochondrial function in cell culture (Levine S.L., Han, Z., Liu, J. Farmer, D.R., and V. Papadopoulos. 2007. Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis. *Cell Biol Toxicol.* 23:385-400).

It is stated that the adjuvant 'most probably' results in bioaccumulation of glyphosate or gene disruption. No substantiation/evidence for this statement is provided. Glyphosate has a negative log octanol-water coefficient and has empirically been shown not to bioaccumulate. Glyphosate has been reviewed by numerous regulatory/scientific groups globally is not considered to be genotoxic. The author repeatedly references his/her group's previously discussed *in vitro* results which can be dismissed as the effects of surface acting agents on cell membranes, as would be noted if testing surfactants in hand soaps and baby shampoos (see Levine, S.L., Han, Z., Lui, J., Farmer, D.R and V.Papadopoulos . 2007. Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis. *Cell Biol Toxicol.* Vol. 23(6):385-400).

Page 17. The author attempts to link the test items with genotoxic and carcinogenic endpoints, yet global regulatory authorities have reviewed extensive *in vivo* data and concluded glyphosate exhibits no genotoxicity or carcinogenicity – see previously mentioned regulatory authority reviews.

Hydroxylated metabolites of PCBs [OH-PCBs] have been shown to have agonist or antagonist interactions with hormone receptors (HRs) or hormone-receptor mediated responses. No data has

been demonstrated that glyphosate or a roundup branded formulation directly interacts with hormone receptors or that these are receptor mediated responses. The effects seen in the series of studies of Richard, Benachour and this current publication can be explained by the nonspecific effect of a surfactant on exposed cells *in vitro*. This is very different than what would be observed in an intact animal.

Study conclusion - Page 17. The statement that toxic effects are observed below environmental exposures is inaccurate. The Acquavella *et al.* citation cited by the authors in the Introduction revealed that 40% of the farmers did not have detectable levels in their urine despite some of them having made applications to fields of over 100 acres. Furthermore, 90% of the applicators in the study had systemic exposures below 0.001 mg/kg (0.001 ppm), which are several orders of magnitude below the EC/LC50 values reported in this study.

The authors referring to one of the Hep cultures used in this study coming from young boys does not belong in the conclusion. The source of the cells used to establish this immortal cell line must be described in the materials and methods section. It appears that the authors are inappropriately attempting to imply a concern for children's health.

The authors' statement "Of course G can be metabolized and excreted out of the body" is incorrect. Glyphosate is not metabolized and is rapidly excreted unchanged in mammals. There are no data to support that glyphosate penetrates mammalian cells in culture or *in vivo*. Likewise, there is no data to support the authors' claim that glyphosate bioaccumulates.

Page 18. The statement that D most likely stimulates detoxification is not supported by any data.

