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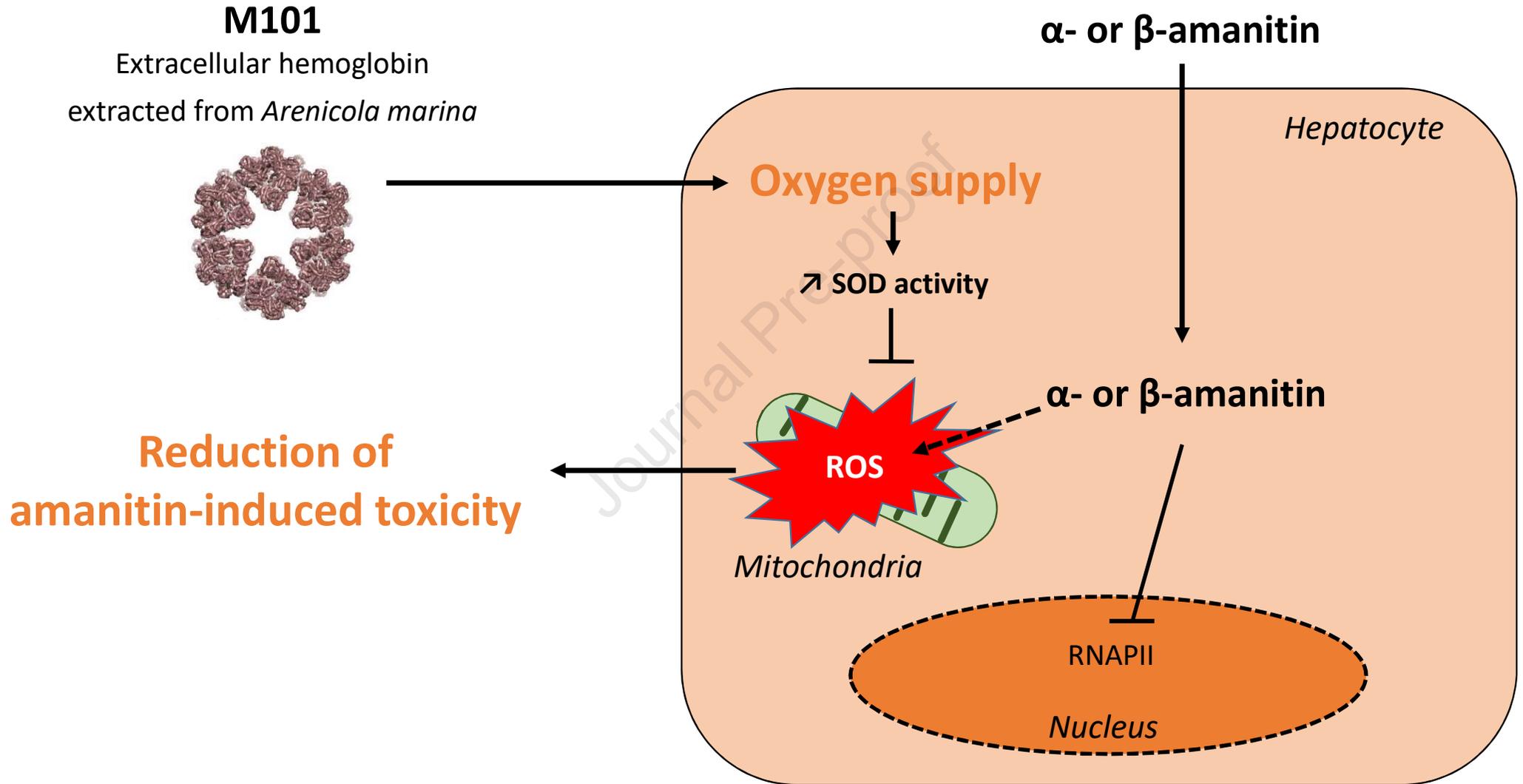
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1 **Short communication**

2 **A therapeutic oxygen carrier isolated from *Arenicola marina* decreases amanitin-**
3 **induced hepatotoxicity**

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6

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11

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25 **ABSTRACT**

26

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

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

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46 1. INTRODUCTION

47 Amanitins are high-molecular-weight bicyclic octapeptides present in certain mushrooms. When
48 ingested, these compounds are responsible for potentially lethal hepatotoxicity. The best-characterized
49 amanitins are α -amanitin and β -amanitin because they are present at high levels in the mushrooms
50 involved in poisonings (Kaya et al., 2013). After amanitin accumulation in the liver *via* uptake through
51 the organic anion transporter OATP1B3 in the hepatocyte's sinusoidal membrane (Letschert et al.,
52 2006), the compounds inhibit the RNA polymerase II in the nucleus and thus jeopardize mRNA
53 translation and protein synthesis (Wieland, 2009). The amanitin-induced stress signal has been shown
54 to induce p53 protein, which then forms complexes with protective proteins (Bcl-X_L and Bcl-2) and
55 subsequent triggers apoptosis via release of mitochondrial cytochrome c into the cytosol (Arima et al.,
56 2005; Leu and George, 2007; Ljungman et al., 1999). Accordingly, Wang et al. (2018) showed that α -
57 amanitin induces significant changes in the mitochondrial proteome and might destroy the mitochondrial
58 membrane potential (Wang et al., 2018). It has also been suggested that oxidative stress is important in
59 the development of severe hepatotoxicity, since α -amanitin accumulation leads to an increase in the
60 activity of superoxide dismutase (SOD) and glutathione peroxidase, the production of malondialdehyde,
61 and lipid peroxidation, and inhibits catalase activity (DüNdar et al., 2017; Zheleva et al., 2007).
62 Furthermore, it has been reported that α -amanitin forms phenoxy free radicals that might be involved
63 in the production of reactive oxygen species (ROS) (Zheleva, 2013). Interestingly, most effective
64 antidotes (including N-acetylcysteine, silymarin and silibinin) have antioxidant properties (Magdalan et
65 al., 2011; Tavassoli et al., 2019; Ye and Liu, 2018). Thus, avoiding oxidative stress appears to be the
66 most promising treatment option.

67 M101 is an extracellular hemoglobin extracted from the marine worm *Arenicola marina*. It is
68 currently used in medicine as an oxygen carrier in organ preservation solutions. This class III medical
69 device (commercialized as HEMO₂life[®] by Hemarina, Morlaix, France) was found to improve the
70 preservation of kidney grafts (Mallet et al., 2014; Thuillier et al., 2019), hearts (Teh et al., 2017) lungs
71 (Ali et al., 2020; Glorion et al., 2017) and liver transplants (Alix et al., 2020) *in vivo*. Furthermore, *in*
72 *vitro* studies have demonstrated that M101's intrinsic Cu/Zn-SOD-like activity can protect against the
73 damage potentially caused by ROS release (Lemaire et al., 2019; Rousselot et al., 2006; Thuillier et al.,
74 2019). Moreover, M101's good safety profile has been demonstrated in preclinical studies - prompting

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

78

79 **2. MATERIAL AND METHODS**

80 **2.1 Reagents:** Phosphate-buffered saline (PBS), Williams's E medium, Hanks' balanced salt solution
81 (HBSS), penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Eugene,
82 OR, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Insulin,
83 hydrocortisone, α - and β -amanitin, SOD inhibitors (diethylthiocarbamic acid [DETC] and 1,4,5-
84 dichloro-2-m-tolylpyridazin-3(2H)-one [LCS]) and SOD assay kit were from Sigma Aldrich (St. Louis,
85 MO, USA). The luminescent ATP detection assay kit was purchased from Abcam (Cambridge, MA,
86 USA). MitoSOX red mitochondrial superoxide indicator was from ThermoFisher Scientific (Waltham,
87 MA, USA). M101 (HEMO₂life[®]) was provided by Hemarina as a frozen stock solution (50 g/L) and
88 used in these experiments at a final concentration in culture medium of 1 g/L (the concentration used in
89 organ preservation solutions). Carboxy-M101 (M101-CO) was obtained by bubbling carbon monoxide
90 gas 99.997% (Rapid'Gaz, Verdun, France) for 10 minutes in the M101 solution.

91

92 **2.2 Cell cultures:** Progenitor HepaRG cells were cultured as described elsewhere (Aninat et al., 2006).
93 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
94 supplemented with 10% FBS, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 5 $\mu\text{g}/\text{mL}$ insulin, 2 mM
95 glutamine, and 50 μM sodium hydrocortisone hemisuccinate. After two weeks, cells were cultured for
96 a further two weeks in the same medium supplemented with 2% DMSO in order to promote hepatocyte
97 differentiation into both cholangiocyte- and hepatocyte-like cells (Cerec et al., 2007).

98

99 **2.3 Measurement of amanitin-induced mitochondrial stress:** MitoSOX Red Mitochondrial Superoxide
100 Indicator (ThermoFisher Scientific, MA, USA) was used to measure mitochondrial ROS production.
101 Briefly, cells were incubated in the dark with 100 μL of MitoSOX (5 μM) probes for 30 minutes at 37°C
102 and with 5% CO₂. After the required incubation time, the medium was removed and the cells were
103 washed once with 100 μL of HBSS at room temperature. The wells were then read quickly using a

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

107

108 **2.4 Cell viability:** Cytotoxicity was assessed using a Luminescent ATP Detection Assay Kit (Abcam),
109 according to the manufacturer's instructions. Plates were read using a microplate reader (POLARstar
110 Omega®, BMG labtech).

111

112 **2.5 SOD activity measurement:** SOD activity was assessed using SOD assay kit (Sigma Aldrich, St.
113 Louis, MO, USA) according to the manufacturer's instructions. HepaRG cell lysis was performed with
114 RIPA buffer (Sigma Aldrich) and SOD activity was evaluated in 40 µl of cell lysate.

115

116 **2.6 Statistical analysis:** Data were expressed as the mean ± standard error of the mean (SEM).
117 Intergroup differences as a function of the treatment were probed in a one-way analysis of variance
118 (ANOVA), with a Bonferroni *post hoc* test for group comparisons. All analyses were performed using
119 Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). All tests were two-sided, and the
120 threshold for statistical significance was set to $p < 0.05$. Differences between α - and β -amanitin were
121 probed in a two-way ANOVA, with a bonferroni *post hoc* test.

122

123 3. RESULTS

124 Since amanitin exposure is known to be hepatotoxic, we looked at whether these toxins were able to
125 dose-dependently decrease viability in a cell-based model of the liver (i.e. differentiated HepaRG cells).

126 We found that amanitin significantly decreased the viability of differentiated HepaRG cells in a dose-
127 dependent manner, starting at 1 µM - showing that the model was sensitive (Figure 1A). α -amanitin was
128 more potent than β -amanitin in terms of loss of viability at 1 µM, but not at other concentrations.

129 Furthermore, we observed that α - or β -amanitin increased mitochondrial ROS generation in a dose-
130 dependent manner, starting at 2 µM. Mitochondrial ROS production was also higher with α -amanitin
131 compared to β -amanitin at 2 µM ($p < 0.05$), but not at other concentrations. Taken together, these results

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

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

148 Since M101 is capable of delivering oxygen to the surrounding environment, we aimed to evaluate
149 whether M101 without its oxygen carrier properties (M101-CO) impacted intracellular SOD activity.
150 HepaRG cells were incubated with M101 (1 g/L) or M101-CO (1 g/L) with or without SOD1 inhibitors
151 (DETC 100 μ M or LCS 10 μ M) (Figure 2C). We found that M101 was able to increase intracellular
152 SOD activity ($p < 0.01$), even in the presence of DETC ($p < 0.01$). Similar trend was observed using
153 LCS without reaching statistical significance. This effect was no longer present using M101-CO (Figure
154 2C). Interestingly, none of these conditions resulted in cell death (Figure 2D).

155

156 4. DISCUSSION

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

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

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

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

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

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216 **Contributors**

217 All authors contributed significantly, and all authors agree with the content of the manuscript:

218 Conception/Design: Brendan Le Daré, Thomas Gicquel, Vincent Lagente

219 Collection and/or assembly of data: Brendan Le Daré, Thomas Gicquel, Vincent Lagente, Nessrine

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221 Data analysis and interpretation: All authors

222 Manuscript writing: All authors

223 Final approval of manuscript: All authors.

224

225 **Conflict of Interest Statement:**

226 Franck Zal is the founder of and holds stock in Hemarina, the company that produces M101 and

227 commercializes it as HEMO₂life®. Eric Delpy is an employee of Hemarina. The other authors declare

228 that they have no conflicts of interest.

229

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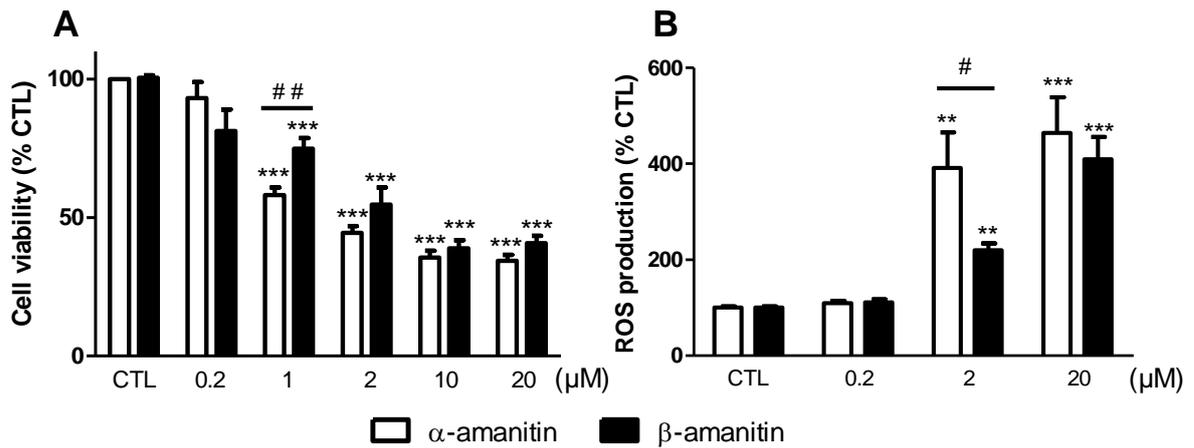
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356 **Figure 1: Effect of α - and β -amanitin on the viability of differentiated hepatocytes.** Differentiated357 HepaRG cells were cultured with α - or β -amanitin (0.2 to 20 μ M) or medium for 24 h. Cell viability was

358 measured using an extracellular ATP assay and expressed relative to the value determined after

359 treatment with medium alone (arbitrarily set to 100%). Levels of mitochondrial ROS 24 h after amanitin

360 treatment were detected using a MitoSOX (5 μ M) probe and normalized against the protein content. The361 data are quoted as the mean \pm SEM from at least three independent experiments performed in triplicate.362 ** $p < 0.01$ *** $p < 0.001$: the control condition compared with α - or β -amanitin at different363 concentrations. # $p < 0.05$ ## $p < 0.01$: α -amanitin compared with β -amanitin.

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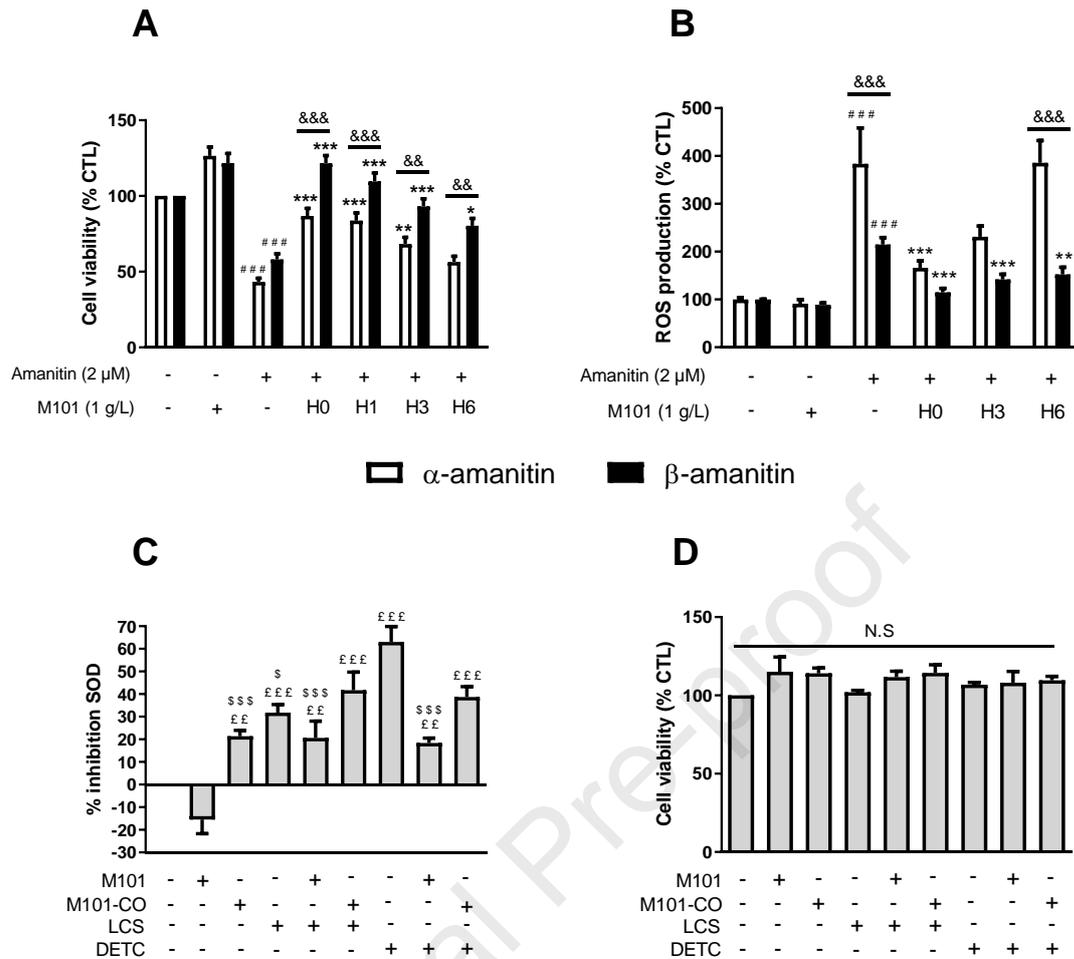
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376 **Figure 2: Effect of M101 on amanitin-induced hepatotoxicity.** (A and B) Differentiated HepaRG
 377 cells were cultured with α - or β -amanitin (2 μ M) or medium for 24 h at 37°C and with 5% CO₂ in the
 378 absence or presence of M101 (1 g/L) added up to 6 h after amanitin treatment. (A and D) The viability
 379 of differentiated HepaRG cells was measured using an extracellular ATP assay and expressed relative
 380 to the value determined after treatment with medium alone (arbitrarily set to 100%). (B) Levels of
 381 mitochondrial ROS 24 h after amanitin treatment were detected using a MitoSOX (5 μ M) probe and
 382 normalized against the protein content. (C) SOD activity in the presence of M101 (1 g/L), M101-CO (1
 383 g/L), DETC (100 μ M) and/or LCS (10 μ M) was determined using SOD assay kit and expressed relative
 384 to the value determined after treatment with medium alone (arbitrary set to 0%). The data are quoted as
 385 the mean \pm SEM from three independent experiments performed in triplicate. ## $p < 0.01$ ### $p < 0.001$:
 386 Control condition compared with α - or β -amanitin alone. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$: α - or β -
 387 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.