The DNA binding polyamine moiety in the vectorized DNA topoisomerase II inhibitor F14512 alters reparability of the consequent enzyme-linked DNA double-strand breaks

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ABBREVIATIONS LIST

DNA-PK: DNA-dependent protein kinase
DSB: DNA double-strand breaks
HR: homologous recombination
IC50: 50% inhibitory concentration
ICE: In vivo Complex of Enzyme bioassay
LIG4: DNA ligase IV
MRN: MRE11/RAD50/NBS1
NHEJ: non-homologous end-joining
PARP: poly (ADP-ribose) polymerase
PTS: polyamine transport system
TOP2: Topoisomerase II
TOP2cc: TOP2 cleavage-complex
TOP2α cc: TOP2α cleavage-complex
XLF: Cernunnos-XLF
ABSTRACT

Poisons of Topoisomerase II (TOP2) kill cancer cells by preventing religation of intermediate DNA breaks during the enzymatic process and thus by accumulating enzyme-drug-DNA complexes called TOP2 cleavage-complex (TOP2cc). F14512 is a highly cytotoxic polyamine-vectorized TOP2 inhibitor derived from etoposide and currently in clinical trials. It was shown in vitro that F14512 has acquired DNA binding properties and that the stability of TOP2cc was strongly increased. Paradoxically, at equitoxic concentrations in cells, F14512 induced less DNA breaks than etoposide. Here we directly compared etoposide and F14512 for their rates of TOP2cc production and resolution in human cells. We report that targeting of TOP2α and not β impacts cell killing by F14512, contrarily to etoposide that kills cells through targeting both isoforms. Then we show that despite being more cytotoxic, F14512 is less efficient than etoposide at producing TOP2αcc in cells. Finally, we report that compared to TOP2α cc mediated by etoposide, those generated by F14512 persist longer in the genome, are not dependent on TDP2 for cleaning break ends from TOP2α, are channelled to a larger extent to resection-based repair processes relying on CtIP and BRCA1 and promote RAD51 recruitment to damaged chromatin. In addition to the addressing of F14512 to the polyamine transport system, the properties uncovered here would be particularly valuable for a therapeutic usage of this new anticancer compound. More generally, the concept of increasing drug cytotoxicity by switching the repair mode of the induced DNA lesions via addition of a DNA-binding moiety deserves further developments.
INTRODUCTION

Topoisomerase II (TOP2) promotes DNA decatenation or DNA supercoils relaxation through generation of transient DNA double-strand breaks (DSB) mediated by enzyme homodimers, which engage reversible covalent tyrosyl-phosphodiester bonds (1-4). TOP2 poisons prevent religation of the DNA break by targeting and stabilizing these open intermediates as enzyme-drug-DNA complexes called TOP2 cleavage-complex (TOP2cc) (5,6). TOP2 poisons, as generators of protein-linked DNA breaks that effectively block transcription and replication, are widely used as anticancer drugs (5-7). For example, treatment with the TOP2 poison etoposide is still a current therapy in several solid or haematological tumors (7).

Two TOP2 isoforms operate in human cells (TOP2α and TOP2β) and etoposide has been shown to target efficiently both (8). Since the trapping of TOP2β has been linked to site-specific chromosomal translocations and to secondary leukemia arising after etoposide treatment (9), research has recently focussed on the isolation of isoform-selective TOP2 poisons (5). Moreover, in order to increase selectivity towards tumor cells, new derivatives of TOP2 poisons have been developed. Thus the molecule F14512 aims at exploiting the overexpression of the polyamine transport system (PTS) in tumours (10) through the addition to the epipodophyllotoxin core of a spermine moiety intended to change its cellular uptake properties (11). Indeed, in several leukemia cell lines, it was established a correlation between F14512 cytotoxicity and the PTS expression measured by flow cytometry with a spermine-based fluorescent probe (12). Notably, the presence of a polyamine tail in F14512 has a supplementary effect, namely an increased stability of the TOP2-drug-DNA complex which correlates with an enhanced activity of F14512 as a TOP2 poison in biochemical assays (11,13). The stabilisation of the TOP2cc by F14512 most probably depends on a combination of drug-protein interactions coupled to polyamine DNA interactions within the TOP2-drug-DNA complex (13,14). Interestingly, F14512 exhibited a marked increase in cytotoxic potency compared to etoposide in cell proliferation assays on a large panel of human cancer cell lines (average IC50 values ~8 fold lower for F14512) (11,12) and showed also potent anti-tumour activity in various in vivo mice models, including acute myeloid leukemia (15,16) and in canine lymphoma (17). Therefore a phase I study of F14512 has been completed in human haematological pathologies (18) and a clinical phase II study is ongoing.

At the cellular level, TOP2cc are repaired by sequential processes that are required first, for DNA-ends clearing from the blocked TOP2 that prevents direct ligation and then, repair of
the remaining DSB (6,19). Two different enzymatic processes can operate to remove TOP2 from the DNA ends. In the first one, TOP2 removal is achieved by proteasomal degradation (20-23) followed by hydrolysis of the tyrosyl-phosphodiester link between a residual TOP2 peptide and DNA by the phosphodiesterase TDP2 (24), and then DNA ends in the remaining DSB are ligated by non-homologous end-joining (NHEJ) (25). In another process, TOP2 is removed by a nucleolytic attack of the DNA flanking the break ends by the MRE11/RAD50/NBS1 (MRN) complex together with the CtIP and BRCA1 proteins (26-31) and ATM as facilitator (32), which is then followed by homologous recombination (HR) with the intact sister chromatid (6,19). A composite pathway mixing nucleolytic attack by MRN and ligation by NHEJ also exists in human cells (26,33,34).

Among these various mechanisms, what is dictating the repair choice of TOP2cc in human cells is not yet clear, nor are the consequences of this choice on cell survival. In this regard, the stability of TOP2cc may be an important parameter. Biochemical assays clearly established that F14512 is a more potent stabilizer of TOP2cc than its cognate molecule etoposide (11,13). Therefore, we decided to compare etoposide and F14512 for the rates of TOP2cc production and resolution in human cells, for their susceptibility to repair by TDP2-dependent or endonucleolytic processes and the susceptibility of the associated DSB to repair by end-joining or resection-based processes.

We report here that targeting of TOP2α and not TOP2β impacts cell killing by F14512, contrarily to etoposide that kills cells through targeting both isoforms. Then we show that despite being more cytotoxic, F14512 is less efficient than etoposide at producing TOP2α cleavage-complex (TOP2α cc) in cells. Finally, we report that TOP2α cc mediated by F14512 persist longer in the genome than those mediated by etoposide and that, as compared to etoposide, their repair relies more on CtIP- and BRAC1-dependent resection-based repair including homologous recombination.

Altogether, our data show that the DNA binding polyamine moiety in F14512 not only prevents targeting of TOP2β but also alters the reparability of TOP2α cc, both traits that, in addition to the addressing to the PTS, would be particularly advantageous for a therapeutic usage of this new anticancer compound. More generally, we believe that the concept of increasing drug cytotoxicity by switching the repair mode of induced DNA lesions via addition of a DNA-binding moiety deserves further developments.
MATERIAL AND METHODS

Cell Culture
All cell lines were sourced from collaborators or repositories. The lung adenocarcinoma A549 human cell line was obtained from Pierre Fabre Laboratories in 2013 and maintained in MEM Earle’s medium (Gibco) supplemented with 10 % foetal calf serum (Lonza), 2 mM glutamine, 25 mM Hepes, 100 U/ml penicillin and 100 µg/ml streptomycin. The osteosarcoma U2OS human cell line was kindly provided by Gaëlle Legube (LBCMCP, University of Toulouse, France) in 2010 and maintained in RPMI-1640 medium (Gibco) supplemented with 10 % foetal calf serum and penicillin-streptomycin. The fibrosarcoma HT1080 human cell line was kindly provided by Pierre Lutz (IPBS, University of Toulouse, France) in 2015 and the cervical cancer HeLa cells were initially obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) in 2002 and maintained in DMEM medium (Gibco) supplemented with 10% foetal calf serum and penicillin-streptomycin. The LIG4-defective and parental HCT116 cell lines were a kind gift from Eric Hendrickson in 2012 (University of Minnesota Medical School, Minneapolis, USA) and the XLF-defective and parental HCT116 cell lines were purchased from Horizon in 2013 (Cambridge, UK). HCT116 cell lines were maintained in RPMI-1640 medium supplemented with 10 % foetal calf serum and penicillin-streptomycin. All cells have been stored in the lab as cryopreserved aliquots. After thawing, a single cryovial is propagated for a maximum of 10 weeks. Cells were routinely tested for lack of mycoplasma contamination and grown in a humidified atmosphere, at 37°C with 5% CO2. Their identity was not formally verified for this study by the authors.

Compounds
F14512 was provided by Institut de Recherche Pierre Fabre and etoposide was purchased from Euromedex, France (S1225).

siRNA sequences and references
siRNA Control [CGUACCGCCAAUACUUCGA-dTdT] and siRNA CtIP [GCUAAAACAGGAACGAAUC-dTdT] were purchased from Sigma Aldrich.
siRNA directed against TDP2 (L-017578-00-0005) and BRCA1 (L-003461-00-0010) were siRNA SMARTpool ON-TARGETplus from Dharmacon (ThermoFisher Scientific).
The siRNA TopoIIα (Hs-TOP2A-6 Flexitube siRNA, SI02665068) and the siRNA TopoIIβ (Hs-TOP2B-5 Flexitube siRNA, SI02780736) were purchased from Qiagen.
siRNA Transfection

Cells were plated in 6 well-plates on day D0. On day D+1, cells were transfected with siRNA at 50 nM final concentration using INTERFERIn® (Polyplus Transfection, POL409-10) according to the manufacturer’s recommendations. On day D+2, cells were transfected again under the same conditions.

Clonogenic assay

Cells were seeded at low density (500 cells/well) in 6-well plates and allowed to plate overnight. Cells were then exposed to various concentrations of etoposide or F14512 for 3h and washed three times with phosphate-buffered saline 1X (Euromedex, ET330-A). Cells were post-incubated 10 days and stained with a 0.2% crystal violet (Sigma Aldrich, C3886) solution for 10 min on an orbital shaker. After extensive washes, groups of at least 50 cells were manually counted and data were normalized relative to untreated controls. The curve fitting analysis (non-linear regression curve fit) was performed with the algorithm provided by the GraphPad Software and allowed to calculate the mean IC50 value (50% inhibitory concentration) under each condition.

FACS analysis

Facs analysis on A549 cells was as previously described (35). Primary antibodies used were anti- γ H2AX (1 :100, Cell Signaling, #2577) and anti-RPA32 (1:200, Abcam, ab2175). Secondary antibodies were AlexaFluor®-488 anti-mouse IgG and -647 anti-rabbit IgG (1:100, Cell Signaling).

ICE experiment

Preparation of TOP2α cleavage-complexes (TOP2Acc)

The ICE protocol was adapted from a published assay (36). Briefly, A549 cells were seeded at 5x10⁶ cells/140cm dish and allowed to attach overnight. Cells were treated with the appropriate dose of etoposide or F14512 for the time required and then harvested with 2 ml of lysis buffer (1% of sarkosyl (N-Laurylsarcosine sodium salt solution, Sigma-Aldrich)) in TE [10 mM Tris-HCl (pH 7.5) - 1 mM EDTA]). The lysates were loaded onto Cesium Chloride (ThermoFisher Scientific) density gradients prepared as follows: a stock solution of CsCl (density = 1.86 g/ml) was diluted with TE to give four CsCl solutions of 1.82, 1.72, 1.50, 1.37
g/ml from which 2 ml were sequentially poured in a polyallomer tube (UltraClear Centrifuge Tube 14*89 mm, Beckman Coulter). The tubes were centrifuged in a Beckman SW41 rotor at 125,000 g during 20 h at 20°C.

From the tube bottom, fractions of ~ 0.4 ml were collected and DNA concentration was measured by fluorescent spectroscopy using Quant-iT™ PicoGreen® reagent (ThermoFisher Scientific) according recommendations from the manufacturer. Fractions 1-10 and 11-20 were pooled as «DNA-bound TOP2α fraction » and « free TOP2α fraction », respectively.

**Quantification of TOP2α by dot-blotting**

Hybond-C nitrocellulose membranes (Amersham) cut to fit the Bio-Dot Apparatus (Bio-Rad) were equilibrated in 25 mM sodium phosphate buffer (pH 6.5) at room temperature for 30 min. Two Whatman 3mm papers were first placed in the Bio-Dot apparatus under the equilibrated membrane. Aliquots of each fraction (100 µl for DNA-bound TOP2α fraction and 5 µl for free TOP2α fraction) completed to 200 µl with 25 mM sodium phosphate buffer (pH 6.5) were loaded on membrane under a mild vacuum. Membrane was then rinsed in PBST [PBS 1X – 0.1% Tween20 (Euromedex)] followed by incubation in PBS containing 5% non-fat dried milk overnight at 4°C. The membrane was washed three times (10 min/wash) in PBST and incubated with an anti-TOP2α antibody (sc-165986, Santa Cruz) diluted 1:200 with PBST/1% BSA (Sigma-Aldrich) for at least 2 h. The membrane was washed three times in PBST, followed by a 45 min incubation with IRDye® 800CW Goat anti-Mouse IgG (H + L) (Tebu-Bio, 610-731-127). The membrane was then rinsed twice with PBST, at least four times with H2O and scanned with LI-COR Odyssey quantitative scanning system (Li-Cor® Biosciences). Quantitative analysis of the scan was performed with the ImageJ software (NIH) and was expressed as the percentage of TOP2α linked to DNA versus total amount of TOP2α present in the both fractions.

**Western Blot**

Whole-cell extracts were prepared by cells scraping in SDS-lysis buffer and disruption through a 25 G needle and then western blotting was performed as previously described (37). Antibodies against TOP2α (sc-165986), TOP2β (sc-13059), TDP2 (sc-135214), BRCA1 (sc-6954) were purchased from Santa Cruz, anti-DNA-PKcs (MS-369-P0) from Thermo Scientific, anti-Phospho-DNA-PKcs (ser 2056; ab18192), anti-KAP1 (ab10483) from Abcam, anti-Phospho-chk1 (Ser 345; #2348S) from Cell Signaling Technology, anti-Phospho-KAP1
(ser 824; IHC-00073) from Bethyl Laboratories, anti-KU80 (NB500-703) from Novus Biological, anti-Actin (A-2066) and anti-HSP60 (H-3524) from Sigma-Aldrich and anti-CtIP is a kind gift of Richard Baer (Columbia University, NY, US). Secondary peroxydase-conjugated antibodies (goat anti-mouse IgG (H+L), 446331 and goat anti-rabbit IgG (H+L), 511380) were from Interchim.

**Cytotoxicity measurement**

The cytotoxic activity of compounds was measured using the ATPlite assay (Perkin Elmer) on WT and NHEJ-deficient HCT116 cell lines. The curve fitting analysis (non-linear regression curve fit) was performed with the algorithm provided by the GraphPad Software and allowed to calculate the mean IC50 value (50% inhibitory concentration) ± 95% confidence interval under each condition.

**Immunostaining**

Cells were pre-extracted, fixed and permeabilized as previously described (38). Incubation with relevant primary and secondary antibodies was carried out sequentially for 1 h each at room temperature. The primary polyclonal antibody used to detect Rad51 was from Santa Cruz (sc8349, 1:200). The secondary antibody was the Alexa Fluor® 488 anti-rabbit IgG (1:1000; Molecular Probes). Images were captured using a confocal lasermicroscope (FV1000 Olympus) equipped with 40X/0.95 lens. Images were taken with the same exposure time when comparing experimental conditions and were analyzed using Image J software (NIH).

**Statistical analysis**

Results are presented as the mean ± SD with significance calculated by Student’s t, Chi-square or Mann-Whitney tests with standard software (GraphPad Prism, GraphPad Software, La Jolla, CA, USA). Significance was assigned for a p-value<0.05.

**RESULTS**

**Targeting TOP2α but not TOP2β impacts cell survival to F14512**

F14512 has been shown to target both TOP2α and TOP2β *in vitro* to generate TOP2cc (13). Whether the two isoforms exhibit a differential susceptibility to F14512 in cells has not been assessed. Therefore, we depleted cells for either TOP2α or TOP2β by siRNA (Fig1A) and
measured cell survival after treatment with either etoposide or F14152. Depletion for either TOP2 isoforms partially rescued cell sensitivity to etoposide, indicating that targeting of the two isoforms contributes to etoposide toxicity, as previously published (8) (Fig. 1BC). In contrast, only depletion for TOP2α partially rescued cell survival to F41512 while depletion for TOP2β had no effect (Fig. 1DE). Therefore, we concluded that contrarily to etoposide, only the targeting of TOP2α isoform significantly contributes to F14512 toxicity in human cells.

**F14512 is less efficient than etoposide at production of TOP2α cleavage-complexes in cells.**

To compare the signalling kinetics of DNA lesions due to etoposide or F14512 in cells, we measured the kinetics of appearance of the DSBs marker γH2AX by flow cytometry analysis. As shown in Fig. 2A, after treatment of A549 cells with an equimolar dose of compound, γH2AX production was delayed in cells exposed to F14512 as compared to etoposide and after 8 h of continuous exposure to F14512 did it reach nearly the level obtained after only 1 h of etoposide treatment. The use of a spermine-based fluorescent probe has allowed estimating that the cellular uptake of F14512 reaches nearly the plateau after 4 h (12). However, the level of γH2AX after 4 h in the presence of F14512 did not reach the one obtained after only 1 h with a ten fold lower dose of etoposide (Fig. 2B). In addition, when two cell lines were exposed for 1 h to an equimolar dose of compound followed by washing, the level of γH2AX still increased during seven more hours post-washing with F14512 while it decreased with etoposide, probably due to breaks repair in the latter case (Fig. S1AB).

The signalling defect for F14512 compared to etoposide was not limited to γH2AX since the level and kinetics of other DSBs markers with this drug treatment was also altered as compared to the effect of an equimolar dose of etoposide (Fig. S2): namely, the ATM substrate KAP1, the ATR substrate Chk1 and an auto-phosphorylation site on DNA-PK were maximally phosphorylated at 4 h with etoposide. By contrast, following F14512 treatment, their phosphorylation was delayed until 8 h and at this late time point, remained lower than the level reached at 4 h with etoposide.

To circumvent a possible impairment of the signalling machinery at the breaks mediated by F14512, we directly addressed the production of TOP2cc through the use of a protocol adapted from the In vivo Complex of Enzyme (ICE) bioassay (36,39,40). Briefly, DNA and free proteins, including TOP2α, isolated from untreated cells partition in different fractions
of an equilibrium density gradient; upon covalent attachment of TOP2 to DNA with TOP2 poisons, a fraction of free TOP2 α moves into the DNA fractions in proportion to the formation of TOP2 α cleavage-complexes (TOP2 α cc) in the treated cells and can be quantified (Fig. S3ABC). Indeed, nearly no TOP2 α protein was detected in the DNA fraction from A549 cells without treatment but some moved into this fraction upon drug treatment, corresponding to formation of TOP2 α cc (Fig. 2C). Both etoposide and F14512 induced a dose-dependent production of TOP2 α cc within 1 h treatment but the two concentrations of F14512 tested yielded only 8 to 15% of the TOP2 α cc amount obtained with equimolar doses of etoposide (Fig. 2CD). When cells were treated continuously over 8 h with an equimolar dose of either etoposide or F14512, the kinetics of TOP2 α cc formation was clearly different with the two molecules, since the amount TOP2 α cc already reached a plateau at 1 h with etoposide but slowly increased up to 8 h with F14512 (Fig. 2EF). Therefore we concluded that the kinetics of TOP2 α cc production in cells is slower with F14512 than with etoposide.

**TOP2 α cleavage-complexes mediated by F14512 persist longer in the genome than those mediated by etoposide.**

The ICE assay allows analysis of the kinetics of production but also of resolution of TOP2cc. Knowing the difference in the kinetics of TOP2 α cc formation between etoposide and F14152, we set up a protocol for cell treatment that induced similar amounts of cleavage-complexes with both molecules at the first time point of the repair kinetics (namely, treatment for 1 h with 3 µM etoposide versus 4 h with 5 µM F14512) (Fig. S4A). Using this protocol, we measured the same initial intensity as well as the same cell-cycle distribution of γH2AX staining following both treatments in A549 cells (Fig. S4BC). Then cells were post-incubated and TOP2 α cc amount was quantified at time 0, 4 and 8 h after cells treatment (Fig. 3A). Despite similar initial yields of TOP2 α cc for etoposide and F14512, their kinetics of resolution in cells were very different since almost background values were reached as early as 4 h post-treatment with etoposide while 40% TOP2 α cc still remained 8 h post-treatment with F14512 (Fig. 3BC). Strikingly, similar differences in repair profiles were obtained for both molecules upon quantification by flow cytometry of the cells positive for the DSBs marker γH2AX (Fig. 3D). We concluded that compared to etoposide, F14512 mediates TOP2 α-linked DNA lesions that persist much longer in cells.
HR participates in repair of F14512-mediated DSB.

Since the kinetics of TOP2αcc removal was very different between etoposide and F14512, we wondered whether the repair mechanisms involved were different. First, we checked TOP2α degradation following cell treatment with either etoposide or F14512. Although etoposide and F14512 yielded similar amounts of TOP2αcc at 8 h incubation time (Fig. 2F), TOP2α was clearly degraded within 10 h in the presence of etoposide, but remained unchanged during the same period in the presence of an equitoxic or equimolar concentration of F14512 (Fig. 4A). TOP2α degradation could only be detected at much later time in the presence of F14512 (Fig. 4B, t=18 h). It has been proposed that TDP2 requires TOP2 degradation for the repair of TOP2-induced DNA damage through its phosphodiesterase activity (22). Strikingly, while TDP2 knock-down by siRNA reduced cell survival to etoposide, as previously published (24,25,41), it did not impact cell survival to F14512, more likely excluding the TOP2 degradation-TDP2 axis for early processing of F14152-mediated DNA damage, in contrast to etoposide (Fig. 4CDE).

Then, we checked whether components of the NHEJ or HR pathways were differentially involved in resistance to etoposide and F14512. Cell viability with either molecule was similarly impacted by a defect in the Cernunnos-XLF (XLF) or DNA ligase IV (LIG4) components of the NHEJ ligation complex, which led to a ~6 to 7-fold increase in sensitivity towards both molecules (Fig. 4F and Table 1), implying that NHEJ was equally involved in the repair of etoposide- or F14512-mediated DSB. Then we knocked down the key HR proteins CtIP or BRCA1 (Fig. 5AB) and checked for an effect on cell survival to etoposide and F14512. While knock-down of CtIP did not further sensitize A549 to etoposide, it did increase cell mortality in the presence of F14512 (Fig. 5CD). A similar preferential sensitization to F14512 compared to etoposide by CtIP knock-down was observed in U2OS, HeLa and HT1080 cells (Fig. S5A-H). For the four cell lines tested the negative impact of CtIP depletion on cell survival was more pronounced with F14512 than with etoposide (Supplementary Table S1). Similarly, knock-down of BRCA1 did not further sensitize A549 to etoposide but increased cell mortality in the presence of F14512 (Fig. 5EF). Then, γH2AX staining was analysed by flow cytometry in A549 cells 8 h after cell treatment inducing similar initial amounts of TOP2αcc with both molecules (cf Fig. S4 and 3A). We found that CtIP knock-down had no impact on the repair of etoposide-mediated DSB but further significantly compromised the poor repair of F14512-mediated DSB (Fig. 5G).
DSB repair by HR comprises at least two essential steps: first resection at the break ends, consisting in the exonucleolytic processing of the 5' end to generate free 3’ single-stranded DNA, a process dependent on CtIP and BRCA1 among other proteins; the single-stranded overhang is coated with replication protein A (RPA) which, in a second step is exchanged with the RAD51 recombinase necessary to initiate strand invasion of the homologous template (for a recent review, (42)). Since we found that cell survival to F14512 required specifically CtIP, we then analyzed both HR steps using dedicated assays in A549 cells treated with F141512 in comparison with etoposide, under conditions set up to induce similar initial amounts of TOP2\(\alpha\)cc with both molecules. First, we used a flow cytometry-based assay allowing measurement of RPA accumulation as a readout for the early HR step, DNA-end resection (43). As shown in Fig. 6AB, F14512 induced a strong RPA signal in the treated cells that further increased over 8 h post-treatment, very far above the signal observed with etoposide. In addition, analysis of the signal distribution within the cell-cycle phases showed that RPA accumulation following treatment with F14512 was confined to cells in S- and G2, as expected for HR-mediated DSB repair which takes place after DNA replication (Fig. 6B). Finally, under the same experimental conditions, we addressed a later step of HR by measuring the extent of RAD51 accumulation in the damaged chromatin of A549 cells treated with either etoposide or F14512. Fig. 6CD show that F14512 but not etoposide provoked a clear chromatin enrichment in RAD51 at the 8 h post-treatment time point, indicative of DSB processing by HR. Similarly, HeLa, U2OS and HT1080 cells exhibited a mobilisation of RAD51 to chromatin that was more pronounced after treatment with F14512 than with etoposide (Fig. 6E).

Altogether, these data demonstrate that in cells, F14512 triggers a stronger commitment of DSB repair towards HR as compared to etoposide.

**DISCUSSION**

The molecule F14512 was initially developed to change the cellular uptake properties of the widely used TOP2 poison etoposide through the addition of a spermine moiety, to benefit from the overexpression of the polyamine transport system (PTS) in tumours (11). These new formula led to an enhancement in toxicity on several human cancer cell lines (11), that we confirm here on A549, HCT116, U2OS, HeLa and HT1080 cell lines (Fig. 1, Table 1 and Supplementary Table S1). Moreover, at least in a panel of human leukemia cell lines, PTS expression tended to correlate with the preferential killing activity of F14512 versus etoposide.
However, PTS negative cells yet preferentially sensitive to F14512 were characterized. More puzzling, the paradox emerged that at equitoxic concentrations, F14512 induced less DNA damage than etoposide in cells, as assessed by measurement of either γH2AX production or genome breakage (11,44). Therefore, the aim of our study was to understand the basis of the cytotoxicity of the DNA damage generated by F14512.

In contrast to etoposide, F14512 is a DNA binder through the interaction of its spermine moiety with the DNA minor groove (11,14). Our present results indicate that the sole DNA binding property of F14512 cannot account for its cellular toxicity and that its TOP2 poisoning activity is still involved. Indeed, TOP2cc stabilisation by F14512 highly contributes to if not determines its cytotoxic activity since TOP2α knock-down reduces F14512 cytotoxicity (Fig. 1D) and dose- and time-dependent F14512-mediated TOP2α cc are generated (Fig. 2C-F).

It has been shown that the polyamine moiety strongly influences the compound properties by sharply increasing the stability of the TOP2-cc and thereby the activity of F14512 as TOP2 poison in reconstituted assays with purified enzymes (11,13).

Here, we have adapted the ICE bioassay to measure directly the kinetics and extent of TOP2cc production and repair in cells. We found that etoposide generates TOP2α cc more rapidly and at a higher extent than F14512 (Fig. 2 D-F). The delay in generation of TOP2α-linked breaks in cells observed with F14512 strikingly parallels a delay in the kinetics of γH2AX production (Fig. 2A). This allows excluding a defect in detection or signalling at F14512 mediated-DSB as a cause for a delay in H2AX phosphorylation and rather points out the blockage of TOP2α by F14512 as the true limiting step for DSB production.

The slow and low DNA breaking activity of F14512 could rely non exclusively on a delayed drug uptake due to the use of the PTS and/or the requirement of special activation pathways or intracellular traffic routes; indeed, an intermediate storage step could explain that DSB still accumulated during the recovery time after F14512 removal from the culture medium while they were lost after removal of etoposide (Fig. S1BC). Further experiments are needed to determine the respective contribution of these steps to the kinetics and extent of the F14512 DNA breaking activity.

Nevertheless, our data clearly demonstrated that in cells, equitoxic concentrations of F14512 induced less TOP2α cc than etoposide. Therefore we directly explored the reparability of F14512 mediated-DSB, a defect of which could reasonably account for the higher cell killing power of this molecule. It has been shown that TOP2cc cannot be processed by the TDP2...
phosphodiesterase unless TOP2 is first degraded (20-23). Here, we confirm that the processing of etoposide-mediated TOP2αcc comprises early TOP2α degradation and TDP2 activity (Fig. 4A-D). In contrast, we also show that TDP2 activity does not impact cell survival to F14512 (Fig. 4D) and that TOP2α degradation takes place much later with F14152 than with etoposide (Fig. 4AB). This suggests that the TOP2αcc generated by F14512 may be structurally different from the TOP2-linked DNA breaks mediated by etoposide, as recently proposed from data of molecular modeling of F14512 and other related etoposide derivatives bound to TOP2cc (14). Also, TOP2cc formed by F14512 in vitro persisted 5-10 fold longer than that induced by etoposide (13). This increased stability of TOP2cc with F14512 may additionally contribute to their resistance to the processing that normally operates at etoposide-generated TOP2cc.

We report that compared to etoposide, TOP2αcc generated by F14512 are repaired much slower (Fig. 3CD) and that they are channelled to a larger extent to repair pathways depending on the DNA resection-activating CtIP and BRCA1 proteins (Fig. 5 and Fig. S5). One of the repair pathways involved in F14512-mediated DSB is clearly HR because we established that, i) in contrast to etoposide, F14512 mobilizes on chromatin the early HR protein RPA in S- and G2-cells (Fig. 6AB); ii) in several cellular models, the accumulation in damaged chromatin of the late HR protein RAD51 was greater with F14512 than with etoposide (Fig. 6DE).

Yet a NHEJ defect leads to a ~6-fold increase in cell sensitivity to F14512 (Table 1). This suggests that a subset of TOP2αcc generated by F14512 are still repaired by NHEJ, that may require the TOP2α degradation that we observed at late incubation time, but not TDP2-dependent processing. Alternatively, repair of some F14512-induced DSBs may rely on the composite pathway mixing NHEJ with CtIP- and MRN-dependent resection and that has been described in G1 cells for a sub-fraction of DSB generated by etoposide (33,34). The proportion of this fraction of DSB may vary with the cellular model. Indeed, we found that CtIP defect had a variable impact on cell survival to etoposide in different cell models (Fig. 5C and Fig. S5C, S5E and S5G). Nevertheless, in all the cells tested, CtIP contribution to survival was greater for F14512 than for etoposide (Supplementary Table S1), most likely corresponding to its role in HR-dependent repair of a fraction of the DSB induced by F14512.

The fact that TOP2α removal from F14512-stabilized TOP2αcc preferentially relies on resection at the DNA ends likely explains the slow repair kinetics observed for TOP2αcc and DSB created by F14512, given that resection-based pathways at DSB are slower that direct
ligation by NHEJ (45-47). Concerning the repair pathway choice at two-ended DSBs and whatever the cell cycle phase, the current view is that the first cellular attempt is to directly reseal the break by NHEJ before engagement to resection in case of ligation failure, depending mostly on the break complexity or chromatin context such as heterochromatin ((48) and references therein). Aside TOP2cc stability and/or conformation specific to F14512 that may switch repair to resection as discussed above, it is notable that this molecule may target a subset of sites in chromatin (49), the location of which could also contribute to its unique repair mode. Altogether, our findings on the peculiar repair mode and kinetics of F14512-generated DSB reveal the overall repair refractory trait of the TOP2cc that F14512 induces, more likely explaining its higher cytotoxicity compared to etoposide.

Phase I clinical trials of F14512 have been completed (17,18) and this promising drug has entered clinical phase II. Our finding that contrarily to etoposide, only targeting TOP2α impacts cell survival with F14512 would be particularly advantageous for its therapeutic usage, since TOP2β is strongly believed to be responsible for translocations at the origin of secondary cancers after treatment with etoposide (9). In addition, since resection capacity of the cell specifically dictates its resistance to F14512, it can be anticipated that targeting a resection-based repair such as HR could improve the drug efficacy. Interestingly, inhibitors of histone-deacetylase, otherwise known to impair HR (50), exhibit synergistic effects on cell killing when combined with F14512 (15). Recently, it has been established that the sensitivity of subsets of acute myeloid leukaemia to inhibitors of poly (ADP-ribose) polymerase (PARP) was explained by their intrinsic defect in HR (51). Whether these subsets of leukaemia are particularly sensitive to F14512 deserves further investigations.

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| Compounds | HCT116 NHEJ-def cell line | IC_{50} (nM ; 95% CI) | Ratio IC_{50} WT/IC_{50} NHEJ-def |
|-----------|-------------------------|---------------------|-------------------------------|
| etoposide |                         |                     |                               |
| XLF-/−    | 172 (92-324)            | 30 (14-64)          | 5.73                          |
| LIG4-/−   | 871 (469-1618)          | 123 (63-240)        | 7.08                          |
| F14512    |                         |                     |                               |
| XLF-/−    | 20 (12-32)              | 3 (2-4)             | 6.67                          |
| LIG4-/−   | 27 (18-39)              | 4 (2-6)             | 6.75                          |

Table1. Evaluation of cytotoxicity of etoposide and F14512 against WT and LIG4 and XLF KO cell lines.

Cell viability was assessed by ATPlite proliferation assay as exemplified in Fig. 4F. IC_{50} values were calculated using a non-linear regression analysis and are reported as the mean with 95% confidence interval (CI). n=6 (F14512) to 9 (etoposide) independent experiments.
FIGURE LEGENDS

Figure 1. Role for TOP2α and TOP2β in the cell toxicity of etoposide and F14512.
A/ Immunoblotting analysis of whole-cell extracts from A549 cells transfected with the indicated siRNAs. Ctrl stands for control. Relative protein levels of TOP2α and TOP2β were quantified, normalized to actin level and set to 1 in cells transfected with control siRNAs. B, C, D and E/ Graphs representing cell survival as assessed by clonogenic assays on A549 cells transfected with control (Ctrl), TOP2α (B and D) or TOP2β (C and E) siRNAs and treated with etoposide (B and C) or F14512 (D and E). Error bars represent the standard deviation from the means, n=3 independent experiments. p values were calculated using a paired Student’s t-test.

Figure 2. Kinetics of production of DSB and TOP2αcc in A549 cells after treatment with etoposide and F14512.
A and B/ The bar graphs represent analysis of DSB production through the mean percentage of γH2AX-positive cells measured by flow cytometry in a population of A549 cells treated with 5 μM of compound as indicated and incubated for 1, 4 or 8 h (A), or incubated for 1h with etoposide or 4 h with F14512, at the indicated drug concentration (B). Error bars represent the standard deviation from the means, n=3 independent experiments. C and D/ The ICE bioassay was used to monitor levels of TOP2α cleavage complexes in A549 cells treated with the indicated compounds. Pooled DNA-bound or free TOP2α fractions from cells incubated for 1 h in the absence of compound or in the presence of 5 or 50 μM etoposide or F14512 were dot-blotted onto a nitrocellulose membrane. Immunoblots were probed with a polyclonal antibody directed against human TOP2α. C/ A representative immunoblot is shown. D/ The bar graph showing quantification of the percentage of DNA-bound TOP2α indicates the mean±SD from three independent experiments as in C. E and F/ The ICE bioassay as above was used to monitor levels of TOP2αcc in A549 cells untreated (NT) or incubated with 5 μM of either compound as indicated for 1, 4 or 8 h. E/ A representative immunoblot is shown. F/ The bar graph represents quantification with standard deviation from the means, n=3 ICE experiments as in E. All p values were calculated using a Mann-Whitney test.
Figure 3. Repair kinetics of TOP2αcc and DSB in A549 cells after treatment with etoposide and F14512.

A/ Schematic of the repair kinetics experiment. B and C/ The ICE bioassay was used to monitor levels of TOP2αcc in A549 cells untreated (NT) or treated as in A and postincubated for 0, 4 or 8 h. B/ A representative immunoblot is shown. C/ The bar graph represents the ratio of the amount of DNA-bound TOP2α at the indicated post-treatment time versus the number of DNA-bound TOP2α at T0 h with standard deviation from the means, n=3 ICE experiments as in B. D/ A549 cells were treated as in A and post-incubated for 0, 4 or 8 h. The bar graph represents analysis of DSB repair through the ratio of the number of γH2AX-positive cells at the indicated post-treatment time versus the number of γH2AX-positive cells at T0 h as measured by flow cytometry. Error bars represent the standard deviation from the means, n=3 independent experiments. All p values were calculated using a Mann-Whitney test.

Figure 4. Role for TOP2α degradation, TDP2- and NHEJ-dependent DNA repair in cell survival to etoposide and F14512.

A / Immunoblotting analysis of whole-cell extracts from A549 cells treated with equitoxic (left and middle panels) or equimolar (left and right panels) doses of compounds for the indicated times. B/ Immunoblotting analysis of whole-cell extracts from A549 cells treated with 50 µM of compounds for the indicated times. C/ Immunoblotting analysis of whole-cell extracts from A549 cells transfected with the indicated siRNAs. Ctrl stands for control. The relative protein level of TDP2 was quantified, normalized to Ku80 level and set to 1 in cells transfected with control siRNA. D and E/ Graphs representing cell survival as assessed by clonogenic assays on A549 cells transfected with control (Ctrl) or TDP2 siRNA and treated with etoposide (C) or F14512 (D). Error bars represent the standard deviation from the means, n=3 independent experiments. p values were calculated using a paired Student’s t-test. F/ Graph representing cell viability as assessed by ATPlite proliferation assay on wild-type (WT) and LIG4 KO HCT116 cells following a 72 h treatment with etoposide. Data points on the graph are reported as means ± 95% confidence interval, n=9 independent experiments. Non-linear regression was performed to model the data (solid lines).
Figure 5. Role for HR-dependent DNA repair in cell survival to etoposide and F14512.

A and B/ Immunoblotting analysis of whole-cell extracts from A549 cells non-transfected (nt) or transfected with the indicated siRNAs. Ctrl stands for control. Relative protein levels of CtIP and BRCA1 were quantified, normalized to actin and HSP60 levels, respectively and set to 1 in untransfected cells. C, D, E and F/ Graphs representing cell survival as assessed by clonogenic assays on A549 cells transfected with control (Ctrl), CtIP (C and D) or BRCA1 (E and F) siRNAs and treated with etoposide (C and E) or F14512 (D and F). Error bars represent the standard deviation from the means, n=3 independent experiments. p values were calculated using a paired Student’s t-test. G/ The bar graph represents the analysis of DSB repair through the ratio of the number of γH2AX-positive cells at T8h versus the number of γH2AX-positive cells at T0h as measured by flow cytometry in a population of A549 cells transfected with the indicated siRNAs, treated with 3 μM etoposide for 1 h or with 5 μM F14512 for 4 h and then released for 8 h. Error bars represent the standard deviation from the means, n=4 independent experiments. p values were calculated using a Chi-square test.

Figure 6. Analysis of two homologous recombination steps in cells after treatment with etoposide or F14512.

A/ Analysis of cell-cycle distribution and RPA chromatin staining of A549 cells treated with 3 μM etoposide for 1 h or with 5 μM F14512 for 4 h and post-incubated for 0, 4 and 8 h. Numbers in blue indicate the percentage of RPA-positive cells. B/ The bar graph represents the mean percentage of cells that are positive for RPA chromatin staining as measured by flow cytometry in A549 cells after etoposide or F14512 treatment as in A. Error bars represent the standard deviation from the means, n=3 independent experiments. p values were calculated using a Chi-square test. C/ Representative images of Rad51 foci fluorescence signal (green) detected in untreated A549 cells (Ctrl) and in cells treated with etoposide or F14512 as in A and released for 8 h. D/ Rad51 relative fluorescence was detected at the indicated time-points by confocal microscopy in nuclei of A549 cells treated with etoposide or F14512. The scatter plots show the overall levels of Rad51 intensity per individual nucleus (A.U., arbitrary units). n>120 nuclei for each condition per independent experiment. Bars indicate the means. The experiment was repeated 3 times. p values were calculated using a Mann-Whitney test. E/ Rad51 relative fluorescence was detected by confocal microscopy in nuclei of untreated cells (NT) and of cells treated with etoposide or F14512 as in C and released for 8 h. The scatter plots show the overall levels of Rad51 intensity per individual
nucleus in HeLa cells (n ≥ 250 for each condition), U2OS cells (n ≥ 130 for each condition) and HT1080 cells (n ≥ 150 for each condition) treated as indicated (A.U., arbitrary units). Significant differences in nuclear Rad51 levels were assessed using a Mann-Whitney test and are indicated by p values. Experiments were repeated three times with HeLa and HT1080 cells and twice with U2OS cells.
Figure 1

(A) Western blot analysis showing the expression levels of TOP2α and TOP2β under different conditions. The molecular weights (MW) are indicated in kDa.

(B, C) Graphs illustrating the cell survival percentages in response to etoposide. The survival rates are compared between siCtrl and siTOP2α (B) or siTOP2β (C) groups. The p-values are indicated for each comparison.

(D, E) Graphs showing the cell survival percentages in response to F14512. The survival rates are compared between siCtrl and siTOP2α (D) or siTOP2β (E) groups. The p-values are indicated for each comparison.
Figure 2
Figure 3

(A) Schematic diagram illustrating the experimental design for studying the effects of etoposide and F14512 on TOP2α. Etoposide (3 μM, 1h) and F14512 (5 μM, 4h) were applied, followed by washing and post-treatment at T0h, T4h, and T8h.

(B) Western blot analysis showing the DNA-bound and free TOP2α fractions at different post-treatment times (NT, T0h, T4h, T8h).

(C) Bar graph showing the DNA-bound TOP2α levels (ratio T/T0h) at T0h, T4h, and T8h.

(D) Bar graph showing the γH2AX-positive cells (ratio T/T0h) at T0h, T4h, and T8h.
Figure 4

A

| MW (kDa) | etoposide (50 μM) | F14512 (5 μM) | F14512 (50 μM) |
|----------|-------------------|----------------|-----------------|
| 250      | ![Image](TOP2α)   | ![Image](TOP2α) | ![Image](TOP2α) |
| 100      | ![Image](Ku80)    | ![Image](Ku80) | ![Image](Ku80)  |

B

| MW (kDa) | etoposide | F14512 |
|----------|-----------|--------|
| 250      | ![Image](TOP2α) | ![Image](TOP2α) |
| 198      | ![Image](KAP-1) | ![Image](KAP-1) |
| 64       | ![Image](hsp60) | ![Image](hsp60) |

C

| MW (kDa) | siCtrl | siTDP2 |
|----------|--------|--------|
| 55       | ![Image](TDP2) | ![Image](TDP2) |
| 100      | ![Image](Ku80) | ![Image](Ku80) |

D

% cell survival vs [etoposide] (μM)

- siCtrl
- siTDP2

E

% cell survival vs [F14512] (μM)

- siCtrl
- siTDP2

F

% cell proliferation vs [etoposide] (μM)

- HCT116 WT
- HCT116 LIG4-/-
Figure 5
The DNA binding polyamine moiety in the vectorized DNA topoisomerase II inhibitor F14512 alters reparability of the consequent enzyme-linked DNA double strand breaks

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