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Differential and Common DNA Repair Pathways for Topoisomerase I- and II-Targeted Drugs in a Genetic DT40 Repair Cell Screen Panel

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Differential and Common DNA Repair Pathways for Topoisomerase I- and II-Targeted Drugs in a Genetic DT40 Repair Cell Screen Panel

Yuko Maede1, Hiroyasu Shimizu4, Toru Fukushima2, Toshiaki Kogame1, Terukazu Nakamura3, Tsuneharu Miki4, Shunichi Takeda1, Yves Pommier5, and Junko Murai1,5

Abstract
Clinical topoisomerase I (Top1) and II (Top2) inhibitors trap topoisomerases on DNA, thereby inducing protein-linked DNA breaks. Cancer cells resist the drugs by removing topoisomerase-DNA complexes, and repairing the drug-induced DNA double-strand breaks (DSB) by homologous recombination and nonhomologous end joining (NHEJ). Because numerous enzymes and cofactors are involved in the removal of the topoisomerase-DNA complexes and DSB repair, it has been challenging to comprehensively analyze the relative contribution of multiple genetic pathways in vertebrate cells. Comprehending the relative contribution of individual repair factors would give insights into the lesions induced by the inhibitors and genetic determinants of response. Ultimately, this information would be useful to target specific pathways to augment the therapeutic activity of topoisomerase inhibitors. To this end, we put together 48 isogenic DT40 mutant cells deficient in DNA repair and generated one cell line deficient in autophagy (ATG5). Sensitivity profiles were established for three clinically relevant Top1 inhibitors (camptothecin and the indenoisoquinolines LMP400 and LMP776) and three Top2 inhibitors (etoposide, doxorubicin, and ICRF-193). Highly significant correlations were found among Top1 inhibitors as well as Top2 inhibitors, whereas the profiles of Top1 inhibitors were different from those of Top2 inhibitors. Most distinct repair pathways between Top1 and Top2 inhibitors include NHEJ, TDP1, TDP2, PARP1, and Fanconi Anemia genes, whereas homologous recombination seems relevant especially for Top1 and, to a lesser extent, for Top2 inhibitors. We also found and discuss differential pathways among Top1 inhibitors and Top2 inhibitors. Mol Cancer Ther; 13(1); 214–20. ©2013 AACR.

Introduction
DNA topoisomerases are the target of widely used anticancer and antibacterial drugs (1–3). Topoisomerase I (Top1) releases DNA torsional stress during replication and transcription by cleaving one strand of duplex DNA and generating Top1-DNA cleavage complexes (Top1cc) that allow the untwisting of supercoiled DNA. Top1 inhibitors kill cycling malignant cells by trapping Top1cc, leading to their conversion to DNA double-strand breaks (DSB; ref. 4) when replication forks encounter the single-strand breaks (SSB). Although there are two major repair pathways, homologous recombination and nonhomologous end joining (NHEJ) for DSB repair, studies in yeast demonstrate that the DSBs induced by Top1 inhibitors are repaired by homologous recombination during S phase (5). Other repair pathways for Top1cc are excision of the 3′-tyrosyl-DNA bond by tyrosyl-DNA phosphodiesterase 1 (TDP1; refs. 6–8) and endonuclease cleavage and elimination of the DNA strand attached to the Top1 by Mus81-Eme1 (9), XPF-ERCC1 (10), and ChP (11). Two camptothecin derivatives, topotecan and irinotecan, are approved by the FDA and across the world as anticancer agents. However, because of their limitations (chemically instability, limited drug accumulation in drug efflux overexpressing cells, and reversible trapping of Top1cc ref. 12), noncamptothecin Top1 inhibitors, such as the indenoisoquinolines [LMP400 (NSC 743400) and LMP776 (NSC 725776)], are in clinical trial (13, 14). We previously reported that 1 μmol/L camptothecin generates similar amounts of Top1cc as 1 μmol/L of LMP400 or LMP776 (14).

Although topoisomerase II (Top2α and -β), like Top1, relaxes DNA supercoiling by cleaving the DNA backbone and forming transient tyrosyl-DNA cleavage complex intermediates (Top2cc), it differs from Top1 in several
ways (1, 15–17). They cleave both strands of one DNA duplex simultaneously to allow another duplex to pass through Top2-linked DSB. This enables Top2 not only to relax supercoiling but also to disentangle DNA knots and catenanes at the end of DNA replication. Thus, Top2 generates DSBs in cycling cells especially during mitosis phase, in which both homologous recombination and NHEJ are available for repair (18). Additional notable differences between Top2 and Top1 include the covalent linkage of Top2 at the 5'-rather than 3'-DNA ends of the breaks, and ATP requirement for catalysis. Top2 inhibitors are divided into two classes, catalytic inhibitors and poisons that trap Top2cc (1, 3, 19). A classic Top2 catalytic inhibitor is ICRF-193 (20), which blocks ATP hydrolysis and inhibits reopening of the ATPase domain in Top2, thereby trapping the crossing DNA inside the enzyme. Top2 poisons, on the other hand, inhibit the religation of Top2cc. They are widely used clinically and belong to two subclasses, nonintercalating and intercalating drugs. Nonintercalating drugs primarily represented by etoposide (VP-16) are highly specific for Top2cc (4, 21). Intercalators, such as doxorubicin, on the other hand, not only trap Top2cc but also kill cells by intercalation and generation of oxygen radicals (22). The removal of Top2cc primarily involves tyrosyl-DNA phosphodiesterase 2 (TDP2), which was discovered recently as the prevalent primary enzyme for excising Top2cc (23). Accordingly, knocking out TDP2 in human (23) or chicken DT40 cells (24) increases the cytotoxicity of etoposide.

Thus, various repair pathways related to numerous genes are required for cellular tolerance to topoisomerase inhibitors (25–28). However, it is hard to tell which genes are more important than others due to a lack of comprehensive system to compare the contribution of genes with drug sensitivity in vertebrate cells. Furthermore, it is not certain whether intercalators and nonintercalating Top2 inhibitors, or camptothecin and non–camptothecin Top1 inhibitors induce similar DNA lesions and are repaired by different pathways. The comprehensive analysis of DNA repair gene mutants would allow the characterization of the drug-induced DNA lesions, and identify genes that could be targeted to augment the selectivity and overcome resistance to topoisomerase inhibitors.

To this end, we organized a panel of isogenic DNA repair mutant chicken DT40 cell lines. Indeed, DT40 cells provide the largest collection of isogenic DNA repair mutant clones in vertebrate cells, due to high gene targeting efficiency and stable karyotype of the chicken B lymphocyte line (29). Furthermore, because DT40 cells lack G1/S checkpoint due to impaired p53 (30) and proliferate at an extremely high rate (>8 hours doubling time), DT40 and malignant cancer cells may share comparable characteristics in cellular responses to chemotherapeutic agents. Taking advantage of the panel of repair-deficient DT40 cells, we recently demonstrated the importance of PARP trapping for the anticancer activity of clinical PARP inhibitors (19). We also showed the relevance of such an approach with a small DT40 mutant library to screen for genotoxic agents (31).

In the present study, we profiled and compared the sensitivity of 49 DT40 mutant cells with three Top1 inhibitors (camptothecin and the two indenoisoquinolines in clinical trial, LMP400, and LMP776) and three Top2 inhibitors (the nonintercalator etoposide, the intercalator doxorubicin, and ICRF-193, a Top2 catalytic inhibitor). We included in the panel a novel knockout cell line (ATG5−/− cells) to examine the effect of autophagy in comparison with DNA repair.

Materials and Methods

Cell lines and cell culture

The DT40 cell lines used in this study were obtained from the Laboratory of Radiation Genetics, Graduate School of Medicine, Kyoto University (Kyoto, Japan) in 2011 to 2012. All the mutant cell lines, except for the ATG5−/− cell line, were previously authenticated by Southern blotting and/or real-time PCR and/or Western blotting (see the references of Supplementary Table S1). The gene disruption of ATG5 in ATG5−/− cells was authenticated in this study by Southern blotting (Supplementary Fig. S1). DT40 cells were cultured at 37°C with 5% CO2 in RPMI-1640 medium (11875-093; Invitrogen) supplemented with 1% chicken serum (16110-082; Invitrogen), 10−5 mol/L β-mercaptoethanol (M-3148; Sigma-Aldrich), penicillin–streptomycin (15140-122; Invitrogen), 1% FBS (100-106; Gemini Bio-Products).

Drug preparations

Camptothecin, LMP400 (NSC 743400), and LMP776 (NSC 725776) were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Drug stock solutions were made in dimethyl sulfoxide (DMSO) at 10 μmol/L for camptothecin and 100 μmol/L for LMP400 and LMP776. Etoposide (E1383; Sigma-Aldrich) and ICRF-193 (I4659; Sigma-Aldrich) were dissolved in DMSO at 1 mmol/L. Doxorubicin (D1515; Sigma-Aldrich) was dissolved in distilled water at 100 μmol/L. Paclitaxel (Taxol, T1912; Sigma-Aldrich) was dissolved in DMSO at 1 μmol/L. All stock solutions were stored at −20°C in dark. We diluted the stock solutions with culture medium. Maximum concentrations were 40 nmol/L for camptothecin, 240 nmol/L for LMP400, 120 nmol/L for LMP776, 800 nmol/L for etoposide, 1,600 nmol/L for ICRF-193, 50 nmol/L for doxorubicin, and 10 nmol/L for paclitaxel. Because of the hyper-resistance of NHEJ mutants (KU70−, LIGIV−, and DNA-PK−deficient cells) to camptothecin, we used 320 nmol/L camptothecin as a maximum concentration. We prepared five different concentrations by 1:2 serial dilution.

Measurement of cellular sensitivity

Two hundred DT40 cells in 20 μL of culture medium were seeded into 384-well white plates (#6007680; Perkin Elmer Life Sciences), and then added 20 μL of culture...
medium containing drugs. Most outer wells were not used to avoid error associated with an evaporation issue while PBS or culture medium was added in the most outer wells. Plates were incubated at 37°C for 72 hours, allowing untreated wild-type cells to divide ~9 times. Cell survival was determined using the ATPlite 1-step kit (Perkin Elmer Life Sciences). In brief, a 20-μL ATPlite solution was directly added to each well of 384-well white plates. Five minutes after adding the ATPlite solution, luminescence was measured by Envision 2104 Multilabel Reader (PerkinElmer). All procedures were performed in triplicate.

Evaluation of relative cellular sensitivity

One 384-well plate allowed us to examine sensitivity to two kinds of drugs in seven different cell lines at once. Wild-type cells were always included in each plate. To evaluate the relative cellular sensitivity of each mutant to wild-type cells, sensitivity curves were drawn by setting the survival of untreated cells as 100%, under conditions in which cell number per well was linearly correlated with fluorescence signal until 100,000 cells per well (Supplementary Fig. S2). IC₉₀ values (inhibition concentration 90%; IC₃₀) for each drug and cell line were determined as the crossing points between the 10% viability line and survival curve connecting average points for each drug concentration (see Supplementary Fig. S3A and S3B). The IC₃₀ of each mutant was divided by the IC₃₀ of wild-type cells that were cultured on the same plate, and then the quotient was converted into logarithmic scale (base 2). Each score was plotted on the same bar graph.

Flow cytometry

Cells were fixed with 70% ethanol, stained with propidium iodide, and then analyzed.

Statistical analyses

A Pearson correlation analysis was used to examine the correlation between drugs.

Generation of the ATG₅⁻/⁻ DT40 mutant cells

To generate ATG₅⁻/⁻ DT40 mutant cells, we constructed ATG₅-targeting vectors to replace exon 3 with puromycin and blasticidin resistance gene cassettes (puro and bsr; Supplementary Fig. S1). The primers used to amplify the left arm, including the probe sequence from DT40 genomic DNA, were 5'-GGGATGGCAGTGTCTCTTTATTTACTTCAAG-3' and 5'-TGAGGTCACTCCATGAATGAGAACGGTTT-3'. The amplified PCR product was digested with KpnI restriction enzyme, and the 5'-fragment was used as the probe for Southern blot whereas the 3'-fragment was used was for the Southern blot in which cell number per well was linearly correlated with fluorescence signal until 100,000 cells per well (Supplementary Fig. S2). IC₉₀ values (inhibition concentration 90%; IC₃₀) for each drug and cell line were determined as the crossing points between the 10% viability line and survival curve connecting average points for each drug concentration (see Supplementary Fig. S3A and S3B). The IC₃₀ of each mutant was divided by the IC₃₀ of wild-type cells that were cultured on the same plate, and then the quotient was converted into logarithmic scale (base 2). Each score was plotted on the same bar graph.

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Unexpected function of TDP2 and NHEJ for ICRF-193–induced lesions and complex effects of Top1cc repair

A closer analysis of the profiling patterns among Top2 inhibitors showed that the etoposide dependency for Top1 inhibitors was very similar to each other (Fig. 1 and Table 1). Although Top1 and Top2 inhibitors were both more active in homologous recombination–deficient cells, one of the most striking differences was their opposite effects in NHEJ-deficient cells. Indeed, KU70 knockout cells (and, to a lesser extent, LIGASE IV or DNA-PKcs knockout cells) were more than 10-fold resistant to camptothecin, whereas they were more than 10-fold hypersensitive to etoposide (black bars at the top of Fig. 1). Doxorubicin and ICRF-193 behave comparably with etoposide with respect to NHEJ, reinforcing the finding that NHEJ is specifically required for the repair of DSBs induced by Top2 inhibitors (33, 34).

Overall, the profiling patterns of the three Top1 inhibitors were very similar to each other (Fig. 1 and Table 1). Hypersensitivity to homologous recombination- (blue), RAD18-, PARP1-, TDP1-, CtIP-, and Fanconi Anemia (pink)–deficient/mutant cells underlines the importance of these genes for Top1cc repair (reviewed in ref. 28). Previous studies indicated that NHEJ has a toxic effect on competing DNA ends, and thereby the loss of NHEJ increases the cellular tolerance to camptothecin (33). Interestingly, mutants in core NHEJ factors (KU70, LIGASE IV, and DNA-PKcs shown with black bars) displayed different responses to the three Top1 inhibitors. Although all NHEJ mutants were resistant to camptothecin and weakly resistant to LMP776, there was neither sensitivity nor resistance to LMP400. These results suggest that targeted Top1 inhibitors can damage chromosomal DNA in a slightly different manner such that accessibility of NHEJ factors to the DSBs may differ for each Top1 inhibitor.

### Differential contribution of NHEJ to Top1cc and Top2cc repair

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![Figure 1: Sensitivity profiles of the indicated topoisomerase inhibitors in the selected DNA repair–deficient DT40 panel. Negative (left) and positive (right) scores show that the indicated cells are sensitive and resistant to the drug, respectively. The bars are colored according to the main DNA repair pathway of the indicated topoisomerase inhibitors in the selected DNA repair–deficient DT40 panel.](www.aacrjournals.org/MolCancerTher/article-pdf/13/1/217/4304872/MCT-13-0551.pdf)
Discussion

Usefulness of the DT40 repair panel profiling for categorizing DNA-damaging and genotoxic agents

In this study, we describe a comprehensive genetic analysis of multiple DNA repair pathways in response to topoisomerase inhibitors in 50 cell lines constituting a DT40 repair panel (DR panel). This is the first report comparing quantitatively and in parallel the significance of as many as 48 repair and one autophagy genes for drug sensitivity in a panel of vertebrate cells. The profiles of topoisomerase inhibitors targeting Top1 versus Top2 were highly correlated among each other. On the other hand, the profiles to Top1 inhibitors were very different from those of Top2 inhibitors. Extension of the DR panels to other classes of DNA-targeted drugs will probably further reveal its value by showing differences between drugs with different targets and mechanism of action. Using a subset of the DR panel, we recently discovered that clinical PARP inhibitors, such as olaparib and niraparib, target PARP-DNA complexes by trapping them on DNA and that not only homologous recombination but also replication bypass and Fanconi pathways are critical for the survival of cells treated with these PARP inhibitors (19). Because the DR panel includes multiple repair pathways, it could be used as a sensitive tool to screen potential genotoxic agents (31). Indeed, one would expect genotoxics to be selectively more cytotoxic in at least a subset of DNA repair–deficient cell lines. This point is supported by our demonstration that the microtubule inhibitor, paclitaxel (Taxol), showed no significant hypersensitivity in any of the DR panel lines.
Differences among topoisomerase inhibitors from the same group

Our comprehensive profiling revealed previously unappreciated differences within drug classes. Indeed, for the Top1 inhibitors, the sensitization conferred by NHEJ genes was different for camptothecin and the two indenoisoquinolines. Biochemical studies show that the indenoisoquinolines induce Top1cc at different genomic sites compared with camptothecin and tend to induce persistent Top1cc that can target transcription in addition to replication complexes (14, 40), suggesting that the accessibility of the NHEJ machinery to DSB ends might be different depending on the types of lesion induced by the indenoisoquinoline Top1 inhibitors.

For the Top2 inhibitors, it is unexpected that etoposide and ICRF-193 are more highly correlated with each other than etoposide and doxorubicin because etoposide and doxorubicin are both categorized as Top2 poisons, whereas ICRF-193 is categorized as a catalytic Top2 inhibitor (1, 3, 17, 20, 35). Considering that TDP2-deficient cells were weakly sensitive to doxorubicin but hypersensitive to etoposide, the contribution of Top2cc for cell killing seems higher for etoposide than doxorubicin. This result is supported by the fact that doxorubicin is not only a Top2cc-targeting drug but also a powerful DNA intercalator and inducer of oxygen radicals (22). On the other hand, the fact that ICRF-193 was significantly more cytotoxic in TDP2-deficient cells suggests the formation of Top2cc and induction of DNA breaks by ICRF-193, which is also consistent with the critical involvement of NHEJ for ICRF-193–treated cells.

Determination of targeting genes for synthetic lethality

The DR panel profiling enabled us to list up the priority genes to be targeted for synthetic lethality. For instance, the PARP1-deficient cells are among the most sensitive mutants to the three Top1 inhibitors, confirming the synergistic combination of Top1 and catalytic PARP inhibitors such as veliparib (10, 19). Likewise, inhibition of homologous recombination, TDP1, RAD18, as well as CtIP–BRCA1 binding, will synergize the cytotoxicity of Top1 inhibitors. On the other hand, the sensitivity of PARP1-deficient cells was equivalent to those of wild-type to the three Top2 inhibitors, suggesting that PARP inhibitors would not induce synthetic lethality with Top2 inhibitors. The profiles indicate that NHEJ genes, especially the KU70 gene, are the prior targets for Top2 inhibitors. Because many key genes involved in DNA damage and repair responses are often mutated in human cancer, if the information about the defective genes in each tumor cells is available in advance, we can expect more effective cell-killing effect by choosing the anticancer drug that induces the DNA damage repaired by the defective gene. The data presented in this study thus provide a rational approach to measure the importance of individual repair pathways and genes as targets in chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Maede, T. Nakamura, T. Miki, S. Takeda, Y. Pommier, J. Murai
Development of methodology: Y. Maede, T. Kogame, S. Takeda, Y. Pommier, J. Murai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Maede, T. Fukushima, T. Kogame, Y. Pommier, J. Murai
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Maede, H. Shimizu, Y. Pommier, J. Murai
Writing, review, and/or revision of the manuscript: Y. Maede, Y. Pommier, J. Murai

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Maede
Study supervision: Y. Maede, Y. Pommier, J. Murai

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