Involvement of the arachidonic acid cytochrome P450 epoxygenase pathway in the proliferation and invasion of human multiple myeloma cells

Jing Shao, Hongxiang Wang, Guolin Yuan, Zhichao Chen, Qiubai Li

Cytochrome P450 (CYP) epoxygenases and the metabolites epoxyeicosatrienoic acids (EETs) exert multiple biological effects in various malignancies. We have previously found EETs to be secreted by multiple myeloma (MM) cells and to be involved in MM angiogenesis, but the role of the arachidonic acid cytochrome P450 epoxygenase pathway in the proliferation and mobility of MM cells remains unknown. In the present study, we found that MM cell lines generated detectable levels of 11,12-EET/14,15-EET and that increased levels of EETs were found in the serum of MM patients compared to healthy donors. The addition of exogenous EETs induced significantly enhanced proliferation of MM cells, whereas 17-octadecynoic acid (17-ODYA), an inhibitor of the CYP epoxygenase pathway, inhibited the viability and proliferation of MM cells. Moreover, this inhibitory effect could be successfully reversed by exogenous EETs. 17-ODYA also inhibited the motility of MM cells in a time-dependent manner, with a reduction of the gelatinolytic activity and protein expression of the matrix metalloproteinases (MMP)-2 and MMP-9. These results suggest the CYP epoxygenase pathway to be involved in the proliferation and invasion of MM cells, for which 17-ODYA could be a promising therapeutic drug.

1   **Author Cover Page**

2 **Title**

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4 and invasion of human multiple myeloma cells

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31 **Abstract**

32   Cytochrome P450 (CYP) epoxygenases and the metabolites epoxyeicosatrienoic acids

33 (EETs) exert multiple biological effects in various malignancies. We have previously found

34 EETs to be secreted by multiple myeloma (MM) cells and to be involved in MM angiogenesis,

35 but the role of the arachidonic acid cytochrome P450 epoxygenase pathway in the proliferation

36 and mobility of MM cells remains unknown. In the present study, we found that MM cell lines

37 generated detectable levels of 11,12-EET/14,15-EET and that increased levels of EETs were

38 found in the serum of MM patients compared to healthy donors. The addition of exogenous

39 EETs induced significantly enhanced proliferation of MM cells, whereas 17-octadecynoic acid

40 (17-ODYA), an inhibitor of the CYP epoxygenase pathway, inhibited the viability and

41 proliferation of MM cells. Moreover, this inhibitory effect could be successfully reversed by

42 exogenous EETs. 17-ODYA also inhibited the motility of MM cells in a time-dependent manner,

43 with a reduction of the gelatinolytic activity and protein expression of the matrix

44 metalloproteinases (MMP)-2 and MMP-9. These results suggest the CYP epoxygenase pathway

45 to be involved in the proliferation and invasion of MM cells, for which 17-ODYA could be a

46 promising therapeutic drug.

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60 **Introduction**

61 It is well known that arachidonic acid (AA) is converted to eicosanoid mediators by the

62 cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) epoxygenase

63 pathways to generate hundreds of metabolites that have different biological activities and

64 contribute to the pathogenesis of numerous diseases. The changes in the expression level and

65 distribution pattern of enzymes involved in eicosanoid biosynthesis may be especially relevant in

66 carcinogenesis (Koch et al. 2011; Wasilewicz et al. 2010). Multiple myeloma (MM) is an

67 incurable plasma cell malignancy characterized by the accumulation of long-living plasma cells

68 in the bone marrow. Current evidence supports the notion of an association between deranged

69 endogenous fatty acids and MM pathogenesis, while the abnormal fatty acid profile may

70 contribute to cancer-associated inflammation through an abnormal arachidonic acids metabolism

71 (Jurczyszyn et al. 2014; Jurczyszyn et al. 2015). AA metabolism pathways are also involved in

72 the pathogenesis of multiple myeloma (MM). COX-2 overexpression is associated with reduced

73 estimated survival of MM patients and unfavorable prognostic factors such as LDH, age, and β_2-

74 microglobulin (Cetin et al. 2005). 12-LOX is also detected in MM cells, and the inhibitor

75 Baicalein suppresses the proliferation of MM cells (Li et al. 2006). However, the role of the

76 cytochrome P450 epoxygenase pathway in the disease progression of MM remains poorly

77 understood.

78

79 Primary metabolic products of CYP epoxygenases include four epoxyeicosatrienoic acid (EET)

80 regioisomers: 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. Upon hydration by soluble

81 epoxide hydrolase (sEH), EETs are converted to more stable and less biologically active

82   metabolites, dihydroxyeicosatrienoic acids (DHETs) (Capdevila et al. 1992; VanRollins et al.

83   1993). Although each purified CYP epoxygenase converts arachidonic acid to all four EET

84   regioisomers, the main products in many cases are 11,12-EET and 14,15-EET (Capdevila et al.

85   2000). Studies have shown that small amounts of 8,9-, 11,12-, and 14,15-EETs are present in the

86   plasma, the liver, and the kidney, with 14,15-EET being the most abundant regioisomer (Karara

87   et al. 1991; Karara et al. 1990). EETs are autocrine and paracrine mediators that function

88   primarily in the cardiovascular and renal system, and play a key role in inflammation and tissue

89   homeostasis in the vascular system (Campbell & Harder 1999). Recently, there is increasing

90   evidence suggesting potential roles of CYP epoxygenases and EETs in tumors (Chen et al. 2011;

91   Jiang et al. 2005; Nithipatikom et al. 2010). EETs dramatically enhance the proliferation and

92   motility of tumor cells, and overexpression of CYP epoxygenases has the same effects in tumor

93   cells *in vitro* and *in vivo*.

94

95   We previously demonstrated that MM cells secrete EETs into the supernatant and that the CYP

96   epoxygenase pathway participates in the MM cell-induced angiogenesis, which can be inhibited

97   by 17-octadecynoic acid (17-ODYA), an inhibitor of CYP epoxygenase pathway (Shao et al.

98   2011). In this study, we further investigated the role of the CYP epoxygenase pathway in cell

99   proliferation, apoptosis, migration and invasion of MM.

100

101   **Materials and Methods**

102   **Materials.**   Cell culture medium (RPMI 1640 medium) and fetal bovine serum (FBS) were

103   purchased from Invitrogen (Carlsbad, CA, USA). The ELISA kits for 11,12-EET and 14,15-EET

104   were obtained from Detroit R&D (Detroit, MI, USA). 11,12-EET, 14,15-EET, and 17-ODYA

105   were purchased from Cayman Chemical (Ann Arbor, MI, USA). 3-(4, 5-dimethyl-2-thiazy1)-

106   2,5-diphenyl-2H-tetrazolium bromide (MTT) and DMSO were obtained from Sigma Chemical

107   Co. (St. Louis, MO, USA). PI and Annexin V/FITC detection kits were purchased from Bender

108   Medsystems Inc. (Burlingame, CA, USA). Antibodies against CyclinD1, Bax, Bcl-2, MMP-2,

109 and MMP-9 were purchased from Epitomics Inc. (Burlingame, CA, USA). Horseradish

110 peroxidase (HRP)-conjugated secondary antibodies (goat-anti-rabbit IgG) were purchased from

111 KPL (Gaithersburg, MA, USA). Enhanced chemiluminescence reagents were purchased from

112 Pierce, Inc. (Rockford, IL, USA). The Transwell plates were obtained from Corning Costar

113 (Cambridge, MA, USA), and the Matrigel was purchased from BD Biosciences (Bedford, MA,

114 USA).

115

116 **Patient samples.** The human study was approved by the Institutional Review Board (Review

117 Board of Tongji Medical College, Huazhong University of Science and Technology, No

118 2012S119). Sixteen patients diagnosed with MM were selected for the present study after

119 providing informed consent. Three healthy subjects were recruited as controls. Six ml of

120 peripheral blood was collected from all cases. Serum was isolated from peripheral blood by

121 centrifugation at 2,000 rpm for 10 minutes and was frozen at -80℃ for the EET Elisa assay.

122 Twelve patients were men, and 4 patients were women (mean age 51±10 years, range 34-66).

123 Fifteen patients were diagnosed as stage Ⅲ MM, and 1 patient was stage Ⅱ. Nine patients were

124 group A, and 7 were group B. Eight patients were newly diagnosed, and 3 patients were relapse

125 cases (Table 1).

126

127 **Cell lines and cell culture.** Multiple myeloma cell lines U266 and RPMI8226 were obtained

128 from the American Type Culture Collection (ATCC, Rockville, MD, USA). U266 and RPMI

129 8226 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100

130 units/ml penicillin and 100 μg/ml streptomycin at 37℃ in a humidified 95% air/ 5% CO_2

131 atmosphere incubator.

132

133 **EET ELISA.** For the measurement of EETs in MM cells, cells (1×10^6/ml) were plated in 6-

134 well plates in the presence or absence of 17-ODYA (100 μmol/L). Cells were collected by

135 centrifugation at 2,000 rpm for 10 minutes. EETs in the MM cells and serum of multiple

136   myeloma patients were determined using an ELISA kit according to the manufacturer's

137   instructions.

138

139   **Cell viability and proliferation assays.**   MM cells were synchronized by incubation in FBS-

140   free RPMI 1640 overnight. 11,12-EET, 14,15-EET and 17-ODYA were added at various

141   concentrations to the media. The cells were cultured for 24, 48 or 72 hours. Because of the

142   instability of EETs, they were added to the media every 4-6 hours. DMSO was used as a vehicle

143   for all compounds. After treatment with MTT (20 µl per well) for 4 hours, DMSO was added to

144   dissolve the crystalline reaction products. The plates were read at a wavelength of 490 nm. Each

145   group measurement was repeated in five duplicate wells.

146

147   **Determination of cell apoptosis and cell cycle by flow cytometry.**   Cells were synchronized

148   by incubation in FBS-free RPMI 1640 overnight. These cells (1×10^6) were plated in 6-well

149   plates and treated with 17-ODYA at 100 µmol/L for 48 hours. Subsequently, the cells were

150   harvested and incubated with FITC-conjugated Annexin Ⅴ and propidium iodide, according to

151   the manufacturer's protocol, and analyzed using a flow cytometer (FACScan, Becton Dickinson,

152   USA).

153   To analyze the cell cycle distribution, cells (1×10^6) were cultured with 17-ODYA (100

154   µmol/L) for 48 hours. Thereafter, the cells were harvested and fixed with 75% ethanol at -20℃

155   overnight. After removing the ethanol, the cells were incubated with RNase (250 µg/ml) at 37℃

156   for 30 minutes followed by propidium iodide (50µg/ml) at 4℃ for 30 minutes. Cell cycle

157   analysis was determined by flow cytometry.

158

159   **Western blotting.**   Proteins from the cell lysates were separated by SDS-PAGE

160   electrophoresis and transferred to a nitrocellulose filter membrane. The membrane was blocked

161   with 5% nonfat milk and incubated with an antibody against a specific protein antigen. The

162   location of the antibody-antigen complex on the Western blot was revealed by incubation with a

163   peroxidase-conjugated secondary antibody. The bands were visualized using the enhanced

164   chemiluminescence method.

165

166   **Migration assay.**   Migration assays were conducted as previously described in detail (Fuchida

167   et al. 2008). Briefly, the assay was performed using a 24-well Transwell plates with a

168   polycarbonate membrane with a pore size of 8 μm. Before the migration assay, cells were treated

169   with 17-ODYA (100 μmol/L) for 24 or 48 hours. The cells were centrifuged and suspended in

170   serum-free RPMI 1640 at a density of 1×10^6/ml. Volumes of 1×10^5 cells were seeded into the

171   upper chambers, and other cells were cultured concurrently in the mother liquor to calculate the

172   migration ratio. The lower chamber contained RPMI 1640 with 10% FBS as a chemoattractant.

173   After 24 hours of incubation at 37℃, the numbers of cells transmigrating into the lower chamber

174   and in the mother liquor were counted using a flow cytometer (FACScan) gated for 20 s at a high

175   flow rate. The experiments were performed in triplicate. The migration ratio was calculated as

176   the migrated cells as the percentage of cells in the mother liquor.

177

178   **Invasion assay.**   The invasion assay was performed as the migration assay, except for the fact

179   that the upper surface of the polycarbonate membranes was coated with Matrigel and dried

180   overnight at 37℃. The Transwell plates were incubated at 37℃ for 24 or 48 hours. The cell

181   number was counted by flow cytometry. The invasion ratio was calculated as the migrated cells

182   as the percentage of cells in the mother liquor.

183

184   **Gelatin zymography.**   Gelatin zymography was performed using the supernatant of cultured

185   MM cells to evaluate the activity of secreted MMP-2 and MMP-9. Volumes of 1×10^6 cells were

186   incubated with or without 17-ODYA (100 μmol/L) for 24 hours. The culture supernatants were

187   collected and centrifuged at 2000rpm for 10 minutes. Supernatants containing the same amount

188   of proteins from each group were applied to 10% SDS-PAGE (containing 0.1% gelatin). After

189   electrophoresis, the gels were washed in 2.5% Triton X-100 for 80 minutes to remove SDS. The

190   gels were incubated for 20 hours at 37℃ and stained with 0.5% Coomassie brilliant blue for 3

191   hours. After staining, the gels were destained with 30% methanol and 10% acetic acid.

192

193   **Statistics.**   The data are presented as the mean ± SE. All experiments were done with triplicate

194   replications of each treatment group (n = 3). Student's t test or ANOVA was performed as

195   appropriate to determine the statistical significance of differences among different groups. The

196   Mann-Whitney two-sample test was used to investigate the significant differences of EET

197   concentration between MM patients and healthy controls. Correlation analysis was used to

198   examine the correlation between sets of variables in MM patients. In all cases, statistical

199   significance was defined by $P < 0.05$.

200

201   **Results**

202   **EETs in MM cell lines and the peripheral blood serum of MM patients**

203   We previously found secreted 11,12-EET and 14,15-EET in the supernatant of MM cell lines

204   (Shao et al. 2011), but the expression levels of 11,12-EET and 14,15-EET in MM cells is

205   unknown. Therefore, we directly assessed the levels of these EETs in MM cells. Based upon the

206   results of EET ELISA assay, we found both MM cell lines U266 and RPMI 8226 produced

207   11,12-EET and 14,15-EET (Figure 1A). The levels of 14,15-EET were much higher than those

208   of 11,12-EET in the two cell lines, suggesting that 14,15-EET was the most abundant

209   regioisomer (Karara et al. 1991; Karara et al. 1990). We also evaluated the levels of EETs in

210   MM patients after collecting the peripheral blood serum of 16 patients and 3 healthy volunteers,

211   as shown in Table 1. The results showed that the concentrations of 11,12-EET and 14,15-EET

212   were significantly higher in patients than in healthy donors (Figure 1B). The mean concentration

213   of 11,12-EET in patient serum was 291.94 ± 383.98 ng/ml (range from 0.78 to 1,193.36 ng/ml),

214   which was significantly different from the control (5.10 ± 2.31 ng/ml, range from 2.86 to 7.47

215   ng/ml,) ($P < 0.01$). The mean concentration of 14,15-EET in MM serum was 1,056.48 ± 906.47

216   ng/ml (range from 0.61 to 2,754.99 ng/ml), which was markedly higher than the control with

217   4.92 ± 2.32 ng/ml (range from 2.54 to 7.18 ng/ml) ($P < 0.01$). Meanwhile, we analyzed the

218   correlations of EET concentrations with the prognostic factors, such as LDH and β2-

219   microglobulin, but no statistically significant correlation was found. With regard to LDH and β2-

220   microglobulin, the results for 11,12-EET were r = -0.139, $P > 0.05$ and r = -0.27, $P > 0.05$,

221   respectively, and for 14,15-EET, r = -0.395, $P > 0.05$ and r = -0.114, $P > 0.05$, respectively.

222

223   **17-ODYA suppressed EET levels and the proliferation of MM cells**

224   Based on previous studies (Chen et al. 2011; Jiang et al. 2005; Nithipatikom et al. 2010) and our

225   above results, we hypothesized that EETs may contribute to the neoplastic phenotype of MM.

226   First, we determined the effect of exogenous EETs on the proliferation of MM cells and found

227   that concentrations of 11,12-EET and 14,15-EET in the range of 100 nmol/L to 400 nmol/L

228   increased the proliferation of U266 and RPMI 8226 cells *in vitro* for 24, 48 and 72 hours (Figure

229   2A). These EEts also promoted cell viability in a dose and time dependent-manners. Because of

230   the instability of EETs, they should be added to the medium every 4-6 hours, but the vehicle

231   (DMSO) significantly affected the viability of the cells (Fig.S1). Thus, we analyzed the

232   differences between the vehicle and experimental groups. As 17-ODYA is the inhibitor of CYP

233   epoxygenases, we measured the levels of EETs in MM cells in the presence or absence of 17-

234   ODYA to further characterize the effect of 17-ODYA on the biosynthesis of eicosanoids in MM

235   cells. Our findings revealed that 17-ODYA decreased both the levels of 11,12-EET and 14,15-

236   EET in U266 and RPMI 8226 cells (Figure 2B). Meanwhile, the addition of the epoxygenase

237   inhibitor 17-ODYA decreased the proliferation of MM cells, and the inhibition ratio increased

238   with the dose and duration of treatment (Figure 2C). Importantly, exogenous EETs reversed the

239   17-ODYA-mediated decrease in proliferation of the MM cell lines compared to the vehicle

240   (DMSO) group (Figure 2D). These results indicate that CYP epoxygenase-derived EETs

241   promote the viability of MM cells and that CYP epoxygenases may play an important role in the

242   proliferation of multiple myeloma.

243

244 **17-ODYA induced apoptosis and cell cycle arrest of MM cells**

245 We next investigated whether 17-ODYA induced cellular apoptosis or disrupted the cell cycle.

246 After treatment with 17-ODYA for 48 hours, the ratios of apoptotic cells were assessed using

247 Annexin V/PI staining. Treatment of both U266 and RPMI 8226 cells with 100 μmol/L 17-

248 ODYA increased apoptosis compared with control (blank) group ($P < 0.05$; Figure 3A).

249 Treatment with 17-ODYA (100 μmol/L) for 48 hours also increased the percentage of cells in

250 G_0/G_1 phase in both MM cell lines compared to the control (blank) and vehicle (DMSO) group

251 ($P < 0.05$; Figure3B). We next measured apoptosis and cell cycle-related proteins by Western

252 blot analysis, and the results revealed that treatment with 17-ODYA decreased the levels of the

253 G_1 phase regulatory protein cyclin D1 and antiapoptotic protein Bcl-2, while increasing the level

254 of proapoptotic protein Bax (Figure 4). Collectively, these results suggest that the epoxygenase

255 pathway is possibly involved in enhancing the proliferation of myeloma cells by protecting cells

256 from apoptosis and promoting the cell cycle progression.

257

258 **17-ODYA inhibited the motility of MM cells**

259 Migration and invasion of MM cells were evaluated using a Transwell model *in vitro*. 17-ODYA

260 significantly decreased cell migration of both MM cell lines, and the migration ratio decreased in

261 a time-dependent manner (Figure 5A). The migration ratio of 17-ODYA treatment for 24 h was

262 0.93 ± 0.07% compared to 1.37 ± 0.13% for the control (blank) group ($P < 0.05$), and 0.10 ±

263 0.02% for the 48 h group compared with the control (blank) group ($P<0.05$) in U266 cells. The

264 results of RPMI 8226 cells were 1.07 ± 0.36% for the 24 h group and 0.48 ± 0.04% for the 48 h

265 group compared to 1.57 ± 0.06% for the control (blank) group ($P < 0.05$). 17-ODYA also

266 inhibited the invasion of MM cells (Figure 5B). As MMPs play an important role in tumor

267 metastasis, we used gelatin zymography assays and Western blotting to investigate the activity

268 and protein content of MMPs in MM cells. As shown in Figure 5C, 17-ODYA treatment

269 suppressed the activity of MMPs in the supernatant. To investigate whether the 17-ODYA-

270 induced inhibition of MMP-2 and MMP-9 activity was caused by changes in the protein levels,

271   MM cells were also analyzed by Western blot. The results showed that the levels of MMP-2 and

272   MMP-9 were decreased by 17-ODYA treatment in both MM cell lines (Figure 5D). Altogether,

273   the inhibitor 17-ODYA suppressed the motility of MM cells and reduced the activity and protein

274   levels of secreted MMPs.

275

276   Discussion

277   As the main products of CYP epoxygenases in many MM cases are 11,12-EET and 14,15-EET

278   (Capdevila et al. 2000), we measured these two EETs in MM cells and in the serum of MM

279   patients. In the present study, we found that both MM cell lines (U266, RPMI 8226) produced

280   different levels of 11,12-EET and 14,15-EET and that EETs were also detected in high

281   concentrations in the serum of MM patients. Accordingly, in leukemia and lymphoma, two other

282   hematological malignancies, high levels of EETs in the urine and blood of patients compared to

283   the healthy controls were reported by Chen et al. (Chen et al. 2011). These findings indicate that

284   high levels of the EETs are one of the pathological characteristics of hematological malignancies,

285   including MM. The clinical role of EETs in MM stratification and prognosis is to be determined

286   in future studies.

287

288   EETs are the major biologically active metabolites of CYP epoxygenases and are locally active

289   small molecule lipid mediators that play a central role in various cellular functions, including

290   proliferation, migration and angiogenesis (Chen et al. 2001; Enayetallah et al. 2006; Jiang et al.

291   2005; Michaelis et al. 2005; Node et al. 1999; Wei et al. 2014). In this study, we found that both

292   11,12-EET and 14,15-EET significantly promoted the proliferation of MM cells, while the

293   inhibitor 17-ODYA suppressed EET levels and the viability of all tested MM cells in a dose- and

294   time-dependent manner. Additionally, the 17-ODYA-mediated decrease of proliferation was

295   reversed by exogenous EETs. These findings support the notion that the elevation of EETs

296   promotes the viability of MM cells. To be noted, 17-ODYA is also considered as an inhibitor of

297   cytochrome P-450 omega-hydroxylase (Ohata et al. 2010), but 20-HETE, the product of

298   cytochrome P-450 omega-hydroxylase, was not considered in the present study. Though the

299   expression and role of 20-HETE is unknown in MM cells, it is important to be further

300   demonstrated using other specific epoxygenase inhibitors, such as MS-PPOH (Wang et al. 1998).

301

302   In the present study, 17-ODYA increased the apoptosis and induced cell cycle arrest at the G_0/G_1

303   phase in MM cells, suggesting that 17-ODYA-induced suppression of MM cells proliferation is

304   possibly mediated through the induction of apoptosis and cell cycle arrest. Our results also

305   showed that the G_1 phase regulatory protein cyclin D1 and Bcl-2 antiapoptotic protein were

306   markedly down-regulated by 17-ODYA, along with up-regulation of Bax proapoptotic protein.

307   These proteins have been shown to play a key role in MM pathogenesis (Marsaud et al. 2010).

308   Thus, together with our previous findings about EET-induced angiogenesis in MM, the present

309   study supported the hypothesis that EETs and CYP epoxygenase pathway contribute to the

310   neoplastic phenotype of MM cells.

311

312   Although a prominent feature of MM consists in the localization of MM cells in the bone

313   marrow, a few MM cells can also be detected in the peripheral circulation. These observations

314   suggest that MM cells have the capacity to circulate, invade and home to the bone marrow

315   (Alsayed et al. 2007; Vande Broek et al. 2007). In the end stage of MM, the circulating plasma

316   cells increase and grow at extramedullary sites (Pour et al. 2014). Clearly, the motility of MM

317   cells is related to the disease progression. Numerous evidence has confirmed that EETs induce

318   endothelial cell migration,even enhancing hematopoietic stem and progenitor cell homing and

319   engraftment (Li et al. 2015). The present study showed that 17-ODYA can significantly inhibit,

320   in a time-dependent manner, the motility of MM cells, including the migration and invasion of

321   all tested MM cells. These results demonstrated that the epoxygenase pathway may be involved

322   in the regulation of the motility of MM cells through the metabolites EETs.   MM cells can

323   localize in the bone marrow, which consists of extracellular matrix (ECM) and stromal cells, and

324   MM cells have the capacity to invade and constitutively produce MMPs that are essential for

325 matrix degradation (Barillé et al. 1997; Hecht et al. 2007; Vande Broek et al. 2004). In the

326 present study, both U266 and RPMI 8226 cells expressed and secreted MMP-2 and MMP-9

327 proteins that can hydrolyze gelatin, but 17-ODYA reduced the activity of MMPs and suppressed

328 the protein levels of both MMP-2 and MMP-9 in MM cells. These data revealed the role of the

329 epoxygenase pathway in 17-ODYA-mediated inhibition of invasion and MMP protein levels.

330

331 Combined with our previous findings (Shao et al. 2011), the present study supports the

332 involvement of the CYP epoxygenase pathway and the elevated levels of EETs in the

333 proliferation, angiogenesis and motility of MM cells. We have identified the role of the third

334 pathway of the arachidonic acid metabolism, the CYP epoxygenase pathway, in the pathogenesis

335 of MM. It is not clear how active MM develops from the "dormancy period" of monoclonal

336 gammopathy of undetermined significance (MGUS). Recently, EETs were found to stimulate

337 tumor cells to escape from tumor dormancy in several tumor models (Panigrahy et al.). Thus, we

338 can reasonably hypothesize that EETs may promote MM to escape from this "dormancy period"

339 and that the CYP epoxygenase pathway may be substantially involved in this progression. In

340 conclusion, the CYP pathway could be an important therapeutic target, and the inhibitor 17-

341 ODYA appears to be an attractive candidate for MM therapy.

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466   **Figure legends**

467

468   **Figure 1.** Levels of EETs in MM cells and serum of MM patients. 11,12-EET and 14,15-EET

469   levels were determined by ELISA following the instruction of manufacturers. A, MM cell lines

470   (1×10^6 cells) expressed 11, 12-EET and 14, 15-EET. B, 11, 12-EET and 14, 15-EET levels from

471   healthy volunteers and patients with multiple myeloma (the ordinate was for log transformation;

472   EETs in MM patients serum were significantly higher compared with healthy subjects, $P < 0.01$).

473

474   **Figure 2.** 17-ODYA decreases EETs level in MM cells and suppresses MM cell viability. A,

475   once addition of exogenous 11,12-EET or 14,15-EET increased the viability of U266 and

476   RPMI8226 ($^*P < 0.05$ versus DMSO vehicle group). B, 17-ODYA (100μmol/L) treated U266

477   and RPMI8226 for 24h or 48h, and Elisa assay was used to detect the 11, 12-EET and 14, 15-

478   EET levels in MM cells ($^*P<0.05$ versus control). C, 17-ODYA (100μmol/L) decreased cell

479   viability of U266 and RPMI8226 in dose and time dependent manners ($^*P < 0.05$ versus control).

480   D. 17-ODYA (100μmol/L) suppressed the proliferation of U266 and RPMI8226 cells, which

481   could be reversed by exogenous EETs (all groups were treated with 100 μmol/L 17-ODYA for

482   24 hours; $^*P < 0.05$ versus vehicle group).

483

484   **Figure 3.** Epoxygenase inhibitor 17-ODYA enhances MM cell apoptosis and induces cell arrest

485   at G_0/G_1 phase. A, apoptosis of MM cells was increased by 17-ODYA (100μmol/L) treatment for

486   48h ($^*P < 0.05$ versus control). B, cell arrest at G_0/G_1 phase was induced by 17-ODYA

487 (100μmol/L) treatment for 48h (*$P < 0.05$ versus control).

488

489 **Figure 4.** Effect of 17-ODYA on levels of Bax, Bcl-2 and cyclin D1 protein in MM. Treatment

490 of both U266 and RPMI 8226 cells with 17-ODYA at 100μmol/L for 48 h decreased levels of

491 cyclin D1, the G_1 phase regulatory protein, and antiapoptotic protein Bcl-2, while increased the

492 level of Bax, a proapoptotic protein.

493

494 **Figure 5.** 17-ODYA inhibits MM cell mobility through reducing MMP activity and protein

495 levels.   MM cells were treated by 17-ODYA (100μmol/L) for 24h or 48h, and DMSO was used

496 for the vehicle. A, 17-ODYA inhibited the migration of MM cells (*$P > 0.05$ versus control; **P

497 < 0.05 versus control). B, 17-ODYA inhibited the invasion of MM cells (*$P > 0.05$ versus

498 control; **$P < 0.05$ versus control). C, 17-ODYA reduced MMPs activity in the supernatant of

499 MM cells (*$P < 0.05$ versus control). D, 17-ODYA reduced MMPs protein level in MM cells (*P

500 < 0.05 versus control).

501

1

Levels of EETs in MM cells and serum of MM patients.

11,12-EET and 14,15-EET levels were determined by ELISA following the instruction of manufacturers. A, MM cell lines (1×10^6 cells) expressed 11, 12-EET and 14, 15-EET. B, 11, 12-EET and 14, 15-EET levels from healthy volunteers and patients with multiple myeloma (the ordinate was for log transformation; EETs in MM patients serum were significantly higher compared with healthy subjects, $P < 0.01$).

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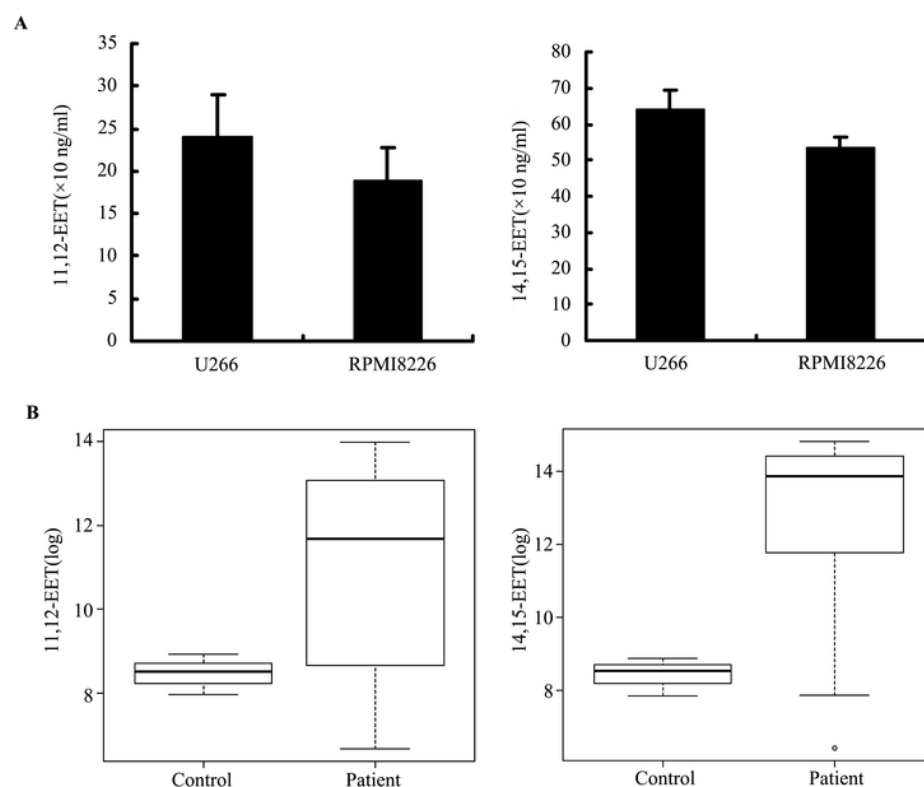
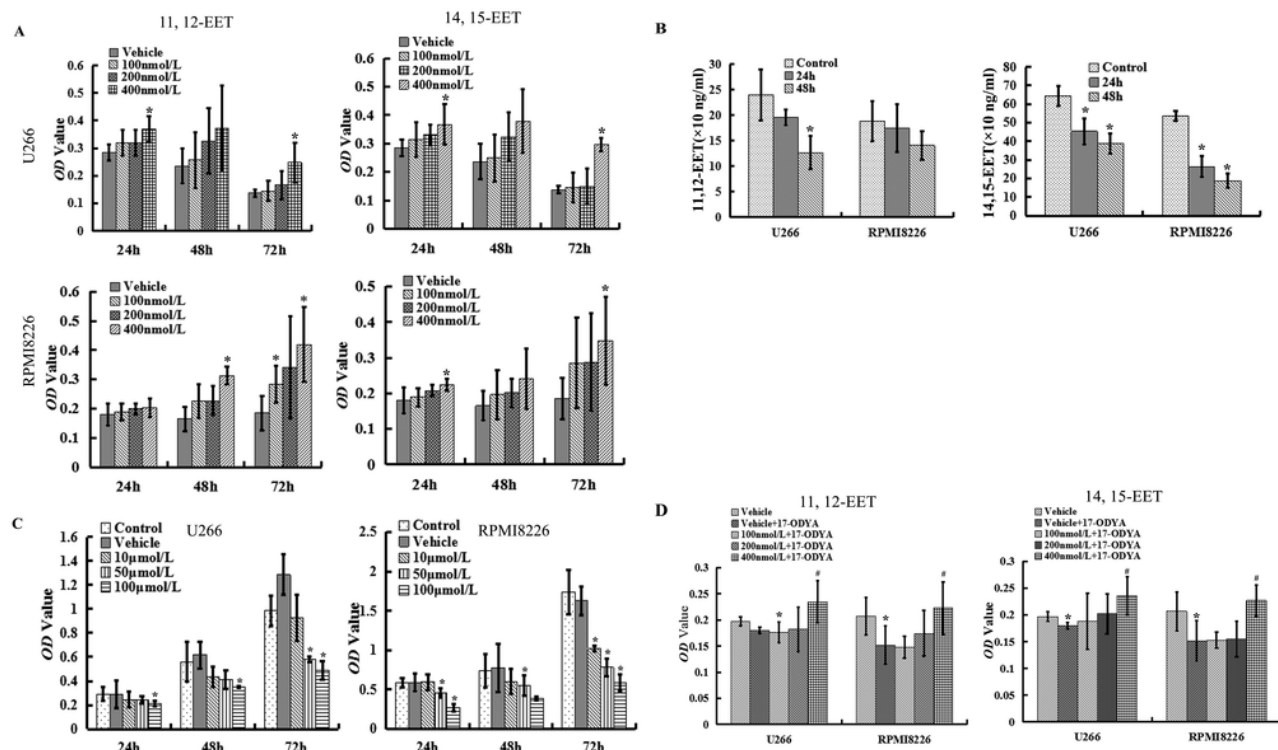
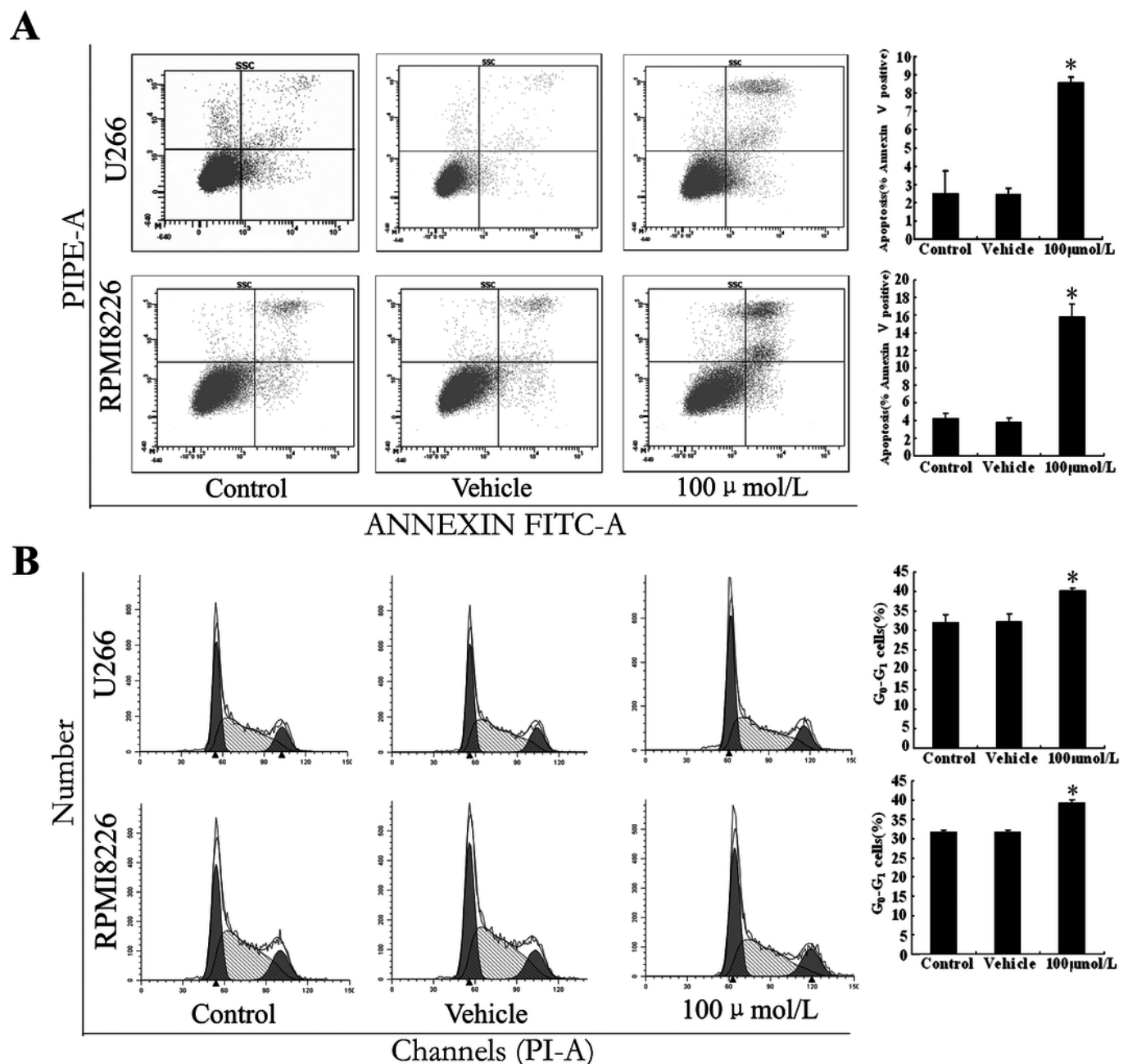

17-ODYA decreases EETs level in MM cells and suppresses MM cell viability.

A, once addition of exogenous 11,12-EET or 14,15-EET increased the viability of U266 and RPMI8226 (*$P < 0.05$ versus DMSO vehicle group). B, 17-ODYA (100µmol/L) treated U266 and RPMI8226 for 24h or 48h, and Elisa assay was used to detect the 11, 12-EET and 14, 15-EET levels in MM cells (*$P<0.05$ versus control). C, 17-ODYA (100µmol/L) decreased cell viability of U266 and RPMI8226 in dose and time dependent manners (*$P < 0.05$ versus control). D. 17-ODYA (100µmol/L) suppressed the proliferation of U266 and RPMI8226 cells, which could be reversed by exogenous EETs (all groups were treated with 100 µmol/L 17-ODYA for 24 hours; *$P < 0.05$ versus vehicle group).

Epoxygenase inhibitor 17-ODYA enhances MM cell apoptosis and induces cell arrest at G_0/G_1 phase.

A, apoptosis of MM cells was increased by 17-ODYA (100μmol/L) treatment for 48h (*P < 0.05 versus control). B, cell arrest at G_0/G_1 phase was induced by 17-ODYA (100μmol/L) treatment for 48h (*P < 0.05 versus control).

Effect of17-ODYA on levels of Bax, Bcl-2 and cyclin D1 protein in MM.

Treatment of both U266 and RPMI 8226 cells with 17-ODYA at 100μmol/L for 48 h decreased levels of cyclin D1, the G_1 phase regulatory protein, and antiapoptotic protein Bcl-2, while increased the level of Bax, a proapoptotic protein.

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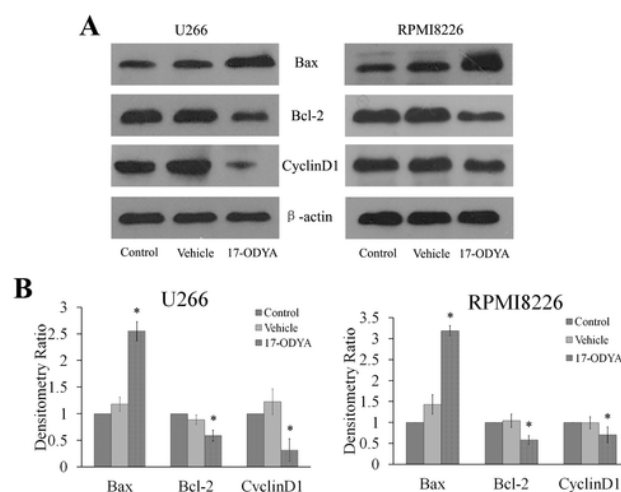

17-ODYA inhibits MM cell mobility through reducing MMP activity and protein levels.

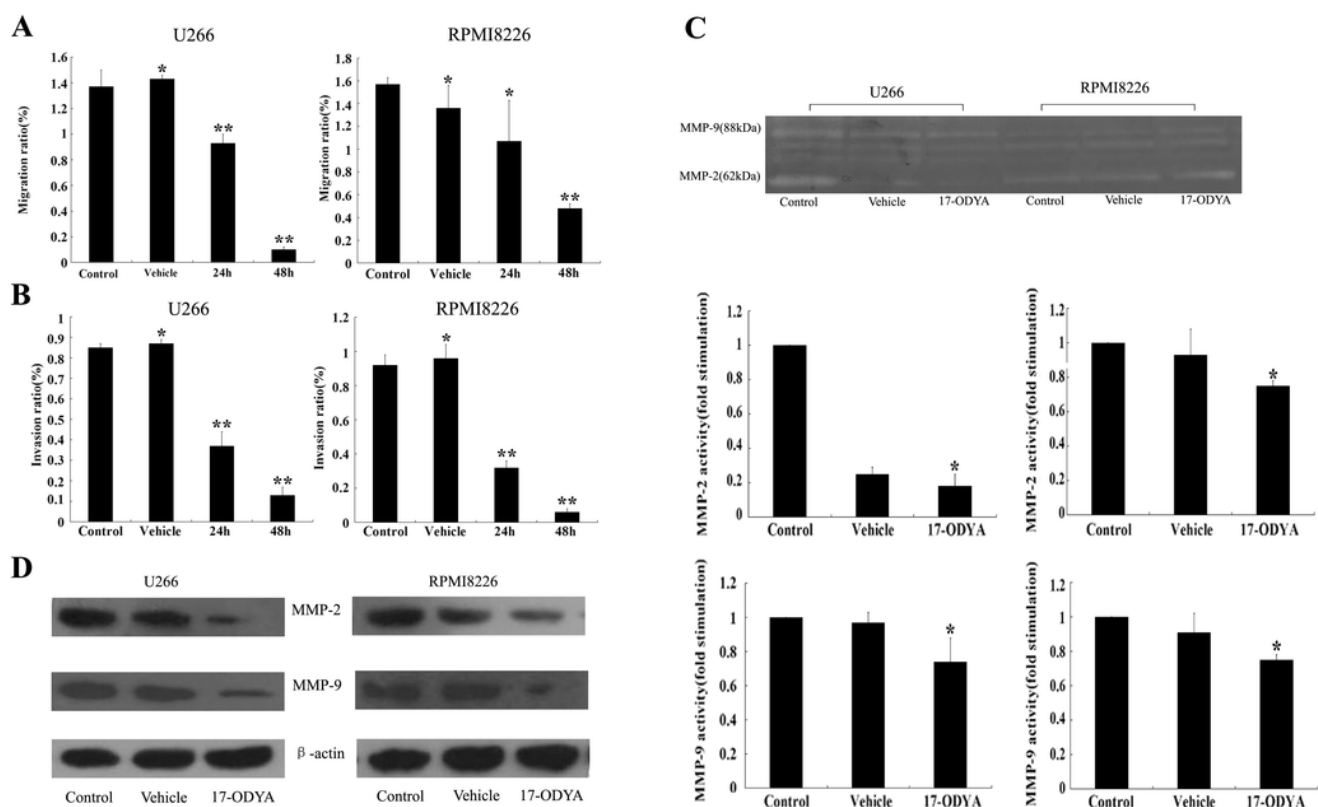
MM cells were treated by 17-ODYA (100μmol/L) for 24h or 48h, and DMSO was used for the vehicle. A, 17-ODYA inhibited the migration of MM cells (*P > 0.05 versus control; **P < 0.05 versus control). B, 17-ODYA inhibited the invasion of MM cells (*P > 0.05 versus control; **P < 0.05 versus control). C, 17-ODYA reduced MMPs activity in the supernatant of MM cells (*P < 0.05 versus control). D, 17-ODYA reduced MMPs protein level in MM cells (*P < 0.05 versus control).

Clinical features of patients with multiple myeloma.

Table1. Clinical features of patients with multiple myeloma

Patient no.	Age/Sex	Durie-Salmon stage	Paraprotein type	β_2-M(mg/L)	LDH(U/L)	Cytogenetic feature
1	58/F	stageIII*	IgG, λ	4.9	220	1q21
2	48/M	stageIII*	IgD, λ	18	164	14q32;13q14.3;1q21
3	62/M	stageIII*	IgG, κ	2.9	NA	13q14.3
4	66/M	stageIII*	IgD, λ	35.5	188	Negative
5	47/M	stageIII	BJP, κ	12.6	205	NA
6	39/M	stageIII	IgD, λ	4.5	201	14q32
7	60/M	stageIII*	IgG, λ	15.1	177	17p13.1
8	60/M	stage II *	IgD, λ	3.3	257	Negative
9	35/M	stageIII*	Nonsecreting type	11.3	177	13q14
10	46/M	stageIII	IgA, κ	3.5	263	NA
11	58/F	stageIII	BJP, κ	1.3	200	NA
12	60/F	stageIII	IgA, λ	2.8	NA	14q32
13	34/F	stageIII*	Nonsecreting type	9.3	159	Negative
14	40/M	stageIII	IgA, λ	1.5	234	17p13.1/17p11.1-q11.1
15	53/M	stageIII	IgG, λ	1.8	106	14q32;13q14
16	44/M	stageIII	BJP, λ	10.1	176	Negative

Abbreviations: M, male; F, female; Ig, immunoglobulin; BJP, Bence Jones protein, β_2-M, β_2-microglobulin; NA, not available;*,newly diagnosised.