Original Article

The antiangiogenic phloroglucinol hyperforin inhibits the secretion of proMMP-2, proMMP-9 and VEGF-A during apoptosis of primary acute myeloid leukemia cells

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33

34 **Abstract**

35 **Aim:** Angiogenesis is observed in acute myeloid leukemia (AML). AML cells

36 abnormally proliferate and are resistant to death. Positive regulators of angiogenesis,

37 VEGF-A and matrix metalloproteinases (MMPs) -2 and -9 are markers of disease status

38 in AML. The natural phloroglucinol hyperforin (HF) displays antitumoral properties of

39 potential pharmacological interest. Herein, we investigated the effects of HF on

40 MMP-2/-9 and VEGF-A expression and survival of primary AML cells.

41

42 **Methods:** Blood and bone marrow samples were collected in 45 patients with distinct

43 subtypes defined by French American British classification, i.e. M0, M1, M2, M3, M4

44 and M5. Levels of MMPs and VEGF-A in leukemic blood cells and culture

45 supernatants were determined by RT-PCR, ELISA, and gelatin zymography (MMPs).

46 The balance between cell death and survival was assessed by flow cytometry with

47 analysis of phosphatidylserine externalization and caspase-3 activation.

48

49 **Results:** The administration of HF promoted a caspase-associated apoptosis in primary

50 AML blasts (from blood and bone marrow), but not normal blood cells and monocytes.

51 In addition, HF inhibited the levels of secreted proMMP-2, proMMP-9 and VEGF-A

52 whithout altering transcripts. The induction of apoptosis by HF significantly paralleled

53 the inhibition of MMP-2/-9 and VEGF-A release by HF. No differences were seen in

54 response to the deleterious effects of HF between AML cells of distinct subtypes.

55

56   **Conclusion:** Our results suggest that HF, through its proapoptotic and potential

57   antiangiogenic properties (by inhibiting MMP-2/-9 and VEGF-A) on primary AML

58   cells, might be a useful experimental agent, in combination with existing drugs, for new

59   therapeutic approaches in the treatment of this incurable disease.

60

61   **Keywords:** Acute myeloid leukemia, apoptosis, hyperforin, matrix metalloproteinase,

62   VEGF, secretion

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65

66   **INTRODUCTION**

67   Angiogenesis, characterized by the formation of new vessels from preexisting blood

68   vessels, participates in the progression of many malignant tumors by supplying oxygen

69   and nutrients [1]. Angiogenesis induction is observed in several hematologic

70   malignancies including acute myeloid leukemia [2-4]. Endothelial cells (ECs) and tumor

71   cells secrete proangiogenic molecules [5,6]. Among the positive regulators of

72   angiogenesis, vascular endothelial growth factors (VEGFs) and more specifically

73   VEGF-A, play a prominent role to sustain angiogenesis [1,5,6]. By binding to VEGF

74   receptors at the surface of ECs, VEGF activates cell signallings involved in the

75   activation of the proliferation and survival of ECs [1,2,7]. In addition to VEGF, matrix

76   metalloproteinases (MMPs) are regulators of pathologic angiogenesis [8-10]. The MMPs

77   enable extravasation and migration of newly formed ECs, and release VEGF bound to

78   the extracellular matrix [8-11]. In peculiar, the proteolytic activities of MMP-2 and

79   MMP-9 are implicated in tumour-associated processes such as cell growth, survival,

80   migration, invasion and angiogenesis [8,12,13]. Moreover, by binding cell surface proteins,

81   the proforms of MMP-2 and MMP-9 can directly trigger intracellular signalling

82   pathways involved in the modulation of cell growth and survival, migration, or

83   angiogenesis [8,14].

84

85   Of particular interest, some natural products display antiangiogenic effects and

86 represent potential interest for the search of novel anti-cancer drugs [15-18]. Among these

87 biologically active compounds, the phloroglucinol hyperforin (HF) isolated from the

88 plant St John's wort *Hypericum perforatum* (Figure 1) acts as a multi-targeting agent

89 [15,19-21]. HF displays anti-depressant, antibacterial, antioxidant and anti-inflammatory

90 properties [15,19-22]. Moreover, HF exhibits anti-proliferative and proapoptotic activities

91 towards a number of mammalian cancer cell lines *in vitro* [15,23-28]. The antiangiogenic

92 properties of HF have been demonstrated *in vitro* and *in vivo* [29,30]. HF's targets include

93 MMP-2, MMP-9 and VEGF-A, all involved in cell survival and migration, and

94 angiogenesis [31-33].

95

96 Acute myeloid leukemia (AML) is a heterogeneous haematopoietic cancer

97 characterized by the accumulation of malignant precursors of the myeloid lineage in the

98 bone marrow (BM), interfering with the production of normal blood cells [34]. The latter

99 are used to define distinct AML subfamilies [34]. Human AML cells show abnormally

100 high levels of proliferation and survival, disseminate from bone marrow into peripheral

101 blood and extramedullary organs [34]. The major reason of mortality of AML patients

102 after allogeneic transplantation is graft versus host disease [4]. The conventional

103 chemotherapeutic approach for AML patients is based on a treatment combinating an

104 anthracycline with cytarabine[35-37]. However, a majority of patients with AML are

105 refractory to primaries therapies or relapse later [35-37]. Both resistance and relapse are

106 due to the heterogeneity of the disease, where high variability both among and within

107 individual patients exist [35-37]. This underscores the need for alternative treatment

108 options for AML patients, with increased tolerability and improved efficacy. Today,

109 alternative strategies for the treatment of newly diagnosed AML patients include a new

110 liposomal formulation of cytarabine and daunorubicin, or the combination of

111 venetoclax (a BH3 mimetic that inhibits the survival function of the B-cell lymphoma-2

112 (Bcl-2) anti-apoptotic protein) with hypomethylating agents or low dose of cytarabine

113 [36-38]. Unfortunately, these therapies are still often accompanied by adverse effects or

114 favored mutations associated to drug resistance [36-38]. Therefore, novel therapies are

115   needed to overcome resistance to these drugs, and the identification of new drugs in

116   AML therapy is of great interest.

117

118   Increased BM angiogenesis in AML correlates with high levels of VEGF-A [39,40]. BM

119   blasts from patients with AML express and secrete VEGF-A [39-42]. The transcript and

120   protein levels of VEGF-A are highly variable in circulating AML cells from pediatric

121   patients [43]. The enhanced VEGF-A levels observed in plasma from AML patients

122   might be explained as the expression of VEGF-A release from blood AML cells, and

123   appeared associated with worse event-free survival and poor overall survival in AML

124   [44]. In contrast to normal BM immature CD34+ progenitor cells, BM AML blasts

125   express MMP-2 and MMP-9 transcripts and release detectable levels of MMP-2 and

126   MMP-9 proteins [45-47]. Similarly, blood AML cells express and secrete MMP-2 and

127   MMP-9 [48]. To the best of our knowledge, there are no literature data reporting the

128   coexpression of VEGF-A and MMP-2/-9 in circulating AML blasts. In this study, we

129   first analyzed the expression status of these factors in myeloid blasts from peripheral

130   blood as a function of the latter's French-American-British (FAB) subtype (M0, M1,

131   M2, M3, M4, M5). In addition, we investigated the ability of HF to exhibit anti-cancer

132   activity in AML disease through the modulation of AML cell survival process and

133   secretion of MMP-2/-9 and VEGF-A. Understanding HF's anti-leukemic activity in

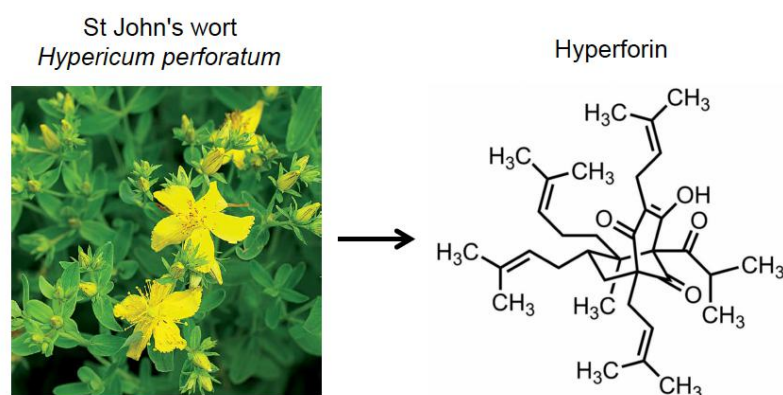134   AML may provide a new model for the treatment of this disease.

136   **Figure 1. Hyperforin (HF) is a bio-active acylphloroglucinol abundantly present in**

137   **the apical flowers of St John's wort (SJW)** ***Hypericum perforatum L.*** **SJW is a**

138   perennial flowering plant. HF is the primary active compound responsible for both the

139   antidepressant and the anti-inflammatory properties of SJW. Chemical structure of HF:

140    $C_{35}H_{52}O_4$; 536 g/mol.

141

142   **METHODS**

143   **Patients, AML samples and cell cultures**

144   Peripheral blood was collected from 45 patients with AML according to standard

145   clinical criteria and the FAB (French-American-British) Committee's cytological

146   criteria ($\geq 80\%$ peripheral blood AML blasts $CD33^+$ $CD13^+$) [49]. The biological and

147   clinical characteristics of AML patients are listed in Table 1. Bone marrow (BM)

148   samples were collected at the time of diagnosis from three AML patients. Control

149   blood samples were collected from healthy, fully anonymized donors. Peripheral blood

150   mononuclear cells (PBMCs) were isolated after Ficoll separation. More than 80% of

151   AML PBMCs were $CD33^+$ $CD13^+$. Monocytes were isolated from normal PBMCs by

152   adherence as described [50]. More than 95% of monocytes were $CD14^+$.

153

154   Freshly isolated cells (10^6/mL) were cultured in RPMI 1640 medium (Life

155   Technologies, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum

156   (FCS) (Gibco; lipopolysaccharide levels < 0.1 ng/ml), 2 mM L-glutamine, 1 mM

157   sodium pyruvate, and 40 μg/ml gentamicin, in a 5% CO_2 humidified atmosphere at

158   37°C. The cells were then treated with purified hyperforin (1.4, 2 and 3 g/mL

159   corresponding to 2.5, 3.6 and 5.4 M respectively) (Cayman Chemical Company, Ann

160   Arbor, Michigan, USA) for 24-72 h. In negative control experiments with HF, cells

161   were treated with the same volume of MeOH alone. After incubation, the cells were

162   collected, washed once, and then used for flow cytometry assays and RT-PCR analyses.

163   The culture supernatants from AML blood cells were harvested under sterile conditions

164   and frozen before MMP-2, MMP-9 and VEGF-A contents were determined by ELISA

165   and zymography. Cell morphology was assessed as previously described [51].

166

167   **Table 1. Clinical characteristics of patients with AML.**

Characteristic	No (%)
Total	45 (100)

Age, years	
Median (range)	60 [19-81]
Male	24 (53)
FAB subfamily	
M0	3 (7)
M1	14 (31)
M2	11 (24)
M3	1 (2)
M4	7 (14)
M5 (including 2 M5b*)	9 (20)

168 FAB, French-American-British. Two AML patients (M0 and M4) were in relapse at the

169 time of our analysis and responded to the effects of HF. M0: undifferentiated blast; M1:

170 undifferentiated myeloblast; M2: myeloblast; M3: promyelocyte; M4: myelomonocyte;

171 M5: monoblast; *M5b: monoblast with differentiation.

172

173 **Flow Cytometry**

174 Intact cells were directly immunostained as previously described [52]. The balance

175 between cell apoptosis and survival was assessed using the annexin V-FITC/propidium

176 iodide (PI) cell death detection kit (Beckman-Coulter, Les Ullis, France). Intracellular

177 active caspase-3 was detected in permeabilized cells as described in [53]. Stained cells

178 were analyzed with a Coulter Epics XL flow (Beckman-Coulter, Les Ullis, France)

179 cytometer. Data were analyzed using LYSYS (Beckman-Coulter) software.

180

181 **Reverse Transcriptase PCR Assays**

182 RNA extraction from treated cells and cDNA synthesis were performed as described

183 previously [52]. The cDNAs coding for human MMP-2, MMP-9, VEGF-A and

184 β2-microglobulin were amplified in PCRs, using primers synthesized by Sigma-Proligo

185 according to the published sequences [52,54,55]. The PCR products were visualized as

186 described previously [56].

187

188 **Measurement of MMP-2/-9 gelatinolytic activity by zymography**

189   Analysis of MMP-2/-9 activities in culture supernatants was carried out in 7.5% (w/v)

190   SDS-polyacrylamide gels containing 0.1% gelatin (w/v) as described in [52].

191   Gelatinolytic activities of MMPs were detected as transparent bands on the background

192   of Eza-blue stained gelatin. The bands were acquired in a densitometer (Oncor).

193

194   **ELISA analysis**

195   Total MMP-2 (pro + active forms), total MMP-9 (pro + active forms) and VEGF-A

196   (containing 125 amino acid residues, $VEGF_{125}$) contents in culture supernatants were

197   determined using commercial ELISA kits provided by R&D (Abingdon, UK). Controls

198   included culture medium alone incubated under the same conditions. Detection levels

199   for MMP-2/-9 was 1 ng/mL and for VEGF-A 5 pg/mL.

200

201   **Statistics**

202   Statistical analyses were performed using GraphPad Prism software (version 7.0,

203   GraphPad Software, La Jolla, CA, USA). Groups were compared using Mann–Whitney

204   tests or unpaired or paired Student's t-tests. Correlations between variables were tested

205   by calculating Spearman's coefficient (r) and the *P*-value. All tests were two-tailed for

206   greater stringency.

207

208   **RESULTS**

209   *Expression of proMMP-2, proMMP-9 and VEGF-A in primary AML cells*

210   We first examined the levels of transcripts of MMP-2/-9 and VEGF-A in leukemic

211   blood cells from 15 AML patients with different FAB subtypes M0, M1, M2, M4 and

212   M5 (FAB M3 is not represented because of its low frequency in the cohort).

213   Representative examples of PCR products are shown in Figure 2A. The PCR products

214   for MMP-2/-9 and VEGF-A were detected at various degrees in all the AML samples

215   tested (Figure 2A and data not shown). MMP-2 and VEGF-A transcripts were detected

216   in all tested samples whereas MMP-9 transcript was detected in 12 of the 15 tested

217   samples (Figure 2A and data not shown). The transcripts patterns showed no

218   associations with the FAB subtype (Figure 2A and data not shown). As exemplified in

219   Figure 2B, zymography analysis of the supernatants of AML cells (72 h of culture)

220   showed the presence of proMMP-9 and proMMP-2 proteins at 92 kDa and 72 kDa

221   respectively (Figure 2B) whereas the active forms of MMP-9 (at 82 kDa) and MMP-2

222   (at 65 kDa) were not detected (Figure 2B). In some cases, an intermediate ≃ 85 kDa

223   MMP-9 form was seen (Figure 2B). The release of these proteins by primary AML cells

224   *in vitro* and under basal conditions, was quantified in ELISAs: the mean (range) of total

225   MMP-2, total MMP-9 and VEGF-A concentrations (after a 72 h of culture) released by

226   AML cells were respectively 4.44 (1-13) ng/mL for 10^6 cells, 16.04 (1-51) ng/mL for

227   10^6 cells and 57.71 (5-288) pg/mL for 10^6 cells (Figure 2C) and were not correlated

228   with the FAB subtype ($P > 0.05$). Next, we tested for associations between the levels of

229   released MMP-2, MMP-9 and VEGF-A. No correlations were found for MMP-2 and

230   MMP-9 (r = 0.2859, $P = 0.1483$), for MMP-2 and VEGF-A (r = 0.1046, $P = 0.6867$) or

231   for MMP-9 and VEGF-A (r = 0.1817, $P = 0.4811$) (Figure 2D).
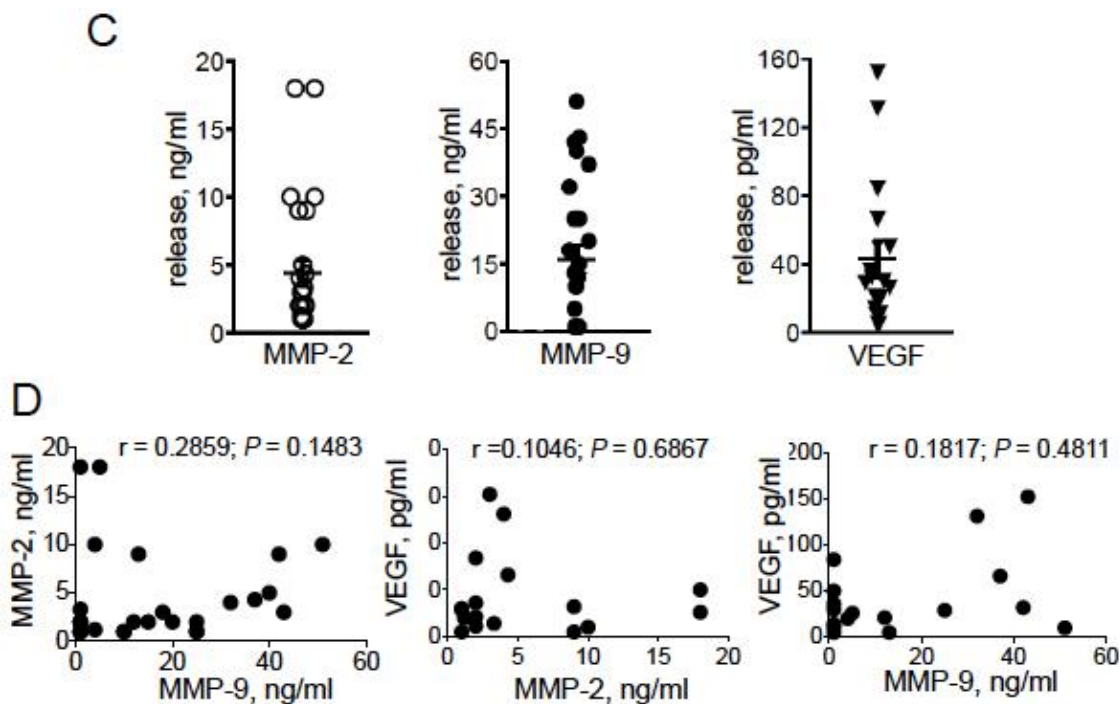
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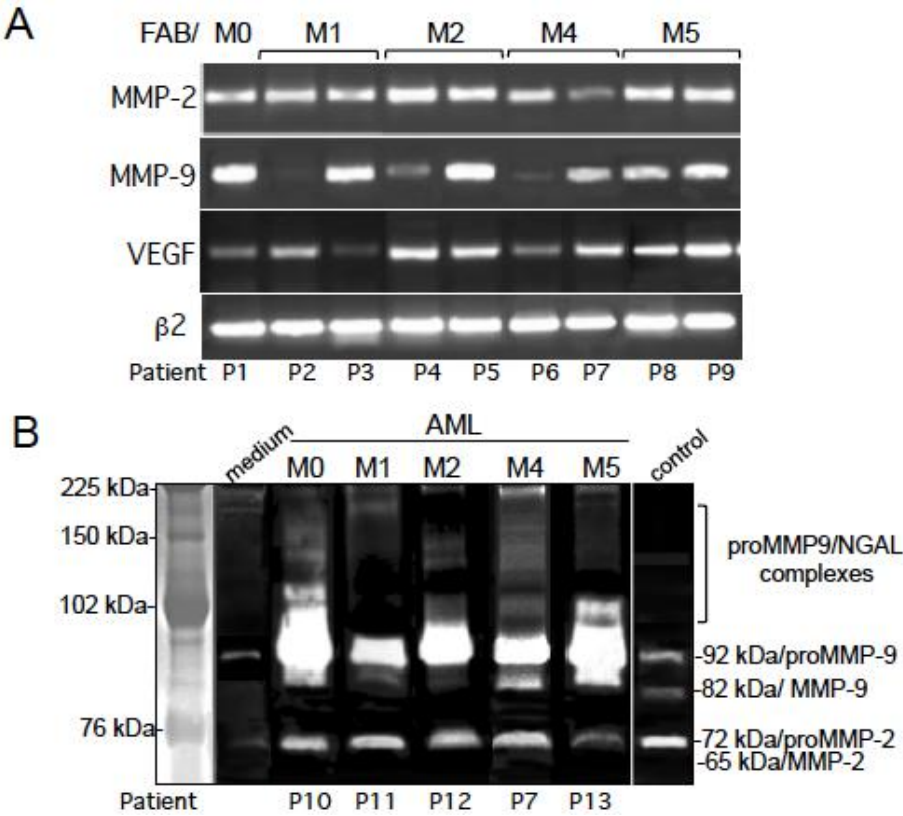
Figure 2. Expression of MMP-2, MMP-9 and VEGF-A in primary AML cells. (A)
PCR analyses of MMP-2/-9 and VEGF-A transcripts. Samples were standardized for
total cDNA content by assessing the presence of identical amounts of 2-microglobulin
transcripts. (B) Expression profiles of MMP-2/-9 (pro and active forms) were analyzed
by zymography using 7.5% (w/v) SDS-polyacrylamide gels containing 0.1% gelatin
(w/v) in the 72 h-conditioned media (supernatant) from AML cells. Control medium is
a FCS-supplemented culture medium alone incubated under the same conditions;
culture supernatant from U937 (M5) cells was used as a positive control for
proMMP-2/-9 and active MMP-9 proteins. The assay's sensitivity for MMP-2/-9
gelatinolytic activity is 25 ng/ml. (C) Total MMP-2 (first column, n = 27 samples), total
MMP-9 (second column, n = 27 samples) and VEGF-A (third column, n = 17 samples)
productions in the 72 h-culture supernatants from AML cells were determined by
ELISA. Mean concentrations ± SEM are indicated. Spots are superposed in all columns.
(D) Correlations between MMP-2, MMP-9 and VEGF-A levels in the 72 h-culture
supernatants from AML cells. Spearman's correlation coefficient (r) and the *P*-value are
shown. MMP-2 vs MMP-9 (n = 27), MMP-2 vs VEGF-A (n =17) and MMP-9 vs
VEGF-A (n = 17).

252

HF induces apoptosis in primary AML cells independently of the FAB subtype

We assessed the effects of HF on the viability of blood PBMCs obtained from 37 AML

patients. Cell death was assessed by determining phosphatidylserine exposure at the

cell surface (using annexin-V-FITC binding) and cell membrane disruption (using

propidium iodide labeling). Initial studies showed that HF's optimal effects in inducing

death of AML cells occurred in a time-dependent manner (24-72 h). Consequently, the

lethal effect of HF at 72 h was investigated in all subsequent experiments. As

exemplified in Figure 3A, the proportion of total annexin-V$^+$ cells (dead cells) by 72 h

enhanced after treatment with increasing doses of HF (1.4, 2 and 3 µg/mL) compared to

control (untreated) experiments. Accordingly, light microscopy observations revealed

that AML cell treatment with HF led to a deterioration of the cell morphology, with a

decrease in the nuclear/cytoplasmic ratio at 1.4 µg/mL HF, an apparition of an enlarged

cytoplasm at 2 µg/mL HF, and appearance of shrunken cells at 3 µg/mL HF, in a

manner indicative of a typical apoptotic morphology (Figure 3B). In contrast, and as

previously reported for normal PBMCs [24], HF (2 µg/mL, 72 h) showed no marked

toxic effects on normal PBMCs and isolated monocytes (Figure 3C). As exemplified in

Figure 3D, HF-mediated cell death similarly increased in blood cells and BM cells

from a same patient with AML. In summary, the effects of HF (2 g/mL, 72 h) were

assessed on the viability of 37 AML samples with M0, M1, M2, M3, M4 and M5 FABs.

Two samples respectively from M1 and M4 patients exhibited high levels of basal

death (> 70% Annexin-5$^+$ cells) and did not respond to HF treatment (see Figure 3H left

panel for M4 patient). For the other 35 samples, cultured leukemic cells exhibited

variable baseline levels of spontaneous death (Figure 3E). Exposure to HF increased

death in 33 of the 35 blood samples (Figure 3E). The paired-t test confirmed the

significant enhanced death in HF-treated AML cells (Figure 3E). The lethal effect of

HF was found independent of the FAB subtype ($P = 0.0735$) (Figure 3F). These data

collectively indicate that HF induces apoptosis in cultured AML cells independently of

FAB status.

281

282 We previously showed that HF induced caspase-dependent apoptosis in AML cell lines

283 representative of primary AML cells through the mitochondrial (intrinsic) pathway [26].

284 Caspase -3 is the executioner caspase of intrinsic apoptosis [57]. To confirm the caspase

285 pathway's involvement in HF-induced apoptosis of primary AML cells, we investigated

286 the level of active caspase-3 expression in two AML samples that respectively respond

287 (Figure 3G) or not (Figure 3H) to the lethal action of HF. As expected, AML responder

288 cells displayed higher levels of active caspase-3 than untreated cells (Figure 3G); in

289 contrast, the levels of active caspase-3 did not change in non responder AML cells

290 (Figure 3H). Altogether, these results indicate that HF induces a caspase-associated

291 apoptosis in primary AML cells, independently of the FAB subtype.

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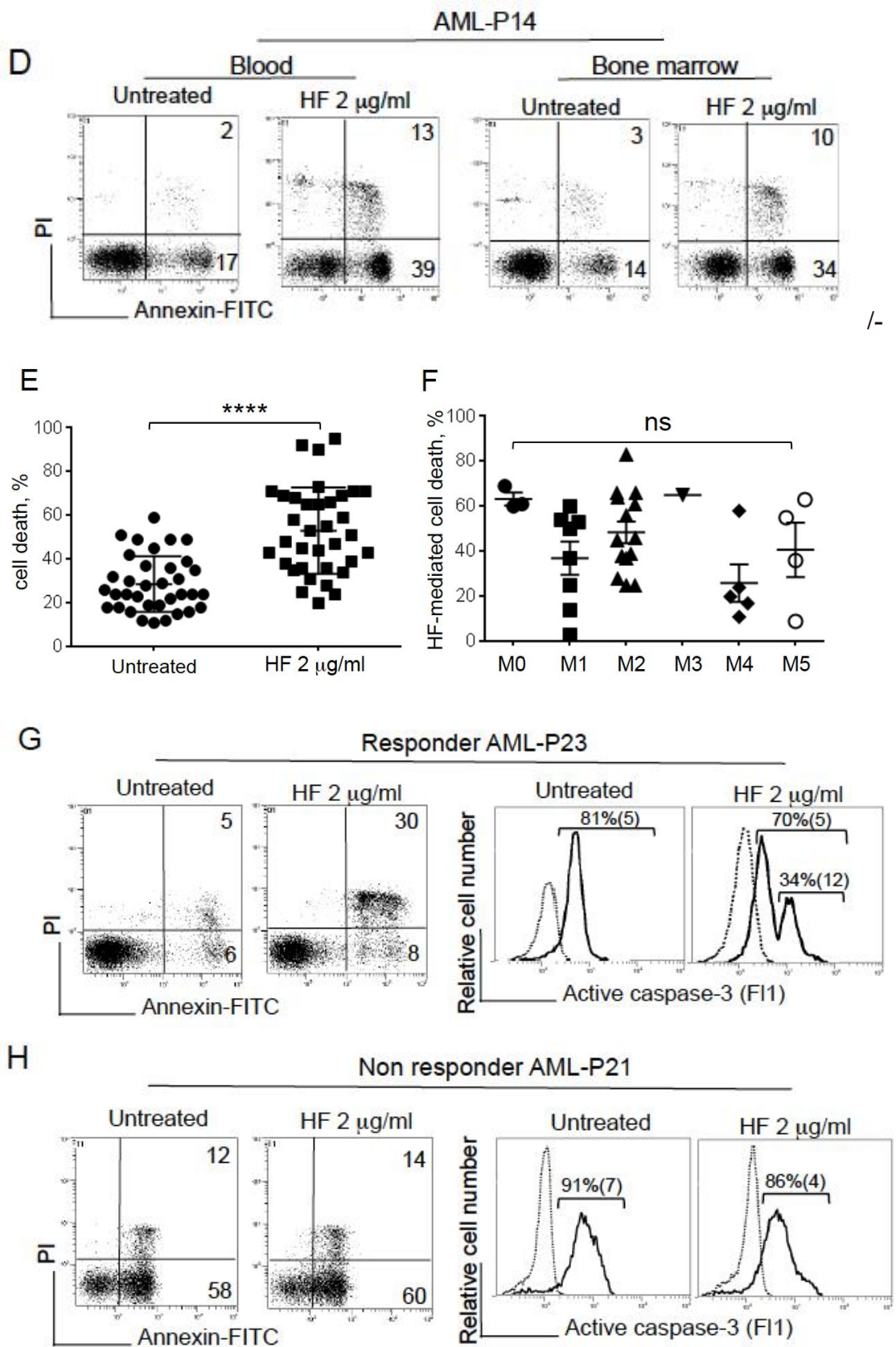

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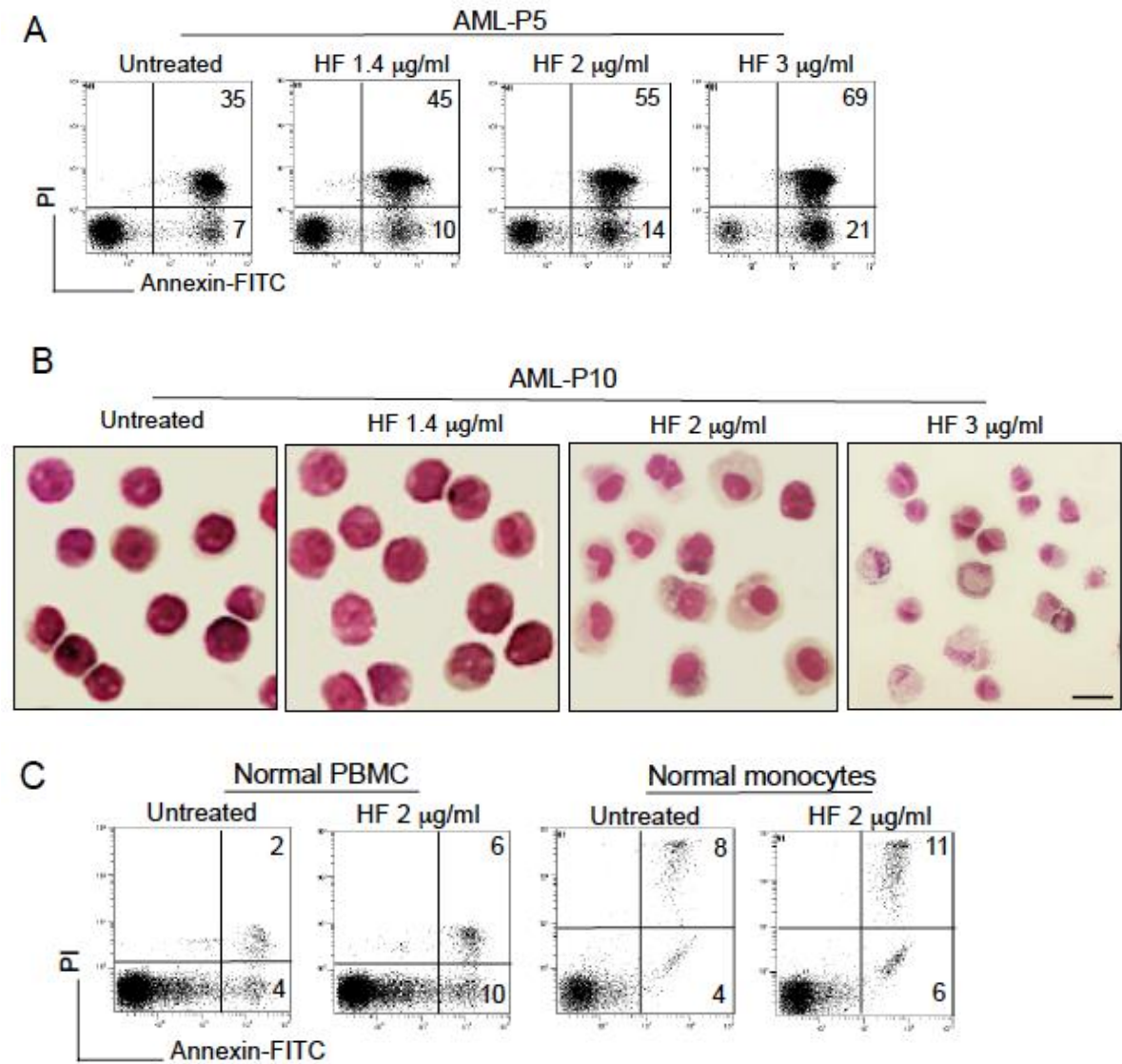
300   **Figure 3. HF induces resistance to apoptosis in AML cells.** (A) Representative

301   cytograms of AML blood cells treated for 72 h with HF (1.4, 2 and 3 g/mL); detection

302   of dead cells after annexin-V-FITC/PI staining and flow cytometry. The percentage of

303   annexin-V-positive cells is shown. (B) Light microscopy of non-treated and AML cells

304   treated with HF. Original magnification: x 600. May Grünwald stain. Scale bar: 10   m.

305   (C) Representative cytograms of PBMCs and isolated monocytes from two healthy

306   donors, treated with 2 g/mL HF for 72 h, and cell death was assessed as described in

307   (A). (D) Representative cytograms of AML cells isolated from blood and bone marrow

308   of one AML patient, treated with 2 g/mL HF for 72 h, and cell death was assessed as

309   described in (A). (E) Cell death levels (2 g/mL, 72 h) were determined in untreated and

310   HF-treated maligant cells from 35 AML patients; Mean concentrations ± SD are

311   indicated; *P* value was calculated using a Mann-Whitney U-test; **** *P* < 0.0001. (F)

312   The percentage of HF-mediated AML cell death was determined for all FAB subtypes

313   by subtracting the percentage of annexin-positive cells in the absence of HF from the

314   percentage of annexin-positive cells in the presence of HF, and divided by the

315   percentage of annexin-positive cells in the presence of HF x 100. Data are the mean ±

316   SEM (M0, n = 3; M1, n = 8; M2, n = 14; M3, n = 1; M4, n = 5; M5, n = 4). *P* values

317   were calculated using one-way ANOVA (without M3 value); ns, not significant. (G&H)

318   AML cells were treated with HF (2   g/mL, 72 h) and the percentage of

319   annexin-V-positive cells was determined as decribed in (A) (left panel). In parallel,

320   active caspase-3 expression was measured by flow cytometry (right panel); cells were

321   stained with FITC-rabbit Ig (control, dashed line) or anti-active caspase-3-FITC (solid

322   line); the percentages refer to the percentage of active caspase-3. (G) responder AML

323   cells sensitive to HF treatment with death induction (38% vs untreated 11%) and

324   caspase-3 activation (34%). (H) non responder AML cells do not respond to HF

325   treatment (no cell death and no caspase-3 activation).

326

327   **HF inhibits the secretion of proMMP-2/-9 and VEGF-A in primary AML cells**

328   We assessed the ability of HF (2 g/mL, 72 h) to modulate the spontaneous release of

329   MMP-2, MMP-9 and VEGF-A by primary AML cells. ELISA data showed that HF did

330   not affect the basal levels of MMP-2/-9 ≤ 1 ng/mL and VEGF-A ≤ 5 pg/mL. In contrast,

331   in the group of AML samples with detectable concentrations of MMP-2/-9 (> 1 ng/mL)

332   and VEGF-A (>5 pg/mL), levels of proteins release fell after treatment with HF (Figure

333   4A). This decrease appeared to be independent of FAB subtype ($P > 0.05$). The

334   Mann-Whitney test confirmed the significant downregulation of the release of

335   MMP-2/-9 and VEGF-A by HF-responsive AML cells (Figure 4A). Decrease of

336   MMP-9 levels by HF was associated with reduced gelatinolytic activity of the 92 kDa

337   form of proMMP-9 accompanied by the accumulation of the truncated 85 kDa form

338   independently of the FAB subtype tested (Figure 4B and data not shown); the increased

339   amounts of 85 kDa form seemed to be inversely associated with the amounts of the 92

340   kDa form in HF-treated AML samples. PCR reactions indicated that HF did not

341   markedly affect the transcription levels of MMP-2/-9 and VEGF-A (when normalized

342   to 2-microglobulin levels) in representative AML samples from distinct FAB subtype

343   (Figure 4C). Altogether, these experiments strongly suggest that HF inhibits, in a

344   post-transcriptional manner, MMP-2/-9 and VEGF-A production in AML cells.

345   Furthermore, the correlations between the levels of released proteins on one hand and

346   cell death on the other were evaluated in both the group of untreated AML samples and

347   the HF-treated group. The levels of released MMP-2/-9 and VEGF-A were not

348   correlated with basal apoptosis in the group of unstimulated AML cells ($P > 0.05$). In

349   contrast, we identified in the group of HF-treated AML cells a negative correlation

350   between HF-mediated proteins' release and HF-mediated apoptosis (Figure 4D).

351   HF-mediated AML cell death was significantly (negatively) correlated with the release

352   of MMP-2 (r = - 0.856; $P = 0.0016$) and MMP-9 (r = -0.881, $P = 0.0072$) but not with

353   that of VEGF-A (r = - 0.542, $P = 0.0588$) (Figure 4D). These results indicate that HF

354   induces the release of MMP-2/-9 and VEGF-A in AML cells.

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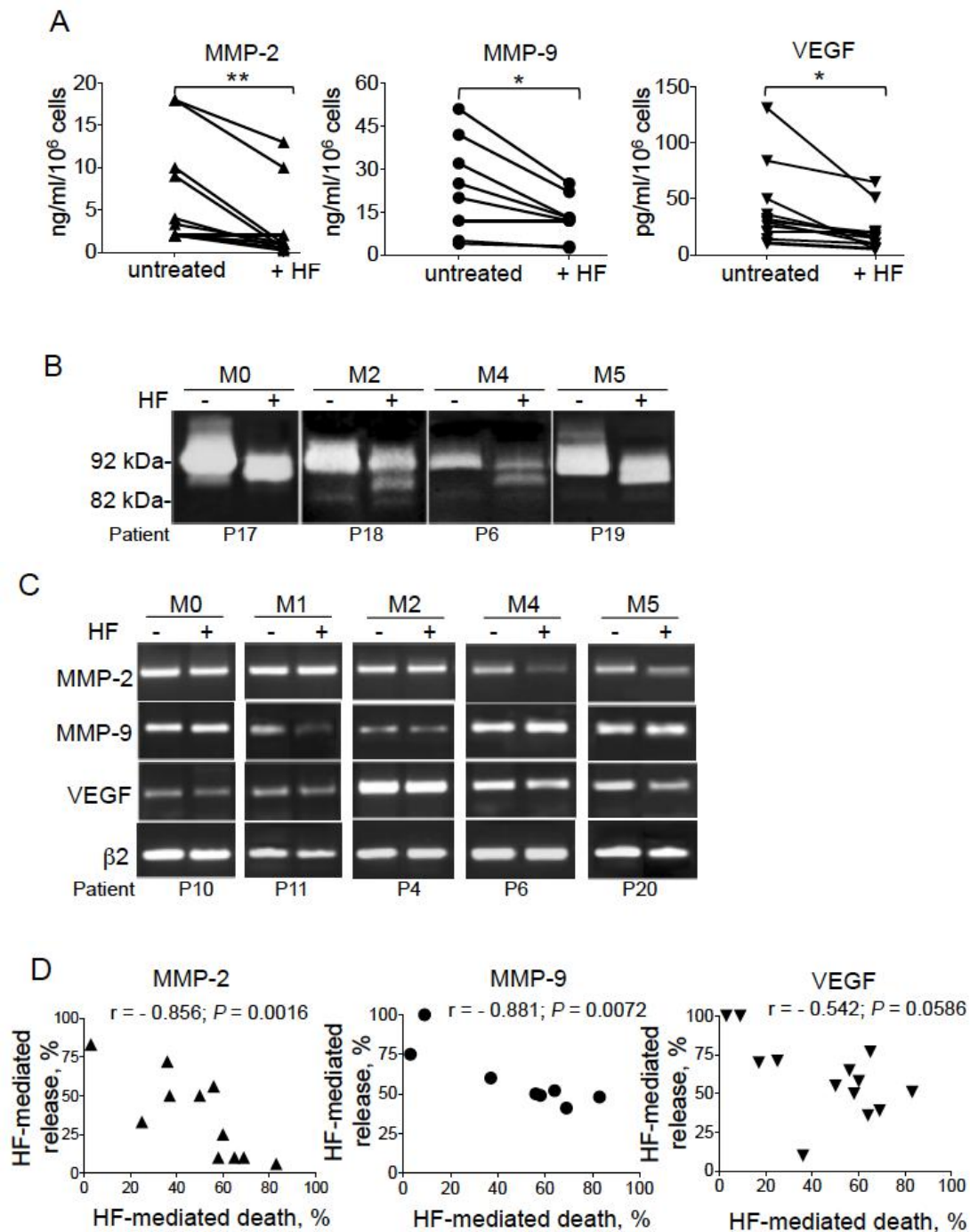
Figure 4. HF inhibits the production of proMMP-2/-9 and VEGF-A by AML cells.
(A) Total MMP-2 (n = 11), total MMP-9 (n = 8) and VEGF-A (n = 13) productions in
the 72 h-culture supernatants from AML samples untreated or treated with HF (2 g/mL)
were determined by ELISA. *P* value was calculated using a Mann-Whitney U-test; * *P*
< 0.05; ** *P* < 0.01. (B) The gelatinolytic activities of MMP-2/-9 were analyzed using
zymography (see Figure 2), in the supernatants from four AML cell samples treated or
not with HF (2 g/mL, 72 h). (C) PCR analyses of MMP-2, MMP-9, VEGF-A and

366   2-microglobulin transcripts from five AML samples treated or not with HF (2 g/mL, 24

367   h). (D) Correlations between the levels of HF-mediated release of MMP-2/-9 or

368   VEGF-A and HF-mediated death in AML cells (n = 11 for MMP-2; n = 8 for MMP-9;

369   n = 13 for VEGF-A); the percentage of HF-mediated AML cell death was determined

370   as described in Figure 3; the percentage of HF-mediated release was determined by

371   subtracting the percentage of MMP/VEGF-A release in the absence of HF from the

372   percentage of MMP/VEGF-A release in the presence of HF, and divided by the

373   percentage of MMP/VEGF-A release in the absence of HF x 100. Spearman's

374   correlation coefficient (r) and the *P*-value are shown.

375

376   **DISCUSSION**

377   Enhanced angiogenesis participates in the progression of AML, and pharmacological

378   targeting of pro-angiogenic proteins (including MMPs and VEGF-A) might be a

379   possible antileukemic strategy. HF exhibits antiangiogenic and proapoptotic activities

380   towards various cancer cells [15,19,29,30]. AML cells abnormally proliferate and escape

381   apoptosis. Our present study provides evidence that primary blood AML cells

382   coexpress proMMP-2, proMMP-9 and VEGF-A proteins. The major finding of our

383   study indicates that HF is able to inhibit two biological events related to AML

384   progression and angiogenesis, i.e. survival and secretion of MMP-2/-9 and VEGF-A in

385   AML patients' cells.

386

387   Peripheral blood blasts from AML patients express detectable levels (transcript and

388   secreted protein) of MMP-2, MMP-9 and VEGF-A. As previously reported for BM

389   AML blasts [45], circulating AML cells release the proforms of MMP-2 (72 kDa) and

390   MMP-9 (92 kDa) proteins. An additional 85 kDa form of MMP-9 is observed in some

391   AML samples (independently of the FAB subtype). A previous study reported that BM

392   AML blasts with the subtypes M4/M5 expressed an ≃ 85 kDa MMP-9 at their cell

393   surface [58]. Breast cancer cells express an 85 kDa MMP-9 devoid of complex

394   carbohydrates, which might result from deglycosylation [59]. Interestingly, active

395   MMP-3 and active MMP-13 are capable to cleave proMMP-9 to generate an 86 kDa

396   intermediate [60,61]. The origin of this MMP-9 variant and its role in AML remain to be

397   defined.

398

399   We observed no intercorrelations between the levels of MMP-2, MMP-9 and VEGF-A

400   secreted by AML cells, likely reflecting their variable expression both among and

401   within individual AML cells. MMP-2 transcription is mainly regulated by STAT3 [62,63]

402   whereas MMP-9 transcription is regulated via NF-κB, AP-1 and SP-1 [64]. VEGF

403   transcription can be regulated by STAT3 and HIF [62,63,65]. Our data strongly suggest that

404   the heterogeneity found in the released profiles of MMP-2/-9 and VEGF-A proteins

405   may be related to the distinct gene regulations of MMP-2/-9 and VEGF in AML cells.

406   Moreover, the released levels of MMP-2/-9 and VEGF-A do not appear to depend on

407   the differentiation stage of AML cells. Our data agree with studies in which the levels

408   of MMP-2 (plasma), MMP-9 (BM) or VEGF-A (BM) and FAB subtypes are not

409   associated [39,40,45,46,66]. In contrast, other studies have shown an association between

410   MMP-9 or VEGF-A (BM) and AML-M4/M5 [43,46], or VEGF-A (BM) and AML-M0 [42].

411   Larger studies are mandatory to fully address this question.

412

413   We previously showed that HF exhibits proapoptotic activities in AML cell lines of

414   distinct FAB phenotype [26]. In peculiar, HF did not activate the enzymatic activities of

415   caspase-3/-8/-9 directly (B Bauvois, unpublished results) but promoted

416   caspase-mediated apoptosis involving BAD and Noxa activation in AML U937 (M5)

417   cells [26]. BAD is a direct downstream target of Akt1 [67]. We previously presented first

418   evidence that HF, by directly inhibiting Akt-1 kinase activity, prevents BAD

419   phosphorylation which in turn activates its proapoptotic role in U937 cells [26]. Primary

420   AML cells overexpress Akt1 [67]. Activated Akt signalling protects AML cells from

421   apoptosis [67]. In this study, the proapoptotic effect of HF in AML cell lines is confirmed

422   in primary AML cells, independently of the latter's FAB subtype; HF-mediated AML

423   cell death occurred in 33 of the 35 AML samples tested, accompanied by caspase-3

424   activation. Whether HF activates the proapoptotic function of BAD through Akt1

425   inhibition in primary cells remains to be confirmed. HF exerts selective cytotoxicity on

426   AML cells but not on normal PBMCs and monocytes. Importantly, BM AML cells

427   appear as sensitive as circulating AML cells toward the apoptotic effect of HF,

428   suggesting that HF might be of interest for the therapy of AML through its potential

429   capacity to eradicate BM leukemic stem cells. Moreover, the induction of apoptosis by

430   2 g/mL HF parallels the inhibition of secretion of MMP-2/-9 and VEGF-A by HF at the

431   same concentration. Suppression of released MMP-2/-9 and VEGF-A proteins by HF

432   does not appear correlated with their transcript levels suggesting a post transcriptional

433   inhibition. Our observations strongly suggest that HF induces simultaneously (i)

434   suppression of MMP-2/-9 and VEGF-A production and (ii) apoptosis in primary AML

435   cells. However, to what extent are apoptosis and secretion interdependent or related? A

436   previous study showed that the stable dicyclohexylammonium salt of HF (HF-DCHA,

437   0.5 M) inhibits MMP-2 release by two human epithelial cell lines (HT-1080 and

438   SK-N-BE) while apoptosis in these cells is induced at concentration 16-fold higher (IC

439   ≥8 M), suggesting that HF-DCHA induces two separate events in these tumor models

440   [68]. The effect of HF at ≤ 2 g/mL on MMP-2/-9 and VEGF-A release remains to be

441   assessed in AML cells. Furthermore, the decreased production of these hemoregulators

442   might result from the apoptotic process. Conversely, the inhibition of MMP-2/-9 and

443   VEGF-A might impact the survival of AML cells. Indeed, VEGF-A signalling (via

444   PI3K/Akt activation) favors autocrine AML cell proliferation, survival, and

445   chemotherapy resistance [69,70]. Moreover, proMMP-2 and proMMP-9 have the ability

446   to directly activate signalling pathways modulating cell survival, migration and

447   angiogenesis [8]. For example, the binding of proMMP-9 to its docking receptors 4 1

448   integrin and CD44 induces an intracellular signalling pathway (STAT3/Lyn/Mcl-1) that

449   favours the survival of circulating chronic lymphocytic leukemia (CLL) cells [71].

450   Similarly, the binding of proMMP-2 or proMMP-9 to the integrins L2 and M2 induces

451   the migration of human AML cell lines [72]. Although AML cells from the majority of

452   patients (at diagnosis or relapse) express / chains of integrins and CD44 [73], the

453   potential roles of proMMP-2/-9 in AML cell survival remain to be shown. Alternatively,

454   HF might simultaneously induce two simultaneous but distinct events (apoptosis and

455   secretion inhibition) in AML cells. Due to its physicochemical properties, HF is easily

456   incorporated into the membrane lipid bilayer, where it can function as a protonophore

457   [74]. Lipid rafts modulate Akt/BAD signalling in cancer cells [75,76] and regulate

458   exocytosis/endocytosis pathways [77-80]. Whether HF disrupts lipid rafts in AML cells,

459   which in turn simultaneously lead to cell apoptosis (through activation of Akt1/BAD)

460   and blockade of secretion of MMP-2/-9 and VEGF-A (through exocytosis inhibition)

461   remains to be established. In view of this possibility, HF could join the family of

462   natural compounds (including quercetin, epigallo catechin-3-gallate, resveratrol,

463   genistein) that induce apoptosis of tumor cells and block cytokine secretion through

464   alteration of lipid rafts [75,76,81-84].

465

466   **CONCLUSIONS AND PERSPECTIVES**

467   Among the search for novel therapeutics for AML, there is a need for the development

468   of drugs that block angiogenesis associated with the AML disease. The inhibition of

469   VEGF-A and/or MMP-2/-9 may provide a therapeutic benefit in AML. Antiangiogenic

470   strategies have sought to target the MMPs' catalytic activity [85]. The failure of MMP

471   inhibitors as cancer drugs in the clinic may be explained by their lack of selectivity

472   towards MMPs (including MMP-2/MMP-9). New approaches are focusing on more

473   selective MMP inhibitors that target motifs outside the active site (the "exosite") of

474   individual MMPs; newly designed inhibitors include peptides that block

475   exosite-mediated cell surface interactions and function-blocking anti-MMP antibodies

476   [85-87] to target proMMP-9-mediated cell migration *in vitro* [88-90]. To date, no efficient

477   therapy based on MMP inhibition has been made available for targeting angiogenesis in

478   cancers including AML, and new strategies are currently developing MMP-responsive

479   drug delivery vehicles [87]. Studies with various VEGF inhibitors are currently

480   underway in a number of tumors [1,91,92]. Bevacizumab, a humanized monoclonal

481   antibody, binds to all circulating forms of VEGF, and thus prevents VEGF binding to

482   VEGF receptors. Apart bevacizumab, other drugs have been reported to inhibit the

483   binding of VEGF to its receptors (aflibercept) or block the signalling pathway mediated

484   by VEGF receptors (ramucirumab, pazopanib, and axitinib) [1,91]. Bevacizumab

485   treatment of AML patients with refractory/relapsing disease reduced VEGF expression

486   at the level of BM but failed to show any significant clinical antileukemic activity [36].

487   Other clinical trials incorporating antibodies against VEGF or VEGF receptors-2 have

488   not produced results supporting a significant clinical benefit [36]. These inhibitors share

489   toxicity and other side effects [1,91,92]. Therefore, the development of new efficient

490   antiangiogenic therapies is necessary. Utilization of natural compounds can be an

491   alternative and/or an additional possibility to inhibit tumor development and associated

492   angiogenesis [75,76,82-84]. By blocking *in vitro* the proliferation of primary blood

493   endothelial cells, and reducing tumor vascularization and tumor-induced

494   lymphangiogenesis in rat models [30,32,93], HF could be already qualified as a putative

495   antiangiogenic agent. Pharmacokinetics studies in humans have demonstrated oral

496   bioavailability of HF [94]. However, two aspects limit the pharmacological use of

497   purified HF which concern (i) its poor solubility and instability [30,95], and (ii) its

498   interference with other therapeutic drugs *via* activation of liver CYP450 enzymes

499   involved in the metabolism of pharmaceutical drugs (notably CYP3A4) [94,96,97]. Oral

500   administration by healthy volunteers of SJW extracts (300-2700 mg containing 14.8-70

501   mg HF) results in the detection of 0.15-1.45 g/mL (0.28-2.68 M) HF concentrations in

502   blood 3h after absorption [94,98-100]. SJW treatment period of 12.5-21 days using high HF

503   extracts (> 27 mg/day) demonstrates a significant CYP3A induction [97]. Altogether,

504   these data strongly suggest that the maximal concentration of HF in blood should not

505   exceed 0.3 to avoid the frequency of drug interactions. With regard to our data obtained

506   *in vitro*, it remains to examine whether or not apoptosis and inhibition of secretion of

507   AML cells *in vivo* can be triggered by a such low dose of HF. In the last years, various

508   chemical derivatives of HF have been developed with improved stability and solubility,

509   and with the hope to remove HF drug metabolism stimulatory activities without

510   affecting its antitumor properties. Thus, HF-DCHA, tetrahydro-HF-DCHA,

511   octahydro-HF-DCHA and O-(carboxy methyl)-HF were shown to retain the antitumor

512   and antiangiogenic properties of HF without inducing toxicity in laboratory animals

513   [28,68,101,102]. Whether these HF derivatives retain the antileukemic properties of parental

514   HF *in vitro* in AML primary cells and in experimental animals should be established. In

515   parallel, the effects of these analogs on CYP3A activity have yet to be evaluated on

516 healthy volunteers. Ongoing therapeutic strategies involve encapsulation of HF into

517 polymeric nanoparticles for overcoming hurdles that hamper the bioactivity and

518 bioavailability of the drug [103-106]. In conclusion, although further studies are

519 undoubtedly required, all these data highlight that HF and its derivatives deserve

520 further study to evaluate them in association with chemotherapy in the treatment of

521 AML.

522

523 **DECLARATIONS**

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528

529 **Authors' contributions**

530 Conceptualization: FM, BB;

531 Formal analysis: FM, RT, BB

532 Investigation: FM, BB

533 Methodology: RT, FM, BB

534 Resources, OL

535 Writing, review and editing:FM, RT, OL, SAS, BB.

536 All authors have read and agreed to the published version of the manuscript

537

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539 Not applicable.

540

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544

545 **Conflicts of interest**

546 All authors declared that there are no conflicts of interest.

547

548 **Ethical approval and consent to participate**

549   In line with the ethical tenets of the Declaration of Helsinki, all the patients diagnosed

550   with AML provided their written and informed consent to participation in the study.

551   Ethics approval was given by the independent ethics committees at Saint-Antoine

552   Hospital (Paris, France) and the French National Institute of Cancer ("Tumorothèque

553   Hématologie" Paris-Saint-Antoine Hospital COHO0203 INCA 2007). Control blood

554   samples were collected from healthy, fully anonymized donors (no special written

555   informed consent) in accordance with the institutional review boards of the

556   Etablissement Français du Sang (Hôtel-Dieu Hospital, Paris, France) and the Institut

557   National de la Santé et de la Recherche Médicale, Paris, France.

558

559   **Consent for publication**

560   Not applicable.

561

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