A Galactosidase-Responsive Doxorubicin-Folate Conjugate for Selective Targeting of Acute Myelogenous Leukemia Blasts

ARTICLE *in* LEUKEMIA RESEARCH · MAY 2013

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A galactosidase-responsive doxorubicin-folate conjugate for selective targeting of acute myelogenous leukemia blasts

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ARTICLE INFO

Article history:
Received 23 September 2012
Received in revised form 24 April 2013
Accepted 28 April 2013
Available online xxx

Keywords:
Acute myelogenous leukemia
Doxorubicin
Vectorization
Chemotherapy
Folate receptor

ABSTRACT

Cytarabine combined with an anthracycline or an anthracenedione represents the usual intensive induction therapy for the treatment of AML. However, this protocol induces severe side effects and treatment-related mortality due to the lack of selectivity of these cytotoxic agents. In this paper, we present the study of the first galactosidase-responsive molecular "Trojan Horse" programmed for the delivery of doxorubicin exclusively inside AML blasts over-expressing the folate receptor (FR). This targeting system allows the selective killing of AML blasts without affecting normal endothelial, cardiac or hematologic cells from healthy donors suggesting that FDC could reduce adverse events usually recorded with anthracyclines.

1. Introduction

Acute myelogenous leukemia (AML) is the most common leukemia in adults [1]. Since many years, cytarabine combined with an anthracycline or an anthracenedione represents the usual intensive induction therapy for the treatment of AML [2]. Such a protocol results in 70% complete remission (CR) but long-term survival is observed in less than 20% of patients [3–5]. Further studies have shown that increased doses of anthracyclines improved the rate of CR and the duration of overall survival, as compared with the standard dose [6,7]. However, the lack of selectivity of cytotoxic agents employed in the induction therapy induces severe side effects and high treatment-related mortality. In order to enhance the selectivity of AML chemotherapy, newer less-aggressive approaches are under investigation [8] including the development of targeting system such as antibody-drug conjugates (ADC) programmed for the delivery of potent cytotoxics exclusively inside malignant cells [9]. Within this framework, Gemtuzumab ozogamicin [10] was the first targeting system to prove the ADC concept in the clinic for the treatment of AML [11]. In recent studies, fractioned doses of gemtuzumab ozogamicin combined to standard chemotherapy improved event free survival and to a less degree overall survival in AML patients aged 50–70 years-old [12–14].

To date, although numerous efforts have been devoted to the discovery of new anticancer drugs, efficient treatment of AML patients whilst limiting adverse events still remains challenging and the development of new therapeutic approaches is warranted. Recently, we designed the first galactosidase-responsive "Trojan Horse" programmed for the selective targeting of folate-receptor (FR) positive tumor cells [15]. This molecular system includes a folate ligand, a galactoside trigger and the potent doxorubicin articulated around a central self-immolative linker (Supplementary Figure 1A) [16]. The folate ligand allows the selective recognition of FR-positive tumor cells and the subsequent internalization of the whole device by endocytosis. The intracellular β-galactosidase-catalysed cleavage of the carbohydrate unit triggers the release of

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http://dx.doi.org/10.1016/j.leukres.2013.04.026

doxorubicin which induces cell death after further translocation into the nucleus.

In this paper, we report the evaluation of our folate-doxorubicin conjugate (named FDC) as a potential new drug for the targeted treatment of AML. Since the FR is expressed in approximately 70% of AML blasts whereas it is present in low or not-detectable quantities in most normal tissues [17,18], this targeting system should allow the selective delivery of doxorubicin inside FR-positive leukemic blasts, thereby avoiding side effects on non-targeted tissues. The results obtained on both leukemic cell lines and 31 AML patient samples show that the FDC exhibits a significant cytotoxic activity which is correlated to FR expression. In contrast to the free doxorubicin, our galactosidase-responsive conjugate does not affect endothelial, cardiac or hematologic cells from healthy donors suggesting that FDC could reduce adverse events usually recorded with anthracyclines.

2. Design and methods

2.1. Cell culture and patients

KG-1 and HL-60 AML cell lines were grown in IMDM supplemented by 20% fetal calf serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 (endothelial cell growth medium-2, Clonetics) with 2% Fetal Bovine Serum. All experiments were performed with HUVECs with less than 5 passages. Human bronchial epithelial cells (16HBE) were cultured in RPMI1640 medium supplemented by 10% Fetal Bovine Serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Blood and bone marrow samples were obtained from 31 AML patients or 8 healthy donors. All patients gave a written informed consent in accordance with the Declaration of Helsinki and the protocol was accepted by the IRB of the CIC 0802. All these samples were collected at diagnostic, before treatment initiation.

2.2. Mononuclear cell preparation

Bone marrow or blood cells were diluted in serum-free medium and layered over Ficoll-Hypaque (Sigma–Aldrich) solution. After 20 min centrifugation at 2500 rpm without brakes, mononuclear cells are removed from the interface and washed twice with PBS before dilution in folate-free RPMI-1640 supplemented by 10% human serum for assay.

2.3. Cell viability

The Cell Proliferation Kit II (XTT, Roche) was used to assess *in vitro* cell viability after 4 days of treatment as previously described [19]. IC_{50} values were determined using GraphPad software. Experiments were performed 5 times in triplicate for KG-1 and HL-60 and 2 times in triplicates for 16HBE.

2.4. Quantitative RT-PCR

Total RNA was extracted using the RNA Total Isolation Kit (Promega), reverse transcription was done with SuperScript II (Invitrogen) and qPCR experiment was performed as previously reported using GAPDH, FOLR1 and FOLR2 specific primers [15,20]. Experiments were done 3 times in duplicate.

2.5. Flow cytometry

5.10^5 cells were transferred to V-bottomed plates and incubated 3 h with indicated compounds at 10 μM. Cells were then washed twice in ice-cold PBS-4% BSA and stained for 30 min at 4 °C with CD3-FITC and CD34-APC (BD Biosciences). After two additional washes, cells were suspended in 7-aminoactinomycinD-containing PBS, a cell viability marker (BD Biosciences). Live cells initially gated by forward and side scatter were analyzed for CD3 and CD34 expression and doxorubicin internalization using FACS CantoII machine and FACS DivaII software (BD Biosciences). Anti-isotype antibodies (BD Biosciences) were used in parallel, for specificity control.

2.6. Tube formation assay

Twenty-four-well plates were coated with 10 mg/mL Matrigel© (BD Biosciences) and kept at room temperature for 4 hours to allow gel formation. 1.10^5 HUVECs per well were seeded in EGM-2 with 2% fetal bovine serum in the presence of 10 μM doxorubicin or FDC for 1 or 4 days. Each well was washed in PBS, fixed in 4% paraformaldehyde for 1 h at 4 °C and stored in 0.02% NaN_3-containing PBS prior to visualization under a MVX10 macroscope (Olympus). Quantification of the vascular network was performed using Angioquant [21]. Images are representative of 2 independents experiments.

2.7. Immunofluorescence

Immunofluorescence was performed as previously reported [15]. Samples were observed with a confocal microscope (FV 1000, Olympus IX-81). The excitation wavelength of 488 nm was used for acquisition of images in red channel (590 nm). Images are representative of 3 independents experiments.

2.8. Western blotting

Immunoblots were done as previously reported [22]. The primary antibodies anti-α-tubulin (1:2000, Sigma), anti-phospho-ERK1/2 or anti-total ERK (1:1000, Santa-Cruz) were detected using HRP-conjugated goat anti-rabbit or anti-mouse antibodies (1:10,000, Sigma) and enhanced chemiluminescence kit (Pierce). Densitometric analyses were performed using ImageJ software. Experiments were done 3 times.

2.9. Whole-cell patch-clamp

HEK293 stably expressing Kv7.1 (KCNQ1 gene) associated with minK subunit (KCNE1 gene) [23] were grown in DMEM supplemented with 10% Fetal Bovine Serum at 37 °C and 10% CO_2. Cells were harvested by using a Dulbecco's Phosphate-Buffered Saline without calcium and magnesium and trypsin (Lorna) and seeded at a density of 5 to 20.10^4 cells/dish to reach a cell confluence of about 50% in the day of experiment. Patch clamp measurements were carried out at room temperature (≈22 °C). Patch electrodes (≈3 MΩ) were pulled from borosilicate glass capillaries using a vertical micropipette puller (Narishige). Voltage clamp experiments were performed using an Axopatch 200B amplifier with a CV 203BU headstage (Molecular Devices). Voltage command pulses were generated by a personal computer equipped with an analog-digital converter (Digidata 1200, Molecular Devices) using pCLAMP software v8.0 (Molecular Devices). Currents were filtered at 2 kHz and digitized at 10 kHz. The patch pipettes were filled with (mM): 10 NaCl, 130 KCl, 0.5 $MgCl_2$, 1 EGTA, and 10 HEPES (pH 7.2, KOH). The bath solution contained (mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, 11 glucose, and 10 HEPES (pH 7.4, NaOH). Currents of five independents cells were recorded using the whole-cell configuration of the patch clamp technique. Potassium currents were generated by clamping cell membrane from a holding potential of – 80 mV to +100 mV for 2500 ms following by a step to −50 mV for 2500 ms to record tail current with 15 s stimulus intervals.

2.10. Statistical analysis

All patient data are described with median and range. The FOLR2 gene expression is presented using boxplots with the median, the 75th and the 25th percentile. The range is shown as vertical edge. Differences between independent groups were assessed by the use of the Wilcoxon rank sum test or Kruskall–Wallis tests as appropriate. For paired data analyses concerning a potential variation between the CD3 and CD34 levels, the ratio of these two parameters was calculated and then analysed using a Wilcoxon test. Correlation between FOLR2 gene expression and blast internalization was determined by the Spearman rank order method. For comparative analyses, all *p* values were two-sided and the type I error was set at 5 percent. Statistical analyses were performed with use of SAS® v9.2 (SAS Institute Cary).

3. Results

3.1. FDC is efficient against KG-1 and HL-60 AML cell lines

We have previously demonstrated that internalization of the folate-doxorubicin conjugate (FDC) into HeLa FR-positive cells proceeded *via* FR-mediated endocytosis [15]. This was followed by intracellular β-galatosidase-catalysed release of doxorubicin and subsequent nucleus translocation leading to cell death (Supplementary Figure 1). In contrast, the low FR-expressing A549 cells were not sensitive to FDC showing the ability of this compound to selectively kill FR-positive tumor cells. The antiproliferative activity of FDC was assessed on KG-1 and HL-60 FR-β-positive AML cell lines (Fig. 1A). FDC exhibited an IC_{50} value of 200 and 300 nM respectively (Fig. 1B-C). On both cell lines, the IC_{50} values of FDC were lower than that of doxorubicin. Indeed, in contrast with doxorubicin that penetrates passively inside the cell, the amount of the drug internalized using FDC is limited by the number of FR present at the cell membrane. However, this lower cytotoxicity could be balanced by the higher selectivity of the targeting system. We also evaluated the antiproliferative activity of an analogue of FDC which lacks the folate targeting ligand (Supplementary Figure 1C). As expected, this compound was non-toxic (Fig. 1B–C) demonstrating that the

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Table 1
Data list of 31 AML patients with age, sex, subtype of AML, the percentage of blood and bone marrow blasts and leukocyte counts, molecular markers, cytogenetics and molecular abnormalities were reported. (n.d.: not done). FOLR2 mRNA expression results are expressed as percentage of GAPDH.

ID Patient	Age	Gender	AML subtype	Leucocytes (mm^3)	% Blasts		Molecular marker	Abnormalities						
					Blood	Bone Marrow		Pronostic	Cytogenetic	FLT3 D835	FLT3 ITD	MLL	CBF	NPM1
1	65	m	M0	12,100	66%	79%		int.	46,XY	n.d.	n.d.	w.t.	w.t.	n.d.
2	74	m	M0	7700	77%	80%		bad	Comp., −5, −7	w.t.	mut.	w.t.	w.t.	n.d.
3	59	m	M1	17,000	88%	97%		bad	Comp., t(3;8)(p24;q22)	n.d.	n.d.	w.t.	w.t.	n.d.
4	43	m	M1	13,100	72%	86%	Dek-Can	bad	t(6;9)(p22;q34)	w.t.	mut.	w.t.	w.t.	n.d.
5	64	m	M1	7600	65%	77%		int.	46,XY	w.t.	w.t.	w.t.	w.t.	n.d.
6	64	f	M1	2400	64%	79%		int.	46,XX	w.t.	w.t.	w.t.	w.t.	n.d.
7	60	f	M1	17,500	78%	67%		fav.	46,XY	w.t.	w.t.	w.t.	w.t.	mut.
8	38	m	M1	29,600	76%	73%		fav.	del13q[3/40]	w.t.	w.t.	w.t.	w.t.	mut.
9	82	m	M2	90,000	18%	32%		unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	64	m	M2	1800	1%	20%		int.	46,XY	w.t.	w.t.	w.t.	w.t.	w.t.
11	80	f	M2	6900	6%	58%		bad	Comp., del5q, −7, del17p	n.d.	n.d.	n.d.	n.d.	n.d.
12	78	f	M2	2800	9%	30%		bad	t(1;3)(p36;p22), del5q	n.d.	n.d.	w.t.	w.t.	n.d.
13	71	m	M2	7100	56%	62%	AML1-ETO	fav.	t(8;21)(q22;q22)	w.t.	w.t.	w.t.	mut.	n.d.
14	80	f	M2	3400	1%	21%		int.	add(X)(q28)	n.d.	n.d.	n.d.	n.d.	n.d.
15	71	m	M2	1300	9%	38%		bad	Comp.,del5q, −7, −17	n.d.	n.d.	n.d.	n.d.	n.d.
16	72	m	M2	1800	2%	21%		bad	Comp., del5, −7, del12p	n.d.	n.d.	n.d.	n.d.	n.d.
17	56	f	M2	93,300	87%	81%		int.	46,XX	w.t.	mut.	w.t.	w.t.	n.d.
18	64	f	M2	1200	1%	39%		int.	46,XX	n.d.	n.d.	n.d.	n.d.	n.d.
19	65	m	M2	2100	1%	55%		int.	46,XY	n.d.	n.d.	n.d.	n.d.	n.d.
20	53	f	M3	1000	2%	74%	PML-RARA bcr1	fav.	t(15;17)(q22;q12)	n.d.	n.d.	w.t.	w.t.	n.d.
21	39	m	M3	2500	60%	75%	PML-RARA bcr3	fav.	n.d.	n.d.	n.d.	w.t.	w.t.	n.d.
22	73	f	M3	30,000	69%	83%	PML-RARA	fav.	t(15;17), +8	n.d.	n.d.	n.d.	n.d.	n.d.
23	70	f	M3	12,500	81%	93%	PML-RARA bcr3	fav.	t(15;17)(q22;q11)	n.d.	n.d.	n.d.	n.d.	n.d.
24	44	m	M4	273,000	94%	97%		int.	46,XY	w.t.	mut.	w.t.	w.t.	n.d.
25	67	m	M4	1600	0%	20%		int.	46,XY	n.d.	n.d.	w.t.	w.t.	n.d.
26	19	m	M5	9300	4%	40%	CBFB-MYH11A	fav.	inv16(p13;q22)	n.d.	n.d.	w.t.	mut.	n.d.
27	75	f	M5	83,000	86%	89%		unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
28	80	m	M5	42,300	12%	48%		int.	del6q, +8	n.d.	n.d.	n.d.	n.d.	n.d.
29	60	f	M5	32,400	55%	88%		int.	46,XX	w.t.	mut.	w.t.	w.t.	n.d.
30	47	m	M5	110,400	95%	90%	MLL-AF9	int.	t(9;11)(p21;q23)	w.t.	w.t.	mut.	w.t.	n.d.
31	57	m	M7	1700	30%	39%		bad	del5q,−17	n.d.	n.d.	n.d.	n.d.	n.d.

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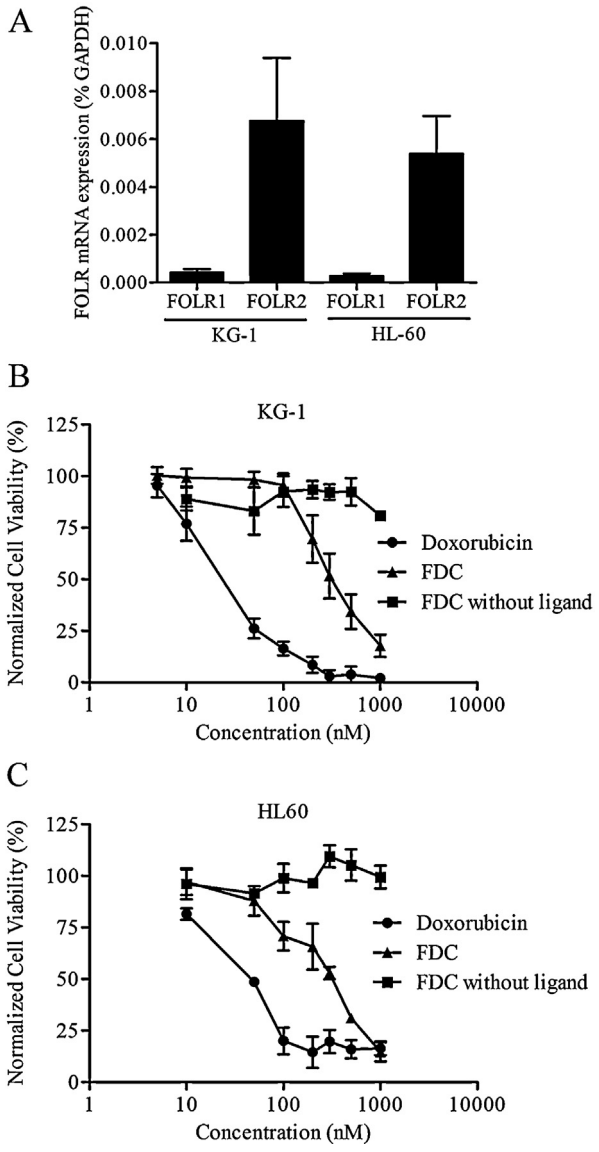

Fig. 1. FOLR1 and FOLR2 expressions in KG-1 and HL-60 (A). The cytotoxic activity of FDC and FDC without targeting ligand was evaluated against KG-1 (B) and HL-60 (C) cells and compared to those of doxorubicin.

interaction of the folate ligand with the corresponding receptor is necessary to achieve toxicity.

3.2. Blasts of AML patients differentially express FR-β

Folate receptors expression was examined on a cohort of 31 AML patients including 12 women aged from 53 to 80 years-old and 19 men aged from 19 to 82 years-old (Table 1). Expression of FOLR1 and FOLR2 genes, encoding the alpha and beta isoforms of folate receptor respectively, was measured by quantitative real-time RT-PCR in AML patient samples. While FOLR1 was never detected, FOLR2 expression was observed at various levels in almost all blasts and was dependent of the AML type (Fig. 2A). These results allowed us to define two groups of patients based on their FOLR2 expression level (Wilcoxon test $p = 0.0029$) (Fig. 2B). Thus, the first group includes M0, M1 and M2 AML patients with blasts expressing a low level of FOLR2 (median: 0.0012% of GAPDH). The second group comprises M3, M4, M5 and M7 AML patients which present a higher expression of FOLR2 (median: 0.0129% of GAPDH). Interestingly, this finding correlates with previous results reported by Pan and colleagues who detected the FR-β protein in only 35% of M0, M1 and M2 blasts and in 90% of M3, M4, M5 and M6 blasts respectively [24]. No relationship was found between expression of FOLR2 with age and gender of AML patients.

3.3. FDC penetrates exclusively in blasts

The potential of FDC as a selective anticancer drug was then evaluated for each AML patient sample included in this study. Either FDC or doxorubicin internalization was evaluated by flow cytometry simultaneously in lymphocytes (CD3+) and blasts (CD34+). Untreated cells were used as control to determine the fluorescence background. As shown in Fig. 2C, the internalization of the FDC measured in FR-positive blasts was beyond the threshold of positivity. In contrast, FDC was not internalized in lymphocytes indicating its specific internalization in cells expressing the FR. On the other hand, doxorubicin which penetrates passively through cell membranes was detected in blasts and lymphocytes. In patients with the lowest FOLR2 expression level (M0, M1 and M2 AML types), a significant difference between lymphocytes and blasts internalization was observed ($p = 0.0034$) with medians of 1.1% and 2.2% respectively (Fig. 2D). In patients with the highest level of FOLR2 expression (M3, M4, M5 and M7 AML types), there was also a strong increase of FDC internalization in blasts (median = 60.9%) as compared to lymphocytes (median = 1.4%) ($p = 0.0024$) (Fig. 2E). Interestingly, among the blasts of AML M0, M1 and M2 patient group, the only ones that internalize FDC have a high FOLR2 gene expression. Unlike in the group of patients M3, M4, M5 and M7, blasts that internalize the least FDC have low FOLR2 gene expression. Regardless of the AML type, these observations suggest that there could be a link between FOLR2 expression level and internalization rate of FDC. As previously observed in KG-1 cells, internalization of either FDC or free doxorubicin induced cell toxicity in AML blasts (Fig. 2F).

3.4. FDC efficiency against blasts is strongly linked to FOLR2 expression level

A strong correlation (Spearman $p = 0.0025$) between FOLR2 expression and internalization rate of FDC is found (Fig. 2G). The study of 50% internalization cut-off lead us to define two populations of AML patients closed to a threshold of FOLR2 expression at 0.01% of GAPDH ($p = 0.0022$) (Fig. 2H). These results indicate that the level of FOLR2 expression in blasts could be a good predictive factor for the management of AML patients to define a high-sensitive FDC group. Since current AML treatments are often influence by cytogenetic and molecular abnormalities, we then studied the efficiency of FDC according to several factors implicated in AML.

3.5. FDC efficiency is not affected by cytogenetic and molecular abnormalities

For each patient, clinical data for molecular and cytogenetic abnormalities have been identified and listed in Table 1. Interestingly, no relationship could be found between FDC internalization in blasts and the biological profile, gender or age of patients. Moreover, whatever the prognostic score (based on cytogenetic, molecular markers and molecular abnormalities), internalization ratio between blasts and lymphocytes is ever significant ($p = 0.0078$, 0.0002 and 0.0078 for favorable, intermediate and for unfavorable-prognostic patients respectively). These results indicate that none of studied abnormalities influence the efficiency of FDC and the FOLR2 expression level in blasts remains the main prognosis criterion.

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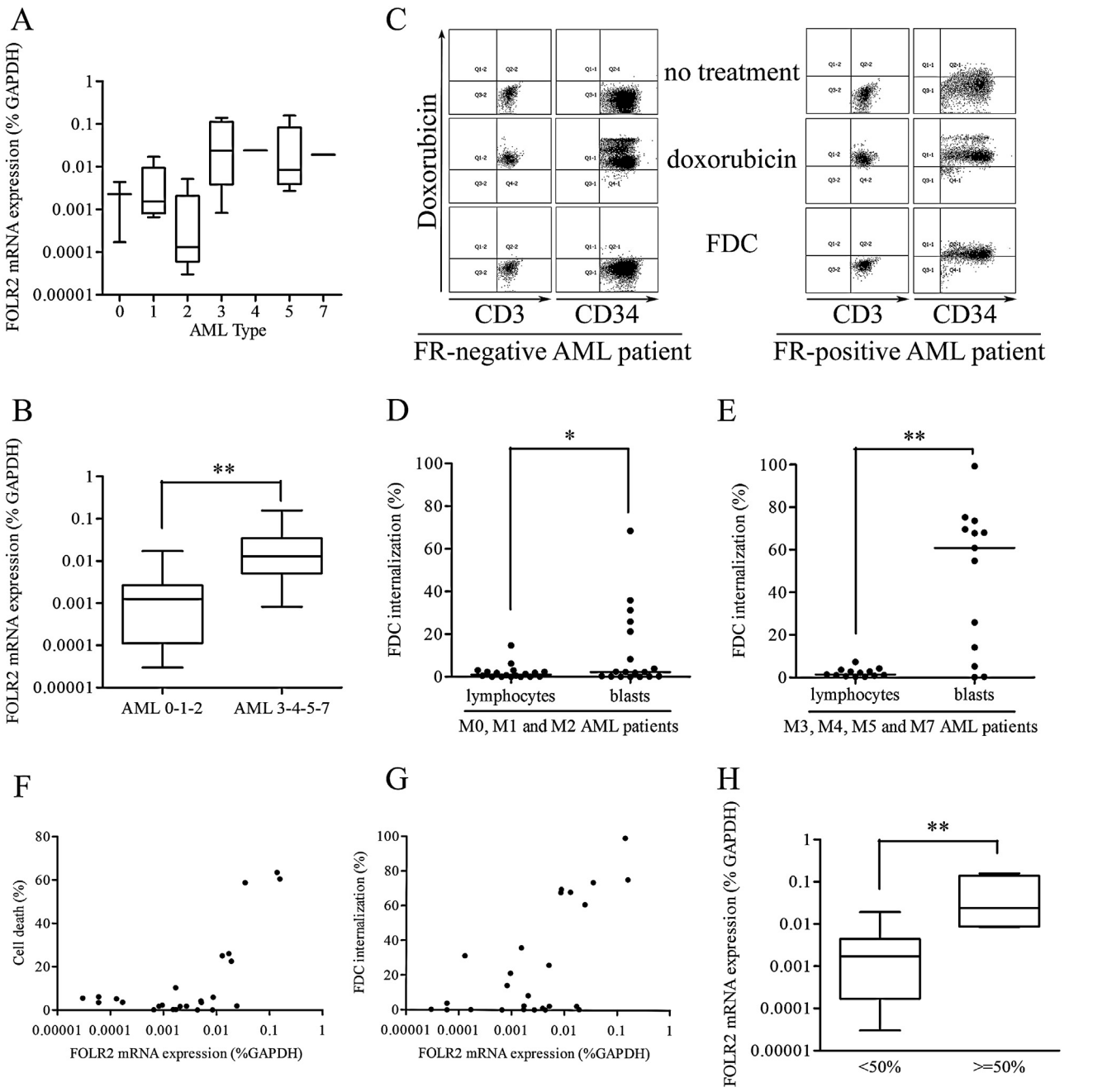

Fig. 2. Expression of beta receptor of folic acid (FOLR2) according to the AML type of patients (A). These results have defined two patient populations as shown in (B): those with low expression (AML 0-1-2) and those with a higher expression (AML 3-4-5-7). Examples of doxorubicin internalization for 2 patients expressing (right) or not FOLR2 (left) in lymphocytes (CD3+) or blasts (CD34+) cells without treatment, treated with doxorubicin or with FDC (C). Plot graph represents FDC internalizations in lymphocytes and blasts in 0-1-2 AML-type (D) or in 3-4-5-7 AML-type patients (E). Scatter-plot represents the toxicity induced by FDC treatment (10 μM) in AML patient cells determined with 7-aminoactinomycinD, a cell viability marker (F). FDC efficiency is highly correlated with FOLR2 expression in blasts, Spearman correlation test $p = 0.0085$ (G). Box-Plot represents respective distribution of the FOLR2 expression according to the 50% FDC internalization threshold in the patient blasts (H).

3.6. FDC has no effects on normal cells

The cytotoxic effect of doxorubicin has been linked by Liu and Colleagues to the ERK1/2 signaling pathway to induce apoptosis in rat cardiomyocytes [25]. Therefore, we investigated the ability of doxorubicin and FDC to penetrate HUVECs and activate this pathway. Predictably, internalization of doxorubicin in HUVECs leads to a 4 fold increase of ERK1/2 phosphorylation (Fig. 3A–C). In contrast, FDC was never detected inside HUVECs and therefore did not activate signaling pathways even after 24 h of treatment (Fig. 3A–C). Moreover, quantification of the tubular networks showed that treatments of HUVECs with FDC did not alter *in vitro* angiogenesis as compared with untreated HUVECs whereas a 25% decreased morphogenesis is observed with doxorubicin (Fig. 3D and Supplementary Figure 2). FDC activity was also investigated on peripheral blood cells from nine healthy donors (4 men and 5 women). Doxorubicin penetrates almost 100% cells whether these are lymphocytes or monocytes. Conversely, FDC never penetrates lymphocytes and only weakly monocytes (Fig. 3E). As lung potentially express folate receptors, FDC toxicity was also investigated on a normal human bronchial epithelial cell line (16HBE). No toxicity

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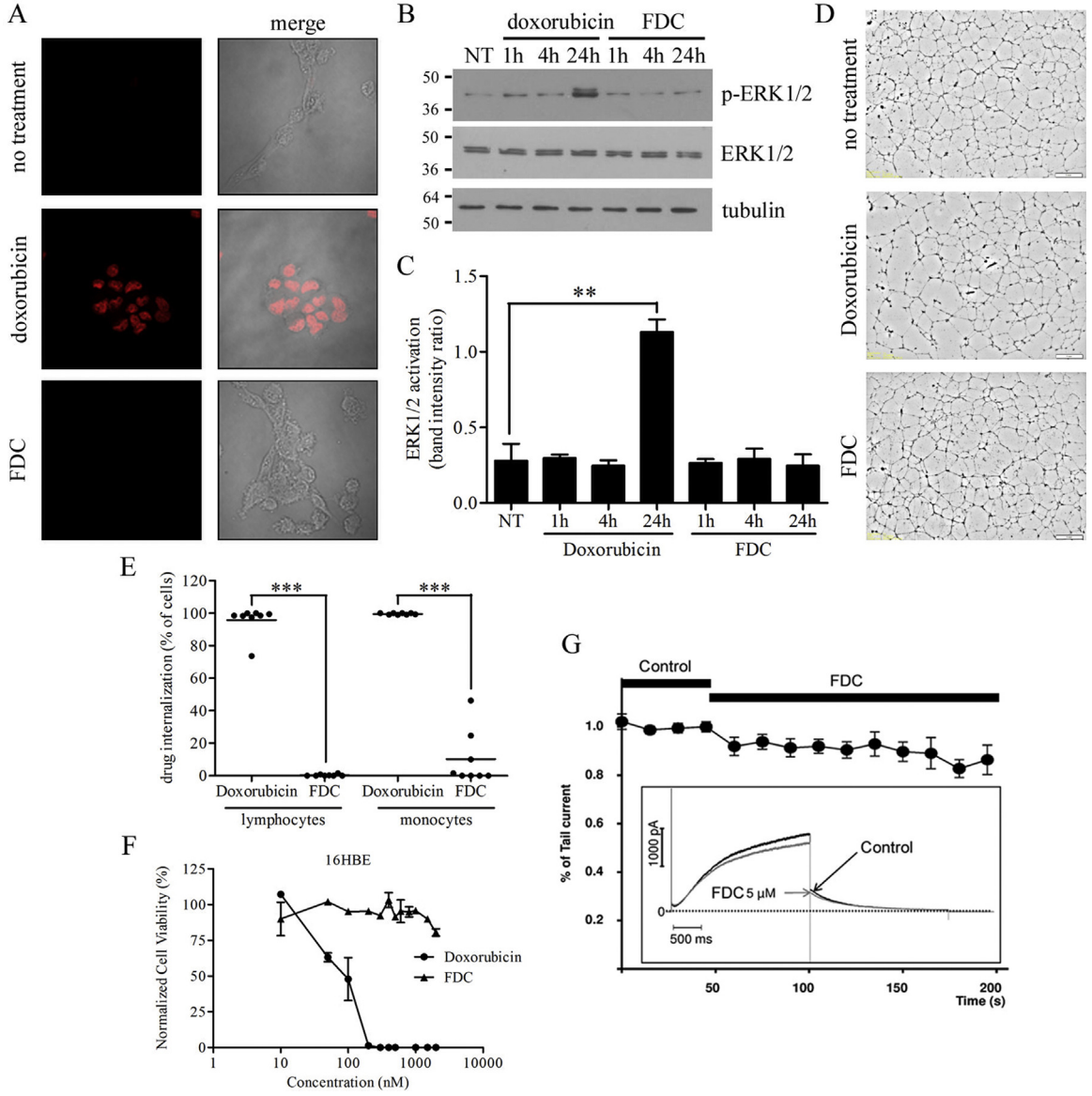

Fig. 3. (A) Internalization of doxorubicin and FDC was evaluated by confocal microscopy in HUVEC endothelial cells thanks to the autofluorescence of doxorubicin. Only doxorubicin is able to penetrate these cells. Representative of 2 independent experiments. (B) ERK1/2 phosphorylation in HUVECs treated 1, 4 or 24 h with 1 μM doxorubicin or vector. Activation of ERK1/2 was detected after long-time exposure to doxorubicin in contrast to vector. Tubulin was used as loading control. Representative of 3 independent experiments. (C) Western blot quantification. Ratios were done between phosphorylation and total bands intensity of ERK1/2. Student *t*-test was performed. (D) Influence of the vector on the morphogenesis of HUVECs grown on Matrigel©. FDC at 1 μM has no effect on the morphogenesis of HUVECs even after 24 h. Scale Bar: 1 mm. Representative of 2 independent experiments. (E) Internalization of doxorubicin and FDC was assessed on 9 blood samples from healthy donors. Vector is drastically less internalized than doxorubicin in lymphocytes (CD3) and in monocytes (CD14). Histograms represent mean ± SEM of drug internalization compared to untreated cells. The student *t*-test was performed. (F) Cell viability of human bronchial epithelial (16HBE) after 4 days of doxorubicin or FDC treatment (G) FDC effects on Iks potassium current in HEK293. Whole-cell patch clamp was performed on 5 independent cells.

was found even at the highest tested dose (Fig. 3F) [26]. We also investigated the potential cardiotoxicity of FDC. Thus, we examined whether human cardiac slow potassium current (I_{KS}) implicated in action potential repolarization could be modulated by FDC in comparison with doxorubicin. The effects of drug on the potassium current tracings (Iks) obtained from cell line stably expressing KCNQ1/KCNE1 channels is illustrated in Fig. 3G. The superfusion of drug (at 5 μM) reduced I_{KS} amplitude and corresponding tail currents recorded at −50 mV to 86.4 ± 6.0% of control (not significant). Since 5 μM was determined in our laboratory as the IC_{50} of doxorubicin [23], FDC presented a less important inhibitory effect on potassium current. Our results suggest FDC could be less toxic than doxorubicin on both normal endothelial, lung, cardiac and healthy blood cells.

4. Discussion

Nowadays, anthracyclines are still intensively used for the treatment of AML patients [27]. However, systemic administration of anthracyclines induces severe side effects, which represent a major limitation for their clinical use. Therefore, the development of new therapeutic approaches aimed to reduce their unselective toxicity is of great interest.

Recently, several folate-drug conjugates have been developed for the selective deposition of potent anticancer agents at the tumor site [28–30]. The FR is a cell-surface marker [31] over-expressed in a wide range of solid tumors [32]. In contrast, FR is present in low or undetectable quantities in most normal tissues. This tumor specificity allows folate-drug conjugate to discriminate cancer cells

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from healthy ones. In this approach, the folate ligand enables the recognition of FR-positive cells and the subsequent internalization of the whole conjugate through receptor-mediated endocytosis [33–35]. The intracellular activation of the targeting system then triggers the release of the active drug thereby launching its antitumor activity. The best illustration of such concept is probably the folate-desacetylvinblastine monohydrazine conjugate Vintafolide (EC145) developed by Leamon *et al.* that is currently progressing through clinical trials [36].

Previous studies demonstrated that the FR β-isoform is highly expressed in AML blasts [17,18,24]. Therefore, this membrane receptor appears as an attractive target to develop appropriate folate-based drug delivery systems for AML therapy. In this context, we designed the first galactosidase-responsive doxorubicin-folate conjugate programmed for the delivery of doxorubicin in AML leukemic blasts expressing the FR β-isoform. The originality of this approach relies on the β-galactosidase-catalyzed drug release mechanism, which may offer advantages compared to the vast majority of the folate-drug conjugates designed so far that contains hydrazone or disulfide-based linkers which are known to be labile in the bloodstream resulting in unselective release of some drug [37–39]. On the other hand, since β-galactosidase is undetectable in serum and mainly located in the intracellular lysosomal compartment, the release of doxorubicin from FDC should occur in a stringently controlled fashion exclusively inside the targeted tumor cells. Through our experiments, we demonstrated that FDC is selectively internalized into FR-expressing leukemic tumor cells including KG-1 and HL-60 cells as well as blasts of AML patients. In contrast, FDC does not affect untargeted normal cells such as endothelial, lung, cardiac or hematologic cells from healthy donors. Moreover, we highlighted a direct correlation between FR expression in blasts and the toxicity of FDC. FDC exerts its activity in FR-β positive leukemic cells (KG-1 and HL-60) in the same manner as in FR-α expressing cells: A2780, HeLa and KB (Supplementary Figure 3) [15,40]. Although we have shown the importance of the folate receptors for FDC activity by saturation tests with free folic acid [15], further experiments using sensitized cells (by transfection of FOLR1/2 genes) or desensitized cells (siRNA-mediated knockdown) should be conducted.

In conclusion, we developed the first β-galactosidase-responsive doxorubicin-folate conjugate devoted to the selective recognition and killing of FR-positive AML leukemic blasts. Such system exhibits no toxicity towards normal cells included in this study. The level of FR expression in blast seems to be a good predictive factor in the management of AML patients to define the chance of success in a potential FDC-based therapy of AML. As blood and bone marrow samples are readily accessible, the analysis of the FR expression level can be easily carried out to identify AML patients who are most likely to respond to potential treatment with FDC. Thus, our findings could represent an important step in the search for a selective chemotherapy of AML patients. Further *in vivo* experiments are however needed to confirm the potential of this approach, particularly in a comparative study with Vintafolid, a folate-drug conjugate that reached the market very recently for the treatment of ovarian cancer.

Conflict of interest statement

The authors reported no potential conflicts of interest.

Acknowledgments

The authors would like to thank Annie-Claire Balandre for her technical assistance in cell culture. The authors thank Image UP facility for confocal microscopy and flow cytometry.

Funding: This work was supported by a grant from La Ligue contre le Cancer, Cancéropole Grand-Ouest and Association Cent Pour Sang La Vie. Our laboratories received support from the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Centre National de la Recherche Scientifique (CNRS).

Author's contributions: JC performed molecular and cellular studies in cell lines and in patient samples. SF, AM and LC designed and performed experiments in endothelial cells. JG performed statistical analysis. EP performed molecular and cellular biology experiments. ER and FG collected patient samples and data. PB and AC performed patch assays. ITO, MT, BR and SP synthesized the tested compound. JC, SP and FG coordinated the research and wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.04.026.

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