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Short communication**A therapeutic oxygen carrier isolated from *Arenicola marina* decreases amanitin-induced hepatotoxicity**

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ABSTRACT

The amanitins (namely α - and β -amanitin) contained in certain mushrooms are bicyclic octapeptides that, when ingested, are responsible for potentially lethal hepatotoxicity. M101 is an extracellular hemoglobin extracted from the marine worm *Arenicola marina*. It has intrinsic Cu/Zn-SOD-like activity and is currently used as an oxygen carrier in organ preservation solutions. Our present results suggest that M101 might be effective in reducing amanitin-induced hepatotoxicity and may have potential for therapeutic development.

KEYWORDS: amanitin, oxygen carrier, M101, hepatocyte, hepatotoxicity

1. INTRODUCTION

Amanitins are high-molecular-weight bicyclic octapeptides present in certain mushrooms. When ingested, these compounds are responsible for potentially lethal hepatotoxicity. The best-characterized amanitins are α -amanitin and β -amanitin because they are present at high levels in the mushrooms involved in poisonings (Kaya et al., 2013). After amanitin accumulation in the liver *via* uptake through the organic anion transporter OATP1B3 in the hepatocyte's sinusoidal membrane (Letschert et al., 2006), the compounds inhibit the RNA polymerase II in the nucleus and thus jeopardize mRNA translation and protein synthesis (Wieland, 2009). The amanitin-induced stress signal has been shown to induce p53 protein, which then forms complexes with protective proteins (Bcl-X_L and Bcl-2) and subsequent triggers apoptosis via release of mitochondrial cytochrome c into the cytosol (Arima et al., 2005; Leu and George, 2007; Ljungman et al., 1999). Accordingly, Wang et al. (2018) showed that α -amanitin induces significant changes in the mitochondrial proteome and might destroy the mitochondrial membrane potential (Wang et al., 2018). It has also been suggested that oxidative stress is important in the development of severe hepatotoxicity, since α -amanitin accumulation leads to an increase in the activity of superoxide dismutase (SOD) and glutathione peroxidase, the production of malondialdehyde, and lipid peroxidation, and inhibits catalase activity (DüNdar et al., 2017; Zheleva et al., 2007). Furthermore, it has been reported that α -amanitin forms phenoxyl free radicals that might be involved in the production of reactive oxygen species (ROS) (Zheleva, 2013). Interestingly, most effective antidotes (including N-acetylcysteine, silymarin and silibinin) have antioxidant properties (Magdalan et al., 2011; Tavassoli et al., 2019; Ye and Liu, 2018). Thus, avoiding oxidative stress appears to be the most promising treatment option.

M101 is an extracellular hemoglobin extracted from the marine worm *Arenicola marina*. It is currently used in medicine as an oxygen carrier in organ preservation solutions. This class III medical device (commercialized as HEMO₂life® by Hemarina, Morlaix, France) was found to improve the preservation of kidney grafts (Mallet et al., 2014; Thuillier et al., 2019), hearts (Teh et al., 2017) lungs (Ali et al., 2020; Glorion et al., 2017) and liver transplants (Alix et al., 2020) *in vivo*. Furthermore, *in vitro* studies have demonstrated that M101's intrinsic Cu/Zn-SOD-like activity can protect against the damage potentially caused by ROS release (Lemaire et al., 2019; Rousselot et al., 2006; Thuillier et al., 2019). Moreover, M101's good safety profile has been demonstrated in preclinical studies - prompting

its therapeutic development. In this context, the objective of the present study was to evaluate M101's effect on the putative mechanisms of amanitin-induced hepatotoxicity and thus its potential for therapeutic development.

2. MATERIAL AND METHODS

2.1 Reagents: Phosphate-buffered saline (PBS), Williams's E medium, Hanks' balanced salt solution (HBSS), penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Eugene, OR, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Insulin, hydrocortisone, α - and β -amanitin, SOD inhibitors (diethylthiocarbamic acid [DETC] and 1,4,5-dichloro-2-m-tolylpyridazin-3(2H)-one [LCS]) and SOD assay kit were from Sigma Aldrich (St. Louis, MO, USA). The luminescent ATP detection assay kit was purchased from Abcam (Cambridge, MA, USA). MitoSOX red mitochondrial superoxide indicator was from ThermoFisher Scientific (Waltham, MA, USA). M101 (HEMO₂life®) was provided by Hemarina as a frozen stock solution (50 g/L) and used in these experiments at a final concentration in culture medium of 1 g/L (the concentration used in organ preservation solutions). Carboxy-M101 (M101-CO) was obtained by bubbling carbon monoxide gas 99.997% (Rapid'Gaz, Verdun, France) for 10 minutes in the M101 solution.

2.2 Cell cultures: Progenitor HepaRG cells were cultured as described elsewhere (Aninat et al., 2006). Briefly, the cells were seeded at a density of $2.6 \times 10^4/\text{cm}^2$ in 96-well plates in William's E medium supplemented with 10% FBS, 50 U/mL penicillin, 50 $\mu\text{g/mL}$ streptomycin, 5 $\mu\text{g/mL}$ insulin, 2 mM glutamine, and 50 μM sodium hydrocortisone hemisuccinate. After two weeks, cells were cultured for a further two weeks in the same medium supplemented with 2% DMSO in order to promote hepatocyte differentiation into both cholangiocyte- and hepatocyte-like cells (Cerec et al., 2007).

2.3 Measurement of amanitin-induced mitochondrial stress: MitoSOX Red Mitochondrial Superoxide Indicator (ThermoFisher Scientific, MA, USA) was used to measure mitochondrial ROS production. Briefly, cells were incubated in the dark with 100 μL of MitoSOX (5 μM) probes for 30 minutes at 37°C and with 5% CO₂. After the required incubation time, the medium was removed and the cells were washed once with 100 μL of HBSS at room temperature. The wells were then read quickly using a

fluorescence microplate reader (POLARstar Omega®, BMG labtech, Ortenberg, Germany), and the data were analyzed using MARS software (BMG labtech). The results were normalized for the protein concentration assay (determined with a Pierce™ BCA protein assay Kit, (ThermoFisher Scientific).

2.4 Cell viability: Cytotoxicity was assessed using a Luminescent ATP Detection Assay Kit (Abcam), according to the manufacturer's instructions. Plates were read using a microplate reader (POLARstar Omega®, BMG labtech).

2.5 SOD activity measurement: SOD activity was assessed using SOD assay kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. HepaRG cell lysis was performed with RIPA buffer (Sigma Aldrich) and SOD activity was evaluated in 40 µl of cell lysate.

2.6 Statistical analysis: Data were expressed as the mean ± standard error of the mean (SEM). Intergroup differences as a function of the treatment were probed in a one-way analysis of variance (ANOVA), with a Bonferroni *post hoc* test for group comparisons. All analyses were performed using Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). All tests were two-sided, and the threshold for statistical significance was set to $p < 0.05$. Differences between α - and β -amanitin were probed in a two-way ANOVA, with a bonferroni *post hoc* test.

3. RESULTS

Since amanitin exposure is known to be hepatotoxic, we looked at whether these toxins were able to dose-dependently decrease viability in a cell-based model of the liver (i.e. differentiated HepaRG cells). We found that amanitin significantly decreased the viability of differentiated HepaRG cells in a dose-dependent manner, starting at 1 µM - showing that the model was sensitive (Figure 1A). α -amanitin was more potent than β -amanitin in terms of loss of viability at 1 µM, but not at other concentrations. Furthermore, we observed that α - or β -amanitin increased mitochondrial ROS generation in a dose-dependent manner, starting at 2 µM. Mitochondrial ROS production was also higher with α -amanitin compared to β -amanitin at 2 µM ($p < 0.05$), but not at other concentrations. Taken together, these results

indicate that the amanitins' toxic effect on differentiated HepaRG cells is dependent - at least in part - on mitochondrial ROS production.

In order to investigate M101's effect on amanitin-induced toxicity, all subsequent experiments were performed with the concentration of α - and β -amanitin (2 μ M) that reduced the viability of differentiated HepaRG cells by 50% and induced significant mitochondrial oxidative stress. M101 (1 g/L) was added at the same time as amanitins (H0), 1 h after amanitins (H1), 3 h after amanitins (H3) or 6 h after amanitins (H6). Under all conditions, M101 remained incubated along with amanitins. We found that M101 alone (1 g/L) significantly increased cell viability. We observed that after 24 h of exposure to amanitin, cell viability was significantly higher in the presence of M101 (1 g/L) when added up to 6 h after β -amanitin (H6) and up to 3 h after α -amanitin (H3) (Figure 2A). This effect was associated with a significantly lower level of mitochondrial ROS generation in the presence of M101 (1 g/L) when added at the same time as α -amanitin (H0) and up to 6 h after β -amanitin (H6) (Figure 2B). M101 resulted in an increase in cell viability as well as a greater decrease in ROS production with β -amanitin than with α -amanitin. Taken together, these results show that M101 protects a cell-based hepatic model against amanitin's toxicity. Furthermore, M101 had a protective effect of the cells even when added several hours after amanitin.

Since M101 is capable of delivering oxygen to the surrounding environment, we aimed to evaluate whether M101 without its oxygen carrier properties (M101-CO) impacted intracellular SOD activity. HepaRG cells were incubated with M101 (1 g/L) or M101-CO (1 g/L) with or without SOD1 inhibitors (DETC 100 μ M or LCS 10 μ M) (Figure 2C). We found that M101 was able to increase intracellular SOD activity ($p < 0.01$), even in the presence of DETC ($p < 0.01$). Similar trend was observed using LCS without reaching statistical significance. This effect was no longer present using M101-CO (Figure 2C). Interestingly, none of these conditions resulted in cell death (Figure 2D).

4. DISCUSSION

In this study, we found that a therapeutic oxygen carrier isolated from *Arenicola marina* (M101) had protective effect on amanitin-induced toxicity on *in vitro* hepatic cell model. We first evaluated the amanitins' toxicity on differentiated HepaRG cells *in vitro*; we observed a dose-dependent decrease in cell viability and a concomitant elevation in mitochondrial ROS generation, starting at an amanitin

concentration of 2 μ M. Therefore, this 2 μ M dose was particularly relevant for testing the efficacy of an antidote on these two parameters. These results are in line with the literature data on the involvement of oxidative stress in amanitin toxicity (DüNdar et al., 2017; Zheleva, 2013; Zheleva et al., 2007). Our study showed that differentiated HepaRG cells constitute a sensitive, reproducible model for studying the toxicity of amanitin. We found that M101 alone significantly increased cell viability, probably because M101 has oxygen-carrier properties, resulting in increased mitochondrial activity. Interestingly, M101 was able to restore HepaRG cell viability and decrease mitochondrial ROS generation after α - and β -amanitin exposure. However, it is unknown whether a change in cell viability was due to an increase in ROS production or the changes in ROS was due to reduced viability. Moreover, we showed that the cells' ROS production was greater after α -amanitin exposure than after β -amanitin exposure. This might explain why (i) M101 had a greater effect on β -amanitin-induced effects and (ii) cell viability was even restored when M101 was added several hours after the toxin. Given that α - and β -amanitin are neutral and acid compounds, one can reasonably hypothesize that the differing responses to M101 are related to the toxins' physical-chemical properties (Garcia et al., 2015). Considering M101's macrostructure (3600 kDa, 15 x 25 nm), this hemoglobin is unlikely to enter hepatocytes. However, we hypothesize that M101's oxygen-carrier properties might be partially responsible for the beneficial effect observed *in vitro*. Using M101-CO, which allowed us to observe the effects of M101 without its oxygen-carrier properties (G. Tsai et al., 2012), we showed that M101 lost its intracellular SOD activating effect. Thus, although the precise mechanisms are not elucidated, these results suggest that oxygen supply by M101 is an important feature in its hepatoprotective effect. This does not exclude that other effects may be part the mechanism. For example, another M101 effect on HepaRG cells might be the down-regulation of pro-inflammatory cytokines and chemokine ligands, or the upregulation of pro-healing mediators and immune modulators, as already reported (Batoool et al., 2020). Lastly, further studies would be needed to determine whether M101's antioxidant Cu/Zn SOD-like properties are involved (Rousselot et al., 2006).

Our present results can be considered with regard to the currently available treatments for *Amanita phalloides* poisoning. The death rate associated with this poisoning is still high (between 10% and 20%), despite the use of antidotes such as penicillins, thiocetic acid, N-acetylcysteine, vitamin C, silymarin, and silibinin (Enjalbert et al., 2002; Escudié et al., 2007; Ganzert et al., 2005; Le Daré et al., 2021). The

fact that none of these compounds are highly effective means that *Amanita phalloides* poisoning is still a challenging medical emergency. We consider that there are two therapeutic perspectives for the use of M101. Firstly, intravenous infusion can be considered, since biodistribution studies in mice have shown that M101 reaches the liver easily (Le Gall et al., 2014). It has also been reported that silymarin enhances SOD activity, which enables a fall in oxidative stress through the harvesting and recycling of ROS, and thus an overall decrease in tissue inflammation and cell death. We suggest that M101's SOD-like activity (alone or in combination with currently available antidotes) might be of therapeutic value for promoting hepatocyte survival (Wellington and Jarvis, 2001). Secondly, M101 could be added to an organ preservation solution if liver transplantation is required after *Amanita phalloides* poisoning. Although this requirement is infrequent, there are case reports of re intoxication of the newly transplanted graft – jeopardizing the clinical outcome (Kucuk et al., 2005). Furthermore, this hemoglobin was developed first in the organ preservation indication, and its good safety profile has been reported in many indications - including liver transplant (Alix et al., 2020; Glorion et al., 2017; Mallet et al., 2014; Teh et al., 2017; Thuillier et al., 2019). Lastly, Ye et al. (2018) reported that liver transplantation was considered to be the only approach for guaranteeing survival in *Amanita phalloides* poisoning with fulminant hepatic failure (Ye and Liu, 2018). Our present data suggest that a liver transplant incubated with M101 might offer a better outcome in this context.

In conclusion, the present study is the first to have demonstrated the value of M101 for reducing amanitin-induced toxicity in an *in vitro* hepatic cell model. Further studies are needed to explore the exact mechanisms involved in M101's hepatoprotective effects. *In vivo* studies would be of particular interest.

Contributors

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Data analysis and interpretation: All authors

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Conflict of Interest Statement:

Franck Zal is the founder of and holds stock in Hemarina, the company that produces M101 and commercializes it as HEMO₂life®. Eric Delpy is an employee of Hemarina. The other authors declare that they have no conflicts of interest.

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REFERENCES

- Ali, A., Watanabe, Y., Galasso, M., Watanabe, T., Chen, M., Fan, E., Brochard, L., Ramadan, K., Ribeiro, R.V.P., Stansfield, W., Gokhale, H., Gazzalle, A., Waddell, T., Liu, M., Keshavjee, S., Cypel, M., 2020. An extracellular oxygen carrier during prolonged pulmonary preservation improves post-transplant lung function. *J. Heart Lung Transplant. Off. Publ. Int. Soc. Heart Transplant.* 39, 595–603. <https://doi.org/10.1016/j.healun.2020.03.027>
- Alix, P., Val-Laillet, D., Turlin, B., Ben Mosbah, I., Burel, A., Bobillier, E., Bendavid, C., Delpy, E., Zal, F., Corlu, A., Boudjema, K., 2020. Adding the oxygen carrier M101 to a cold-storage solution could be an alternative to HOPE for liver graft preservation. *JHEP Rep.* 2, 100119. <https://doi.org/10.1016/j.jhepr.2020.100119>
- Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouët, S., Morel, F., Guguen-Guillouzo, C., Guillouzo, A., 2006. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab. Dispos. Biol. Fate Chem.* 34, 75–83. <https://doi.org/10.1124/dmd.105.006759>
- Arima, Y., Nitta, M., Kuninaka, S., Zhang, D., Fujiwara, T., Taya, Y., Nakao, M., Saya, H., 2005. Transcriptional Blockade Induces p53-dependent Apoptosis Associated with Translocation of

- p53 to Mitochondria. J. Biol. Chem. 280, 19166–19176.
<https://doi.org/10.1074/jbc.M410691200>
- Batool, F., Stutz, C., Petit, C., Benkirane-Jessel, N., Delpy, E., Zal, F., Leize-Zal, E., Huck, O., 2020. A therapeutic oxygen carrier isolated from *Arenicola marina* decreased P. gingivalis induced inflammation and tissue destruction. Sci. Rep. 10, 14745. <https://doi.org/10.1038/s41598-020-71593-8>
- Cerec, V., Glaise, D., Garnier, D., Morosan, S., Turlin, B., Drenou, B., Gripon, P., Kremsdorf, D., Guguen-Guillouzo, C., Corlu, A., 2007. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. Hepatology 45, 957–967. <https://doi.org/10.1002/hep.21536>
- DüNdar, Z.D., ErgiN, M., KiliN, İ., Olak, T., Oltulu, P., Cander, B., 2017. The role of oxidative stress in α -amanitin-induced hepatotoxicity in an experimental mouse model. Turk. J. Med. Sci. 47, 318–325. <https://doi.org/10.3906/sag-1503-163>
- Enjalbert, F., Rapior, S., Nouguié-Soulé, J., Guillon, S., Amouroux, N., Cabot, C., 2002. Treatment of Amatoxin Poisoning: 20-Year Retrospective Analysis. J. Toxicol. Clin. Toxicol. 40, 715–757. <https://doi.org/10.1081/CLT-120014646>
- Escudié, L., Francoz, C., Vinel, J.-P., Moucari, R., Cournot, M., Paradis, V., Sauvanet, A., Belghiti, J., Valla, D., Bernuau, J., Durand, F., 2007. *Amanita phalloides* poisoning: Reassessment of prognostic factors and indications for emergency liver transplantation. J. Hepatol. 46, 466–473. <https://doi.org/10.1016/j.jhep.2006.10.013>
- G. Tsai, A., Intaglietta, M., Sakai, H., Delpy, E., Drieu La Rochelle, C., Rousselot, M., Zal, F., 2012. Microcirculation and NO-CO Studies of a Natural Extracellular Hemoglobin Developed for an Oxygen Therapeutic Carrier. Curr. Drug Discov. Technol. 9, 166–172. <https://doi.org/10.2174/157016312802650814>
- Ganzert, M., Felgenhauer, N., Zilker, T., 2005. Indication of liver transplantation following amatoxin intoxication. J. Hepatol. 42, 202–209. <https://doi.org/10.1016/j.jhep.2004.10.023>
- Garcia, J., Costa, V.M., Carvalho, A., Baptista, P., de Pinho, P.G., de Lourdes Bastos, M., Carvalho, F., 2015. *Amanita phalloides* poisoning: Mechanisms of toxicity and treatment. Food Chem. Toxicol. 86, 41–55. <https://doi.org/10.1016/j.fct.2015.09.008>
- Glorion, M., Polard, V., Favereau, F., Hauet, T., Zal, F., Fadel, E., Sage, E., 2017. Prevention of ischemia-reperfusion lung injury during static cold preservation by supplementation of standard preservation solution with HEMO₂ life[®] in pig lung transplantation model. Artif. Cells Nanomedicine Biotechnol. 1–8. <https://doi.org/10.1080/21691401.2017.1392315>
- Kaya, E., Yilmaz, I., Sinirlioglu, Z.A., Karahan, S., Bayram, R., Yaykasli, K.O., Colakoglu, S., Saritas, A., Severoglu, Z., 2013. Amanitin and phalloxin concentration in *Amanita phalloides* var. *alba* mushroom. Toxicon 76, 225–233. <https://doi.org/10.1016/j.toxicon.2013.10.008>
- Kucuk, H.F., Karasu, Z., Kilic, M., Nart, D., 2005. Liver Failure in Transplanted Liver Due to *Amanita Falloides*. Transplant. Proc. 37, 2224–2226. <https://doi.org/10.1016/j.transproceed.2005.03.107>
- Le Daré, B., Ferron, P.-J., Gicquel, T., 2021. Toxic Effects of Amanitins: Repurposing Toxicities toward New Therapeutics. Toxins 13, 417. <https://doi.org/10.3390/toxins13060417>
- Le Gall, T., Polard, V., Rousselot, M., Lotte, A., Raouane, M., Lehn, P., Opolon, P., Leize, E., Deutsch, E., Zal, F., Montier, T., 2014. In vivo biodistribution and oxygenation potential of a new generation of oxygen carrier. J. Biotechnol. 187, 1–9. <https://doi.org/10.1016/j.jbiotec.2014.07.008>
- Lemaire, F., Sigrist, S., Delpy, E., Cherfan, J., Peronet, C., Zal, F., Bouzakri, K., Pinget, M., Maillard, E., 2019. Beneficial effects of the novel marine oxygen carrier M101 during cold preservation of rat and human pancreas. J. Cell. Mol. Med. 23, 8025–8034. <https://doi.org/10.1111/jcmm.14666>
- Letschert, K., Faulstich, H., Keller, D., Keppler, D., 2006. Molecular Characterization and Inhibition of Amanitin Uptake into Human Hepatocytes. Toxicol. Sci. 91, 140–149. <https://doi.org/10.1093/toxsci/kfj141>
- Leu, J.I.-J., George, D.L., 2007. Hepatic IGFBP1 is a prosurvival factor that binds to BAK, protects the liver from apoptosis, and antagonizes the proapoptotic actions of p53 at mitochondria. Genes Dev. 21, 3095–3109. <https://doi.org/10.1101/gad.1567107>

- Ljungman, M., Zhang, F., Chen, F., Rainbow, A.J., McKay, B.C., 1999. Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogene* 18, 583–592. <https://doi.org/10.1038/sj.onc.1202356>
- Magdalan, J., Piotrowska, A., Gomul̓kiewicz, A., Sozański, T., Szeląg, A., Dziegiel, P., 2011. Influence of commonly used clinical antidotes on antioxidant systems in human hepatocyte culture intoxicated with α -amanitin. *Hum. Exp. Toxicol.* 30, 38–43. <https://doi.org/10.1177/0960327110368418>
- Mallet, V., Dutheil, D., Polard, V., Rousselot, M., Leize, E., Hauet, T., Goujon, J.M., Zal, F., 2014. Dose-Ranging Study of the Performance of the Natural Oxygen Transporter HEMO₂ Life in Organ Preservation: Dose-Ranging Study of the Performance of HEMO₂ Life. *Artif. Organs* 38, 691–701. <https://doi.org/10.1111/aor.12307>
- Rousselot, M., Delpy, E., Drieu La Rochelle, C., Lagente, V., Pirow, R., Rees, J.-F., Hagege, A., Le Guen, D., Hourdez, S., Zal, F., 2006. *Arenicola marina* extracellular hemoglobin: a new promising blood substitute. *Biotechnol. J.* 1, 333–345. <https://doi.org/10.1002/biot.200500049>
- Tavassoli, M., Afshari, A., Arsene, A.L., Mégarbane, B., Dumanov, J., Paoliello, M.M.B., Tsatsakis, A., Carvalho, F., Hashemzaei, M., Karimi, G., Rezaee, R., 2019. Toxicological profile of *Amanita virosa* – A narrative review. *Toxicol. Rep.* 6, 143–150. <https://doi.org/10.1016/j.toxrep.2019.01.002>
- Teh, E.S., Zal, F., Polard, V., Menasché, P., Chambers, D.J., 2017. HEMO₂ life as a protective additive to Celsior solution for static storage of donor hearts prior to transplantation. *Artif. Cells Nanomedicine Biotechnol.* 45, 717–722. <https://doi.org/10.1080/21691401.2016.1265974>
- Thuillier, R., Delpy, E., Matillon, X., Kaminski, J., Kasil, A., Soussi, D., Danion, J., Sauvageon, Y., Rod, X., Donatini, G., Barrou, B., Badet, L., Zal, F., Hauet, T., 2019. Preventing acute kidney injury during transplantation: the application of novel oxygen carriers. *Expert Opin. Investig. Drugs* 28, 643–657. <https://doi.org/10.1080/13543784.2019.1628217>
- Wang, M., Chen, Y., Guo, Z., Yang, C., Qi, J., Fu, Y., Chen, Z., Chen, P., Wang, Y., 2018. Changes in the mitochondrial proteome in human hepatocytes in response to alpha-amanitin hepatotoxicity. *Toxicon* 156, 34–40. <https://doi.org/10.1016/j.toxicon.2018.11.002>
- Wellington, K., Jarvis, B., 2001. Silymarin: A Review of its Clinical Properties in the Management of Hepatic Disorders: *BioDrugs* 15, 465–489. <https://doi.org/10.2165/00063030-200115070-00005>
- Wieland, T., 2009. The toxic peptides from *Amanita* mushrooms. *Int. J. Pept. Protein Res.* 22, 257–276. <https://doi.org/10.1111/j.1399-3011.1983.tb02093.x>
- Ye, Y., Liu, Z., 2018. Management of *Amanita phalloides* poisoning: A literature review and update. *J. Crit. Care* 46, 17–22. <https://doi.org/10.1016/j.jcrc.2018.03.028>
- Zheleva, A., 2013. Phenoxyl radicals formation might contribute to severe toxicity of mushroom toxin alpha amanitin - an electron paramagnetic resonance study.
- Zheleva, A., Tolekova, A., Zhelev, M., Uzunova, V., Platikanova, M., Gadzheva, V., 2007. Free radical reactions might contribute to severe alpha amanitin hepatotoxicity – A hypothesis. *Med. Hypotheses* 69, 361–367. <https://doi.org/10.1016/j.mehy.2006.10.066>

Figure 1: Effect of α - and β -amanitin on the viability of differentiated hepatocytes. Differentiated HepaRG cells were cultured with α - or β -amanitin (0.2 to 20 μ M) or medium for 24 h. Cell viability was measured using an extracellular ATP assay and expressed relative to the value determined after treatment with medium alone (arbitrarily set to 100%). Levels of mitochondrial ROS 24 h after amanitin treatment were detected using a MitoSOX (5 μ M) probe and normalized against the protein content. The data are quoted as the mean $\pm$ SEM from at least three independent experiments performed in triplicate. ** $p < 0.01$ *** $p < 0.001$: the control condition compared with α - or β -amanitin at different concentrations. # $p < 0.05$ ## $p < 0.01$: α -amanitin compared with β -amanitin.

Figure 2: Effect of M101 on amanitin-induced hepatotoxicity. (A and B) Differentiated HepaRG cells were cultured with α - or β -amanitin (2 μ M) or medium for 24 h at 37°C and with 5% CO₂ in the absence or presence of M101 (1 g/L) added up to 6 h after amanitin treatment. (A and D) The viability of differentiated HepaRG cells was measured using an extracellular ATP assay and expressed relative to the value determined after treatment with medium alone (arbitrarily set to 100%). (B) Levels of mitochondrial ROS 24 h after amanitin treatment were detected using a MitoSOX (5 μ M) probe and normalized against the protein content. (C) SOD activity in the presence of M101 (1 g/L), M101-CO (1 g/L), DETC (100 μ M) and/or LCS (10 μ M) was determined using SOD assay kit and expressed relative to the value determined after treatment with medium alone (arbitrary set to 0%). The data are quoted as the mean $\pm$ SEM from three independent experiments performed in triplicate. $^{##}p < 0.01$ $^{###}p < 0.001$: Control condition compared with α - or β -amanitin alone. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$: α - or β -amanitin alone compared with α - or β -amanitin + M101 added at a different time point (H0, H1, H3,

388 H6). $\&\& p < 0.01$ $\&\&\& p < 0.001$: α -amanitin compared with β -amanitin. \$ $p < 0.05$ \$\$\$ $p < 0.001$:
389 in comparison with DECT alone. $\&\& p < 0.01$ $\&\&\& p < 0.001$: in comparison with M101 alone.

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HIGHLIGHTS

- α - and β -amanitin lead to cell death and mitochondrial reactive oxygen species production in differentiated HepaRG cells.
- M101 is effective in reducing amanitin-induced cell death and mitochondrial reactive oxygen species production *in vitro*.
- M101 might be effective in reducing amanitin-induced hepatotoxicity and may have potential for therapeutic development.

This material is the authors' own original work, which has not been previously published elsewhere.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Franck Zal is the founder of and holds stock in Hemarina, the company that produces M101 and commercializes it as HEMO₂life®. Eric Delpy is an employee of Hemarina. The other authors declare that they have no conflicts of interest.