

1 **Doxorubicin-loaded human serum albumin nanoparticles overcome transporter-**  
2 **mediated drug resistance**

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19

20 **Abstract**

21 Resistance to systemic drug therapies is a major reason for the failure of anti-cancer therapies.  
22 Here, we tested doxorubicin-loaded human serum albumin (HSA) nanoparticles in the  
23 neuroblastoma cell line UKF-NB-3 and its ABCB1-expressing sublines adapted to vincristine  
24 (UKF-NB-3<sup>r</sup>VCR<sup>1</sup>) and doxorubicin (UKF-NB-3<sup>r</sup>DOX<sup>20</sup>). Doxorubicin-loaded nanoparticles  
25 displayed increased anti-cancer activity in UKF-NB-3<sup>r</sup>VCR<sup>1</sup> and UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells  
26 relative to doxorubicin solution, but not in UKF-NB-3 cells. UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells were re-  
27 sensitised by nanoparticle-encapsulated doxorubicin to the level of UKF-NB-3 cells. UKF-NB-  
28 3<sup>r</sup>DOX<sup>20</sup> cells displayed a more pronounced resistance phenotype than UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells  
29 and were not re-sensitised by doxorubicin-loaded nanoparticles to the level of parental cells.  
30 ABCB1 inhibition using zosuquidar resulted in similar effects like nanoparticle incorporation,  
31 indicating that doxorubicin-loaded nanoparticles circumvent ABCB1-mediated drug efflux.  
32 The limited re-sensitisation of UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells to doxorubicin by circumvention of  
33 ABCB1-mediated efflux is probably due to the presence of multiple doxorubicin resistance  
34 mechanisms. So far, ABCB1 inhibitors have failed in clinical trials, probably because systemic  
35 ABCB1 inhibition results in a modified body distribution of its many substrates including  
36 drugs, xenobiotics, and other molecules. HSA nanoparticles may provide an alternative, more  
37 specific way to overcome transporter-mediated resistance.

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**Keywords:**

Nanoparticles; human serum albumin; transporter; ABCB1; cancer; doxorubicin

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## 40 **Introduction**

41 According to Globocan [1], there "were 14.1 million new cancer cases, 8.2 million cancer  
42 deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012  
43 worldwide." Despite substantial improvements over recent decades, the prognosis for many  
44 cancer patients remains unacceptably poor. The outlook is particularly grim for patients that are  
45 diagnosed with disseminated (metastatic) disease who cannot be successfully treated by local  
46 treatment (surgery, radiotherapy) and depend on systemic drug therapy, because the success of  
47 systemic therapies is typically limited by the occurrence of therapy resistance [2-4].

48 Drug efflux mediated by transporters including ATP-binding cassette (ABC) transporters has  
49 been shown to play a crucial role in cancer cell drug resistance [2,5]. ABCB1 (also known as  
50 P-glycoprotein or MDR1) seems to play a particularly important role in cancer cell drug  
51 resistance as a highly promiscuous transporter that mediates the cellular efflux of a wide range  
52 of structurally different substrates including many anti-cancer drugs . Different studies have  
53 reported that nano-sized drug carrier systems can bypass efflux-mediated drug resistance [6].  
54 This includes various nanoparticle and liposome formulations of the ABCB1 substrate  
55 doxorubicin [7-12].

56 Here, we here investigated the effects of doxorubicin-loaded human serum albumin (HSA)  
57 nanoparticles in ABCB1-expressing neuroblastoma cells. HSA nanoparticles are easy to  
58 produce [13-17], and HSA is a well-tolerated material. It is the most abundant protein in human  
59 blood plasma and used in many pharmaceutical formulations, in particular as part of critical  
60 care treatment [18].

61

## 62 **Materials and methods**

63

## 64 **Reagents and chemicals**

65 HSA and glutaraldehyde were obtained from Sigma-Aldrich Chemie GmbH (Karlsruhe,  
66 Germany). Dulbecco's Phosphate buffered saline (PBS) was purchased from Biochrom GmbH  
67 (Berlin, Germany). Doxorubicin was obtained from LGC Standards GmbH (Wesel, Germany).  
68 All chemicals were of analytical grade and used as received.

69

## 70 **Human serum albumin (HSA) nanoparticle preparation by desolvation**

71 HSA nanoparticles were prepared by desolvation as previously described [13-17]. 100  $\mu$ L of a  
72 1% (w/v) aqueous doxorubicin solution were added to 500  $\mu$ L of a 40 mg/mL (w/v) HSA  
73 solution and incubated for 2 h at room temperature under stirring (550 rpm, Cimatic i  
74 Multipoint Stirrer, ThermoFisher Scientific, Langenselbold, Germany). Then, 4 mL ethanol  
75 96% were added at room temperature under stirring using a peristaltic pump (Ismatec ecoline,  
76 Ismatec, Wertheim-Mondfeld, Germany) at a flow rate of 1 mL/min. After the desolvation  
77 process, the resulting nanoparticles were stabilised/ cross-linked using different amounts of  
78 glutaraldehyde that corresponded to different percentages of the theoretic amount that is  
79 necessary for the quantitative crosslinking of the 60 primary amino groups present in the HSA  
80 molecules of the particle matrix. The addition of 4.7  $\mu$ L 8% (w/v) aqueous glutaraldehyde  
81 solution resulted in a theoretical cross-linking of 40% of the HSA amino groups, the addition  
82 of 11.8  $\mu$ L 8% (w/v) aqueous glutaraldehyde solution in 100% cross-linking, and the addition  
83 of 23.6  $\mu$ L 8% (w/v) aqueous glutaraldehyde solution in 200% cross-linking. After  
84 glutaraldehyde addition, the suspension was stirred for 12 h at 550 rpm. The particles were  
85 purified by repeating three times centrifugation at 16,000 g for 12 min and resuspension in  
86 purified water. During particle purification the supernatants were collected, the drug content

87 was measured by HPLC, and the loading efficiency of doxorubicin to the nanoparticles was  
88 calculated.

89

#### 90 **Determination of particle size distribution**

91 Average particle size and the polydispersity were measured by photon correlation spectroscopy  
92 (PCS) using a Malvern zetasizer nano (Malvern Instruments, Herrenberg, Germany). The  
93 resulting particle suspensions were diluted 1:100 with purified water and measured at a  
94 temperature of 22°C using a backscattering angle of 173°.

95

#### 96 **Doxorubicin quantification via HPLC-UV**

97 The amount of doxorubicin that had been incorporated into the nanoparticles was determined  
98 by HPLC-UV (HPLC 1200 series, Agilent Technologies GmbH, Böblingen, Germany) using a  
99 LiChroCART 250 x 4 mm LiChrospher 100 RP 18 column (Merck KGaA, Darmstadt,  
100 Germany). The mobile phase was a mixture of water and acetonitrile (70:30) containing 0.1%  
101 trifluoroacetic acid [16]. In order to obtain symmetric peaks a gradient was used. In the first  
102 6 min the percentage of A was reduced from 70% to 50%. Subsequently within 2 min the  
103 amount of A was further decreased to 20% and then within another 2 min increased again to  
104 70%. These conditions were held for a final 5 min resulting in a total runtime of 15 min. While  
105 using a flow rate of 0.8 mL/min, an elution time for doxorubicin of  $t = 7.5$  min was achieved.  
106 The detection of doxorubicin was performed at a wavelength of 485 nm [19].

107

108

## 109 **Cell culture**

110 The MYCN-amplified neuroblastoma cell line UKF-NB-3 was established from a stage 4  
111 neuroblastoma patient [20]. UKF-NB-3 sub-lines adapted to growth in the presence of  
112 doxorubicin 20 ng/mL (UKF-NB-3<sup>r</sup>DOX<sup>20</sup>) [20] or vincristine 1 ng/mL (UKF-NB-3<sup>r</sup>VCR<sup>1</sup>)  
113 were established by continuous exposure to step-wise increasing drug concentrations as  
114 previously described [20,21] and derived from the Resistant Cancer Cell Line (RCCL)  
115 collection [22].

116 All cells were propagated in Iscove's modified Dulbecco's medium (IMDM) supplemented  
117 with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C. The  
118 drug-adapted sub-lines were continuously cultured in the presence of the indicated drug  
119 concentrations. Cells were routinely tested for mycoplasma contamination and authenticated  
120 by short tandem repeat profiling.

121

## 122 **Cell viability assay**

123 Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium  
124 bromide (MTT) assay modified after Mosman [23], as previously described [Michaelis et al.,  
125 24].  $2 \times 10^4$  cells suspended in 100 µL cell culture medium were plated per well in 96-well plates  
126 and incubated in the presence of various drug concentrations for 120 h. Then, 25 µL of MTT  
127 solution (2 mg/mL (w/v) in PBS) were added per well, and the plates were incubated at 37°C  
128 for an additional 4 h. After this, the cells were lysed using 200 µL of a buffer containing 20%  
129 (w/v) sodium dodecylsulfate and 50% (v/v) N,N-dimethylformamide with the pH adjusted to  
130 4.7 at 37°C for 4 h. Absorbance was determined at 570 nm for each well using a 96-well  
131 multiscanner. After subtracting of the background absorption, the results are expressed as  
132 percentage viability relative to control cultures which received no drug. Drug concentrations

133 that inhibited cell viability by 50% (IC50) were determined using CalcuSyn (Biosoft,  
134 Cambridge, UK).

135

### 136 **Statistical testing**

137 Results are expressed as mean  $\pm$  S.D. of at least three experiments. Comparisons between two  
138 groups were performed using Student's t-test. Three and more groups were compared by  
139 ANOVA followed by the Student-Newman-Keuls test. P-values lower than 0.05 were  
140 considered to be significant.

141



## 142 **Results**

### 143 **Nanoparticle size, polydispersity and drug load**

144 HSA nanoparticles were prepared by desolvation as previously described [13-17]. The  
145 nanoparticles were stabilised by the crosslinking of free amino groups present in albumin. Three  
146 different nanoparticle preparations were produced using glutaraldehyde at amounts that  
147 corresponded to a theoretical cross-linking of 40% (HSA 40% nanoparticles), 100% (HSA  
148 100% nanoparticles), or 200% (HSA 200% nanoparticles) of the amino groups that are  
149 available in the HSA molecules. A non-stabilised (0% cross-linking) formulation was used as  
150 a control. The resulting particle sizes and polydispersity indices are shown in Table 1. HSA(0%)  
151 nanoparticles displayed a large particle size of almost 1  $\mu\text{m}$  range and a high polydispersity of  
152 0.5, confirming that no stable nanoparticles had formed (Table 1). The three HSA nanoparticle  
153 preparations stabilised by the different glutaraldehyde concentrations displayed similar  
154 diameters between 460 and 500 nm and polydispersity indices in the range of 0.153 and 0.213  
155 indicating a narrow but not monodisperse size distribution (Table 1).

156 While HSA(40%), HSA(100%), and HSA(200%) nanoparticles displayed similar drug loads  
157 between 152 and 191  $\mu\text{g}$  doxorubicin/ mg nanoparticle, HSA(0%) nanoparticles had bound  
158 371  $\mu\text{g}$  doxorubicin/ mg HSA (Table 1). This probably reflected the higher accessibility of  
159 doxorubicin binding sites, which are known to be available on HSA [25], in HSA molecules in  
160 solution compared to the accessible binding sites available in HSA nanoparticles.

161

162

### 163 **Doxorubicin sensitivity of the used neuroblastoma cell lines**

164 The parental neuroblastoma cell line UKF-NB-3 and its doxorubicin- (UKF-NB-3<sup>rDOX</sup><sup>20</sup>) and  
165 vincristine-adapted (UKF-NB-3<sup>rVCR</sup><sup>1</sup>) sub-lines substantially differed in their doxorubicin  
166 sensitivity (Figure 1). UKF-NB-3 displayed the lowest doxorubicin IC<sub>50</sub> (3.8 ng/mL). UKF-  
167 NB-3<sup>rVCR</sup><sup>1</sup> was 4-fold more resistant to doxorubicin than UKF-NB-3 (doxorubicin IC<sub>50</sub>:  
168 15.5 ng/mL). UKF-NB-3<sup>rDOX</sup><sup>20</sup> showed the highest doxorubicin IC<sub>50</sub> (89.0 ng/mL) resulting  
169 in a 23-fold increase in doxorubicin resistance compared to UKF-NB-3 (Figure 1, Suppl. Table  
170 1).

171

### 172 **Effects of doxorubicin-loaded nanoparticles on neuroblastoma cells**

173 The effects of doxorubicin applied in solution or incorporated into HSA(0%), HSA(40%),  
174 HSA(100%), or HSA(200%) nanoparticles on neuroblastoma cell viability are shown in Figure  
175 2. The numerical values are presented in Suppl. Table 1. Empty control nanoparticles did not  
176 affect cell viability in the investigated concentrations.

177 In the neuroblastoma cell line UKF-NB-3, the nanoparticle preparations displayed similar  
178 activity as doxorubicin solution, with doxorubicin-loaded HSA(40%), HSA(100%), and  
179 HSA(200%) nanoparticles potentially showing a trend towards a slightly increased activity  
180 (Figure 2). However, the differences did not reach statistical significance. Similar results were  
181 obtained in the doxorubicin-adapted UKF-NB-3 sub-line UKF-NB-3<sup>rDOX</sup><sup>20</sup>, although the  
182 difference between doxorubicin-loaded HSA(200%) nanoparticles and doxorubicin solution  
183 reached statistical significance (Figure 2). Notably, non-stabilised doxorubicin-bound  
184 HSA(0%) nanoparticles differed in their relative activity and did not reduce UKF-NB-3<sup>rDOX</sup><sup>20</sup>  
185 viability by 50% within the observed concentration range up to 200 ng/mL.

186 The vincristine-adapted UKF-NB-3 sub-line UKF-NB-3<sup>r</sup>VCR<sup>1</sup> displayed decreased  
187 doxorubicin sensitivity. However, doxorubicin-loaded HSA(40%), HSA(100%), and  
188 HSA(200%) nanoparticles displayed a higher relative potency compared to doxorubicin  
189 solution in UKF-NB-3<sup>r</sup>VCR<sup>1</sup> (Figure 2, Figure 3). The fold sensitisation doxorubicin IC50  
190 doxorubicin solution/ doxorubicin IC50 nanoparticle-bound doxorubicin for HSA(40%),  
191 HSA(100%), and HSA(200%) nanoparticles (3.6 - 4.5-fold) was higher than for UKF-NB-3  
192 (1.9 - 2.5-fold), and UKF-NB-3<sup>r</sup>DOX<sup>20</sup> (2.1 - 2.9-fold). The differences between doxorubicin-  
193 loaded HSA(40%) nanoparticles, HSA(100%) nanoparticles, and HSA(200%) nanoparticles  
194 and doxorubicin solution reached statistical significance ( $P < 0.05$ ) (Figure 2, Figure 3).  
195 Doxorubicin encapsulation into HSA(40%), HSA(100%), or HSA(200%) nanoparticles  
196 reduced the doxorubicin IC50 in UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells to the levels of doxorubicin solution  
197 in parental UKF-NB-3 cells (Figure 2, Suppl. Table 1). In contrast, the doxorubicin IC50 of  
198 doxorubicin-loaded HSA nanoparticles remained clearly (8-11-fold) higher in UKF-NB-  
199 3<sup>r</sup>DOX<sup>20</sup> cells than the doxorubicin IC50 of doxorubicin solution in parental UKF-NB-3 cells.

200

#### 201 **Effects of the ABCB1 inhibitor zosuquidar on the efficacy of nanoparticle-bound** 202 **doxorubicin in UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells**

203 Doxorubicin is an ABCB1 substrate, and UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells are characterised by high  
204 ABCB1 expression [20,26]. Vincristine is also an ABCB1 substrate, and vincristine-adapted  
205 cancer cell lines often display enhanced ABCB1 levels [20,26-29]. Accordingly, UKF-NB-  
206 3<sup>r</sup>VCR<sup>1</sup> cells are sensitised by the ABCB1 inhibitor zosuquidar [2-6] to doxorubicin to the level  
207 of parental UKF-NB-3 cells (Suppl. Figure 1), which indicates that ABCB1 expression  
208 contributes to the resistance phenotype observed in UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells.

209 Doxorubicin bound to nano-sized drug carrier systems has been shown to bypass ABCB1-  
210 mediated drug efflux [7-12]. In UKF-NB-3<sup>VCR</sup><sup>1</sup> cells, both zosuquidar and doxorubicin  
211 encapsulation into HSA nanoparticles reduced the doxorubicin IC<sub>50</sub> to the level of parental  
212 UKF-NB-3 cells (Figure 2, Suppl. Figure 1, Suppl. Table 1), which do not display detectable  
213 ABCB1 activity [20,27,29]. Hence, the increased activity of nanoparticle-bound doxorubicin  
214 that we observed in UKF-NB-3<sup>VCR</sup><sup>10</sup> cells is likely to be attributed to the circumvention of  
215 ABCB1-mediated doxorubicin efflux.

216 In UKF-NB-3<sup>DOX</sup><sup>20</sup> cells, however, the differences between doxorubicin solution and  
217 doxorubicin nanoparticles only reached statistical significance for doxorubicin-loaded  
218 HSA(200%) nanoparticles (Figure 2). Reasons for this may include that nanoparticle-  
219 incorporated doxorubicin do not completely avoid ABCB1-mediated efflux from UKF-NB-  
220 3<sup>DOX</sup><sup>20</sup> cells and/ or that doxorubicin resistance is caused by multiple resistance mechanisms  
221 and that avoidance of ABCB1-mediated transport is not sufficient to re-sensitise UKF-NB-  
222 3<sup>DOX</sup><sup>20</sup> cells to doxorubicin to the level of UKF-NB-3 cells.

223 To further study the role of ABCB1 as a doxorubicin resistance mechanism in UKF-NB-  
224 3<sup>DOX</sup><sup>20</sup> cells, we performed additional experiments in which we combined the ABCB1  
225 inhibitor zosuquidar and doxorubicin applied as a solution or nanoparticle preparations in UKF-  
226 NB-3<sup>DOX</sup><sup>20</sup> and UKF-NB-3 cells. Zosuquidar (1 μM) did not affect the efficacy of  
227 doxorubicin solution or nanoparticle-bound doxorubicin in parental UKF-NB-3 cells (Figure  
228 4), which do not display noticeable ABCB1 activity [20,27,29]. These experiments also  
229 confirmed that there is no significant difference in the anti-cancer activity between doxorubicin  
230 solution and doxorubicin nanoparticles in UKF-NB-3 cells, despite an apparent trend in the first  
231 set of experiments (Figure 2).

232 In UKF-NB-3<sup>DOX</sup><sup>20</sup> cells, addition of zosuquidar resulted in an increased sensitivity to free  
233 doxorubicin (Figure 4). The doxorubicin IC<sub>50</sub> decreased by 2.5-fold from 91 ng/mL in the

234 absence of zosuquidar to 37 ng/mL in the presence of zosuquidar, but not to the level of UKF-  
235 NB-3 cells (4.6 ng/mL) (Suppl. Table 2). This confirmed that ABCB1 is one among multiple  
236 resistance mechanisms that contribute to the doxorubicin resistance phenotype observed in  
237 UKF-NB-3<sup>r</sup>DOX<sup>20</sup>.

238 In this set of experiments, doxorubicin-loaded nanoparticles displayed a significantly increased  
239 activity compared to doxorubicin solution in UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells (Figure 4). This finding  
240 together with the non-significant trend observed in the first set of experiments (Figure 2)  
241 suggests that doxorubicin-loaded nanoparticles do indeed exert stronger effects against UKF-  
242 NB-3<sup>r</sup>DOX<sup>20</sup> cells than doxorubicin solution. Zosuquidar only moderately increased the  
243 efficacy of doxorubicin nanoparticles further (1.1 - 1.8-fold) in UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells (Figure  
244 4, Suppl. Table 2). In particular, the anti-cancer effects of doxorubicin-loaded HSA(200%)  
245 nanoparticles, the most active nanoparticle preparation in UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells, displayed a  
246 doxorubicin IC<sub>50</sub> of 20 ng/mL, which was not further reduced by addition of zosuquidar  
247 (doxorubicin IC<sub>50</sub>: 18 ng/mL) (Figure 4, Suppl. Table 2). Hence, the increased anti-cancer  
248 activity of doxorubicin incorporated into HSA nanoparticles appears to be primarily caused by  
249 circumventing ABCB1-mediated doxorubicin efflux in UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells.

250

## 251 **Discussion**

252 The occurrence of drug resistance is the major reason for the failure of systemic anti-cancer  
253 therapies [2]. Here, we investigated the effects of doxorubicin-loaded HSA nanoparticles on  
254 the viability of the neuroblastoma cell line UKF-NB-3 and its sub-lines adapted to doxorubicin  
255 (UKF-NB-3<sup>r</sup>DOX<sup>20</sup>) and vincristine (UKF-NB-3<sup>r</sup>VCR<sup>1</sup>), which both display ABCB1 activity  
256 and resistance to doxorubicin. The HSA nanoparticles were prepared by desolvation and  
257 stabilised by glutaraldehyde, which crosslinks amino groups present in albumin molecules [13-  
258 17]. Glutaraldehyde was used at molar concentrations that corresponded to 40% (Dox  
259 HSA(40%) nanoparticles), 100% (Dox HSA(100%) nanoparticles), or 200% (Dox HSA(200%)  
260 nanoparticles) theoretical cross-linking of the amino groups available in the HSA molecules.  
261 The resulting nanoparticle preparations had similar sizes of about 200 nm and low  
262 polydispersity indices in the range of 0.2.

263 Doxorubicin-loaded nanoparticles displayed similar activity as doxorubicin solution in the  
264 parental UKF-NB-3 cell line, but exerted stronger effects than doxorubicin solution in the  
265 ABCB1-expressing UKF-NB-3 sub-lines. UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells were similarly sensitive to  
266 doxorubicin-loaded nanoparticles as parental UKF-NB-3 cells to doxorubicin solution (and  
267 doxorubicin-loaded nanoparticles). This suggests that the doxorubicin resistance of UKF-NB-  
268 3<sup>r</sup>VCR<sup>1</sup> cells exclusively depends on ABCB1 expression. In concordance, the ABCB1 inhibitor  
269 zosuquidar re-sensitised UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells to the level of parental UKF-NB-3 cells.

270 UKF-NB-3<sup>r</sup>DOX<sup>20</sup> cells displayed a more pronounced doxorubicin resistance phenotype than  
271 UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells and were neither re-sensitised by nanoparticle-encapsulated  
272 doxorubicin nor by zosuquidar to the level of UKF-NB-3 cells. This suggests that UKF-NB-  
273 3<sup>r</sup>DOX<sup>20</sup> cells have developed multiple doxorubicin resistance mechanisms. In contrast,  
274 adaptation of UKF-NB-3<sup>r</sup>VCR<sup>1</sup> cells to vincristine, a tubulin-binding agent with an anti-cancer  
275 mechanism of action that is not related to that of the topoisomerase II inhibitor doxorubicin,

276 did not result in the acquisition of changes that confer doxorubicin resistance beyond ABCB1  
277 expression [2,20,30,31]. This indicates that the personalised use of nanoparticle-encapsulated  
278 transporter substrates will benefit from the use of biomarkers that indicate drug-specific  
279 resistance mechanisms in addition to transporter expression.

280 Furthermore, zosuquidar did not increase the efficacy of doxorubicin-loaded HSA(100%) and  
281 HSA(200%) nanoparticles and only modestly enhanced the efficacy of doxorubicin-loaded  
282 HSA(40%) nanoparticles. Together, these data confirm that administration of doxorubicin as  
283 HSA nanoparticles resulted in the circumvention of ABCB1-mediated drug efflux. The  
284 difference between HSA(40%) nanoparticles and the other two preparations may be explained  
285 by elevated drug release due to the lower degree of cross-linking.

286 Interestingly, high concentrations of the crosslinker glutaraldehyde did not affect the efficacy  
287 of the resulting doxorubicin-loaded nanoparticles although high glutaraldehyde concentrations  
288 might have been expected to affect drug release and/ or to bind covalently to doxorubicin via  
289 its amino group.

290 Notably, the results differ from a recent similar study in which nanoparticles prepared from  
291 poly(lactic-co-glycolic acid) (PLGA) or polylactic acid (PLA), two other biodegradable  
292 materials approved by the FDA and EMA for human use [32,33], did not bypass ABCB1-  
293 mediated drug efflux [34]. Differences in the mode of uptake and cellular distribution of  
294 nanoparticles from different materials may be responsible for these discrepancies. HSA  
295 nanoparticles may be internalised upon interaction with cellular albumin receptors [35,36].  
296 Notably, nab-paclitaxel, an HSA nanoparticle-based preparation of paclitaxel (another ABCB1  
297 substrate [26]), which is approved for the treatment of different forms of cancer [37], had  
298 previously been shown not to avoid ABCB1-mediated drug efflux [38]. However, nab-  
299 paclitaxel is not produced by the use of crosslinkers, and the interaction of paclitaxel with

300 albumin may differ from that of doxorubicin. Hence, variations in drug binding and drug release  
301 kinetics may be responsible for this difference.

302 Despite the prominent role of ABCB1 as a drug resistance mechanism, attempts to exploit it as  
303 drug target have failed so far, despite the development of highly specific allosteric ABCB1  
304 inhibitors (of which zosuquidar is one) [5,26]. A number of reasons seem to account for the  
305 clinical failure of ABCB1 inhibitors. ABCB1 is expressed at various physiological borders and  
306 involved in the control of the body distribution of its many endogenous and exogenous  
307 substrates. Systemic ABCB1 inhibition can therefore result in toxicity as consequence of a  
308 modified body distribution of anti-cancer drugs (and other drugs that are co-administered for  
309 other conditions than cancer), xenobiotics, and other molecules. In addition, cancer cells may  
310 be characterised by multiple resistance mechanisms (including the expression of multiple  
311 reporters) and targeting just one transporter may not be sufficient to overcome resistance (as  
312 supported by our current finding that UKF-NB-3'DOX<sup>20</sup> cells cannot be fully re-sensitised to  
313 doxorubicin by zosuquidar) [2,5,26]. Hence, the use of drug carrier systems to bypass ABC  
314 transporter-mediated drug efflux is conceptually very attractive, because it can (in contrast to  
315 specific inhibitors of ABCB1 or other transporters) overcome resistance mediated by multiple  
316 transporters and does not result in the systemic inhibition of ABC transporter function at  
317 physiological barriers.

318 In conclusion, doxorubicin-loaded HSA nanoparticles produced by desolvation and  
319 crosslinking using glutaraldehyde overcome (in contrast to other nanoparticle systems)  
320 transporter-mediated drug resistance.

321



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325

326 **Declarations of interest**

327 None

328

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- 432

433 **Table 1.** Nanoparticle diameter, polydispersity, and drug load.

<b>Nanoparticles</b>	<b>Diameter (nm)</b>	<b>Polydispersity</b>	<b>Drug load (<math>\mu\text{g}</math> doxorubicin/ mg nanoparticle)</b>
HSA(0%)	848.7	0.500	370.9
HSA(40%)	485.8	0.189	151.9
HSA(100%)	496.4	0.213	190.5
HSA(200%)	463.4	0.153	164.8

434

435

436 **Figure legends**

437 **Figure 1.** Doxorubicin sensitivity of UKF-NB-3, its doxorubicin-adapted sub-line UKF-NB-  
438 3<sup>r</sup>DOX<sup>20</sup> and its vincristine-adapted sub-line UKF-NB-3<sup>r</sup>VCR<sup>1</sup>. A) Doxorubicin concentrations  
439 that reduce cell viability by 50% (IC50) as indicated by MTT assay after 120 h of incubation.  
440 B) Fold change in doxorubicin sensitivity (doxorubicin IC50 UKF-NB-3 sub-line/ doxorubicin  
441 IC50 UKF-NB-3). Numerical values are presented in Suppl. Table 1. \* P < 0.05 relative to  
442 UKF-NB-3

443 **Figure 2.** Effects of doxorubicin (Dox) applied as a solution or incorporated into human serum  
444 albumin (HSA) nanoparticles on neuroblastoma cell viability. The investigated nanoparticles  
445 differed in the amount of the cross-linker glutaraldehyde that was used for nanoparticle  
446 stabilisation. The amount of glutaraldehyde corresponded to 40% (Dox HSA(40%) NP), 100%  
447 (Dox HSA(100%) NP), or 200% (Dox HSA(200%) NP) theoretical cross-linking of the  
448 available amino groups present on HSA. Preparations prepared without glutaraldehyde served  
449 as a control (Dox HSA(0%) NP). Values are expressed as concentrations that reduce cell  
450 viability by 50% (IC50) as determined by MTT assay after 120 h of incubation. Numerical  
451 values are presented in Suppl. Table 1. Empty nanoparticles did not affect cell viability in the  
452 investigated concentrations. \* P < 0.05 relative to doxorubicin solution; # IC50 > 200 ng/mL

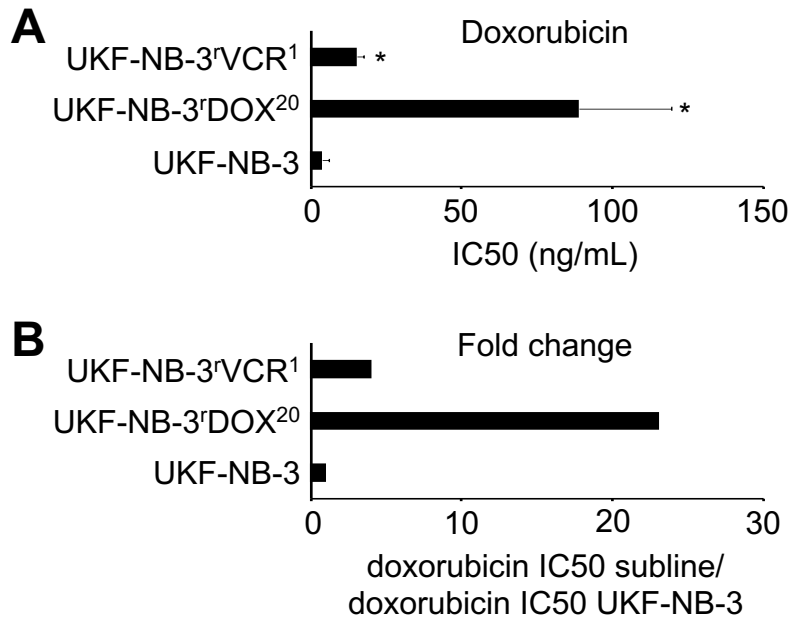
453 **Figure 3.** Fold sensitisation to doxorubicin by doxorubicin-bound nanoparticles (NP). Values  
454 are expressed as fold changes doxorubicin (Dox) IC50 of doxorubicin solution/ doxorubicin  
455 IC50 of doxorubicin-bound nanoparticles (NPs). Human serum albumin (HSA) nanoparticles  
456 were stabilised by glutaraldehyde concentrations corresponding to 40% (Dox HSA(40%) NP),  
457 100% (Dox HSA(100%) NP), or 200% (Dox HSA(200%) NP) theoretical cross-linking of the  
458 available amino groups present on HSA.



459 **Figure 4.** Doxorubicin (Dox) concentrations that reduce neuroblastoma cell viability by 50%  
460 (IC50) in the presence or absence of the ABCB1 inhibitor zosuquidar (1  $\mu$ M) as determined by  
461 MTT assay after 120 h incubation. Doxorubicin was either applied as a solution or incorporated  
462 into human serum albumin (HSA) nanoparticles which had been stabilised by addition of  
463 glutaraldehyde concentrations corresponding to 40% (Dox HSA(40%) NP), 100% (Dox  
464 HSA(100%) NP), or 200% (Dox HSA(200%) NP) theoretical cross-linking of the available  
465 amino groups present on HSA. Zosuquidar (1  $\mu$ M) did not affect cell viability on its own.  
466 Numerical data are presented in Suppl. Table 2. \* P < 0.05 relative to the doxorubicin IC50 in  
467 the absence of zosuquidar; § P < 0.05 relative to doxorubicin solution

468

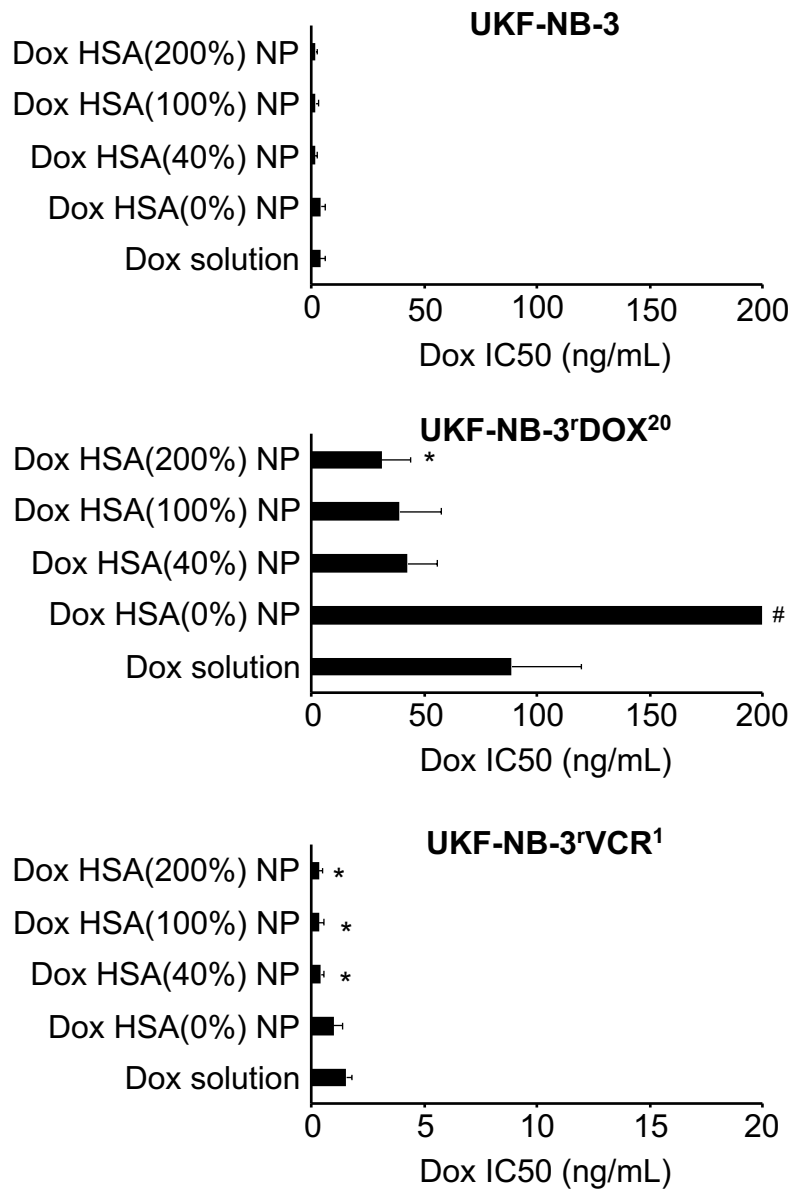
## Figure 1



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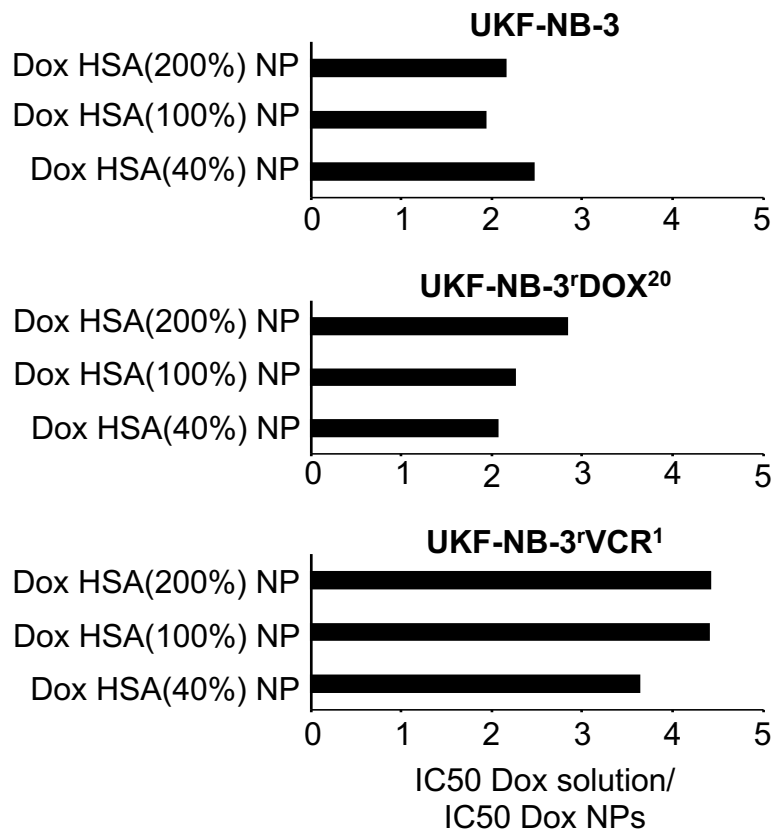
## Figure 2



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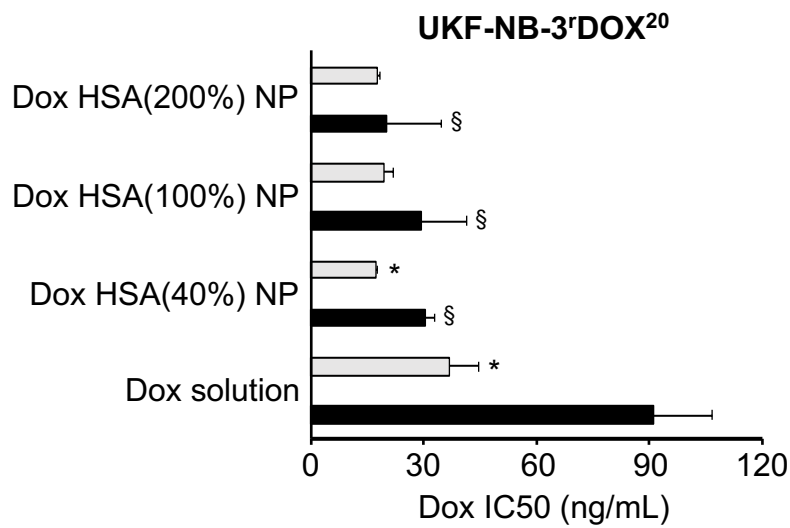
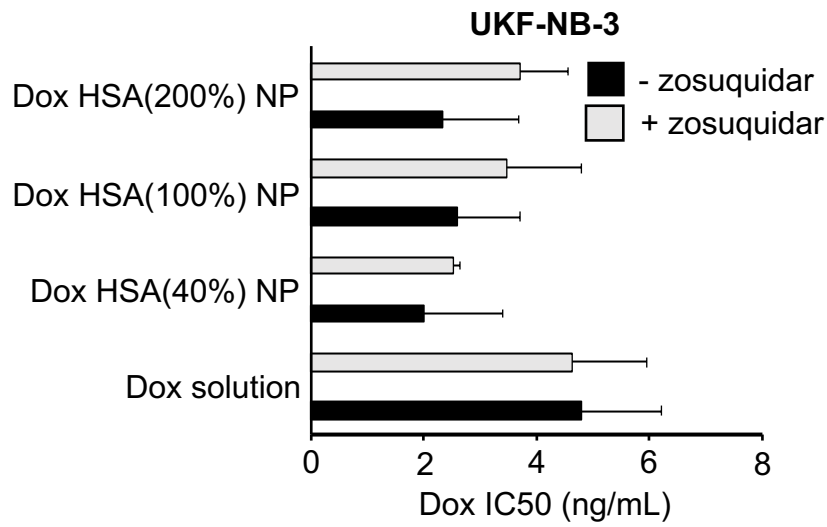
### Figure 3



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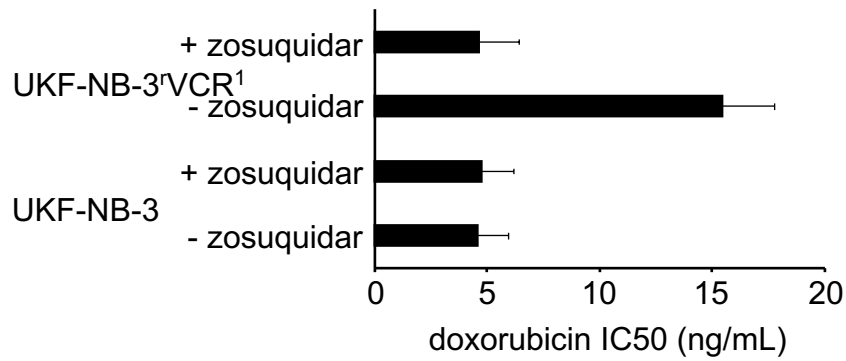
**Figure 4**



475

476

## Suppl Figure 1



**Suppl. Figure 1.** Doxorubicin concentrations that reduce neuroblastoma cell viability by 50% (IC50) in the absence or presence of the ABCB1 inhibitor zosuquidar (1 $\mu$ M).

477

478

479 **Suppl. Table 1.** Effects of doxorubicin (Dox) applied as solution or incorporated into human  
 480 serum albumin (HSA) nanoparticles on neuroblastoma cell viability. The investigated  
 481 nanoparticles differed in the amount of the crosslinker glutaraldehyde that was used for  
 482 nanoparticle stabilisation. The glutaraldehyde amount corresponded to 40% (Dox HSA(40%)  
 483 NP), 100% (Dox HSA(100%) NP), or 200% (Dox HDA(200%) NP) of the theoretical amount  
 484 of available amino groups present on HSA. Preparations prepared without glutaraldehyde  
 485 served as control (Dox HSA(0%) NP). Values are expressed as concentrations that reduce cell  
 486 viability by 50% (IC<sub>50</sub>) as determined by MTT assay after 120h of incubation.

487

	IC <sub>50</sub> doxorubicin (ng/mL)		
	UKF-NB-3	UKF-NB-3 <sup>20</sup> DOX <sup>20</sup>	UKF-NB-3 <sup>1</sup> VCR <sup>1</sup>
Dox solution	3.85 ± 2.46	89.0 ± 30.8 (23.1) <sup>1</sup>	15.5 ± 2.3 (4.03) <sup>1</sup>
DoxHSA(0%)	4.20 ± 1.72 (1.09) <sup>2</sup>	>200 <sup>3</sup> (>2.25) <sup>2</sup>	9.88 ± 3.78 (0.64) <sup>2</sup>
DoxHSA(40%)	1.55 ± 1.00 (0.40) <sup>2</sup>	42.8 ± 13.3 (0.48) <sup>2</sup>	4.25 ± 1.35 (0.27) <sup>2</sup>
DoxHSA(100%)	1.98 ± 1.03 (0.51) <sup>2</sup>	39.1 ± 18.6 (0.44) <sup>2</sup>	3.52 ± 2.00 (0.23) <sup>2</sup>
DoxHSA(200%)	1.78 ± 1.04 (0.46) <sup>2</sup>	31.2 ± 12.9 (0.35) <sup>2</sup>	3.51 ± 1.66 (0.23) <sup>2</sup>

488

489 <sup>1</sup> fold change in doxorubicin sensitivity relative to UKF-NB-3

490 <sup>2</sup> fold change in doxorubicin sensitivity relative to doxorubicin solution

491 <sup>3</sup> cell viability in the presence of doxorubicin 200 ng/mL applied as non-stabilised HSA  
 492 preparation: 81.9 ± 12.9% relative to untreated control

493

494 **Suppl. Table 2.** Effects of doxorubicin (Dox) applied as solution or incorporated into human  
 495 serum albumin (HSA) nanoparticles on neuroblastoma cell viability in the absence or presence  
 496 of zosuquidar (1 $\mu$ M). The investigated nanoparticles differed in the amount of the crosslinker  
 497 glutaraldehyde that was used for nanoparticle stabilisation. The glutaraldehyde amount  
 498 corresponded to 40% (Dox HSA(40%) NP), 100% (Dox HSA(100%) NP), or 200% (Dox  
 499 HDA(200%) NP) of the theoretical amount of available amino groups present on HSA.. Values  
 500 are expressed as concentrations that reduce cell viability by 50% (IC50) as determined by MTT  
 501 assay after 120h of incubation.

502

UKF-NB-3	+ Zosuquidar (1 $\mu$ M)			
	Doxorubicin IC50 (ng/mL)	Zosuquidar alone <sup>1</sup>	Doxorubicin IC50 (ng/mL)	Fold change <sup>2</sup>
Doxorubicin	4.80 $\pm$ 1.41	107 $\pm$ 24	4.64 $\pm$ 1.33	1.04
Dox HSA (40%) NP	2.01 $\pm$ 1.40	107 $\pm$ 24	2.52 $\pm$ 0.11	0.80
DOX HSA (100%) NP	2.61 $\pm$ 1.11	107 $\pm$ 24	3.48 $\pm$ 1.31	0.75
DOX HSA (200%) NP	2.34 $\pm$ 1.35	107 $\pm$ 24	3.70 $\pm$ 0.86	0.63

503

UKF-NB-3 <sup>+</sup> DOX <sup>20</sup>	+ Zosuquidar (1 $\mu$ M)			
	Doxorubicin IC50 (ng/mL)	Zosuquidar alone <sup>1</sup>	Doxorubicin IC50 (ng/mL)	Fold change <sup>2</sup>
Doxorubicin	91.0 $\pm$ 15.9	112 $\pm$ 17	36.9 $\pm$ 7.7	2.47
Dox HSA (40%) NP	30.5 $\pm$ 2.4	112 $\pm$ 17	17.4 $\pm$ 0.3	1.75
DOX HSA (100%) NP	29.3 $\pm$ 12.2	112 $\pm$ 17	19.3 $\pm$ 2.5	1.52
DOX HSA (200%) NP	20.1 $\pm$ 14.4	112 $\pm$ 17	17.7 $\pm$ 0.6	1.14



504

505

506 <sup>1</sup> cell viability in the presence of Zosuquidar (1 $\mu$ M) expressed as % untreated control

507 <sup>2</sup> doxorubicin IC50/ Doxorubicin IC50 in the presence of zosuquidar

508