Suppression of MD2 Inhibits Breast Cancer in Vitro and in Vivo

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1 **Title page**

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3 Suppression of MD2 inhibits breast cancer in vitro and in vivo

4 **Authors' name**

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20

21

22

23 Abstract

24 **Background:** To explore the effects of the intervening measure targeting myeloid differentiation
25 2 (MD2) on breast cancer progression *in vitro* and *in vivo*.

26 **Methods:** The expression of MD2 in normal breast cells (Hs 578Bst) and three kinds of breast
27 carcinoma cell lines (MCF-7, MDA-MB-231s and 4T1) were detected by western blot. MTT
28 assay was used to detect the proliferation of 4T1 cells treated by L6H21, cell migration and
29 invasion was measured by wound healing assay and transwell matrigel invasion assay,
30 respectively. In addition, to further study the role of MD2 in tumor progression, we assessed the
31 effects of inhibition of MD2 on the progression of xenograft tumors *in vivo*.

32 **Results:** The expression of MD2 is much higher in MDA-MB-231s and 4T1cells than that in
33 normal breast cells (Hs 578Bst) or MCF-7 cells ($P<0.05$). *In vitro*, suppression of MD2 by
34 L6H21 has a significant inhibition of proliferation, migration and invasion in 4T1 cells
35 in dose-dependent manner. *In vivo*, L6H21 pretreatment significantly improved survival of
36 4T1-bearing mice ($P<0.05$). Additionally, we also observed that there was none of the mice died
37 from the toxic of $10\text{ mg}\cdot\text{kg}^{-1}$ L6H21 in 60 days.

38 **Conclusion:** Overall, this work indicates that suppression of MD2 shows progression inhibition
39 *in vitro* and significantly prolong survival *in vivo*. These findings provide the potential
40 experimental evidence for using MD2 as a therapeutic target of breast carcinoma.

41 **Key words:** Breast neoplasms, myeloid differentiation 2 (MD2), proliferation, migration,
42 invasion.

43

44

45 **Introduction**

46 Breast cancer is one of the most common cancers affecting women worldwide[1]. Despite
47 intensive efforts and remarkable advances in the management of breast cancer, the physiological
48 conditions that lead to tumorigenesis including breast cancer are not well understood and distant
49 metastasis are still occurred in part of patients after treatments. It was reported that over 90% of
50 the deaths of cancer patients are caused by metastasis[2]. Therefore, Finding new modalities that
51 treat the local and systemic components of the disease has become increasingly important.

52
53 Toll like receptors (TLRs), which are essential components of innate immune system and serve
54 as major contributor to chronic inflammation[3], have garnered an extraordinary amount of
55 interest in cancer research because of their role in tumor progression[4-7]. Among TLRs, TLR4
56 has already been linked to tumors including breast cancer and has been implicated in tumor cell
57 invasion, survival, and metastasis[8-11].

58
59 MD2, well known as an accessory protein of TLR4, plays an essential role in activation of TLR4
60 signaling pathway in inflammatory response[12]. However, whether MD2 has a similar effect on
61 the progression of breast cancer is still poorly understood and there was rare evidence indicating
62 the correlation of MD2 and tumor progression. Previously, our cooperaters synthesized a new
63 chalcone derivative, (E)-2,3-dimethoxy-4'-methoxychalcone (L6H21), which identified MD2 as
64 its molecular target. In addition, they demonstrated that L6H21 shows excellent inhibition of the
65 TLR4-mediated inflammatory response and septic injury both *in vitro* and *in vivo*[13]. Therefore,
66 in order to investigate whether L6H21 has antitumor effects, in this study, we first detected the

67 expression of MD2 in four kinds of cell lines by western blot assay. Next, we aimed to observe
68 and confirm that the effects of L6H21 on 4T1 cells proliferation, migration and invasion *in vitro*.
69 Additionally, model of transplanted tumor on BALB/c nude mouse were used to study
70 the anticancer effect of L6H21 *in vivo*.

71

72 **Materials and methods**

73 ***Cell culture***

74 Normal breast cells (Hs 578Bst) and breast cancer cell lines MCF-7, MDA-MB-231s and 4T1
75 were obtained from ATCC and grown according to ATCC recommended culture conditions. The
76 cells were cultured in DMEM (Gibco) or RPMI 1640 medium (Gibco) with 10% fetal bovine
77 serum (FBS) (Gibco), and then incubated at 37°C in a humidified atmosphere containing 5%
78 CO₂ and 95% air.

79

80 ***Western blot***

81 Cell protein samples (50µg) were subjected to 10% SDS-PAGE and transferred onto a PVDF
82 membrane (Bio-Rad Laboratories). After being blocked in blocking buffer (5% milk
83 intris-buffered saline containing 0.05% Tween 20) for 1.5 h at room temperature, membranes
84 were incubated with different primary antibodies overnight at 4°C. The membranes then were
85 washed in TBS-T and reacted with secondary horseradish peroxidase-conjugated antibody (Santa
86 Cruz, CA, USA;1:5000) for 1-2 h at room temperature. Blots were then visualized using
87 enhanced chemiluminescence reagents (Bio-Rad Laboratories). The density of the
88 immunoreactive bands was analysed using Image J software (NIH, Bethesda, MD, USA).

89

90 ***MTT assay***

91 Cell proliferation was determined using the MTT method. Briefly, 48 h after treatment, the
92 MCF-7, MDA-MB-231s and 4T1 cells were seeded into 96-well culture plates (BD Biosciences,
93 Franklin Lakes, NJ, USA) and incubated overnight at 37°C in 5% CO₂. Cell proliferation was
94 assessed at 24, 48, 72, 96 and 120 h following addition of 0.5 mg/ml MTT (Sigma, USA)
95 solution. After a 4-h incubation, The reaction was stopped by addition of 150 µl DMSO (Sigma).
96 After 10 min of agitation (100 rpm), the optical density (OD) at 490 nm was determined with
97 microplate reader (BioTek). Each sample was tested with six replicates. All experiments were
98 performed in biological triplicate.

99

100 ***Wound migration assay***

101 The 4T1 cells were grown in six-well plates to a confluent monolayer and subsequently wounded
102 with sterile pipette tips. The wounded monolayers were then incubated with lipopolysaccharide
103 (LPS) (Sigma-Aldrich) and corresponding protein as indicated in Figure(3A) for 48 hours. The
104 wound area was measured under microscope. The percentage of wound healing rate was
105 estimated as follows: Wound healing rate % = [1-(wound width at 48 hours/wound width at 0
106 hour)] ×100%. ×100 microscopic fields under microscope.

107

108 ***Transwell Matrigel invasion assay***

109 Tumor cell invasion was performed using Transwell system (Millipore) with 8 µm-pore
110 polycarbonate filter membrane. The upper chamber was covered with Matrigel (Sigma-Aldrich)

111 and incubated at 4°C overnight. The upper chamber was then seeded with 1×10^4 4T1 cells
112 incubated with LPS and corresponding protein as indicated in Figure(3B) and inserted into the
113 lower chamber containing complete medium. After incubation at 37°C in 5% CO₂ for 24 hours,
114 the cells on the interior of upper chamber were removed, and the polycarbonate membranes were
115 stained with 0.1% crystal violet (BASO) for 10 minutes. The number of migrating cells was
116 counted in eight randomly selected $\times 400$ microscopic fields under microscope.

117

118 *Xenograft assays in nude mice*

119 To evaluate *in vivo* tumorigenesis, breast carcinoma xenografting mouse model was used. Male
120 BALB/c mice weighing 18-22g were obtained from the Wenzhou Medical University Animal
121 Centre and prepared for tumor implantation. All experimental procedures involving animals were
122 performed in accordance with animal protocols approved by the Institutional Animal Use and
123 Care Committee of Wenzhou Medical University and performed according to the institutional
124 ethical guidelines for animal experiment. The mice were randomly divided into four groups (n=8
125 per group). 4T1-bearing mice were injected with 3×10^5 4T1 cells (i.v. through the tail vein) 3
126 days after being treated with L6H21 (at 10 mg·kg⁻¹ or 5 mg·kg⁻¹) and saline by intragastric
127 administration, respectively. To investigate the toxicity of L6H21, mice of the fourth group were
128 only treated with L6H21 at 10 mg·kg⁻¹ but without inoculation of 4T1 cells. Each group of mice
129 were then intragastrically administrated per day until death or day 60. The survival curve was
130 made to analyse the survival rate.

131

132 *Statistical analysis*

133 For all data, statistical analysis was performed in SPSS 22.0 for Windows (SPSS Inc.). All data
134 are shown as means±SDs. The statistical significance between groups was obtained by Student's
135 t-test or one-way ANOVA test and the significance level was set at $P < 0.05$.

136

137 **Results**

138 *MD2 expression in four kinds of cell lines*

139 In our experiment, we first used western blot assay to examine the expression of MD2 in
140 normal breast cells (Hs 578Bst) and three kinds of breast carcinoma cell lines (MCF-7,
141 MDA-MB-231s and 4T1). The logarithmic phase of these cells were cracked and the total
142 protein was extracted. MD2 protein expression was then detected by western blot. As shown in
143 Figure 1, MD2 expressed in each kind of cell line, of these, MD2 is highly expressed in
144 highly malignant MDA-MB-231s and 4T1 cells.

145

146 **Figure 1** MD2 is highly expressed in MDA-MB-231s and 4T1 cells ($p < 0.05$ compared to that expressed in Hs 578Bst cells
147 and MCF-7 cells)

148

149 *L6H21 inhibition of breast cancer cells proliferation*

150 To explore the role of MD2 in breast cancer cells proliferation, we assessed the effects of
151 inhibition of MD2 by L6H21 in 4T1 cells by MTT assay. As shown in Figure 2, L6H21

152 significantly inhibited the proliferation of 4T1 cells in dose-dependent manner ($IC_{50}=10.41\ \mu M$) ,
 153 which has a similar inhibition effect in each group by curcumin, respectively.

154
 155 **Figure 2** MTT assay showed that L6H21(10 μM, 30 μM and 100 μM) significantly inhibited the proliferation of 4T1 cells
 156 in dose-dependent manner.

157

158 ***L6H21 inhibition of breast cancer cells migration and invasion***

159 To study the effect of MD2 in cell migration, we adopted a scratch wound model in the
 160 presence of mitomycin C which inhibited proliferation. In these conditions, migration was
 161 significantly decreased in L6H21 group (10 μM) and anti-MD2 group (1 μg/mL) as compared
 162 to the control group. Next, we performed transwell invasion assay to investigate the effect of
 163 L6H21 in 4T1 cell invasion further. As shown in Figure 5, there were significant differences
 164 of effect of invasion in control group, 5 μM L6H21 group and 10 μM L6H21 group. Moreover,
 165 there was almost no cell observed in 10 μM L6H21 group as well as in 1 μg/mL anti-MD2
 166 group. These results indicate that L6H21 has a significant inhibition of migration and

167 invasion in 4T1 cell concentration-dependently.

168

169

170 **Figure 3** L6H21 inhibition of 4T1 cells migration and invasion.(A) L6H21 significantly inhibited the migration of 4T1

171 cells, The images (100×) were obtained by microscope. (B) L6H21 significantly inhibited the invasion of 4T1 cells(400×).

172 In 5μM L6H21 group ,there were a few migrating cells observed compared to that of control group.While in 10μM L6H21

173 group and in 1μg/ml anti-MD2 group, there was almost no migrating cell observed.

174

175 ***L6H21 suppresses tumor progression in the nude mice***

176 To further investigate the role of MD2 in tumor progression, we assessed the effects of

177 inhibition of MD2 on the progression of xenograft tumors *in vivo*. Figure 4 shows the

178 survival rate of 4T1-bearing mice (treated with saline, 5mg·kg⁻¹ and 10 mg·kg⁻¹ L6H21,

179 respectively) and toxic control mice (L6H21 treated but without inoculation of 4T1 cells). As
 180 expected, the 5 mg·kg⁻¹ L6H21- or 10 mg·kg⁻¹ L6H21-treated mice survived significantly (p
 181 <0.05) longer than the saline-treated mice. The mean survival times of 4T1-bearing mice
 182 treated with saline, 5 mg·kg⁻¹ and 10 mg·kg⁻¹ L6H21 were 23.8±4.8, 30.9±8.9, 40.4±12.6
 183 days, respectively. In addition, after treated for 60 days, there were still two mice (25%)
 184 survive in 4T1-bearing mice treated with 10 mg·kg⁻¹ L6H21. Interestingly, we also observed
 185 that there was none of the mice died from the toxic of 10 mg·kg⁻¹ L6H21 in 60 days. These
 186 data indicated that as an inhibitor of MD2, L6H21, could prolong survival efficiently with
 187 reliable security.

188
 189 **Figure 4** L6H21 enhanced survival in nude mice. Male BALB/c mice were randomly divided into four groups (n=8 per
 190 group). 4T1-bearing mice were injected with 3×10^5 4T1 cells (i.v. through the tail vein) 3 days after being treated with
 191 L6H21 (at 10 mg·kg⁻¹ or 5 mg·kg⁻¹) and saline by intragastric administration, respectively, and the mice of the fourth
 192 group were only treated with L6H21 at 10 mg·kg⁻¹ but without inoculation of 4T1 cells. Each group of mice were then
 193 intragastrically administrated per day until death or day 60. The survival curve was made to analyse the survival rate.

194

195 *Statistical analysis*

196 For all data, statistical analysis was performed in SPSS 22.0 for Windows (SPSS Inc.). All
197 data are shown as means±SDs. The statistical significance between groups was obtained by
198 Student's t-test or one-way ANOVA test and the significance level was set at $P < 0.05$

199

200 **Discussion**

201 Diverse studies have shown that TLR4 is associated with tumor development and progression.
202 In breast cancer, TLR4 activation has been linked to both cancer inhibition and
203 growth[10,14-16]. Huan Yang et al reported that TLR4 expressed higher levels than any other
204 TLRs and knockdown of TLR4 could actively inhibit proliferation and survival of human
205 breast cancer cells MDA-MB-231. Functional analyses of ribonucleic acid interference
206 (RNAi) against TLR4 revealed this successfully inhibited the growth and proliferation of
207 MDA-MB-231 cells and resulted in a significant ($P < 0.05$) reduction of inflammatory
208 cytokines[11]. In other work, 4T1 cells challenged with lipopolysaccharide induced tumor
209 growth and metastasis, by increasing angiogenesis, vascular permeability, and tumor
210 invasion[17-18]. A total of 74 breast carcinomas were collected from patients to study the
211 clinical relevance of TLRs in breast cancer. Tumors with high TLR4 expression, in
212 mononuclear cells were found to have a higher probability of metastasis[19]. These studies
213 suggest TLR4 involvement in breast cancer progression.

214

215 Although TLR4 plays an essential part in breast cancer progression, the role of its accessory
216 protein, MD2, known for several years as an essential co-factor for TLR4 signaling, has not

217 yet been clarified. One study highlighted that MD2 was overexpressed in highly invasive
218 colorectal cancer cells (SW837), in poorly differentiated, moderately invasive colorectal
219 cancer cells (HT-29), and in well-differentiated but non-invasive colorectal cancer cells
220 (Caco-2)[17]. Another study reported that serum amyloid A 3, a major component of acute
221 inflammation, binds to MD2 and activates the MyD88-dependent TLR4/MD2 pathway and
222 thus facilitates lung metastasis[18]. Therefore, MD2 could be related to the degree of
223 differentiation, proliferation, and migration capacity of cancers. However, there was few
224 research focus on the relationship between the MD2 expression and the progression of breast
225 cancer.

226
227 In the first stage of our study, we detected the expression of MD2 in several cell lines. As
228 expected, the western blot assay showed MD2 is highly expressed in MDA-MB-231s (the
229 estrogen receptor-negative, progesterone receptor-negative and HER2 negative -human breast
230 cancer cells) and 4T1 cells (spontaneously metastasizing mammary adenocarcinoma). Our
231 preliminary results may probably indicate that MD2 is higher expressed in highly malignant
232 cell lines than that in normal breast cells (Hs 578Bst) or MCF-7 cells ($P<0.05$).

233
234 In order to investigate the role of MD2 in breast cancer progression, we next utilized L6H21,
235 a new MD2 inhibitor, for down-regulating the expression of the MD2. And we performed
236 experiments to observe the change of biological behavior of 4T1 cells by using L6H21. MTT
237 assay was performed and the results showed that L6H21 significantly inhibited the
238 proliferation of 4T1 cells in dose-dependent manner, especially in 100 μ M group. Moreover,

239 scratch wound model and transwell invasion assay demonstrated that L6H21 has a significant
240 inhibition of migration and invasion in 4T1 cell. These results indicated that suppression of
241 MD2 could effective repress tumor cell proliferation, migration and invasion *in vitro*, in part
242 consistent with the findings of Virginie Grondin *et al* which was experimented with HT-29
243 cell[20]. To further determine whether MD2 regulates tumor progression *in vivo*, we used
244 tumor xenografts by inoculating 4T1 cells in L6H21-treated or saline-treated nude mice. And
245 we provided evidence that MD2 suppression by L6H21 prolonged survival in nude mice with
246 hypotoxicity.

247

248

249 **Conclustion**

250 In conclusion, our study indicates that 4T1 cells treated with L6H21 show progression
251 inhibition *in vitro*. Moreover, L6H21 can significantly prolong survival *in vivo*. To our
252 knowledge, this is the first report to describe the significance of MD2 expression to breast
253 caner cells *in vitro* and *in vivo*. Although the precise molecular mechanisms behind the
254 altered expression of MD2 in breast cancer remain poorly understood, our data suggest that
255 MD2 may be a promising candidate as a potential therapeutic target for breast cancer
256 intervention.

257

258 **Abbreviations**

259 MD2: myeloid differentiation 2

260 TLRs: Toll like receptors

261 L6H21: (E)-2,3-dimethoxy-4'-methoxychalcone

262 FBS: fetal bovine serum

263 OD: the optical density

264 LPS: lipopolysaccharide

265 RNAi: ribonucleic acid interference

266

267 **Declarations**

268 **Ethics approval and consent to participate:**

269 The authors are accountable for all aspects of the work in ensuring that questions related to
270 the accuracy or integrity of any part of the work are appropriately investigated and resolved.

271

272 **Consent for publication:**

273 Not applicable

274

275 **Availability of data and materials:**

276 The datasets used and/or analysed during the current study are available from the
277 corresponding author on reasonable request.

278

279 **Competing interests**

280 The authors declare that they have no competing interests.

281

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286

287 **Authors' contributions:**

288 GL G and SR Z designed the study. SR Z and WD F conducted this experiment and RM M
289 helped to design study model. WD F, SR Z, QD H, JW G, JY Z and KK L collected data. SR
290 Z, WD F, RM M and QD H did the statistical analyses and prepared figures. SR Z, WD F and
291 GL G reviewed the results and interpreted data. SR Z and WD F wrote the manuscript. All
292 authors have made an intellectual contribution to the manuscript and approved the submission.
293 WD F and SR Z are co-first authors.

294

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297

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Figures

Figure 1

MD2 is highly expressed in MDA-MB-231s and 4T1 cells ($p < 0.05$ compared to that expressed in Hs 578Bst cells and MCF-7 cells)

Figure 2

MTT assay showed that L6H21 (10μM, 30μM and 100μM) significantly inhibited the proliferation of 4T1 cells in dose-dependent manner.

Figure 3

L6H21 inhibition of 4T1 cells migration and invasion.(A) L6H21 significantly inhibited the migration of 4T1 cells, The images (100×) were obtained by microscope. (B) L6H21 significantly inhibited the invasion of 4T1 cells (400×). In 5μM L6H21 group ,there were a few migrating cells observed compared to that of control group. While in 10μM L6H21 group and in 1μg/ml anti-MD2 group, there was almost no migrating cell observed.

Figure 4

L6H21 enhanced survival in nude mice. Male BALB/c mice were randomly divided into four groups (n=8 per group). 4T1-bearing mice were injected with 3×10^5 4T1 cells (i.v. through the tail vein) 3 days after being treated with L6H21 (at $10 \text{ mg} \cdot \text{kg}^{-1}$ or $5 \text{ mg} \cdot \text{kg}^{-1}$) and saline by intragastric administration, respectively, and the mice of the fourth group were only treated with L6H21 at $10 \text{ mg} \cdot \text{kg}^{-1}$ but without inoculation of 4T1 cells. Each group of mice were then intragastrically administrated per day until death or day 60. The survival curve was made to analyse the survival rate.