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RESEARCH PAPER

Inhibitory effects of marine-derived DNA-binding anti-tumour tetrahydroisoquinolines on the Fanconi anaemia pathway

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BACKGROUND AND PURPOSE

We have previously shown that cells with a defective Fanconi anaemia (FA) pathway are hypersensitive to trabectedin, a DNA-binding anti-cancer tetrahydroisoquinoline (DBAT) whose adducts functionally mimic a DNA inter-strand cross link (ICL). Here we expand these observations to new DBATs and investigate whether our findings in primary untransformed cells can be reproduced in human cancer cells.

EXPERIMENTAL APPROACH

Initially, the sensitivity of transformed and untransformed cells, deficient or not in one component of the FA pathway, to mitomycin C (MMC) and three DBATs, trabectedin, Zalypsis and PM01183, was assessed. Then, the functional interaction of these drugs with the FA pathway was comparatively investigated.

KEY RESULTS

While untransformed FA-deficient haematopoietic cells were hypersensitive to both MMC and DBATs, the response of FA-deficient squamous cell carcinoma (SCC) cells to DBATs was similar to that of their respective FA-competent counterparts, even though these FA-deficient SCC cells were hypersensitive to MMC. Furthermore, while MMC always activated the FA pathway, the DBATs inhibited the FA pathway in the cancer cell lines tested and this enhanced their response to MMC.

CONCLUSIONS AND IMPLICATIONS

Our data show that although DBATs functionally interact with DNA as do agents that generate classical ICL, these drugs should be considered as FA pathway inhibitors rather than activators. Moreover, this effect was most significant in a variety of cancer cells. These inhibitory effects of DBATs on the FA pathway could be exploited clinically with the aim of 'fanconizing' cancer cells in order to make them more sensitive to other anti-tumour drugs.

Abbreviations

DBAT, DNA-binding anti-cancer tetrahydroisoquinoline; FA, Fanconi anaemia; ICL, inter-strand cross link; MM, multiple myeloma; MMC, mitomycin C; SCC, squamous cell carcinoma

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Introduction

Fanconi anaemia (FA) is an autosomal recessive disease (in all subtypes except FA-B, which is X-linked) caused by loss-of-function mutations in any of the 16 *FANC* genes so far identified (see review in Kee and D'Andrea, 2012 and Bogliolo *et al.*, 2013). FA cells are characterized by increased chromosomal instability and are hypersensitive to agents that induce DNA inter-strand cross links (ICLs), such as mitomycin C (MMC; Kee and D'Andrea, 2012). Because of the involvement of the FA pathway in DNA repair, FA patients have a high predisposition to develop acute myeloid leukaemia (AML), and also solid tumours, most frequently squamous cell carcinoma (SCC) (Kutler *et al.*, 2003).

Interestingly, the relevance of disruptions of the FA pathway to malignancies has also been shown in individuals without a family history of cancer (Lyakhovich and Surralles, 2006). Moreover, it has been found that up to 10% of sporadic malignancies contain either genetic or epigenetic modifications in *FANC* genes that lead to a disruption in the FA pathway (Valeri *et al.*, 2011) and thus hypersensitivity to ICL drugs (Taniguchi *et al.*, 2003; van der Heijden *et al.*, 2004; Marsit *et al.*, 2004; Hess *et al.*, 2008; Palagyi *et al.*, 2010; Zhang *et al.*, 2010).

Within the FA pathway, three different protein complexes have been identified. Mutations in any of the eight FANC proteins forming the FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) result in loss of monoubiquitination of FANCD2 and FANCI (the 'FA-ID complex'), which is the central regulatory process in the FA pathway. The activated FA-ID complex is then loaded onto chromatin and binds FAN1, which co-localizes with downstream proteins, including FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, RAD51C (putative FANCO), FANCQ/ERCC4, RAD51 and BRCA1 to form large nuclear foci on damaged DNA (Kee and D'Andrea, 2012).

A few years ago, our group reported that different *FANC*-deficient human cell lines were hypersensitive to trabectedin (Casado *et al.*, 2008), a DBAT of marine origin that binds to the minor groove of selected DNA triplets by forming a covalent bond with a guanine in one strand and establishing one or two hydrogen bonds with bases in the opposite strand (Gago and Hurley, 2002; D'Incalci and Galmarini, 2010). The resulting adducts largely stabilize double-stranded DNA (dsDNA) in melting experiments and give rise to the formation of double-strand breaks (DSBs) *in vivo* during the DNA repair process (Soares *et al.*, 2007; Casado *et al.*, 2008). These experimental findings support the notion that the mono-adducts formed by DBAT can functionally mimic a DNA ICL, which, if left unrepaired, can prevent or hamper both transcription and replication (Casado *et al.*, 2008; Bueren-Calabuig *et al.*, 2011; Feuerhahn *et al.*, 2011). A similar thermal stabilization of dsDNA can be induced by the binding of Zalypsis (Leal *et al.*, 2009) and PM01183 (Leal *et al.*, 2010; Bueren-Calabuig *et al.*, 2011). Following on from these observations, in the present study we aimed to investigate the effects a defective FA pathway has on the responses of different cell types to trabectedin, *Zalypsis* and PM01183, as representative ICL-mimicking monofunctional DBATs, in comparison with bifunctional MMC (Bueren-Calabuig *et al.*, 2012).

Methods

Drugs

The three DBATs investigated in this study, trabectedin (ET-743; Yondelis®), PM00104 (Zalypsis) and PM01183 (lurbinectedin), were provided by Pharmamar (Colmenar Viejo, Spain) as dry powders that were reconstituted in DMSO (Sigma-Aldrich, Saint Louis, MO, USA), diluted to concentrated stock solutions of 1 mM and kept in aliquots at −80°C. MMC (Life Technologies, Grand Island, NY, USA) was kept at −80°C as a 1 mM stock solution.

Mice

As models of FA-A and FA-D1 mice, Fanca$^{-/-}$ (Cheng *et al.*, 2000) and Brca2$^{\Delta27/\Delta27}$ (McAllister *et al.*, 2002) mice were used. As their respective controls, syngeneic WT FVB.129 and Balb/C mice were used (Jackson Laboratory, Bar Harbor, ME, USA) respectively. Twelve- to fourteen-week-old mice were used as donors of haematopoietic progenitor cells. Breeding pairs were raised at the CIEMAT Laboratory Animal Facility (Madrid, Spain; Registration No. 28079-21A) and allowed food (Harlan Teklad Global Diets 2914) and water (50 µm filtered and UV irradiated) *ad libitum*. Mice experiments were conducted in accordance with European and Spanish laws and regulations. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Mouse haematopoietic progenitor cells

Bone marrow (BM) cells were aseptically removed from the central canal of both femora with Iscove's modified Dulbecco's medium (Gibco Life Technologies, Grand Island, NY, USA). An appropriate number of viable BM cells were seeded in dishes containing MethoCult GF M3534 culture media (StemCell Technologies, Vancouver, BC, Canada) in the presence of different concentrations of the drugs tested and plated in triplicate on 35 mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) as previously shown (Pessina *et al.*, 2001). Granulocyte/macrophage colonies, representing the growth of granulocyte/macrophage colony forming units (CFU-GM), were scored under an inverted microscope after 7 days of incubation at 37°C in 5% CO_2 and fully humidified. The percentage of CFU-GM surviving corresponding to each drug exposure was calculated with respect to the control condition (drug solvent); the control number of colonies was taken as 100% of survival. The solvent for all drugs was 0.1% DMSO except for MMC, which was dissolved in saline solution. The number of colonies in control cultures with saline or 0.1% DMSO did not statistically differ.

Human cell lines

Head and neck SCC *FANCA*-deficient EUFA1365 and *FANCC*-deficient EUFA1131 cell lines (van Zeeburg *et al.*, 2005) were kindly provided by Hans Joenje. These cells were cultured as previously described (van Zeeburg *et al.*, 2005). For gene complementation, EUFA1365 and EUFA1131 cells were transduced with *FANCA*/Neo^r and *FANCC*/Neo^r retroviral vectors, respectively (Hanenberg *et al.*, 2002), generously provided by H. Hanenberg, and selected with geneticine. Clonogenic

assays were conducted conventionally in p6 multiwells (Becton Dickinson, Franklin Lakes, NY, USA) previously treated with 0.1% gelatine by incubating the cells for 24 h with drugs. Colonies were scored after 8–12 days at 37°C in 5% CO_2 and fully humidified air under an inverted microscope. Percentage of cell survival corresponding to each drug exposure was calculated with respect to the control condition (exposition to solvent only). The solvent was 0.1% DMSO, except for MMC, which consisted of saline. The number of colonies in the control condition was considered as 100% of survival.

The AML cell line Kasumi-1 (DSMZ, Braunschweig, Germany) and the ovarian cancer cell line IGROV1 (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 with GlutaMAX™ supplemented with 10% FBS and PE. Healthy and FA-A and FA-J lymphoblastic cell lines (LCLs) were subtyped and cultured as previously described (Casado *et al*., 2007). LCLs were exposed to drugs for 5 days and cell viability was tested with propidium iodide by flow cytometry (Casado *et al*., 2007). The percentage of cell survival was calculated; the PI values obtained after incubation with the solvent (0.1% DMSO in all instances except for MMC, which was dissolved in saline) were considered to be 100%.

Analyses of FANCD2 by real-time quantitative PCR, Western blot and immunofluorescence

For real-time (RT) quantitative PCR (qPCR) analyses, total RNA was obtained from cell extracts (Roche, Indianapolis, IN, USA) and 1 μg of RNA was reverse transcribed to cDNA using RETROscript® First-Strand Synthesis kit (Ambion, Life Technologies, San Antonio, TX, USA). qPCR was performed using primers specific for the human *FANCD2* gene (Fw: 5′ CCC AGAACTGATCAACTCTCCT 3′; Rv: 5′ CCATCATCACACG GAAGAAA 3′) and human housekeeping genes *GUS-B* (glucuronidase β) (Fw: 5′ CCTGTGACCTTTGTGAGCAA 3′; Rv: 5′ AACAGATCACATCCACATACGG 3′), *GADPH* (glyceraldehyde 3-phosphate dehydrogenase) (Fw: 5′ GCTCTCTGCTC CTCCTGTTC 3′; Rv: 5′ ACGACCAAATCCGTTGACTC 3′) and β-actin (Fw: 5′ ATTGGCAATGAGCGGTTCC 3′; Rv: 5′ CACA GGACTCCATGCCCA 3′). All of them had similar efficiencies. Quantitative analysis of *FANCD2* mRNA expression was determined using Fast SYBR® Green PCR Master mix (Applied Biosystems, Life Technologies) with the 7500 fast real-time PCR system (Applied Biosystems, Life Technologies). All quantitative data were accrued from three independent experiments, with each reaction performed in triplicate. The relative abundance of FANCD2 expression was calculated by the $2^{-\Delta\Delta Ct}$ algorithm, normalized to the *GUS-B* gene, as it was the best option for a housekeeping gene.

For Western blot analyses, total cell extracts were prepared by lysis of 4×10^6 cells in lysis buffer, HCl (pH 8); 5 M NaCl, 10% Nonidet NP40, supplemented with protease and phosphatase inhibitors (Roche) as described previously (Casado *et al*., 2007). After quantification using the Bradford method, proteins were separated on a 6% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, blocked and incubated with either anti-FANCD2 or anti-vinculin as previously described (Casado *et al*., 2007). For immunofluorescence analyses of FANCD2 foci, cells were seeded in cover slides, fixed and identified with anti-FANCD2 antibody as described previously (Casado *et al*., 2007). Cells with >10 foci were scored as positive (Casado *et al*., 2007).

Quantification of apoptosis by Annexin V staining

Healthy LCLs and Kasumi-1 cells were treated with 40 nM MMC, 10 nM trabectedin, 10 nM PM01183 or 20 nM Zalypsis for 2–24 h at 37°C. For apoptosis analysis, treated cells were stained with anti-Annexin V-PE antibody according to the manufacturer's instructions (BD Pharmingen™, San Antonio, TX, USA). Briefly, cells were washed in PBS after incubation with drugs and resuspended in 100 μL of binding buffer containing anti-Annexin V antibody labelled with phycoerythrin (PE). Fluorescence data were analysed by FACS after the addition of 7-AAD (7-aminoactinomycin D). Results are expressed as the percentage of early apoptotic cells (Annexin V-PE$^+$/7-AAD$^-$) and compared with those obtained in unexposed cells.

Cell cycle analysis

Healthy LCLs and Kasumi-1 cells were exposed to 40 nM MMC, 10 nM trabectedin, 10 nM PM01183 or 20 nM Zalypsis for 24 h at 37°C and cell cycle was assessed as reported previously (Casado *et al*., 2008). Briefly, aliquots of 10^5 cells were washed in PBS and fixed in ice-cold 1% methanol-free formaldehyde in PBS for 15 min on ice. After centrifugation, cells were resuspended in 70% ethanol (0–4°C), washed in BSA-T-PBS and resuspended in propidium iodide solution 5 μg·mL^{-1} and 100 μg·mL^{-1} DNase-free RNase A (Sigma). After 30 min of incubation at 37°C, cells were analysed by flow cytometry from 10 000 events with doublet discrimination. Analyses of cell cycle phases were carried out using the MODFIT LT software (Verity Software House, Topsham, ME, USA).

Combined treatment of cancer cells with DBATs and MMC

FANCA-complemented EUFA1365 FA-A SCC cells were exposed to 20 nM Zalypsis (ZLP), 100 nM MMC and a combination of both (MMC + ZLP). When IGROV1 ovarian cancer cells were studied, drug doses that produced above 70% of growth inhibition were used. The effects of the combined drugs were determined graphically by isobolograms (Williams, 1992; Berenbaum, 1989). After presentation of the isobole (line that joins isoeffect doses of two drugs independently used), combined data points were plotted. According to this model, the effect is defined as antagonistic when the fitted plot is above the isobole; additive, if it is near the isobole; and synergistic, if it is under the isobole.

Statistical methods

In the survival experiments, control cultures were exposed to 0.1% DMSO and, in the other experiments, to culture medium. Differences between groups were assessed using Student's two-tailed *t*-test. Data processing and statistical analyses were performed with the Statgraphics Plus 5.0 package (Manugistics Inc., Rockville, MD, USA). Differences were considered significant when $P \leq 0.05$. Results are presented as mean ± SEM of at least three independent experiments.

Results

Untransformed haematopoietic progenitor cells and lymphoblast cell lines with biallelic mutations in FANC genes are hypersensitive to DBATs

In a first set of experiments, we evaluated the response of primary FA-A and FA-D1 haematopoietic progenitors to DBATs with respect to healthy progenitor cells. As shown in Figure 1, FA-A progenitor cells showed a generalized increased sensitivity to trabectedin, Zalypsis and PM01183, compared with their respective controls. A similar, although even more significant hypersensitive response to these three DBATs was observed in mouse FA-D1 progenitor cells. To assess whether other human *FANC*-deficient cells are also hypersensitive to these drugs (Casado *et al.*, 2008), the response of FA-A and FA-J LCLs was investigated and compared with that of their healthy counterparts. The results shown in Figure 2 demonstrate that human FA LCLs are also hypersensitive to trabectedin, Zalypsis and PM01183, consistent with the response of these cells to agents that give rise to classical ICL such as MMC (see also representative PI plots in Supporting Information Figure S1).

SCC cells have an FA-dependent response to MMC, but not to DBATs

As expected, FA-A (EUFA1365) and FA-C (EUFA1131) SCC cells were significantly more sensitive to MMC than the same cell lines not deficient in *FANCA* and *FANCC* genes respectively. In stark contrast, the sensitivity of FA-A and FA-C SCC cells to each of the different DBATs was comparable to that observed in their respective *FANCA*- and *FANCC*-complemented counterparts (Figure 3).

Differential response of the FA pathway to MMC and DBATs

In the next set of experiments, we investigated whether trabectedin, Zalypsis and PM01183 activated the FA pathway, as described previously for MMC (Kee and D'Andrea, 2010). Because the induction of nuclear FANCD2 foci formation constitutes a critical step in the FA pathway after DNA damage, we first determined the generation of nuclear FANCD2 foci in cells treated with equitoxic concentrations of MMC and the different DBATs. Strikingly, while MMC induced a significant increase in the proportion of either WT LCLs or complemented FA SCC cells with FANCD2 foci, this proportion was unchanged in DBAT-treated cells (Figure 4A

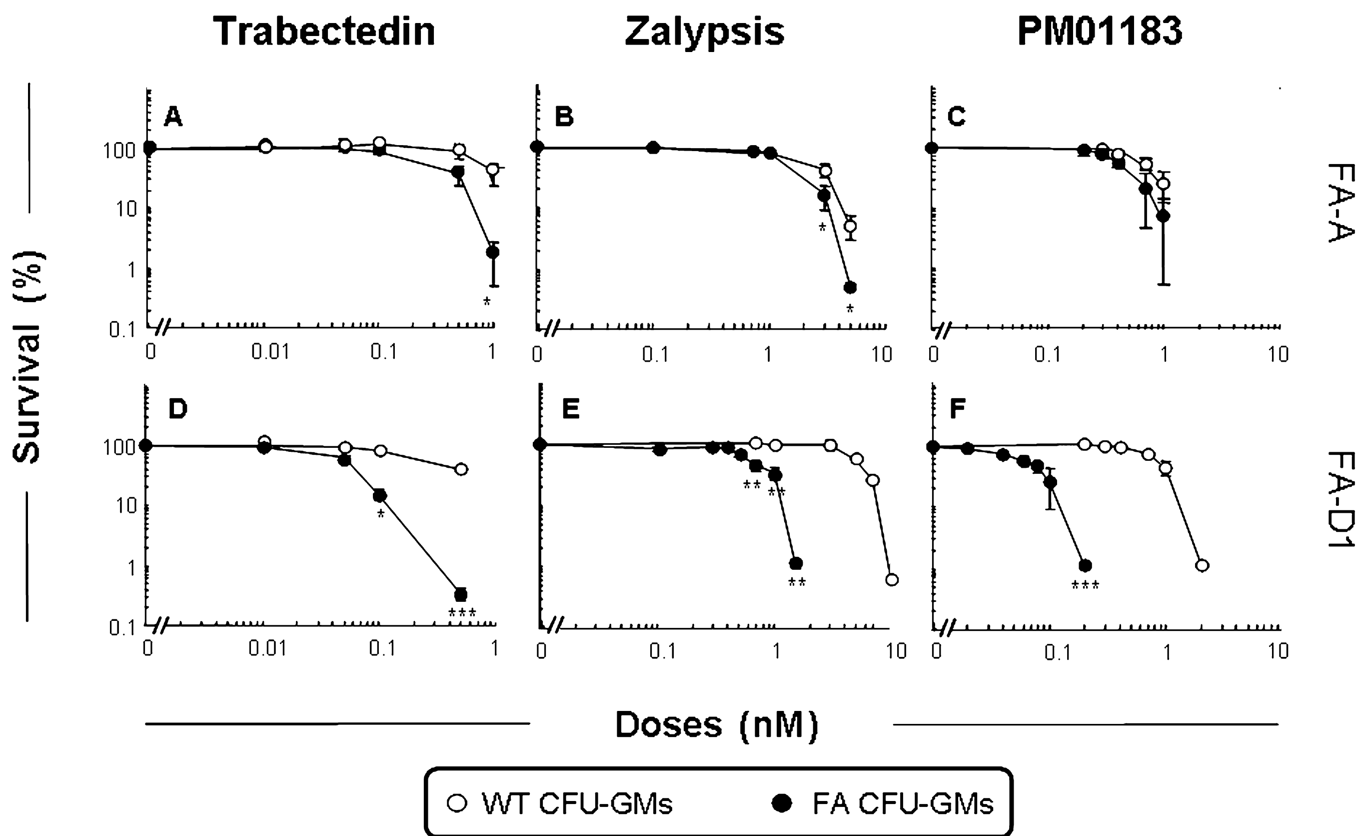

Figure 1

Analysis of the sensitivity of FA-competent and FA-deficient mouse haematopoietic progenitors to DBATs. Bone marrow cells obtained from mice, either FA-deficient or WT, were incubated in semi-solid medium containing increasing concentrations of trabectedin, Zalypsis and PM01183. Panels (A)–(C) show data related to $Fanca^{-/-}$ cells and their corresponding WT controls (FA-A). Panels (D)–(F) present results with $Brca2^{\Delta27/\Delta27}$ cells and their corresponding WT controls (FA-D1). Each point represents the mean ± SEM of colony counts obtained after 7 days of culture in three independent experiments. Differences were significant at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. X and Y axes are logarithmic scales.

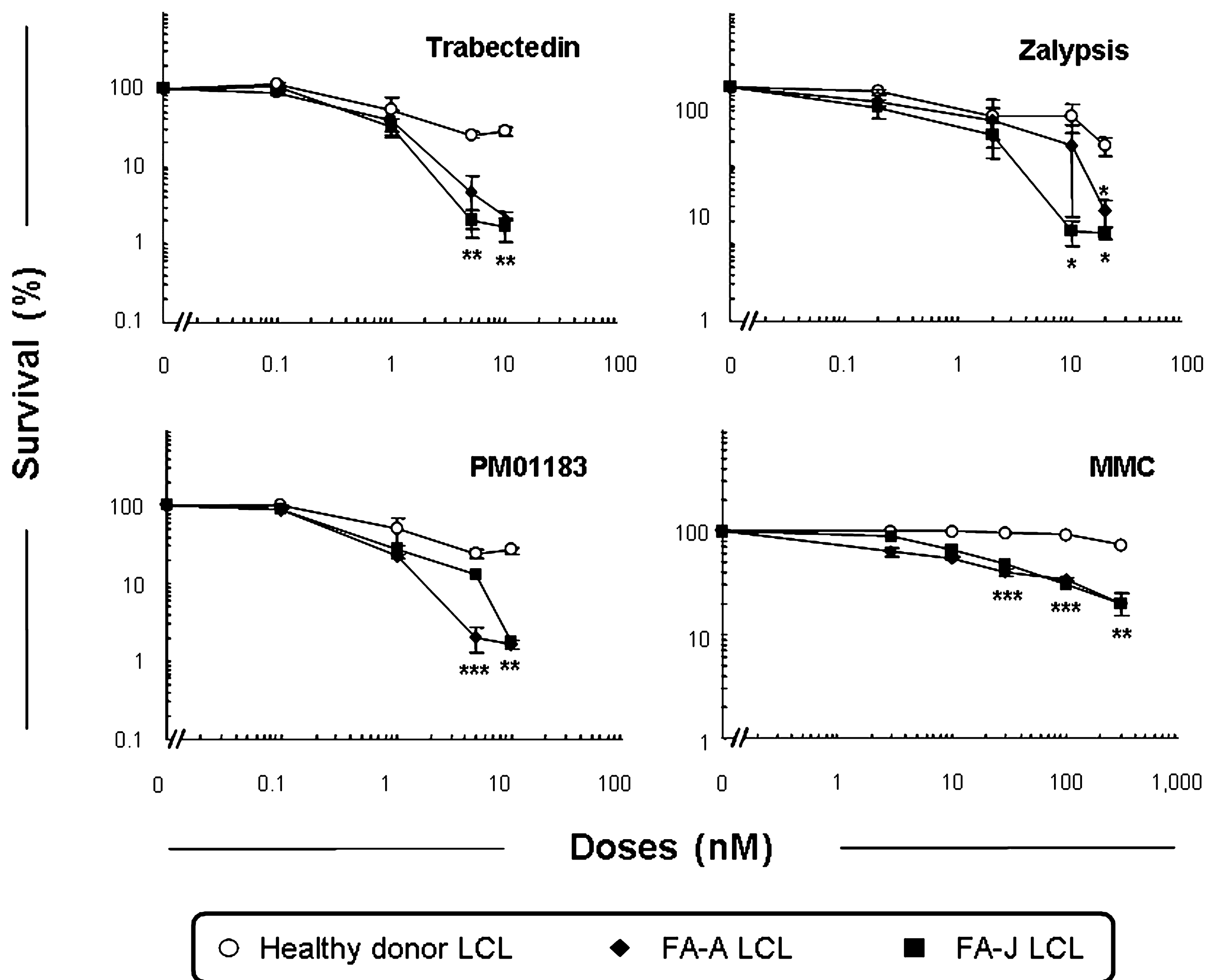

Figure 2

Analysis of the sensitivity of FA-competent and FA-deficient human lymphoblast cells to DBATs and MMC.
Lymphoblast cell lines (LCLs) from a healthy donor and from FA-A and FA-J patients were maintained in liquid cultures containing increasing concentrations of each of the indicated drugs. After 5 days, analyses of cell viability with propidium iodide were performed. Each point represents the mean ± SEM corresponding to three independent experiments. Differences were significant at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *X* and Y axes are a logarithmic scale.

and Supporting Information Figure S4). Moreover, when complemented FA SCC cells were treated with a combination of MMC and Zalypsis, as a representative DBAT drug, the generation of FANCD2 foci induced by MMC was inhibited by Zalypsis (Figure 4B and Supporting Information Figure S4), showing that this DBAT interferes with the activation of the FA pathway.

To understand whether the inhibition of FANCD2 foci formation was a consequence of either reduced expression or deficient monoubiquitination of this protein, the expression of this protein was first determined in FA-proficient LCLs, SCC cells and Kasumi AML cells exposed to moderate doses of either MMC (40 nM), trabectedin (10 nM), Zalypsis (20 nM) or PM01183 (10 nM). FANCD2 expression was markedly down-regulated in SCC and AML cells treated with the DBATs, but not in those exposed to MMC, as assessed by Western blots (Figure 5A). In LCLs, no evident down-regulation was observed, although a reduced ratio (U) between the monoubiquitinated and the non-ubiquitinated forms of FANCD2 was observed after DBAT exposure as compared with MMC. To evaluate whether reduced levels of FANCD2 protein in DBAT-treated cancer cells were associated with reduced FANCD2 mRNA levels, RT-qPCR was performed in these samples. As shown in Figure 5B, mRNA levels of FANCD2 were reduced relative to those of a housekeeping gene (vinculine) both in LCLs and in SCC cells upon

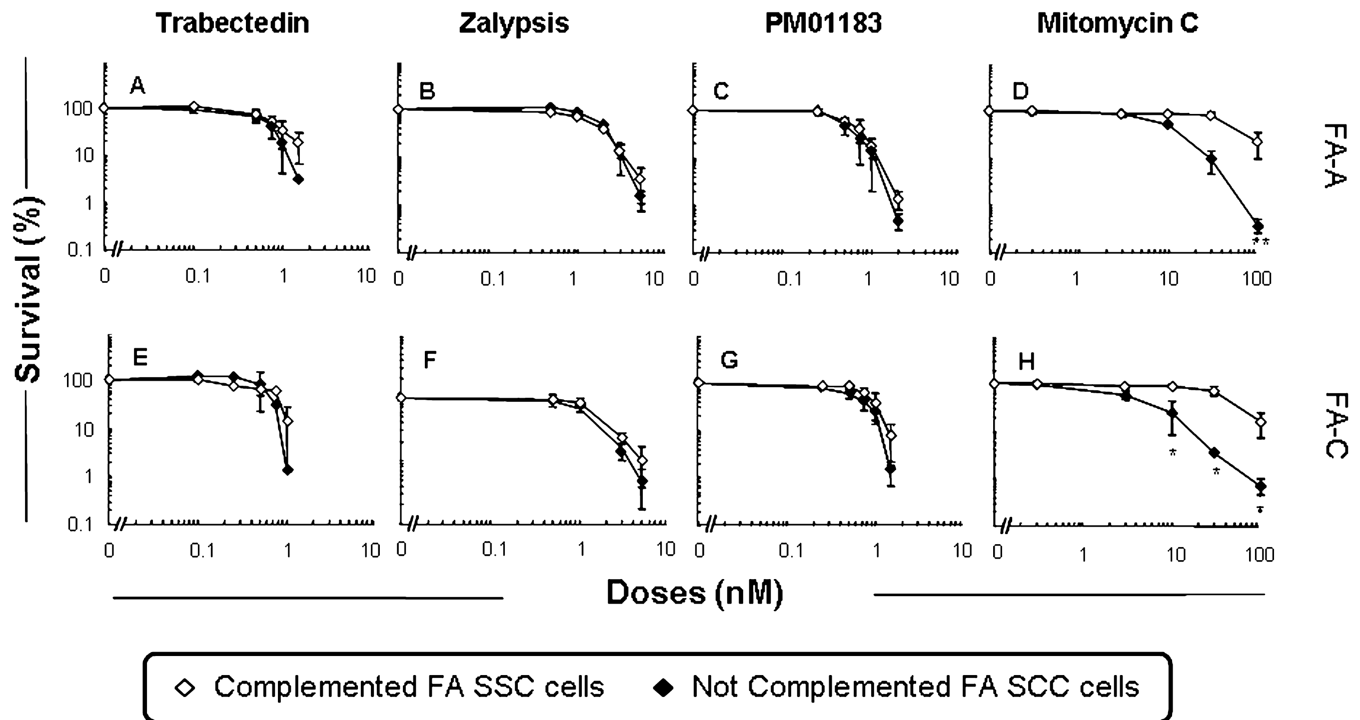

Figure 3

Analysis of the sensitivity of FA-competent and FA-deficient human SCC cells to DBATs and MMC. FA-deficient SCC cells, either uncorrected or complemented with the corresponding *FANC* gene, were cultured in the presence of increasing concentrations of each drug for 24 h in plates pretreated with gelatine. After 7 days, colonies were scored and survival data determined. Panels (A)–(D) show data corresponding to the EUFA1365 cell line (FA-A). Panels (E)–(H) correspond to the EUFA1131 cell line (FA-C). Each point represents the mean ± SEM corresponding to three independent experiments. Differences were significant at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *X* and *Y* axes are a logarithmic scale.

treatment with the DBATs, whereas such an effect was not observed in MMC-treated cells. In the case of Kasumi-1 AML cells, the relative level of FANCD2 mRNA did not decrease after treatment with DBATs. However, this was a consequence of a generalized reduction in mRNA expression in these cells (similar reductions in the levels of FANCD2 and the different housekeeping genes that were used as controls are shown in Supporting Information Table S1).

In parallel experiments, we investigated the induction of apoptosis by DBATs and MMC in untransformed cells and cancer cells. None of the drugs tested induced apoptosis in the untransformed cells, whereas the DBATs but not MMC increased the percentage of apoptotic cancer cells (Supporting Information Figure S2). Additionally, the exposure to clinically relevant doses of DBATs, but not to MMC, resulted in a preferential cell cycle blockade in transformed cells, increasing the percentage of cells in the G_1 phase and reducing those in the G_2-M phase in comparison to untreated cancer cells. No cell cycle arrest was observed in the untransformed cells following treatment with either DBATs or MMC (Supporting Information Figure S3).

Analysis of the combined effect of DBATs and MMC in SCC cells and ovarian cancer cells

Because we showed that the DBATs interfere with activation of the FA pathway, we next investigated the effects of Zalypsis in the presence of MMC, the classical inter-strand cross-linking agent to which FA-disrupted cells are hypersensitive (Kee and D'Andrea, 2012), on complemented SCC cells (EUFA1365 FA-A). As shown in Figure 6A, the combined treatment with these two drugs induced a higher mortality of SCC cells than the individual drugs alone. Likewise, exposure of IGROV1 cells to a combination of MMC with each of the three DBATs studied resulted in additive effects with either PM01183 or Zalypsis and a synergistic effect with trabectedin, as shown in the isobologram analyses displayed in Figure 6B.

Discussion and conclusions

Trabectedin, Zalypsis and PM01183 are three marine-derived tetrahydroisoquinolines endowed with potent anti-tumour activities. Trabectedin is approved for the treatment of advanced soft tissue sarcoma and relapsed platinum-sensitive ovarian cancer in combination with liposomal doxorubicin (Fayette *et al*., 2006; D'Incalci and Galmarini, 2010). Zalypsis can affect multiple myeloma (MM) cells *in vitro* (Ocio *et al*., 2009) and is currently in phase II clinical trials for the treatment of MM. The high potency of PM01183 in several murine models of human cancer (Leal *et al*., 2010) supports its current use as a novel anti-neoplastic agent in phase I and II clinical trials for solid tumours and haematological malignancies.

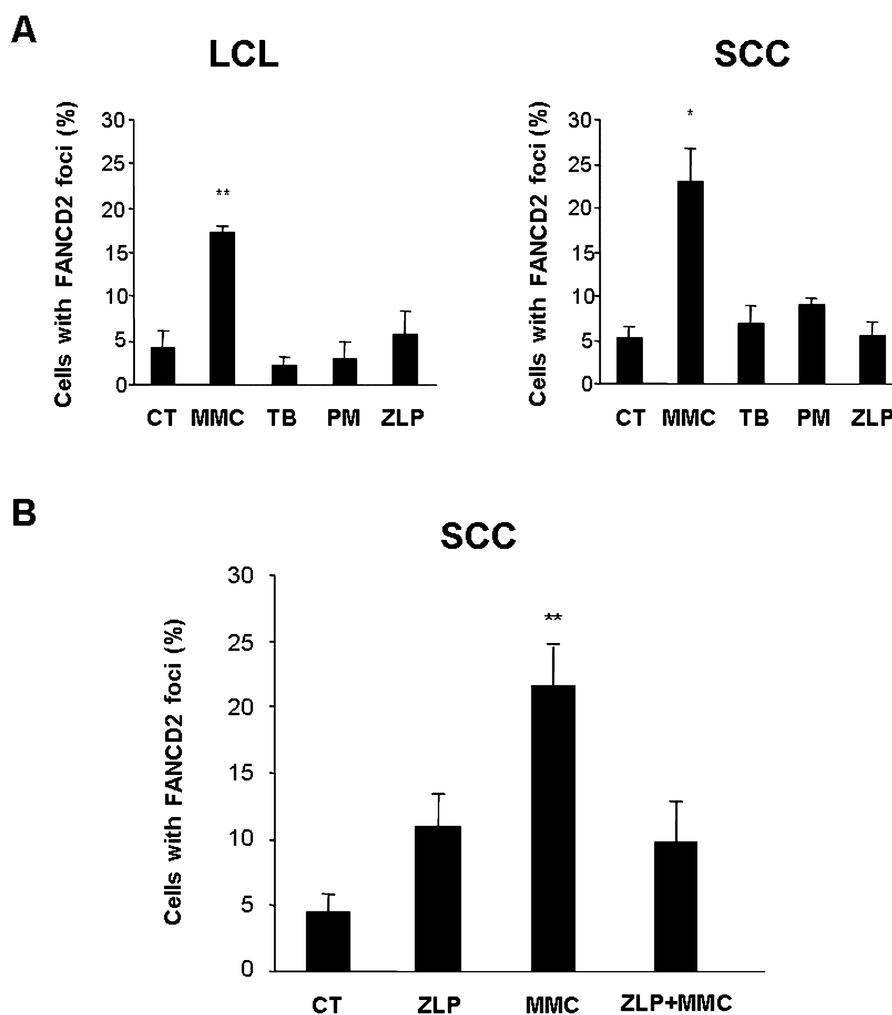

Figure 4

Differential effect of DBATs and MMC on the generation of nuclear FANCD2 foci in FA-competent human LCLs and SCC cells. (A) Analysis of nuclear FANCD2 foci formation in FA-competent SCC and AML cells after 24 h of incubation in the absence of anti-cancer drugs (CT) or in the presence of 40 nM mitomycin C (MMC), 10 nM trabectedin (TB), 10 nM PM01183 (PM) or 20 nM Zalypsis (ZLP). (B) Results of similar experiments conducted with 40 nM MMC and 20 nM ZLP, either individually or in combination (ZLP + MMC). Each bar represents the mean ± SEM corresponding to three independent experiments. Differences with respect to control (CT) samples were significant at $*P < 0.05$; $**P < 0.01$.

It has been shown that covalent binding of these three DBATs in the minor groove of dsDNA (i) hampers strand separation in melting experiments (Soares *et al.*, 2007; Casado *et al.*, 2008; Leal *et al.*, 2009; 2010); (ii) blocks transcription initiation and elongation (Feuerhahn *et al.*, 2011); (iii) gives rise to the generation of DSB *in vivo* (Soares *et al.*, 2007; Casado *et al.*, 2008; Guirouilh-Barbat *et al.*, 2009; Leal *et al.*, 2009; 2010); and (iv) requires homology-directed repair for removal of the damage in treated cells (Herrero *et al.*, 2006; Soares *et al.*, 2007; Casado *et al.*, 2008). Taken together, these findings support the hypothesis that, *inter alia*, the effect of these monofunctional DBATs on dsDNA is similar to that exerted by drugs that generate true ICLs, such as MMC (Gago and Hurley, 2002; Casado *et al.*, 2008; Bueren-Calabuig *et al.*, 2011; Feuerhahn *et al.*, 2011). As a consequence, cells defective in the FA pathway were expected to be more sensitive to DBATs than their FA-proficient counterparts. Earlier studies showed that untransformed FA fibroblasts are indeed hypersensitive to trabectedin (Casado *et al.*, 2008). We then hypothesized that cancer cells with a disrupted FA pathway would also be hypersensitive to DBATs, as is the case for classical ICL drugs (Taniguchi *et al.*, 2003; Koul *et al.*, 2004; Hess *et al.*, 2008; Palagyi *et al.*, 2010).

Our results in this study first confirm that mutations in *FANC* genes in either murine haematopoietic progenitors or human lymphoblasts confer enhanced sensitivity not only to trabectedin (Casado *et al.*, 2008) but also to Zalypsis and PM01183. However, the response of cancer cells was rather unexpected because a similar sensitivity to these DBATs was found in FA-competent and FA-deficient SCC cells, despite their different FA-pathway-dependent responses to MMC.

To elucidate the effect of DBATs on the FA pathway, we next focused on the analyses of the response of FANCD2, a critical protein in the FA pathway, to these drugs (Knipscheer

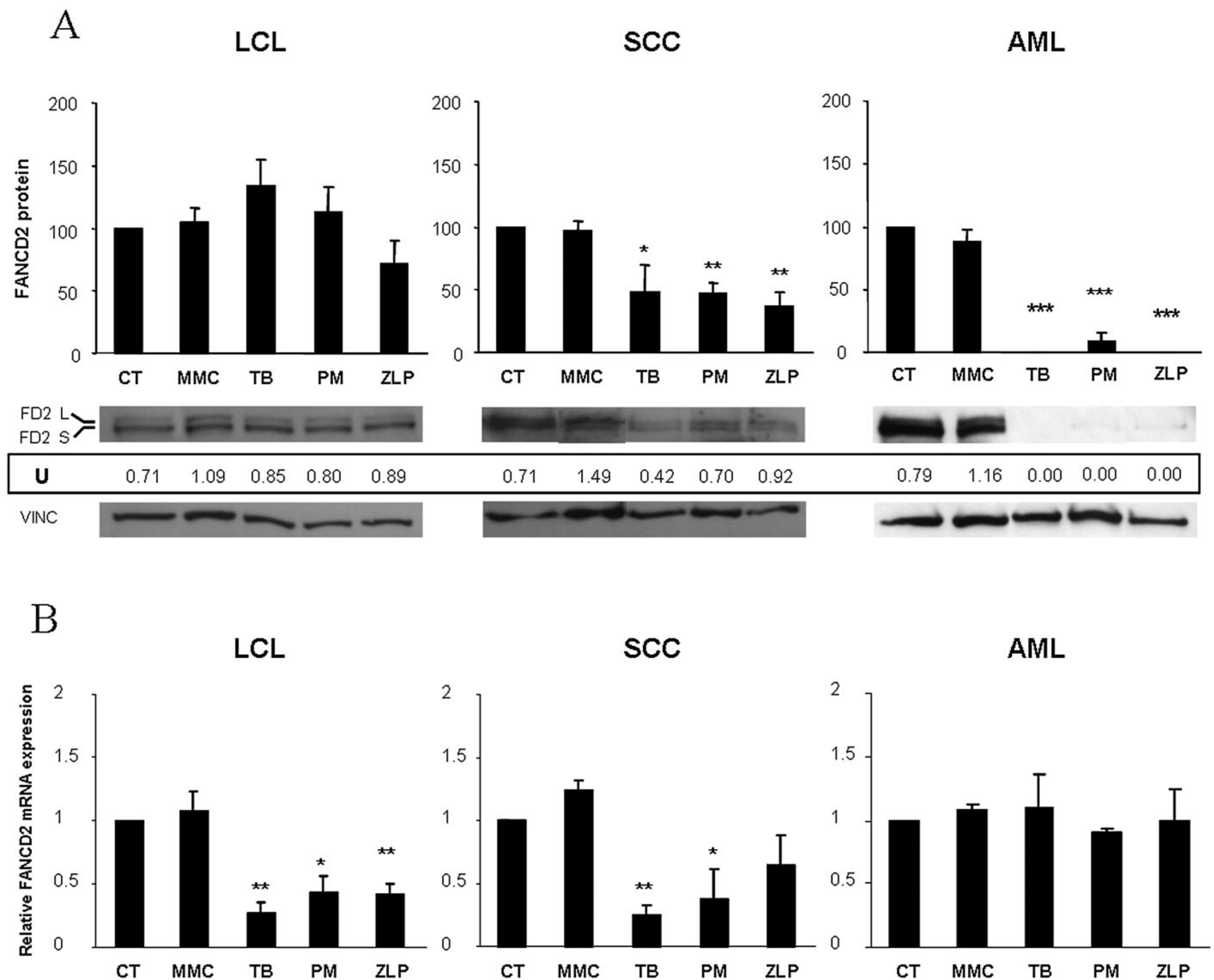

Figure 5

Differential effect of DBATs and mitomycin (MMC) on the expression of FANCD2 in FA-competent human LCL, SCC and AML cells. FA-competent lymphoblasts (LCLs from a healthy donor), FA-competent SCC cells (*FANCA*-complemented FA-A SCC cells) and AML Kasumi-1 cells were treated with 40 nM MMC, 10 nM trabectedin (TB), 10 nM PM01183 (PM) or 20 nM Zalypsis (ZLP). Twenty four hours after exposure, cells were collected and subjected to Western blot analyses with an anti-FANCD2 antibody (A) and FANCD2 mRNA analysis by RT-qPCR (B). Bars represent means ± SEM corresponding to three independent experiments. Differences with respect to CT samples were significant at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. U: ratio between the monoubiquitinated FANCD2 (FD2-L) and non-ubiquitinated FANCD2 (FD2-S).

et al., 2009). Strikingly, our data showed that, in contrast to MMC, none of the DBATs tested was capable of inducing nuclear FANCD2 foci in FA-competent LCLs and SCC cells. Moreover, our results showed that Zalypsis inhibited the MMC-induced formation of nuclear FANCD2 foci in SCC cells. These results strongly suggest that in the case of transformed cells, their response to DBATs is independent of FA proteins because these drugs inhibit the FA pathway *per se*.

When the expression levels of total and monoubiquitinated FANCD2 were investigated after exposure to the drugs, significant differences were again observed. In LCLs, MMC – but none of the DBATs tested – increased FANCD2 monoubiquitination, whereas in SCC cells, the differential effect was even more dramatic because a marked down-regulation of FANCD2 protein was observed in response to DBATs. This decreased expression of FANCD2 was even more noticeable in Kasumi-1 cells, indicating that the DBAT-mediated down-regulation of FANCD2 is not specific for SCC cells. At the mRNA level, marked differences in the effects of DBATs and MMC were also noted. While MMC did not reduce the levels of mRNA FANCD2 in any cell type, all of the DBATs tested reduced the levels of FANCD2, suggesting a predominant effect of these drugs on FANCD2 transcription. In the case of the Kasumi-1 cells, mRNA down-regulation was not specific for FANCD2, denoting a more generalized transcriptional

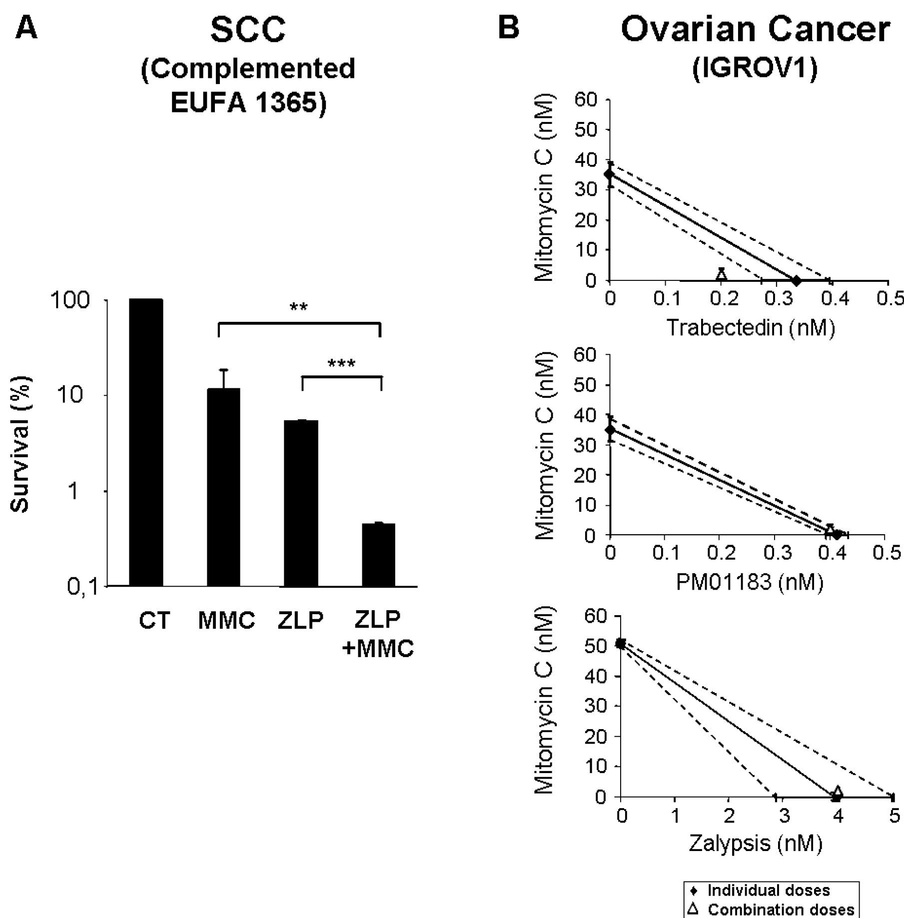

Figure 6

Analysis of the effects mediated by the combination of DBATs and mitomycin C (MMC) in cancer cells. (A) Complemented EUFA1365 cells were exposed to 100 nM MMC, 20 nM Zalypsis (ZLP) or a combination of both drugs for 24 h. Data show mean survival values ± SEM corresponding to three independent experiments. Differences between individual and combined treatments were significant at $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Isobologram plots for combination treatments of DBATs and MMC. The diagonal is the line of additivity. Experimental data points, represented by dots, located below, on or above the line indicate a synergist, additive or antagonist effect respectively.

repression by DBATs in specific cells, consistent with previous observations (Guirouilh-Barbat *et al.*, 2009).

As regards the influence of DBATs on apoptosis and cell cycle progression, our results also show distinct cell type-dependent effects upon treatment with either MMC or DBATs. In this respect, evident apoptosis and cell cycle arrest were induced by DBATs in cancer cells but not in untransformed cells, in good agreement with data published previously (Martinez *et al.*, 2005; Leal *et al.*, 2010; Colado *et al.*, 2011). Although we cannot discard the possibility that the apoptotic and cell cycle effects induced by DBATs could play a role in the down-regulated expression of FANCD2, our results are again consistent with other observations showing both a down-regulated expression of different DNA damage response genes by Zalypsis in two different AML cell lines (Colado *et al.*, 2011) and a more generalized transcriptional regulatory role of certain DBATs (Jin *et al.*, 2000; Minuzzo *et al.*, 2000; David-Cordonnier *et al.*, 2005).

The findings of the present study support the view that the cellular response to DBATs mainly relies on the effects of these drugs on the FA pathway, and that consequences derived from a defective formation of FANCD2 foci will be strongly dependent on the transformation status of the cell. Moreover, our data clearly show that inhibition of the FA pathway induced by exposure to DBATs correlates with additive or synergistic effects of these drugs with MMC on SCC and IGROV1 cells, a relevant finding because trabectedin has already been approved for the treatment of ovarian cancer (Monk *et al.*, 2012).

Taken together, our results lead us to propose that DBATs could be exploited clinically to 'fanconize' FA-competent cancer cells, which would then become hypersensitive to either classical ICL drugs such as cisplatin or MMC, or to drugs (e.g. the topoisomerase II poison etoposide) that are more toxic to cells lacking FANCD2 (Kachnic *et al.*, 2011). In this respect, recent studies on parental and cisplatin-resistant ovarian carcinoma cells (Soares *et al.*, 2011; Vidal *et al.*, 2012) have shown a synergistic effect of PM01183 with cisplatin, a drug whose anti-neoplastic effects are known to depend on the integrity of the FA pathway (Taniguchi *et al.*, 2003) and to

be potentiated by curcumin, a natural product that inhibits FANCD2 monoubiquitination (Chirnomas *et al.*, 2006). Likewise, treatment of MM cells with proteasome inhibitors such as bortezomib leads to a marked reduction in the expression of FANCD2, which results in enhanced sensitivity to the ICL-forming drug melphalan (Yarde *et al.*, 2009).

Finally, although previous data have shown that DBATs generate DNA adducts that mimic classical ICLs (Gago and Hurley, 2002; Casado *et al.*, 2008; Bueren-Calabuig *et al.*, 2011; Feuerhahn *et al.*, 2011), our new data demonstrate marked differences in the effects that DBATs and MMC have on the FA pathway. While MMC is defined as a typical FA-pathway inducer, the three DBATs studied herein can now be defined as inhibitors of this pathway, at least in the SCC and AML models investigated in this study. We expect that this finding will be advantageously used to explore new combination regimes in cancer chemotherapy.

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Conflicts of interest

S. M., C.L.G., M. A. and J. C. T. are Pharmamar employees. The other authors reported no potential conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Representative plots of PI staining experiment in WT and FA LCLs after 5 days of exposure to DBATs and MMC. Percentages of viable cells (orange) are included in the plots.

Figure S2 Effect of DBATs on apoptosis levels in untransformed and cancer cells. WT LCLs and Kasumi-1 AML cells were exposed to 40 nM mitomycin C (MMC), 10 nM trabectedin (TB), 10 nM PM01183 (PM) or 20 nM Zalypsis (ZLP) for 2–24 h, stained by Annexin V/7-AAD and subjected to flow cytometry analysis. (A) Dot plots of a representative 24 h experiment. (B) Percentages of apoptotic cells (Annexin V^+/7-AAD^-) under each condition were expressed as the mean ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ with respect to 0 h.

Figure S3 Cell cycle analysis of untransformed and cancer cells treated with MMC or DBATs. WT LCLs (A) and Kasumi-1 AML cells (B) were exposed to 40 nM mitomycin C (MMC), 10 nM trabectedin (TB), 10 nM PM01183 (PM) or 20 nM Zalypsis (ZLP) for 24 h, fixed, stained with PI and subjected to flow cytometry analysis and compared with untreated cells (CT). Distribution of cells in G_1 phase (open columns), in S phase (striped columns) or in G_2-M phase (black columns) under each condition is expressed as the mean ± SEM of three independent experiments.

Figure S4 Representative images of FANCD2 foci in FA-competent human SCC cells after a 24 h exposure to DBATs and mitomycin C.

Table S1 Effect of DBATs on mRNA FANCD2 expression in untransformed and cancer cells. Cells were exposed for 24 h to the same doses of DBATs and MMC as in Figure 4. Relative FANCD2 mRNA levels were determined by RT-PCR using GUS-B gene as housekeeping ($2^{-(\Delta\Delta Ct)}$). The absolute cycle threshold (CT) of each gene studied is included in the table. The data show the mean ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$ with respect to the control.