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Enhancing tumor response to targeted chemotherapy through up-regulation of folate receptor α expression induced by dexamethasone and valproic acid.

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Short title: Dexa and VPA enhance folate conjugates efficacy

Keywords: Folate receptor; Targeted drug delivery; Dexamethasone; Valproic acid; Monomethyl auristatin E.

Abstract

Several folate-drug conjugates are currently undergoing clinical trials for application in oncology. However, the efficacy of folate-targeted therapy strongly depends on the folate receptor (FR) abundance at the surface of cancer cells. Recently, it has been postulated that up-regulation of FR α by means of chemo-sensitizing agents could enhance the anticancer activity of FR-drug conjugates. In this study, we demonstrate *in vitro* that a combination of dexamethasone (Dexa) and valproic acid (VPA) increases FR α expression selectively at the surface of FR-overexpressing cancer cells. The same stimulation was observed *in vivo* in KB-tumor xenografts when mice are treated with this combined treatment. This effect is reversible since treatment interruption induces the return of FR expression at basal level. When incubated with Dexa and VPA, the β -galactosidase-responsive folate-monomethyl auristatin E (MMAE) conjugate, called MGAF, exhibits higher cytotoxic activity on several FR-positive human cancer cell lines, compared to its administration as a single agent. This improved toxicity results from the enhanced concentration of MMAE released within cancer cells after internalization and subsequent enzymatic activation of MGAF. Higher deposition of MMAE is also observed *in vivo* after up-regulation of FR expression level in tumor xenografts, induced by the prior administration of the Dexa/VPA combination. In this model, MGAF/Dexa/VPA combined therapy results in an 81% inhibition of tumor growth compared to the control group, while MGAF used in monotherapy is inefficient. Since Dexa and VPA are currently used in humans, this finding could be of great interest for further development of folate-drug conjugates, in particular for those that are presently under clinical investigation.

Manuscript

1. Introduction

Over the past years, numerous internalizing ligand- and antibody-drug conjugates have been developed to enhance the selectivity of cancer chemotherapies[1–4]. After recognition of a specific cell surface marker (e.g. antigens and receptors), these drug delivery systems penetrate cancer cells, where they are activated either chemically or enzymatically to release highly toxic compounds. This therapeutic strategy was recently validated in human with the marketing of two antibody-drug conjugates (ADCs), namely, brentuximabvedotin[5] and trastuzumabemtansine[6]. Despite these very promising results, the large size of antibodies restricts the ability of ADCs to penetrate solid tumors, therefore limiting their efficacy for the treatment of these malignancies[7]. Furthermore, ADCs can be immunogenic and exhibit long circulatory half-lives that can lead to undesired side effects. Consequently, several low-molecular weight ligands have been explored as an alternative to antibodies for designing drug delivery systems[3,8]. Within this framework, the targeting of the folate receptor (FR) by the mean of folate-drug conjugates has received considerable attention[3,9–25]. One of the best illustrations of these drug delivery systems is Vintafolide[26,27] that has been investigated in phase II and III studies in non-small cell lung cancer (NSCLC) and ovarian cancer, respectively[28]. However, the efficiency of such a targeting approach strongly depends on the FR expression at the surface of tumor cells. When tumors do not express optimal level of the FR[29], the amount of drug internalized and subsequently released within cancer cells is insufficient to induce tumor shrinkage[30].

Recently, we developed the first generation of β -galactosidase-responsive folate-drug conjugates[31–34]. Following selective internalization inside FR-positive cancer cells, these drug delivery systems are activated by lysosomal β -galactosidase, leading to the release of anticancer agents in a stringently controlled fashion. The efficiency of this targeting strategy was assessed in mice with the galactoside prodrug of monomethyl auristatin E (MMAE), called MGAF, (Fig. S1) that produced a remarkable antitumor effect without any detectable side effects[32]. Moreover, we demonstrated that the β -galactosidase-catalyzed drug release process was sufficiently efficient to produce a bystander effect triggering the destruction of

surrounding FR-negative cancer cells. Folate-drug conjugates inducing bystander effect are of great interest due to the high heterogeneity of cells in tumor tissues[35], including malignant cells that cannot be killed solely by internalized conjugates. In order to increase the concentration of drug released inside tumor cells with moderate FR expression, we also designed dendritic β -galactosidase-responsive folate-drug conjugates leading to improved efficacy compared to the corresponding monomers[34,36].

In 2005, Ratnam's group proposed to enhance the efficiency of FR-targeted therapies by increasing the level of FR alpha isoform (FR α) expression selectively in FR-positive HeLa cancer cells using a combination of dexamethasone (Dexa) and histone deacetylase inhibitors (HDACi)[37]. In this study, authors demonstrate that valproic acid (VPA) potentiate the FOLR1 promoter stimulation induced by Dexa. A few years later, Chen and collaborators reported *in vitro* enhanced uptake and cytotoxicity of folate-conjugated mitoxantrone-loaded micelles via FR α up-regulation in HeLa cancer cell lines pre-treated with Dexa [38]. Herein, we show for the first time that enhancing FR α expression at the surface of cancer cells potentiates *in vivo* therapeutic efficacy of folate-drug conjugates. Administration of a combination of Dexa and VPA to mice bearing FR-positive tumor xenografts increases the level of tumor-associated membrane FR α selectively in tumor tissues. As a result, the subsequent injection of our folate-conjugate MGAF allows higher deposition of the active drug MMAE in the tumor mass, leading to improved antitumor activity.

2. Results and Discussion

2.1 Dexamethasone and valproic acid stimulate FR α expression in cancer cells.

The FR α , encoded by the gene *FOLR1*, is a glycosylphosphatidylinositol (GPI)-anchored membrane protein which binds free folic acid and derivatives with high affinity. FR α expression in normal tissues is notably restricted to the apical surface of polarized epithelial cells of some organs such as the kidney, lung and choroid plexus, where it is not exposed to bloodstream and circulating folates[10,12]. In contrast, FR α is overexpressed in many solid tumors and is often associated with tumor progression[9–18]. As a consequence, FR- α -targeted therapeutic and diagnostic

strategies have been developed for applications in oncology[3,23,25]. Furthermore, up-regulation of FR α has been proposed to enhance the efficacy of FR α -targeted therapies while the validity of this approach was not demonstrated *in vivo* yet. Ratnam *et al.* already reported enhancement of FR α expression in HeLa cells when treated with a combination of Dexa and VPA[37]. In our study, we first investigated the effect of this Dexa/VPA combination on KB cells which exhibit a strong basal *FOLR1*/FR- α expression localized at the membrane and a weak *FOLR2* expression (Fig. S2), as well as on HeLa and A2780 cells which have moderate *FOLR1* and *FOLR2* expressions (Fig. S2). Dexa and VPA toxicities used alone or in combination were first investigated to determine an *in vitro* nontoxic concentration of 0.1 μ M and 200 μ M respectively (Fig. S3). FR stimulation assays were then carried out during 3 days with Dexa and VPA treatments. When incubated separately, Dexa and VPA did not significantly modulate either *FOLR1* or FR α expressions in KB cells (Fig. 1A-B) while the Dexa/VPA combination increased *FOLR1* and FR α expressions by 2.2- and 7-fold, respectively. The combined treatment did not modify the membrane localization of FR α , which remained in its functional compartment (Fig. 1C).

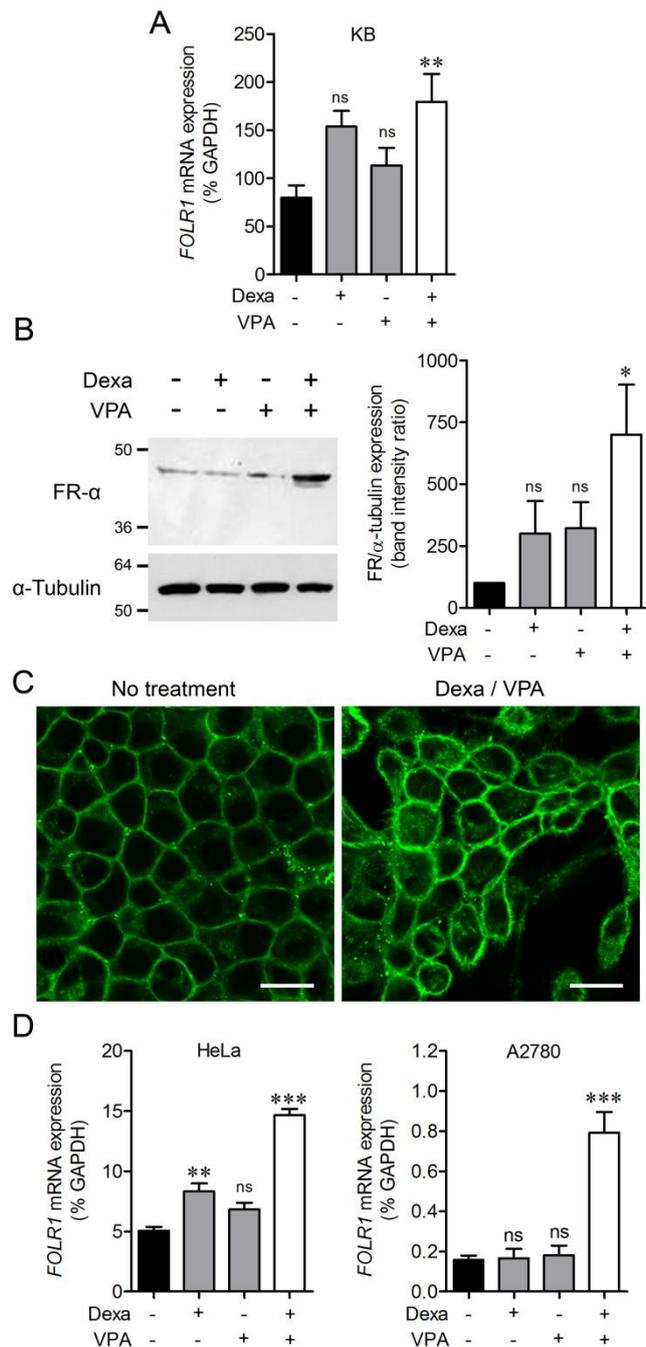


Figure 1: Dexamethasone and valproic acid stimulate *FOLR1* expression in cancer cells. (A) After 3 days of treatment with Dexa (0.1 μ M) alone or in combination with VPA (200 μ M), *FOLR1* expression was assessed relatively to *GAPDH* by quantitative real-time PCR (qPCR) in KB cancer cells. Results are presented as mean values \pm s.e.m from 5 independent experiments in triplicate. (B) Western Blot analysis (left) and ImageJ quantification (right) of FR α expression in KB cells after 3 days of treatment with Dexa (0.1 μ M) alone and/or with VPA (200 μ M). α -tubulin was used as loading control. Representative Western blot of 3 independent experiments is shown. (C) FR α localization in KB cells determined by confocal microscopy. Scale bar: 25 μ m. (D) *FOLR1* expression was assessed relatively to *GAPDH* by quantitative real-time PCR (qPCR) in HeLa (left) and A2780 (right) cancer cell lines after 3 days of treatment with Dexa (0.1 μ M) and VPA (200 μ M). Results are presented as mean values \pm s.e.m from 3 independent experiments in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, ns indicates not significant.

This effect was not the result of a general phenomenon since expressions of other folate receptor (*FOLR2*), and transporters (Reduced Folate Carrier, *RFC* and Proton-Coupled Folate Transporter, *PCFT*) were not modified (Fig. S4A). Comparable results were obtained in HeLa and A2780 cells with 2.9- and 4.9-fold increase of *FOLR1* expression respectively (Fig. 1D). In these two cell lines, Dexa alone, but not VPA, slightly modified *FOLR1* expression while *FOLR2*, *RFC* and *PCFT* were unmodified (Fig. S4B-C). A continuous 3 or 5 days Dexa/VPA treatment enhanced *FOLR1* expression in KB, HeLa and A2780 cell lines whereas, after treatment

interruption, it returned to basal level (Fig. S5). This result indicated that Dexa/VPA combination did not induce sustainable alteration of the genome and that the effect on *FOLR1* expression was reversible. Unfortunately, little is known on the regulation mechanism of the *FOLR1* gene so it is difficult to establish a precise molecular explanation to these results. However, as described by Ratnam's group[37], it is known that Dexa do not act directly on the *FOLR1* promoter because of its delay in action and the fact that cycloheximide treatment inhibits its effect. This is also confirmed by the absence of GRE site in the *FOLR1* promoter. Our hypothesis is that the Dexa increases the expression of a still unknown effector that can bind to the *FOLR1* promoter to activate it. In this context, we hypothesize that VPA, by its HDACi activity, could open chromatin to facilitate the binding of this unknown effector on the *FOLR1* promoter. Thus, treatment arrest should decrease gene expression of this effector and close the chromatin to return at a basal level of *FOLR1* expression. The implication of this unknown effector could explain why the Dexa/VPA treatment stimulates the expression of some genes that have the effector binding site in their promoter (such as *FOLR1*), while others (*FOLR2*, *RFC*, *PCFT*), lacking this binding site, are not sensitive. In contrast to results obtained in the three FR-positive tumor cell lines, Dexa/VPA did not modify *FOLR1* expression in A549 lung cancer cells or in human endothelial cells in which *FOLR1* and *FOLR2* expressions were almost undetectable (Fig 2A and Fig. S2A). Moreover, Dexa/VPA did not affect endothelial cell proliferation (Fig. 2B) and tube formation (Fig. 2C-D). These results were consistent with previous ones showing that Dexa/VPA treatments increased FR expression selectively in cell lines for which the gene is transcriptionally active[37].

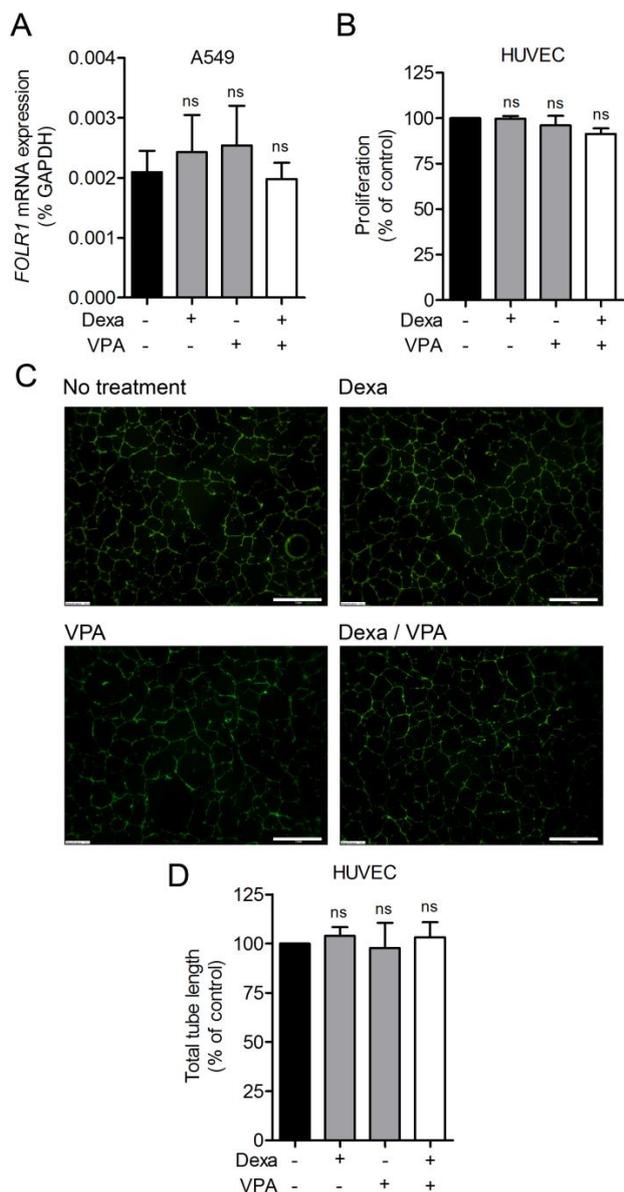


Figure 2: Effect of dexamethasone and valproic acid on FR-negative cells.

(A) After 3 days of treatment with dexamethasone (0.1 μ M) alone or in combination with valproic acid (200 μ M), *FOLR1* expression was assessed relatively to *GAPDH* by quantitative real-time PCR (qPCR) in A549 cancer cells. Results are presented as mean values \pm s.e.m from 5 independent experiments in triplicate. **(B)** HUVECs proliferation measurement after treatment by dexamethasone (0.1 μ M) alone or in combination with valproic acid (200 μ M). Data obtained from 5 independent experiments performed in duplicate and results are expressed as percentages of untreated cells \pm s.e.m. **(C)** *In vitro* morphogenesis assay with HUVECs treated for 16 h with dexamethasone (0.1 μ M) and/or valproic acid (200 μ M). Scale bar: 1 mm. Images are representative of 3 independent experiments. **(D)** Quantification of total tube length of capillary-like structures by automatic counting using the AngioQuant software. Data obtained from 3 independent experiments are expressed as percentages of untreated cells \pm s.e.m. ns indicates not significant.

2.2 Co-treatment of Dexa and VPA potentiates MGAF toxicity selectively on FR-expressing cancer cells.

We next investigated the effect of Dexa/VPA on the cytotoxic activity of the β -galactosidase-responsive folate-MMAE conjugate MGAF. We previously demonstrated that MGAF killed selectively FR-positive tumor cells with an efficacy which depends on the FR expression level[32,33]. Therefore, we hypothesized that Dexa/VPA should improve toxicity of MGAF for FR-overexpressing cancer cells through up-regulation of FR α . First, MGAF (5 nM) was incubated with KB cells treated with or without Dexa/VPA. As shown by LC/MS experiments, the amount of MMAE released from the intracellular β -galactosidase-catalyzed activation of MGAF was significantly higher (1.9-fold) within KB cells co-treated with Dexa/VPA (Fig. 3A).

To determine the biological significance of this increase, MGAF toxicity was then evaluated in KB cells under anchorage-independent conditions. Either Dexa/VPA or MGAF did not modify colony formation in soft agar assay after 3 or 6 days of treatment (Fig 3B-C-D and Fig. S6A).

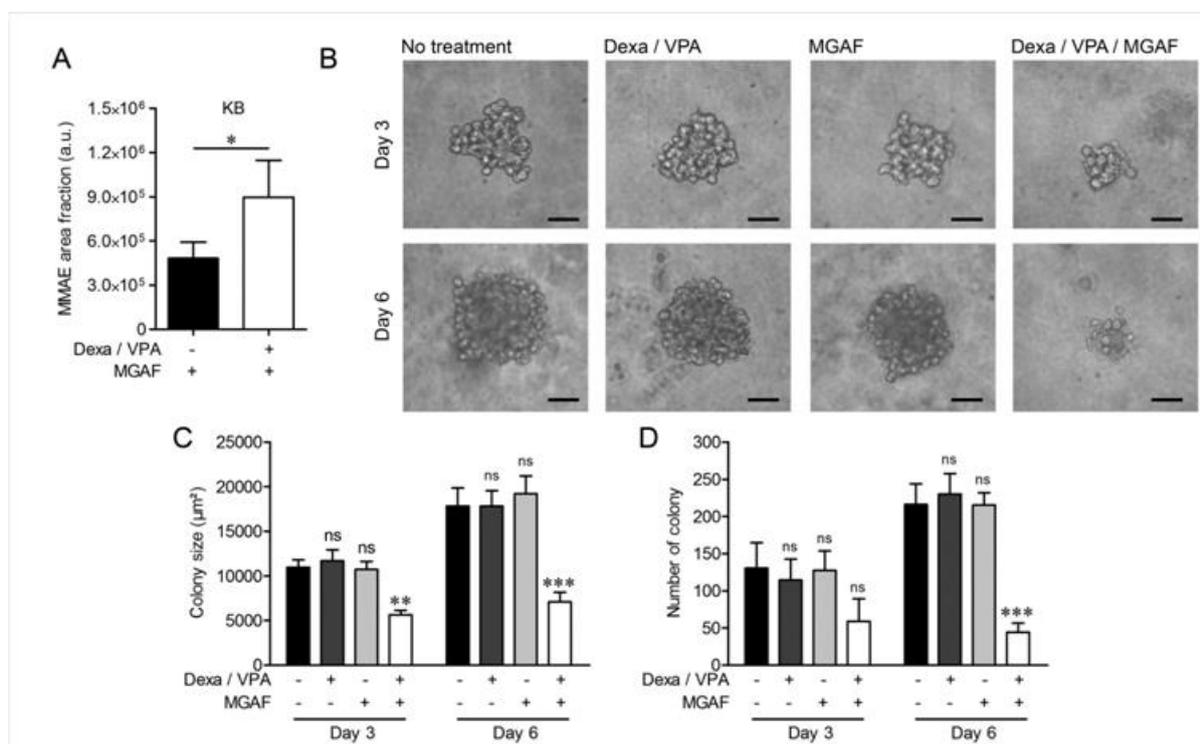


Figure 3: Anchorage-independent growth of KB cells decreased after dexamethasone, valproic acid and MGAF treatments. (A) Release of MMAE in KB tumor cells was measured by mass spectrometry. Each bar shows mean \pm s.e.m. from 6 independent experiments. (B) Representative images of KB colonies in a soft-agar colony formation assay after 3 or 6 days with MGAF (5 nM) and/or dexamethasone (0.1 μ M) and valproic acid (200 μ M). Scale bar: 100 μ m. Images are representative of 5 independent experiments. Quantitative analysis of KB colonies size (C) and number (D) in a soft-agar colony formation assay after 3 or 6 days with MGAF (5 nM) and/or dexamethasone (0.1 μ M) and valproic acid (200 μ M). Results are presented as mean values \pm s.e.m from 5 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001, ns indicates not significant.

On the other hand, Dexa/VPA/MGAF reduced KB colony size and number. Similar results were obtained with HeLa and A2780 FR-positive cells; while no difference was observed in FR-negative A549 cells (Fig. S6B). To get further insight to this effect, we performed a complementary experiment on poly-HEMA coated plate, a surface that prevents adherence of tumor cells[39]. In these suspension conditions, increase of *FOLR1* expression by Dexa/VPA was equivalent to that observed under the standard adherent conditions (Fig. 1A and 4A). MGAF stimulated apoptosis in similar manner under both adherent and suspension conditions (23.8% and 22.5%

respectively, Fig. 4B-C). However, the percentage of apoptotic cells cultured in poly-HEMA-coated plate rose to 59.3% when MGAF was used in combination with Dexa/VPA (Fig. 4B-C).

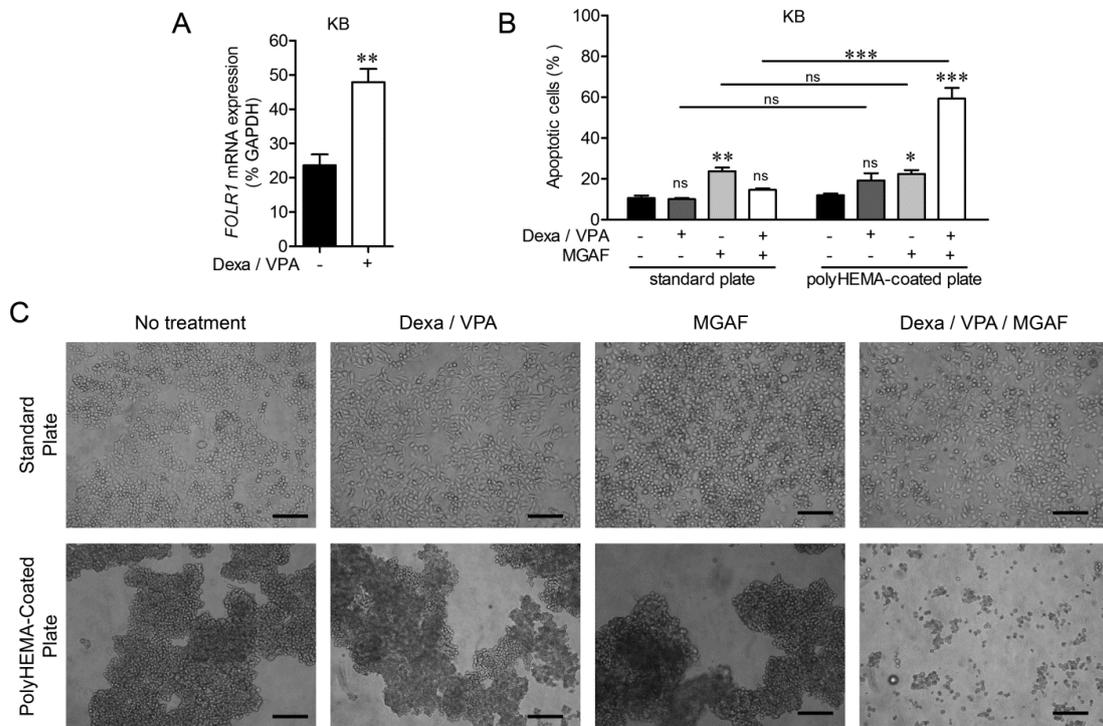


Figure 4: Anchorage independent growth of KB cancer cells is abolished after Dexa/VPA/MGAF treatment. (A) *FOLR1* expression was assessed relatively to *GAPDH* (RTqPCR) in KB cancer cells cultured on polyHEMA-coated plate and after 3 days of treatment with dexamethasone (0.1 μ M) and valproic acid (200 μ M). Results are presented as mean values \pm s.e.m from 4 independent experiments in triplicate. (B) Quantification of apoptosis in KB cells treated 3 days with Dexa (0.1 μ M), VPA (200 μ M) and/or MGAF (5 nM) was performed in adherent (standard plate) or in non-adherent conditions (polyHEMA coated plate). Results are presented as mean values \pm s.e.m from 4 independent experiments. (C) Representative images of KB cells after 3 days of treatment with MGAF (5 nM) and/or dexamethasone (0.1 μ M) and valproic acid (200 μ M) and on standard (top) or polyHEMA-coated (bottom) plates. Scale bar: 200 μ m. Images are representative of 4 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, ns indicates not significant.

It's worth mentioning that this outcome was not observed in standard culture conditions. These results clearly demonstrated that Dexa/VPA increased the MGAF-induced apoptosis under anchorage independent conditions. Comparable effects were obtained with other tubulin destabilizing agents[40,41]. In these reports, the authors demonstrated that staurosporin or paxillin inhibited the anoikis resistance of cancer cells which is a programmed cell death induced by cell detachment from extracellular matrix[42,43]. Resistance to anoikis is a critical step for cancer cells to

allow anchorage-independent growth and induce metastatic colonization. Since MMAE released from MGAF is also a well-known tubulin destabilizing agent[44] and able to inhibit colony formation in dose-dependent manner (Fig. S7), one may hypothesize that Dexa/VPA/MGAF provoked apoptosis through anoikis initiation.

2.3 Dexamethasone and valproic acid stimulate folate receptor expression and improve MGAF in vivo efficacy

Modulation of *FOLR1* expression by Dexa/VPA was next assessed in mice bearing KB-tumor xenografts. Dexa and VPA mix were intraperitoneally administered at 2 and 300 mg/kg/day respectively. Previous studies reported that such doses did not affect animal behavior when these two compounds were employed separately[45–47]. In our experiments, tumor cells from mice treated with Dexa/VPA exhibited a significant higher *FOLR1* expression compared to untreated ones (Fig. 5A). This result was confirmed at the protein level as evidenced by FR α immunodetection assays (Fig. 5B). In contrast to tumor tissues, no significant changes in *FOLR1* expression were detected in ovary, uterus, kidney or heart (Fig. 5C). Additionally, *FOLR2* expression was unmodified in tumors or organs after Dexa/VPA treatments (Fig. S8A-B). In order to evaluate the effect of FR α enhancement on *in vivo* antitumor efficacy of MGAF, the folate-MMAE conjugate was injected at non-optimal doses of 0.5 mg/kg. We previously demonstrated that MGAF strongly reduced KB tumor growth in mice when administered twice a week at 5 mg/kg[32]. As expected, administration of 10-fold lower doses of MGAF did not modify growth of KB tumors (Fig 5D). On the other hand, the Dexa/VPA combination induced a 54% reduction of tumor growth 18 days after tumor transplantation. These results were in agreement with the well-known anticancer properties of Dexa and VPA[45–47]. At the same time, co-treatment with Dexa/VPA and MGAF (0.5 mg/kg) resulted in a higher inhibition of tumor growth (81% compared to the control group at day 18 post tumor transplantation, Fig. 5D). Comparison of tumor weights confirmed the therapeutic benefit brought by the Dexa/VPA/MGAF association, since with this combined therapy tumor weights were 40% lower than with Dexa/VPA (Fig. 5E). To verify that this enhanced efficacy was not only the result of an additive effect, the relative concentrations of MMAE released in the tumor from MGAF and Dexa/VPA/MGAF treatments were quantified. As shown in Fig 5F, the amount of drug delivered at the tumor site was 1.5-fold higher following the administration of the combined Dexa/VPA/MGAF therapy than that of MGAF

alone. Taken together, these results demonstrated that up-regulation of $FR\alpha$ by Dexamethasone (Dexa)/Valproic Acid (VPA) allowed higher deposition of MMAE *in vivo* through enhanced uptake of MGAF in tumor cells, thereby leading to a better anticancer activity.

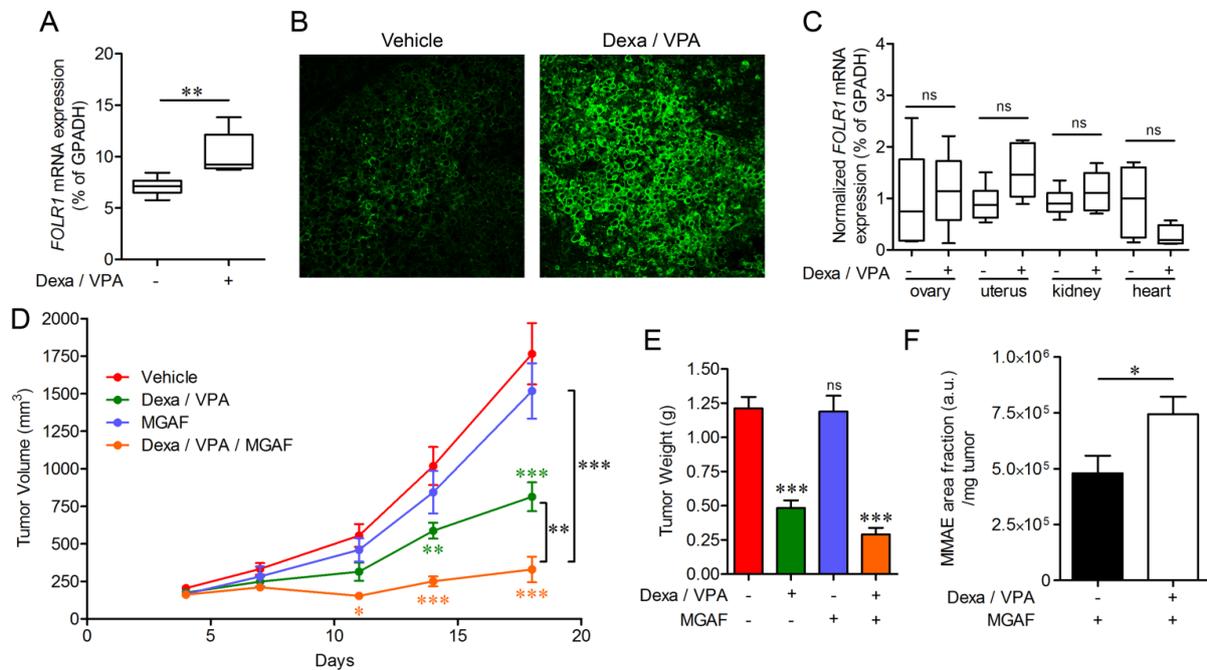


Figure 5: MGAF antitumor activity is improved by *FOLR1* overexpression driven by dexamethasone and valproic acid treatments. *FOLR1* expression (A and C) and $FR\alpha$ localization (B) were assessed by RTqPCR or confocal microscopy in KB tumors (A and B) and organs (C) of untreated or dexamethasone (2 mg/kg) and valproic acid (300 mg/kg) treated mice (6 mice/group). Scale bar: 50 μ m. (D) Tumor growth inhibition of subcutaneous KB xenografts under treatment with Dexa (2 mg/kg) and VPA (300 mg/kg) and/or MGAF (0.5 mg/kg). Each point represents mean \pm s.e.m. from 6 mice. (E) Tumor weights at day 18 of mice bearing KB xenografts treated with vehicle, MGAF (0.5 mg/kg) and/or dexamethasone (2 mg/kg) and valproic acid (300 mg/kg). Each bar represents mean \pm s.e.m. from 6 mice. (F) Release of MMAE in KB tumors was measured by mass spectrometry. Each bar shows mean \pm s.e.m. from 6 mice. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, ns indicates not significant.

The weight of mice treated with Dexa/VPA (alone or in co-treatment with MGAF) did not evolve and stabilized around 17.5 g while for the other groups, body weight increased regularly during the study (Fig S8C). This effect could be explained by the administration of Dexa which, like other glucocorticoids, can cause growth retardation, muscular atrophy or osteoporosis[48–51]. This result was in accordance with observation reported for patients treated with Dexa[52]. However, no change in animal behavior or activity was recorded between the different groups of animals. Furthermore, an extensive histopathological analysis carried out on various organs

such as heart, liver and kidneys revealed no toxicity due to the different treatments (i.e. kidney staining Fig. S9). However, further complementary biochemistry tests linked to auristatins associated toxicity are needed to confirm conclusions of histopathological analysis. Thus, it appeared that the Dexa/VPA/MGAF treatment produced a significant anticancer activity without damages for healthy tissues.

3. Conclusions

In summary, our study demonstrates that Dexa and VPA, used in combination, enhance FR α expression selectively at the surface of FR-overexpressing cancer cells, both *in vitro* and *in vivo*. The FR α up-regulation potentiates the effects of folate-drug conjugates such as MGAF, leading to the release of higher concentrations of the cytotoxic within cancer cells. Therefore, MGAF exhibits superior anticancer efficacy when administered in combination with Dexa and VPA compared to monotherapy, for the treatment of FR-positive tumor xenografts in mice. Since Dexa and VPA are currently used in human, and folate-drug conjugates already under evaluation in clinic, the potential of such a combined therapy could be estimated relatively rapidly for the treatment of patients with FR-expressing tumors. Thus, the results of this study could have a significant impact for the chemotherapy of these malignant pathologies.

Material and Methods

1. Cell Culture

KB (human mouth epidermal carcinoma-ATCC CCL-17), HeLa (human cervix adenocarcinoma-ECACC 93021013), A2780 (human ovarian carcinoma-ECACC 93112519), and A549 (human lung carcinoma-ECACC 86012804) cells were grown in RPMI 1640-GlutaMax without folic acid (Life Technologies, Paisley, UK) supplemented by 10% fetal bovine serum (Lonza, Verviers, Belgium), and 100 u/ml Penicillin/Streptomycin (Life Technologies, Paisley, UK) in a humidified incubator at 37 °C and 5% CO₂. KB cells were purchased from American Type Culture Collection (ATCC) and HeLa, A2780 and A549 cells were from European Collection of Cell Cultures (ECACC). Human umbilical vascular endothelial cells (HUVEC, Lonza,

Verviers, Belgium) were grown in EGM-2 complete medium (Lonza, Verviers, Belgium). All cells were used at early passages (<10) and cultured as recommended by the manufacturers.

2. Quantitative real-time PCR

Total RNA was extracted using SV Total RNA Isolation System (Promega, Charbonnière-les-Bains, France) for cancer cells or NucleoSpin RNA XS for normal cells (Macherey-Nagel, Hoerd, France) as previously described[33]. Reverse transcription was performed with qScript cDNA Synthesis Kit (VWR, Fontenay-sous-Bois, France) from 1.5 to 1.8 µg of total RNA according to the manufacturer's instructions. Expression levels were assessed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by quantitative real-time PCR with the GeneAmp 7500 Fast Sequence Detection System and SYBR Green chemistry (Applied Biosystems, Illkirch, France). Primers were synthesized by Invitrogen: human and mouse GAPDH, forward 5'- TGC ACC ACC AAC TGC TTA GC-3' and reverse 5'- GGC ATG GAC TGT GGT CAT GAG-3'; human FOLR1, forward 5'- AGC ACC ACA AGG AAA AGC CAG G-3' and reverse 5'- GTG CCA TCT CTC CAC AGT GGT T-3'; human FOLR2, forward 5'- CCA CTT CAT CCA GGA CAC CTG T-3' and reverse 5'- CAT CCA GGA AGC GTT CTT TGC G-3'; mouse Folr1, forward 5'-GGA CTG AAC TTC TCA ATG TCT GC-3' and reverse 5'- CTT CCT GGC TTG TGT TCG TGG A-3'; mouse Folr2, forward 5'- CTG ACC TAG GGA GAG GCC AA-3' and reverse 5'- TGT CTG TTT CCA GGC CAT GT-3'; human RFC, forward 5'- CTT TGC CAC CAT CGT CAA GAC C-3' and reverse 5'- GGA CAG GAT CAG GAA GTA CAC G-3'; human PCFT, forward 5'- CCT TTG CCA CTA TCA CGC CTC T-3' and reverse 5'- ACC AGC TTG GAG AGT TTA GCC C-3'. Sensitivity and specificity of each primer couple were previously checked[32,33].

3. Western Blotting

After 24 h of culture, cells were exposed to 0.1 µM Dexamethasone (Sigma-Aldrich, St-Quentin-Falavier, France) and/or 200 µM Valproic Acid (Sigma-Aldrich) for 3 days. Cells were collected by scraping and lysed with a non-reducing buffer (10 mM Tris, 150 mM NaCl, 1% Triton X100, 0.5% NP40, 1 mM EDTA, 1 mM EGTA, 0.2 mM Sodium orthovanadate). 15 µg or 100 µg of proteins (for α-tubulin and FRα detection respectively) were analyzed by SDS-PAGE. Proteins were then transferred onto

nitrocellulose membrane and probed with the anti- α -tubulin (Sigma-Aldrich St-Quentin-Falavier, France, 1:2000) or anti-FR α (Santa-Cruz, Heidelberg, Germany, 1:500) primary antibodies. Detection was performed using HRP-conjugated goat anti-rabbit or anti-mouse antibodies (Sigma-Aldrich, St-Quentin-Falavier, France) and Luminata chemiluminescence kit (Merck, Molsheim, France).

4. Confocal Microscopy

Immunodetection of FR α was performed on KB and A549 cells that were fixed in 3.7% formaldehyde in PBS at room temperature (RT) for 20 min. After incubation in a blocking solution (10% goat serum, 0.3% Triton X-100 in PBS, 45 min at RT), cells were incubated 3 h at RT with a monoclonal anti-FR α antibody (1:50, R&D Systems, Lille, France). Alexa Fluor 488 goat anti-mouse antibody (1:250 in 1% Bovine Serum Albumin, 10% Goat Serum, 0.3% Triton X-100 in PBS, Invitrogen, excitation/emission wavelengths of 488 and 590 nm) was then applied at RT for 1 h. Cover slips were mounted with Mowiol prior to observation with a confocal microscope. All controls, performed by omitting the primary antibody, were negative.

5. Cell Viability Assay

The Cell Proliferation Kit II (XTT, Sigma-Aldrich, St-Quentin-Falavier, France) was used to assess cell viability as previously described[32]. Briefly, 2×10^3 tumor cells/well were plated in a 96-well plate. After 3 days of treatment, 25 μ l of the XTT labeling mixture were added per well. Cells were further incubated for additional 4 h at 37 °C before determination of the absorbance at 490 nm on a 96-well microplate reader. IC₅₀ values were determined using Graphpad software.

6. BrdU incorporation assay

Cells were plated in 96-well plates at a density of 2×10^3 cells/well in RPMI-1640 medium supplemented by 10% FBS. After 24 h, cells were exposed to 0.1 μ M Dexa and 200 μ M VPA for 3 days. BrdU incorporation rate was measured using “Cell Proliferation ELISA, BrdU colorimetric kit” according of the manufacturer’s instructions. Absorbance was determined at 370 nm on a 96-well microplate reader.

7. In vitro morphogenesis assay

This assay was performed as previously described[53]. Wells of a 48-well plate were coated with Matrigel® (Corning, Brumath, France) and kept at room temperature for 4 hours to allow gel formation. HUVECs (5×10^4 cells/well) were then plated onto the Matrigel®. After 16 hours, cells were stained with 25 μ M calcein-AM then fixed in 2% formaldehyde in PBS. The 3-dimensional cell organization was photographed using Olympus MVX10 microscope. Capillary-like structures were quantified by automatic counting using the AngioQuant software.

8. Soft agar colony formation assay

Soft agar colony formation assay is a relevant method to monitor anchorage-independent growth of transformed cells[41,54]. Briefly, after coating a 3.5 mm diameter culture dish with folic acid-depleted RPMI-1640 medium supplemented by 10% fetal bovine serum and containing 0.6% agarose, 5×10^3 isolated KB cells were plated in a 0.35% agarose comparable medium. After 4 days, cells were treated with indicated compounds for 3 or 6 days. Number and size of colonies are assessed using a Olympus MVX10 microscope and ImageJ software.

9. Poly-HEMA Culture

Cells were cultured in standard adherent or in low-adherent conditions using polyHEMA [poly(2-hydroxyethyl methacrylate), Sigma-Aldrich]. Briefly, 10 mg/cm² of poly-HEMA, dissolved in 95% ethanol, were added to a 6-well culture plate and allowed to dry out overnight. KB cells (1.5×10^5 cells/well) were then seeded in folic acid-depleted RPMI-1640 medium supplemented by 10% FBS and 1% penicillin/streptomycin. After 24 h, cells were treated with the indicated compound for 3 additional days, collected and stained for apoptosis assay, or lysed for quantitative real-time PCR as described above.

10. Apoptosis assay

Proportion of apoptotic cells was assessed with Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell, Heidelberg, Germany) according to the manufacturer's instructions. Cells, cultured in adherent or low-adherent conditions, were collected using Versene (Life Technologies, Paisley, UK). After a wash in PBS, 2×10^5 cells were stained during 15 min in the dark with 5 μ L of FITC-Annexin V, 5 μ L of Ethidium

Homodimer III and 5 μ L of Hoechst 33342 and analyzed by flow cytometry using a BD FACS Verse analyzer.

11. In vivo experiments

Female BALB/c nu/nu mice (Janvier Labs, St-Berthevin, France), purchased from Janvier Labs, were the only animals used in this study. All experimental procedures (n°02028.01) involving animals were validated by the regional ethical committee and carried out in accordance with the guidelines of the French Agriculture and Forestry Ministry (decree 2013-118) and of the European Communities Council Directive (2010/63/UE). Seven-week-old mice were anesthetized with 2% vaporized isoflurane and 1.10^6 KB cells were transplanted subcutaneously in PBS containing 50% of growth factor reduced Matrigel® into the dorsal flank (day 0). Animals were then randomly distributed in 4 experimental groups of 6 mice. From day 4 to 21, they were daily treated by intraperitoneal injection of 2 mg/kg dexamethasone and 300 mg/kg valproic acid in PBS – 5% ethanol (Dexa/VPA and Dexa/VPA/MGAF groups) or PBS – 5% ethanol (vehicle and MGAF groups). In addition, mice were treated intravenously on days 6, 8, 11, 13, 15 and 18 with either DMSO 5% in PBS buffer (vehicle and Dexa/VPA groups) or MGAF (0.5 mg/kg) in DMSO 5% - PBS (MGAF and Dexa/VPA/MGAF groups). Tumor sizes were measured three times a week in two dimensions using caliper. Individual tumor volumes (V) were calculated by the formula $V = [\text{length} \times (\text{width})^2] / 2$. Mice weights were evaluated every day. Mice were euthanized when tumor exceeded a volume of 2 cm³. After euthanasia, tumors were removed and weighted. Pieces of tumor and organs were directly immersed in liquid nitrogen for RNA and LC/MS analyses or preserved in 4%-formol for histological examination. RNA extraction and quantification were performed as previously described. 4 μ m thick tissue sections were mounted on glass slides and stained with hemalun-eosin (Novaxia) before blind examination by independent pathologist (Le Net Pathology Consulting). The quality of the histological sections, tissue accountability, slides labeling and tissue placement were considered adequate for the purposes of the study. Tissue sections were monitored by light microscopy on a Leica Diaplan microscope. All histopathological findings on the organs were graded in severity using a five point system of minimal, slight, moderate, marked or severe.

12. LC/MS analyses

The relative quantity of MMAE was determined in 1×10^6 KB cells treated 3 days with dexamethasone (0.1 μM), valproic acid (200 μM) and/or 24 h with MGAF (100 nM) and grown in RPMI-1640 depleted in folic acid and supplemented by 10% fetal bovine serum and 1% Penicillin/Streptomycin. Culture medium was removed and cells were collected with Versene, pelleted by centrifugation, lysed using a micropestle in 0.5 ml of 0.3 M sodium acetate and centrifuged for 5 min at 1500 g. The supernatant was transferred in 1 ml of cold ethanol and incubated for 1 h at -20 °C. After centrifugation at 17000 g and 4 °C for 20 min, 0.5 ml of acetonitrile-methanol (2:1) (v:v) was added to the supernatant and incubated for 1 h at -20 °C. The sample was then centrifuged for 20 min at 17000 g and 4 °C. The supernatant was transferred to 2 ml microcentrifuge tubes and centrifuged again at 17000 g and 4 °C for 10 min. The same extraction was performed from 40-80 mg of KB tumors. Supernatants were analyzed by HPLC/HRMS. One milliliter of sample was injected and desalted through on-line trap columns at a flow rate of $0.5 \text{ mL} \cdot \text{min}^{-1}$ for 2 min with water-0.1% formic acid as the loading eluent. Trapped analytes were then back flushed to the analytical column. Target selected ion monitoring data dependent-MS/MS (t-SIM-ddMS/MS) (ESI+) was used to relatively quantify MMAE in samples. Targeted MS parameters were optimized at resolution 70000 for precursor ion and 17500 for product ions, AGC target 10^5 (precursor ion) and $2 \cdot 10^5$ (product ions), max IT 100 ms (precursor ion) and 50 ms (product ions), MSX count 1, and isolation window 2.0 m/z. The normalized collision energy was set at 35%. The precursor ion selected for MMAE identification was $[\text{M} + \text{H}]^+ \text{ m/z } 718.509$. The product ions m/z 86.097, 134.096 and 154.123 were selected for verification. Peaks integration and MS spectra acquisition was performed with ThermoXcalibur Qualitative Browser. A mass tolerance of 10 ppm was applied for the extraction of target product ions. The relative area of MMAE peak was determined and MMAE ratio was calculated.

13. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. ANOVA followed by the Bonferroni post-test was used to analyze 3 or more experimental groups and the Student t-test was used for 2 groups with three significant thresholds : $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Abbreviations

FR, folate receptor; Dexa, dexamethasone; VPA, valproic acid; ns, not significant; MMAE, monomethyl auristatin E; MGAF, β -galactosidase-responsive folate-monomethyl auristatin E conjugate; RT, room temperature;

Conflict of interest statement

The authors reported no potential conflict of interest.

Author Contributions

E.P. and J.C. conceived the project and designed the experiments. E.P. CM and JC performed molecular and cellular *in vitro* experiments. E.P., L.C. and AM. performed *in vivo* experiments. B.R and I.T-O synthesized and characterized chemical compounds. FG gave clinical advices. P.P performed mass spectrometry analyses. S.P. and J.C. supervised the whole project and wrote the manuscript.

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