NK314, a Topoisomerase II Inhibitor That Specifically Targets the α Isoform*

Received for publication, May 22, 2008, and in revised form, June 27, 2008. Published, JBC Papers in Press, July 2, 2008, DOI 10.1074/jbc.M803936200

Eriko Toyoda†§, Shigehide Kagaya‡, Ian G. Cowell†, Aya Kurosawa†, Keiichi Kamoshita§, Kiyohiro Nishikawa†, Susumu Iizumi‡, Hideki Koyama‡, Caroline A. Austin,§ and Noritaka Adachi†∥

From the †International Graduate School of Arts and Sciences, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan, the ‡Pharmaceutical Research Laboratories, Nippon Kayaku Co., Ltd., 31-12, Shimo 3-chome, Kita-ku, Tokyo 115-8588, Japan, and the §Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle-upon-Tyne, NE2 4HH United Kingdom

Topoisomerase II (Top2) is a ubiquitous nuclear enzyme that relieves torsional stress in chromosomal DNA during various cellular processes. Agents that target Top2, involving etoposide, doxorubicin, and mitoxantrone, are among the most effective anticancer drugs used in the clinic. Mammalian cells possess two genetically distinct Top2 isoforms, both of which are the target of these agents. Top2α is essential for cell proliferation and is highly expressed in vigorously growing cells, whereas Top2β is nonessential for growth and has recently been implicated in treatment-associated secondary malignancies, highlighting the validity of a Top2α-specific drug for future cancer treatment; however, no such agent has been hitherto reported. Here we show that NK314, a novel synthetic benzo[c]phenanthridine alkaloid, targets Top2α and not Top2β in vivo. Unlike other Top2 inhibitors, NK314 induces Top2-DNA complexes and double-strand breaks (DSBs) in an α isoform-specific manner. Heterozygous disruption of the human Top2α gene confers increased NK314 resistance, whereas TOP2β homozygous knock-out cells display increased NK314 sensitivity, indicating that the α isoform is the cellular target. We further show that the absence of Top2β does not alleviate NK314 hypersensitivity of cells deficient in non-homologous end-joining, a critical pathway for repairing Top2-mediated DSBs. Our results indicate that NK314 acts as a Top2α-specific poison in mammalian cells, with excellent potential as an efficacious and safe chemotherapeutic agent. We also suggest that a series of human knock-out cell lines are useful in assessing DNA damage and repair induced by potential topoisomerase-targeting agents.

DNA topoisomerase II (Top2)² is a ubiquitous nuclear enzyme that alters the topological structure of DNA and chromosomes through a transient DNA double-strand break (DSB) and subsequent religation of the DSB (1, 2). The enzyme has been implicated in many aspects of DNA metabolism, including DNA replication, repair, transcription, and chromosome condensation/segregation (1, 3). Top2 has been of considerable interest to human medicine, because it is an important target for cancer chemotherapy (4). Top2-targeting agents, involving etoposide, doxorubicin, and mitoxantrone, are among the most effective and widely used anticancer drugs in cancer chemotherapy (5, 6). These agents are referred to as “Top2 poisons,” because they convert the essential enzyme into a highly cytotoxic DNA-damaging agent through the formation of “cleavage complex” (also called “cleavable complex”), in which a Top2-linked DNA strand-passing intermediate is stabilized, allowing the generation of a DSB (7, 8).

Mammalian cells possess two genetically distinct Top2 isoforms (9, 10). Despite their similar structural features (~70% identity at the amino acid level) and biological properties, the two isoforms are differentially regulated and play different roles in living cells. Top2α is most abundantly expressed in rapidly growing tissues and its expression is cell cycle-regulated, peaking in G2/M, whereas Top2β is expressed in virtually all tissues and it is expressed throughout the cell cycle (11–13). Top2α has been shown to be essential for cell proliferation and embryonic development (14, 15). In mitosis, only Top2α associates with chromosomes, playing a unique role in chromosome segregation that cannot be substituted by Top2β (14–18). By contrast, despite its apparent roles in transcription (for example, Ref. 19), Top2β is dispensable for cell survival (20), although it has been implicated in neuronal differentiation (20–24).

The fact that Top2α, relative to Top2β, is highly expressed in tumor cells (21, 23, 25, 26) implies the validity of α isoform-specific Top2 inhibitor in cancer treatment. Indeed, two studies suggested that Top2α, rather than Top2β, was the determinant of cytotoxic effects of etoposide (27, 28). Even more intriguingly, Azarova et al. (27) suggested that Top2β may be responsible for the development of secondary malignancy associated with etoposide treatment. Hence, it is quite reasonable to expect that α isoform-specific Top2 poisons will be efficacious

MEF, mouse embryonic fibroblast; NHEJ, non-homologous end-joining; PFGE, pulsed-field gel electrophoresis; siRNA, small interfering RNA; TARDIS, trapped in agarose DNA immunostaining; PBS, phosphate-buffered saline.

*This work was supported in part by Yokohama City University Strategic Research Project Grants W18006 and K19009 (to N. A.), and by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan Project numbers 18590063, 18058019, and 18018034 (to N. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: International Graduate School of Arts and Sciences, Yokohama City University, Seto 22-2, Kanazawa-ku, Yokohama 236-0027, Japan. Fax: 81-45-787-2228; E-mail: nadachi@yokohama-cu.ac.jp.

‡The abbreviations used are: Top2, topoisomerase II; DMSO, dimethyl sulfoxide; DSB, double-strand break; ICE, in vivo complex of enzyme;
A Topoisomerase IIα-specific Drug

and safe chemotherapeutic agents with reduced risk of treat-
ment-related secondary malignancies. To our knowledge, how-
ever, no such agent has been reported thus far.

NK314 is a novel synthetic benzo[c]phenanthridine alkaloid
that exhibits strong antitumor activity (29). We previously
reported that the drug stabilizes Top2 cleavage complexes and
induces rapid DSBs to cause G2 arrest in tumor cells (29, 30).
In this article, we find that NK314 acts as an α isoform-specific
Top2 poison in living mammalian cells. We demonstrate that
NK314 induces Top2-DNA complexes and chromosomal DSBs
in a Top2α-dependent manner. Furthermore, with the use of a
series of human gene knock-out cell lines, we genetically inves-
tigate DNA damage and repair after NK314 treatment. This is
the first report on the discovery and characterization of an α
isoform-specific Top2 poison.

EXPERIMENTAL PROCEDURES

Topoisomerase Inhibitors—NK314 and etoposide were syn-
thesized at Nippon Kayaku (Tokyo, Japan). Doxorubicin hydro-
chloride and mitoxantrone hydrochloride were purchased
from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and Wyeth
K.K. (Tokyo, Japan), respectively. Camptothecin, amsacrine,
and XK469 were purchased from Sigma. ICRF-193 was pur-
based from Funakoshi (Tokyo, Japan). Etoposide, mitox-
antrone, amsacrine, ICRF-193, and XK469 were dissolved in
dimethyl sulfoxide (DMSO). NK314 and doxorubicin were dis-
solved in distilled water. All the drugs were stored frozen
in aliquots at −20 °C.

Cells and Culture Conditions—All cells were maintained at
37 °C in a humidified atmosphere containing 5% CO2. The
human pre-B cell line Nalm-6 and its derivatives were cultured
in ES medium (Nissui Seiyaku Co., Tokyo, Japan) supple-
mented with 10% calf serum (HyClone, Logan, UT) and 50 μM
2-mercaptoethanol. The human cervical carcinoma cell line
HeLaS3 was maintained in Eagle’s minimal essential medium
(Asahi Glass Co., Ltd., Chiba, Japan) containing 10% fetal
bovine serum and 50 μg/ml kanamycin sulfate. The kidney can-
cer cell line ACHN (ATCC CRL 1611) was maintained in
Eagle’s minimal essential medium supplemented with 10% fetal
bovine serum and 1% non-essential amino acids. The non-small
cell lung cancer cell line H460 (ATCC HTB 177) was main-
ained in RPMI 1640 medium (Asahi Glass Co., Ltd.) supple-
mented with 10% fetal bovine serum, 1 mM sodium pyruvate,
and 10 mM Heps. The colorectal adenocarcinoma cell line
DLD-1 was obtained from JCRB Cell Bank (Tokyo, Japan) and
maintained in RPMI 1640 medium supplemented with 10%
fetal bovine serum. Mouse embryonic fibroblasts (MEFs)
(Top2β+/+ and Top2β−/− cells) were cultured in 25-cm² tissue
culture flasks in Dulbecco’s modified Eagle’s medium contain-
ing 10% fetal calf serum and 1× penicillin/streptomycin.

Pulsed-field Gel Electrophoresis (PFGE) Analysis—Cells were
treated with Top2 inhibitor for 1 h, and chromosome-sized
DNA was prepared from the cells using the CHEF Genomic
DNA Plug Kit (Bio-Rad). Briefly, cells (1×10⁶) were embedded
to agarose plugs, and the plugs were treated with lysis buffer (10
mm Tris-HCl (pH 8.0), 500 mm EDTA, 1% Sarkosyl) in the pres-
ence of 1 mg/ml protease K at 50 °C for 2 days, and washed
three times with 50 mm EDTA. Plugs were loaded onto a 1%
attached to a cooled slow scan CCD camera. For each of three randomly chosen fields of view, images of Hoescht 33258 (blue) and fluorescein isothiocyanate (green) fluorescence were captured to give a total of ~100 cells/dose for each antibody. 16-Bit images were then analyzed to quantify the levels of blue and green fluorescence. All images were corrected for stray light and camera background and were subjected to blue and green shade correction to compensate for variations in intensity of illumination and non-uniformities in light transmission (34). Graphing and statistical analysis was carried out using GraphPad Prism software (Cherwell Scientific, Oxford, UK).

**Top2 Assays—** Human Top2α and Top2β were purified as described previously (35) and used for in vitro Top2 assays. Decatenation assay was performed by using a Topo II Assay Kit (TopoGEN, Inc.). Briefly, 0.2 μg of kinetoplast DNA was incubated with Top2α or Top2β at 37 °C for 15 min in 20 μl of 10 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM ATP, and 30 μg/ml bovine serum albumin. One unit of activity is defined as the amount of Top2 enzyme that decatenates 0.2 μg of kinetoplast DNA under standard conditions.

To examine the inhibitory effect of NK314 and etoposide on Top2α or Top2β catalytic activity, 0.2 μg of kinetoplast DNA was incubated with 2 units of Top2α or Top2β in 20 μl of reaction buffer containing 5% DMSO at 37 °C for 15 min in the presence or absence of NK314 or etoposide. The reaction was stopped by adding 5 μl of loading dye (5% Sarkosyl, 0.0025% bromphenol blue, and 25% glycerol) and electrophoresed in a 1% agarose gel containing 0.5 μg/ml of ethidium bromide in TBE buffer.

DNA cleavage assay was performed by using a Topo II Drug Screening Kit (TopoGEN, Inc.). Briefly, 0.2 μg of pRYG plasmid was incubated with 5 units of Top2α or Top2β in 20 μl of assay buffer containing 5% DMSO at 37 °C for 30 min in the presence or absence of NK314 or etoposide. DNA cleavage product was trapped by the addition of 2 μl of 10% SDS, and 2.5 μl of 10 mg/ml proteinase K was added to the sample, which was incubated for 30 min at 37 °C to digest Top2. The samples were mixed with 2.5 μl of loading buffer and cleaned up by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After brief vortex mixing, the sample was spun in a microcentrifuge for 5 s. An aliquot (10 μl) of the upper aqueous phase was electrophoresed in a 1% agarose gel containing 0.5 μg/ml of ethidium bromide in TBE buffer.

**Small Interfering RNA (siRNA)-mediated Gene Knockdown—** Top2 knockdown experiments were carried out in the same manner as described previously (36). The Top2α-targeting siRNA corresponds to nucleotides 76 to 96, and the Top2β siRNA to nucleotides 86 to 106 (relative to the first nucleotide of the start codon). These siRNAs were purchased from Invitrogen. As a nonspecific control, non-silencing control siRNA was used (sense, 5'-UUUCUGCCAGGUUGACAGCAGCAUdTdT-3'; antisense, 5'-ACGGUGACUGUUCCGAAGAdAddTdT-3'; Qiagen, Japan). HeLa cells were transfected with siRNA using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Top2α, Top2β, and non-silencing control siRNAs were achieved to final concentrations of 1, 10, and 100 nM, respectively. Twenty-four hours after transfection, cells were collected by trypsinization and plated at 2.5 × 10⁵ cells in 60-mm dishes and cultured for 48 h, followed by Western blot analysis or clonogenic assays. For clonogenic assays, cells were cultured for 1 h in the presence of NK314. After washing twice with PBS, the cells were trypsinized, collected in 15-ml Falcon tubes, and counted. Cells were plated at 10⁴–10⁵ cells/dish into 60-mm dishes. After a 2-week incubation, colonies were fixed with methanol, stained with methylene blue, and counted. The percent survival was determined by comparing the number of surviving colonies to untreated controls.

**TOP2 Targeting Vectors—** Targeting vectors were constructed by using a simplified construction method based on the MultiSite Gateway® Technology (Invitrogen) as described (37). Genomic DNA fragments were obtained by PCR amplification with ExTaq DNA polymerase (Takara Bio, Otsu, Japan) from Nalm-6 genomic DNA using the following primers: Top2A5F (5'-GGGGGACACGTGTGTATGAAAAGTGTTAAGGGACAACAAACAGG-3') and Top2ASR (5'-GGGGGACGTGTTTGTGTACAAAATGAGTGAAGTCGG-3') for Top2α 5'-arm, Top2AF (5'-GGGGGACACGTGTTTGTGTACAAAATGAGTGAAGTCGG-3') for Top2β 3'-arm, Top2BSF (5'-GGGGGACACGTGTTTGTGTACAAAATGAGTGAAGTCGG-3') and Top2B3R (5'-GGGGGACACGTGTTTGTGTACAAAATGAGTGAAGTCGG-3') or Top2B 3'-arm. The Top2α and Top2β targeting vectors were linearized with I-SceI or AdhI, respectively, prior to transfection.

**Generation of Human Gene Knock-out Cell Lines—** DNA transfection for gene targeting was performed in Nalm-6 cells as described previously (38–40). Briefly, to generate Top2α⁺/- and Top2β⁺/- cells, 4 × 10⁶ wild-type cells were electroporated with 4 μg of linearized targeting vector, cultured for 22–24 h, and replated at a density of 0.5–1 × 10⁶ per 90-mm dish into agarose medium containing 0.4 mg/ml hygromycin B (Wako Pure Chemical, Osaka, Japan). After a 2–3-week incubation, hygromycin-resistant colonies were isolated and genomic DNA was prepared from each clone. Primers used for PCR screening were Top2A5CC (5'-TTATGCGCCTGTTTGTTCG-3') and universal primer A (5'-AGTGTGCAGATAGAGGACCTG-3') (37) for Top2α targeting, and Top2B5CC (5'-AGTGTGCAGATAGAGGACCTG-3') and universal primer B (5'-AGTGTGCAGATAGAGGACCTG-3') for Top2β targeting. The Top2α and Top2β targeting vectors were amplified with I-SceI or AdhI, respectively, prior to transfection.

3 A. Kurosawa, H. Koyama, S. Iiizumi, S. So, S. Nakamura, K. Iwabuchi, M. Lieber, and N. Adachi, unpublished results.
A Topoisomerase IIα-specific Drug

Drug Sensitivity Assays—Clonogenic assays using Nalm-6 and its derivative cell lines were performed as described previously (39). Briefly, exponentially growing cells were plated at 10^5–10^6 cells/dish into 60-mm dishes containing 5 ml of agarose medium with various concentrations of DNA-damaging agents. After a 2–3-week incubation at 37 °C, visible colonies were counted, and the percent survival was determined by comparing the number of surviving colonies to untreated controls. For growth inhibition assays, 1 × 10^4 cells were seeded into 48-well plates and cultured for 96 h in growth medium containing various concentrations of topoisomerase inhibitor. Cell proliferation was measured by using the CellTiter-Glo® Luminescence Viability Assay Kit (Promega) or methylene blue staining. Methylene blue staining was performed as previously described (41). Briefly, cells were stained with 0.05% methylene blue dissolved in 10 mM Tris buffer (pH 8.5) for 30 min, and then washed thoroughly with distilled water. The stained dye was extracted with 3% HCl, and the absorbance at 660 nm was measured.

Survival Assays with MEFs—MEFs (9 × 10^4 cells) at 50–80% confluence were plated in each of a series of 60-mm dishes. Cells were cultured in these dishes for 48 h before adding NK314. Serial dilutions of NK314 were prepared, resulting in final concentrations of 4–1024 nM in the dishes. Two hours after drug addition, the drug-containing medium was removed and plates were washed with PBS. Cells were trypsinized, collected in 15-ml Falcon tubes, and counted. Cells were plated in duplicate at 2 × 10^2–5 × 10^4 cells per 90-mm dish. After 8 days, colonies were fixed with Carnoy’s solution, stained with crystal violet, and counted.

RESULTS

NK314 Specifically Induces Top2α-DNA Complex—We previously showed that NK314 induced Top2 complexes and DSBs in several tumor cell lines (29, 30). In this article, we first performed PFGE analysis of genomic DNA from drug-treated Nalm-6 cells (Fig. 1A). We employed this human cell line for two reasons. First, Nalm-6 expresses roughly equal levels of Top2α and Top2β (Fig. 1B), which should be advantageous to analyze the relative contribution of Top2 isoforms to DNA damage and cytotoxicity. Second, the cell line enables rapid production of gene knock-out cell lines by gene targeting (37, 42) (see below). As expected, we found that NK314 rapidly induces chromosomal DSBs, and this DSB induction was significantly alleviated by pretreatment with ICRF-193 (Fig. 1A, lanes 3 and 4), a Top2 inhibitor that does not stabilize the cleav-
Whole cell extract was prepared from mock-transfected cells (transfected with Top2α resistance. pendent experiments. Where absent, SDS-polyacrylamide gel. Levels of expression were quantified using an image analyzer. Ku70 served as a contrast, similar levels of Top2α complexes were significantly reduced in cells pretreated with ICRF-193 (data not shown). These results indicate that, unlike other Top2 drugs such as etoposide, NK314 induces Top2-DNA complex in a manner highly specific to the α isoform. It should be noted, however, that NK314 at higher concentrations (10 µM in HeLa cells; Fig. 1D) can induce Top2β complexes, although to a much lesser extent than Top2α complexes. In fact, the TARDIS assay (28) on MEFs revealed Top2β complexes in NK314-treated cells, although less than in etoposide-treated cells (Fig. 1E).

We next sought to examine whether NK314 specifically inhibits Top2α in vitro. For this purpose, we performed a in vitro DNA cleavage assay using a plasmid with a Top2 cleavage consensus sequence (45). As shown in Fig. 1F, NK314 induced DNA cleavage in the presence of Top2α (lanes 6–8), as evidenced by the appearance of linear DNA (marked by L). Such Top2-mediated DNA cleavage, however, was hardly observed with the β isoform (lanes 13–15). By contrast, etoposide-induced DNA cleavage was similarly detected with both Top2α (lanes 9 and 10) and Top2β (lanes 16 and 17). We note that DNA binding activity of Top2β is not inhibited by NK314 in vitro, as evidenced by electrophoretic mobility shift assay (data not shown). We then performed a decatenation assay using kinetoplast DNA, a catenated DNA substrate. Etoposide inhibited the decatenation activity of both Top2α and Top2β (data not shown). Perhaps surprisingly, NK314 inhibited the decatenation activity of Top2β, to an extent similar to that of Top2α (Fig. 1G). Additionally, DNA relaxation assays showed that NK314 inhibited the relaxation activity of Top2β as well as Top2α (data not shown). The finding that NK314, in vitro, can inhibit the decatenation and relaxation activity of both Top2 isoforms may possibly explain the results of the above-mentioned DNA cleavage assay, in which similar amounts of supercoiled and relaxed DNA were observed with Top2α and Top2β by NK314 treatment, despite apparently different amounts of linear DNA product (Fig. 1F, lanes 6–8 versus lanes 13–15).

Top2α, Not Top2β, Is Responsible for NK314-induced DNA Damage and Cytotoxicity —Our finding that NK314 preferentially induces Top2α complexes suggests that the expression level of Top2α, rather than Top2β, determines the cytotoxic effect of NK314 in living mammalian cells. To test this, we performed gene knockdown experiments in HeLa cells using siRNA (36). siRNA-transfected cells were subjected to clonogenic survival
assays 3 days after transfection, when optimal silencing of Top2 expression was achieved (Fig. 2A). As expected, cells transfected with Top2α siRNA exhibited increased resistance to NK314, relative to those transfected with control siRNA or Top2β siRNA, which had little or no influence on NK314 cytotoxicity (Fig. 2B).

To further investigate the relative contribution of each Top2 isoform to NK314-induced cytotoxicity, we generated human TOP2α+/− and TOP2β+/− cells by gene targeting in the Nalm-6 cell line (Figs. 3A and 4A). Heterozygous disruption of TOP2α and homozygous disruption of TOP2β were confirmed by Southern and Western blot analysis (Figs. 3, B and C, and 4, B and C; note that Top2α levels in Top2α+/− cells were decreased to ~50% that of wild-type cells). TOP2β gene disruption did not affect Top2α levels, and vice versa; and cell proliferation was marginally affected by these gene-targeting events (Figs. 3, C and D, and 4, C and D). Using these mutant human cell lines, we performed clonogenic survival assays in the presence of NK314 or other Top2-targeting drugs. As shown in Fig. 5, TOP2α+/− cells exhibited increased resistance to NK314 as well as to other Top2 inhibitors, etoposide, doxorubicin, amsacrine, mitoxantrone, and XK469. In sharp contrast, TOP2β−/− cells did not display an increased resistance, but slightly increased sensitivity to NK314. Such increased sensitivity was not observed with other Top2 inhibitors; rather, increased resistance was observed in TOP2β+/− cells (Fig. 5, B–F), confirming that these inhibitors all target Top2β as well as Top2α in a cell. Of note, neither Top2α nor TOP2β gene targeting significantly affected cellular sensitivity to camptothecin, a potent topoisomerase I (Top1) poison (7) (Fig. 5G), serving as controls to indicate that increased sensitivity/resistance to Top2 inhibitors observed in the mutant cell lines results from changes in Top2 expression status itself. To examine whether NK314 actually induces Top2α-dependent chromosomal DSBs, we carried out PFGE analysis using genomic DNA from drug-treated cells. As shown in Fig. 5H, NK314 induced chromosomal DSBs at lower levels in TOP2α+/− cells than in wild-type (TOP2α+/+) or TOP2β−/− cells. (Note that TOP2β−/− cells show wild-type levels of DSBs.) By contrast, etoposide induced lower levels of DSBs in both TOP2α+/− and TOP2β−/− cells than in wild-type cells. Finally, to test the possibility that Top1 could be involved in NK314 cytotoxicity, we performed heterozygous disruption of the human TOP1 gene. The TOP1+/− cells had ~30% decreased Top1 levels and showed increased resistance to the Top1 poison camptothecin (data not shown). However, the TOP1+/− mutant exhibited wild-type levels of sensitivity to NK314 (and etoposide), clearly eliminating the possibility that NK314 targets Top1 in vivo (data not shown).

Together, these results show that Top2α is responsible for NK314-induced cytotoxicity and chromosomal DSBs in living cells. We also performed survival assays for NK314 using MEFs, and confirmed no contribution of Top2β to NK314-induced cytotoxicity (Fig. 6).

Human Cells Deficient in Non-homologous End-joining Are Hypersensitive to NK314, Although to a Lesser Extent Than Etoposide and Doxorubicin—Repair of drug-induced DNA damage, in general, is one of the key factors that determine the cellular sensitivity to, and the efficacy of, the drug. It is thus important to elucidate the repair mechanisms responsible for drug-induced DNA
A Topoisomerase IIα-specific Drug

To investigate repair mechanisms of NK314-induced DSBs in human somatic cells, we employed a series of knock-out cell lines, involving those lacking Rad54 and/or DNA ligase IV (Lig4), which are key components of homologous recombination and NHEJ, respectively (38, 52–54). As shown in Fig. 7A, Rad54−/− cells showed only slightly increased sensitivity to NK314, whereas Lig4−/− cells showed much higher sensitivity to NK314, and Lig4−/−Rad54−/− cells were slightly more hypersensitive than Lig4−/− cells. These results indicate that the NHEJ pathway is important for repairing NK314-induced DSBs, whereas the homologous recombination pathway only plays a minor role in the repair. Similar results were obtained with etoposide and doxorubicin (Fig. 7, B and C), consistent with the notion that Top2-mediated DSBs rely heavily on NHEJ repair in animal cells. It should be noted, however, the NHEJ dependence of NK314 appears to be less prominent than that of other Top2 inhibitors; for instance, IC90 comparison indicated that Lig4−/− cells were ~20 times more sensitive than wild-type cells to etoposide, but only 2.7 times more sensitive to NK314 (Fig. 7, H and I). More specifically, Lig4−/− cells did not show increased resistance to NK314, unlike other inhibitors, at very low concentrations. This may imply that a repair pathway(s) other than NHEJ can efficiently repair NK314-induced DSBs. Alternatively, or additionally, NK314, compared with other inhibitors, might require substantial amounts in a cell to exert cytotoxicity (see “Discussion”).

We next sought to examine whether the less prominent NHEJ dependence of NK314 could result from its nature as an α isoform-specific poison. If NHEJ repair was much more important for Top2β-mediated DNA damage than for Top2α-mediated DNA damage, then NHEJ-deficient cells would show greatly increased etoposide resistance in the absence of Top2β, perhaps with similar survival curves to those for NK314. To test this possibility, we performed Top2α and Top2β gene targeting in a Lig4−/− background (Figs. 3 and 4). The resulting Lig4−/−Top2α−/− and Lig4−/−Top2β−/− cells had normal growth properties (Figs. 3D and 4D). As shown in Fig. 7E, Top2β disruption only slightly alleviated etoposide hypersensitivity of Lig4−/− cells, to an extent similar to that observed in wild-type (Lig4+/+) cells. This finding suggests that Top2β-mediated DNA damage does rely on NHEJ repair, implying that the above-mentioned less prominent NHEJ dependence of
NK314 cannot be attributable to α isoform-specific poisoning by this drug. As shown, TOP2α heterozygous disruption conferred greatly increased resistance to NK314 and etoposide in the Lig4−/− background (Fig. 7, F and G). These observations further support the conclusion that NK314 only targets the Top2α isoform of Top2 in vivo.

NK314 cytotoxicity is less dependent on exposure time than etoposide cytotoxicity. As shown, TOP2β disruption had no effect on NK314 sensitivity of Lig4−/− cells (Fig. 7D), whereas TOP2α heterozygous disruption conferred greatly increased resistance to NK314 and etoposide in the Lig4−/− background (Fig. 7, F and G). These observations further support the conclusion that NK314 only targets the α isoform of Top2 in vivo.

NK314 Cytotoxicity Is Less Dependent on Exposure Time Than Etoposide Cytotoxicity—As the above described clonogenic survival assays were all performed in the presence of relatively low drug concentrations (i.e., continuous drug exposure), we next performed these experiments after a 1-h treatment of cells with NK314 or etoposide. Again, Lig4−/− cells were found to be highly sensitive to NK314 and etoposide, whereas RAD54−/− cells showed no increased sensitivity to these agents (Fig. 8, A and B). These results do confirm that etoposide (B), and doxorubicin (C). D and E, sensitivities of wild-type, TOP2β−/−, Lig4−/−, and Lig4−/−/TOP2β−/− cells to NK314 (D) and etoposide (E). F and G, sensitivities of wild-type, TOP2α−/−, Lig4−/−, and Lig4−/−/TOP2α−/− cells to NK314 (F) and etoposide (G). In all assays, cells were allowed for colony formation in agarose medium containing the indicated concentrations of drugs. Data are the mean ± S.D. of three to six independent experiments. Where absent, error bars fall within symbols. H, summary of sensitivity assays shown in A and B. I, summary of sensitivity assays shown in D–G.
NHEJ is indeed important for repairing Top2-mediated DNA damage. Similarly importantly, it should be emphasized that ~20 times higher concentration was required for etoposide to achieve a 90% inhibition in wild-type Nalm-6 cells (5068 nm for 1-h treatment versus 277 nm for continuous exposure), whereas at most a 5 times higher concentration was required for NK314 (457 versus 98 nm) (Fig. 8C). Interestingly, the difference was more prominent in the LIG4−/− mutant (1329 versus 14 nm for etoposide, and 201 versus 36 nm for NK314) (Fig. 8C), suggesting that the cytotoxic effect of NK314 is less dependent on exposure time than that of etoposide. To further confirm this, we treated various human cancer cell lines with NK314 or etoposide and compared the IC50 values for short (1 h) and long (72 h) exposures. As expected, the concentration of etoposide to achieve 50% growth inhibition was 10–50 times higher for a short exposure than for a long exposure, whereas that of NK314 was only ~2–3 times higher (Fig. 8D). Together, these results suggest that, compared with etoposide, NK314 exerts its cytotoxic effect in an exposure time-independent manner.

DISCUSSION

Etoposide, doxorubicin, and mitoxantrone all target both isoforms of Top2, and are among the most effective anticancer drugs in clinical use; however, these drugs often cause serious side effects, such as secondary malignancies. Recently, Azarova et al. (27) presented evidence that in the absence of Top2β less melanomas developed, suggesting that the β isoform is involved in the development of these malignancies. This, together with the fact that Top2α, the isoform essential for cell growth, is typically highly expressed in rapidly growing cancer cells, strongly supports the idea that Top2α-specific drugs may be a valuable novel approach for cancer treatment. In this article, we have reported for the first time an α isoform-specific Top2 inhibitor, NK314. We have shown that NK314 induces Top2 complexes and chromosomal DSBs in a Top2α-dependent manner. Furthermore, with the use of human gene knock-out mutants, we have genetically investigated isoform specificity of, and the repair mechanisms for DNA damage induced by, NK314. Our results unequivocally indicate that NK314 specifically induces Top2α-mediated DSBs, which are preferentially repaired by the NHEJ pathway. Although further work is required to establish that NK314 is a promising drug candidate for cancer treatment, our finding that NK314 is a specific Top2α poison suggests that NK314 may serve as an anticancer agent that does not cause secondary malignancies. It will thus be particularly interesting to examine whether NK314 treatment does or does not lead to deleterious chromosomal translocations or chromosomal rearrangements.

In the present study, we have conducted genetic analyses using gene knock-out human cell lines to investigate isoform specificity of NK314. Our data suggest that the α isoform is responsible for NK314 cytotoxicity. Additionally, we have examined the relative contribution of Top2 isoforms to cytotoxicity of other Top2 drugs. For instance, our results confirm that the α isoform is the major determinant of etoposide and doxorubicin cytotoxicity; in contrast, the β isoform does significantly contribute to mitoxantrone and XK469 cytotoxicity (see Fig. 5, B–F). (Note that XK469 was reported to be a β isoform-specific inhibitor in previous work (55), but in our cell lines this is not the case.) It should be emphasized that because these human cell lines have been created by targeted gene disruption, the isogenicity between the cell lines is otherwise completely retained. Furthermore, the Nalm-6 cell line has normal p53 status (56) and expresses nearly equal levels of Top2α and Top2β (Fig. 1B). These human cell mutants described here will be invaluable for studying the role of each topoisomerase in the cytotoxicity of potential anticancer agents.

Acknowledgments—We thank Dr. Akihiko Kikuchi for providing the anti-human Top2 monoclonal antibody 7B9. We also thank Haruna Kamekawa for help in performing some of the Top2 assays.

REFERENCES

1. Wang, J. C. (1996) *Annu. Rev. Biochem.* 65, 635–692
2. Dong, K. C., and Berger, J. M. (2007) *Nature* 450, 1201–1205
3. Wang, J. C. (2002) *Nat. Rev. Mol. Cell Biol.* 3, 430–440
4. Li, T. K., and Liu, L. F. (2001) *Annu. Rev. Pharmacol. Toxicol.* 41, 53–77
5. Osheroff, N. (1989) *Biochemistry* 28, 6157–6160
6. Cummings, J., and Smyth, J. F. (1993) *Annu. Oncol.* 4, 533–543
7. Liu, L. F. (1989) *Annu. Rev. Biochem.* 58, 351–375
8. Nitis, J. L., and Wang, J. C. (1996) *Mol. Pharmacol.* 50, 1095–1102
9. Austin, C. A., and Marsh, K. L. (1998) *Bioessays* 20, 215–226
10. Champoux, J. J. (2001) *Annu. Rev. Biochem.* 70, 369–413
11. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth & Differ.* 2, 209–214
12. Padget, K., Pearson, A. D., and Austin, C. A. (2000) *Leukemia* (Basingstoke) 14, 1997–2005
13. Adachi, N., Nomoto, M., Kohno, K., and Koyama, H. (2000) *Gene (Amst.)* 245, 49–57
14. Akimitsu, N., Adachi, N., Hirai, H., Hossain, M. S., Hamamoto, H., Koba- yashi, M., Aratani, Y., Koyama, H., and Sekimizu, K. (2003) *Genes Cells* 8, 393–402
15. Carpenter, A. J., and Porter, A. C. (2004) *Mol. Biol. Cell* 15, 5700–5711
16. Christensen, M. O., Larsen, M. K., Barthelmes, H. U., Hock, R., Andersen, C. L., Kjeldsen, E., Knudsen, B. R., Westergaard, O., Boege, F., and Mielke, C. (2002) *J. Cell Biol.* 157, 31–44
17. Linka, R. M., Porter, A. C., Volkov, A., Mielke, C., Boege, F., and Christensens, M. O. (2007) *Nucleic Acids Res.* 35, 3810–3822
18. Chaly, N., Chen, X., Denty, J., and Brown, D. L. (1996) *Chromosome Res.* 4, 457–466
19. Ju, B. G., Lunyak, V. V., Perissi, V., Garcia-Bassets, I., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2006) *Science* 312, 1798–1802
20. Yang, X., Li, W., Prescott, E. D., Burden, S. J., and Wang, J. C. (2000) *Science* 287, 131–134
21. Tsutsui, K., Kosoya, O., Sano, K., and Tokunaga, A. (2001) *J. Comp. Neurol.* 431, 228–239
22. Tsutsui, K., Sano, K., Kikuchi, A., and Tokunaga, A. (2001) *J. Biol. Chem.* 276, 5769–5778
23. Lyu, Y. L., and Wang, J. C. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 7123–7128
24. Zandvliet, D. W., Hanby, A. M., Austin, C. A., Marsh, K. L., Clark, I. B., Wrighton, N. A., and Poulsom, R. (1996) *Biochim. Biophys. Acta* 1307, 239–247
25. Capranico, G., Tinelli, S., Austin, C. A., Fisher, M. L., and Zunino, F. (1992) *Biochim. Biophys. Acta* 1132, 43–48
26. Watanabe, M., Tsutsui, K., and Inoue, Y. (1994) *Neurosci. Res.* 19, 51–57
27. Azarova, A. M., Lyu, Y. L., Lin, C. P., Tsai, Y. C., Lau, J. Y., Wang, J. C., and Liu, L. F. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 11014–11019
28. Errington, F., Willmore, E., Tilby, M. J., Li, L., Li, G., Li, W., Baguley, B. C., and Austin, C. A. (1999) *Mol. Pharmacol.* 56, 1309–1316
29. Onda, T., Toyoda, E., Miyazaki, O., Seno, C., Kagaya, S., Okamoto, K., and Nishikawa, K. (2007) *Cancer Lett.* 259, 99–110
30. Guo, L., Liu, X., Nishikawa, K., and Plunkett, W. (2007) *Mol. Cancer Ther.*
A Topoisomerase IIα-specific Drug

6. 1501–1508

31. Uegaki, K., Adachi, N., So, S., Iiizumi, S., and Koyama, H. (2006) DNA Repair (Amst.) 5, 303–311

32. Sakaguchi, A., Miyake, M., Kuroda, K., Nozaki, N., Tanaka, M., Hibino, M., Fujiy, Y., Kato, S., and Kikuchi, A. (2002) J. Biochem. (Tokyo) 132, 409–416

33. Trask, D. K., and Muller, M. T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1417–1421

34. Willmore, E., Frank, A. J., Padget, K., Tilby, M. J., and Austin, C. A. (1998) Mol. Pharmacol. 54, 78–85

35. Austin, C. A., Marsh, K. L., Wasserman, R. A., Willmore, E., Sayer, P. J., Wang, J. C., and Fisher, L. M. (1995) J. Biol. Chem. 270, 15739–15746

36. Sakaguchi, A., and Kikuchi, A. (2004) J. Cell Sci. 117, 1047–1054

37. Iiizumi, S., Nomura, Y., So, S., Uegaki, K., Aoki, K., Shibahara, K., Adachi, N., and Koyama, H. (2006) BioTechniques 41, 311–316

38. Adachi, N., Ishino, T., Ishii, Y., Takeda, S., and Koyama, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12109–12113

39. So, S., Adachi, N., Lieber, M. R., and Koyama, H. (2004) J. Biol. Chem. 279, 55433–55442

40. So, S., Nomura, Y., Adachi, N., Kobayashi, Y., Hori, T., Kurihara, Y., and Koyama, H. (2006) Genes Cells 11, 363–371

41. Kobori, O., Vuillot, M. T., and Martin, F. (1982) Int. J. Cancer 30, 65–67

42. Adachi, N., So, S., Iiizumi, S., Nomura, Y., Murai, K., Yamakawa, C., Miyagawa, K., and Koyama, H. (2006) DNA Cell Biol. 25, 19–24

43. Roca, J., Ishida, R., Berger, J. M., Andoh, T., and Wang, J. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1781–1785

44. Andoh, T. (1998) Biochimie (Paris) 80, 235–246

45. Spitzner, J. R., Chung, J. K., and Muller, M. T. (1990) Nucleic Acids Res. 18, 1–11

46. Adachi, N., Iiizumi, S., So, S., and Koyama, H. (2004) Biochem. Biophys. Res. Commun. 318, 856–861

47. Adachi, N., Suzuki, H., Iiizumi, S., and Koyama, H. (2003) J. Biol. Chem. 278, 35897–35902

48. Haber, J. E. (2000) Trends Genet. 16, 259–264

49. Lieber, M. R., Ma, Y., Pannicke, U., and Schwarz, K. (2003) Nat. Rev. Mol. Cell Biol. 4, 712–720

50. Lieber, M. R. (1999) Genes Cells 4, 77–85

51. Lieber, M. R. (2008) J. Biol. Chem. 283, 1–5

52. Heyer, W. D., Li, X., Rolfsmeier, M., and Zhang, X. P. (2006) Nucleic Acids Res. 34, 4115–4125

53. Grawunder, U., Zimmer, D., Fugmann, S., Schwarz, K., and Lieber, M. R. (1998) Mol. Cell 2, 477–484

54. Grawunder, U., Zimmer, D., Kulesza, P., and Lieber, M. R. (1998) J. Biol. Chem. 273, 24708–24714

55. Gao, H., Huang, K. C., Yamasaki, E. F., Chan, K. K., Chohan, L., and Snapka, R. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12168–12173

56. So, S., Adachi, N., and Koyama, H. (2007) DNA Cell Biol. 26, 517–525