XK469, a selective topoisomerase IIβ poison

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XK469 (NSC 697887) is a synthetic quinoxaline phenoxypropionic acid derivative that possesses unusual solid tumor selectivity and activity against multidrug-resistant cancer cells. We report here that XK469 and its S(−) and R(+)-isomers induce reversible protein-DNA crosslinks in mammalian cells. Under protein denaturing conditions, the protein-DNA crosslinks are rendered irreversible and stable to DNA banding by CsCl gradient ultracentrifugation. Several lines of evidence indicate that the primary target of XK469 is topoisomerase IIβ. Preferential targeting of topoisomerase IIβ may explain the solid tumor selectivity of XK469 and its analogs because solid tumors, unlike leukemias, often have large populations of cells in the G_1/G_0 phases of the cell cycle in which topoisomerase IIβ is high whereas topoisomerase IIα, the primary target of many leukemia selective drugs, is low.

XK469 (NSC 697887, Fig. 1) is an analog of the herbicide Assure (DuPont) that was discovered in a screen for solid tumor-selective agents (1–3). The screen is based on an agar diffusion assay in which an agent's relative toxicity for leukemias and solid tumors is determined by comparison of zones of colony inhibition (3, 4). A zone difference of 250 units represents approximately an 8-fold difference in sensitivity, and XK469 gave a leukemia/solid tumor zone difference of 510 (1). XK469 has broad activity against murine solid tumors such as colon 38, pancreatic 03, and mammary 16/C (1). The R(+)- and S(−)-isomers of XK469 (respectively, NSC 698215 and NSC 698216) were found to be equally toxic in studies with animal tumor models (1). XK469 also was found to be highly active against multidrug-resistant tumors (1, 2). The dose-limiting toxicities for XK469 and its analogs were marrow toxicity and epithelial damage in the gastrointestinal tract. XK469 has low toxicity in comparison to other anticancer agents, such as camptothecin. For instance, the average *in vitro* GI_{50} (50% growth inhibitory concentration) for the NCI 60 tumor cell line panel is 7×10^{-5} M for XK469 and 4.5×10^{-8} for camptothecin, a 1,500× difference (National Cancer Institute Developmental Therapeutics Compare web site, http://dtp.nci.gov). The R- and S-isomers of XK469 were well tolerated by tumor-bearing mice at an effective therapeutic dose of 74.4 mg/kg, followed by several lower doses of 47 mg/kg/injection (1). Thus, relatively high levels of XK469 have been tolerated in animal model studies. Extensive pharmacokinetic studies of the S-isomer of XK469 in CD1F2 mice were performed at 100 mg/kg (≈1/6 maximum tolerated total dose) as a single i.v. bolus dose. In these studies, up to 0.48 mg/ml (≈1.4 mM) maximum serum concentrations of XK469 were produced (K.K.C., unpublished data). Thus, millimolar *in vivo* concentrations can be produced at nontoxic doses in rodents. The most active analogs, such as XK469, have a halogen at the 7 position (1). The carboxylic acid forms are water soluble and can be injected. Exposure of human colon carcinoma cells to XK469 for 24 hr resulted in 90% inhibition of DNA synthesis, increased RNA and protein synthesis, and a block at the G_2/M boundary of the cell cycle in association with an increased expression of cyclin B1 (5). The molecular target and mechanism of action of XK469 and its analogs have not been determined, but common mechanisms of anticancer drug action, such as DNA binding, alkylation, tubulin binding, DNA scission, or inhibition of purine/pyrimidine metabolism, have been ruled out (1). XK469 is not yet approved for clinical trials but is expected to move into the clinic pending completion of detailed toxicologic studies.

Topoisomerase II is an essential enzyme that alters DNA topology by carrying out an ATP-dependent reaction in which one DNA double helix is passed through another. In vertebrates, there are genes coding for two separate topoisomerase II isozymes, topoisomerase IIα (p170 isozyme) and topoisomerase IIβ (p180 isozyme). Expression of both isozymes is high in S-phase cells and peaks in G_2/M (6). Expression of the α isozyme is very low in terminally differentiated cells and quiescent cells (6, 7). Although topoisomerase IIβ expression fluctuates slightly during the cell cycle, overall its expression is relatively constant. As cells move from logarithmic growth to plateau phase, topoisomerase IIα levels drop sharply whereas topoisomerase IIβ levels remain constant or increase (8–10).

We report here that XK469 is a selective topoisomerase IIβ poison. Topoisomerase poisons are drugs that stabilize DNA strand passing intermediates in the topoisomerase reaction in which the enzymes are covalently attached to the DNA at the site of a DNA strand break. These drug-stabilized reaction intermediates are toxic to the cells, probably because of collisions with DNA replication forks and transcription complexes. Most topoisomerase IIα poisons also act to a greater or lesser extent as topoisomerase IIβ poisons (6, 11–14). However, no drug highly specific for topoisomerase IIβ has previously been reported. We discuss the topoisomerase IIβ selectivity of XK469 with respect to this drug's unusual solid tumor selectivity.

Materials and Methods

Cells and Virus. African green monkey cells (CV-1) were obtained from the American Type Culture Collection and were maintained in Eagle's minimal essential media (GIBCO/BRL) supplemented with 10% calf serum, 14 mM Hepes (pH 7.2), 4 mM $NaHCO_3$, and penicillin/streptomycin. Confluent CV-1 cells in 35-mm plates were infected with plaque-purified SV40 strain 777 (10 plaque-forming units/cell) for 36 hr before labeling of DNA with ^{3}H-dT.

GM637 cells were obtained from the NIGMS Human Genetic Mutant Cell Repository. The GM637 line was derived by SV40 transformation of skin fibroblasts from an apparently normal woman. The subclone GM637d2 was obtained by plating at low cell density. GM637d2 cells were grown in a 5% CO_2 atmosphere at 37°C in modified Eagle's medium (GIBCO/BRL) or DMEM (GIBCO/BRL) supplemented with 10% fetal calf serum and penicillin/streptomycin. MCF-7 human breast adenocarcinoma cells were obtained from the American Type Culture Collection and were grown in Dulbecco's Modified Eagle's medium with 10% fetal calf serum.

CPTCV10c22 cells are maintained in 1.5 μM camptothecin.

Abbreviations: *m*-AMSA, [4′-(9-acridinylamino)methanesulfon-*m*-anisidide]; GuHCl, guanidinium chloride; CsCl, cesium chloride.

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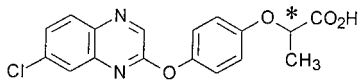

Fig. 1. Structure of XK469. The acid form of XK469 is shown, and the asymmetric center is indicated by an asterisk.

They are CV-1 cells selected stepwise for camptothecin resistance beginning at 8 nM camptothecin after a single exposure to germicidal UV light (254 nm, 30 J/m^2). AMCV1 are CV-1 cells maintained in 3 μM [4′-(9-acridinylamino)methanesulfon-*m*-anisidide] (*m*-AMSA). They were selected stepwise for resistance to *m*-AMSA beginning at 20 nM after a single exposure to UV (30 J/m^2).

Drugs. (±)XK469 (racemic, sodium salt, NSC 656889 or racemic free acid, NSC 697887), S(−)XK469 (free acid, NSC 698216), and R(+)XK469 (free acid, NSC 698215) were provided by the National Cancer Institute Drug Synthesis Branch. Camptothecin lactone (NSC 94600) and VM-26 (teniposide, NSC 122819) were obtained from the National Cancer Institute Division of Cancer Treatment, Natural Products Branch, and *m*-AMSA (NSC 249992) was obtained from the National Cancer Institute Division of Cancer Treatment, Drug Synthesis Branch. DMSO was the solvent for all drug stocks.

Filter Assay for Protein-DNA Crosslinks. The GF/C filter assay for protein-SV40 DNA crosslinks is used to measure topoisomerase poisoning *in vivo*, in cultured cells (15), and *in vitro*, with purified enzymes and DNA substrates (16). Replicating viral DNA was pulse-labeled at 36 hr postinfection (^{3}H-dT, 250 μCi/ml, 30 min), and drugs were added to the labeling medium 15 min after the start of labeling. Labeling and drug exposure were terminated by removal of drug and label-containing medium and addition of Hirt lysing fluid. Hirt lysing fluid contains SDS, which denatures topoisomerases trapped in topoisomerase-DNA cleavage complexes by topoisomerase poisons and thus renders the covalent topoisomerase-DNA crosslinks irreversible. SV40 DNA was then selectively extracted by the Hirt method (17). To assay protein crosslinks to SV40 DNA, duplicate aliquots of the Hirt extract supernatants were mixed with 0.4 M and 4.0 M GuHCl (guanidinium chloride), respectively, then were filtered through prewetted glass fiber filters. In 4.0 M GuHCl (DNA binding conditions), all nucleic acids bind to the filter (equivalent to a trichloroacetic acid precipitation). The radioactivity retained on the filter under DNA binding conditions gives the value for total labeled DNA in the aliquot. In 0.4 M GuHCl (protein binding conditions), the only labeled DNA retained on the filter is DNA crosslinked to protein. The ratio of the radioactivity retained on GF/C filters in 0.4 M GuHCl to the radioactivity retained on filters in 4.0 M GuHCl gives the fraction of labeled DNA that is crosslinked to protein. A single crosslinked protein is sufficient to cause the retention of a DNA molecule on the filter under protein-binding conditions (15).

A modified GF/C assay was used to measure protein crosslinks to cellular DNA. Cells were labeled with ^{3}H-dT (1.0 μCi/ml, 43 hr), and drug treatments were carried out for 15 min after labeling. The medium then was removed, and the cells were lysed with Hirt lysing fluid. The lysate (500 μl) was transferred to a 1.5-ml microcentrifuge tube containing a small stainless steel nut, the tube was capped securely, and the DNA was sheared by vortexing for 15 seconds. The lysate then was heated at 65°C for 10 min to ensure denaturation and removal of noncovalently attached proteins from the DNA. After cooling to room temperature, aliquots of the lysate were assayed with the GF/C filter assay for the percentage of labeled DNA that is crosslinked to protein as in the assay for protein-SV40 DNA crosslinks (15).

CsCl Gradient Ultracentrifugation. The CsCl step gradients (18) were prepared by the successive layering of 2-ml volumes of solutions A through D (solution A, 1.75 g/ml CsCl; solution B, 1.63 g/ml; solution C, 1.42 g/ml; solution D, 1.32 g/ml) into an ultracentrifuge tube (14 × 89 mm). Cell lysate was prepared and loaded on a preformed CsCl gradient. Light mineral oil (Sigma) was used to fill the tube to top. The tubes were centrifuged in an SW41 rotor (Beckman Coulter) at 30,000 rpm for 23 hr at 20°C. Fractions (200 μl) were collected from the bottom of the tubes, and a 5-μl aliquot was taken from each fraction for direct scintillation counting. For GF/C assay of protein-DNA crosslinks, the fractions were dialyzed overnight against TE buffer (10 mM Tris·HCl, pH 7.4/1 mM EDTA) before the assay.

Protein Gel Electrophoresis and Western Blotting. The cells were lysed with SDS sample buffer (4% SDS/2% β-mercaptoethanol/20% glycerol/125 mM Tris·HCl, pH 6.8) and were separated by 7.5% SDS/PAGE (50 or 100 μg of protein/lane) according to the procedure of Laemmli (19) The proteins were transferred to poly(vinylidene difluoride) membrane (Amersham Pharmacia) overnight at 40 volts, 4°C in a model TE 42 Transphor electrophoresis unit (Hoefer). After treatment with blocking buffer (Tris-buffered saline with 10% nonfat dry milk), the blots were probed at room temperature for 1 hr with topoisomerase IIα polyclonal rabbit antibody (TopoGen, Columbus, OH) or topoisomerase IIβ polyclonal rabbit antibody (kindly provided by Daniel M. Sullivan, H. Lee Moffitt Hospital, Tampa, FL). Anti-rabbit IgG–peroxidase antibody (Boehringer Mannheim) then was added as a secondary antibody. SuperSignal chemiluminescent substrate (Pierce) was used to detect binding of antibodies, and the chemiluminescence signals were visualized on CL-Xposure film (Pierce).

Metabolic Labeling of Proteins. Cells were grown to 90% confluence in 10-cm culture plates before labeling. The culture medium was removed, and the cell layer was washed twice with 10 ml of washing medium (lysine- and leucine-deficient DMEM supplemented with 5% dialyzed calf serum). The cells were kept in 5 ml of washing medium in a humidified 37°C, 5% CO_2 incubator for 15 min to deplete intracellular pools of lysine and leucine. After the depletion step, the washing medium was replaced with 1 ml of labeling medium, and labeling was carried out for 3 hr in a humidified 37°C, 5% CO_2 incubator. Labeling medium was washing medium with 200 μCi/ml L-[4,5-^{3}H] leucine (Amersham Pharmacia) and 200 μCi/ml L-[4,5-^{3}H] lysine (Amersham Pharmacia). Fifteen minutes before the end of the labeling procedure, XK469 was added to a final concentration of 1 mM. Labeling and drug treatment were terminated by replacing the labeling medium with 1 ml of 6 M GuHCl. The cell lysate then was layered onto a CsCl step gradient, prepared as described above, and DNA was banded by ultracentrifugation. After centrifugation, aliquots were collected after piercing the bottom of each centrifuge tube.

Enzyme Assay. ATP-dependent relaxation of superhelical DNA (20) was done with purified human topoisomerase IIα and IIβ by using superhelical pBR322 DNA as a substrate. The same assay conditions were used for both topoisomerase II isozymes (50 mM Tris·HCl, pH 8.0/120 mM KCl/10 mM $MgCl_2$/0.5 mM DTT/0.5 mM ATP/0.25 μg of supercoiled pBR322 DNA). Reactions were started by addition of the topoisomerase II and were incubated for 30 min at 37°C. Sufficient enzyme was added to the assay to relax ≈95% of the superhelical DNA in the presence of the drug solvent, DMSO. Various amounts of S(−)XK469 were included in separate reactions, keeping the solvent volume constant. Reactions were stopped by the addition of 2 μl of 10% SDS. Proteinase K (1 mg/ml) then was added, and the

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samples then were incubated at 45°C for 60 min. Finally, 0.1 volume of loading buffer (0.25% bromophenol blue/50% glycerol) was added and mixed with the sample. Protein-free DNA then was prepared by mixing the sample vigorously with an equal volume of chloroform:isoamyl alcohol (24:1), followed by brief centrifugation. The top phase, containing the bromophenol blue tracking dye and DNA, was removed and subjected to electrophoresis on a 1% agarose gel in 1× TAE buffer (40 mM Tris acetate, pH 8.0/1 mM EDTA). DNA bands were stained with 0.5 μg/ml ethidium bromide, were visualized by UV transillumination, and were photographed and scanned on an AlphaImage 2000 Digital Image System (Alpha Innotech, San Leandro, CA) for quantitation.

Results and Discussion

XK469-Induced Protein Crosslinks to Cellular and SV40 DNA. Both the R- and S-isomers of XK469 caused dose-dependent protein crosslinks to ^{3}H-dT-pulse-labeled SV40 DNA in infected CV-1 cells (Fig. 2). The R-isomer was approximately twice as effective as the S-isomer (Fig. 2*A*). The XK469-induced protein-DNA crosslinks are stable to SDS and GuHCl because the cell lysis is done by addition of SDS-containing Hirt lysing fluid and the assays are done in 0.4 M GuHCl. Predigestion of the samples with proteinase K eliminates the signal, showing that filter retention of labeled DNA in 0.4 M GuHCl is attributable to protein attached to the DNA. Removal of XK469 results in rapid reversal of the protein-SV40 DNA crosslinks (Fig. 2*B*). The slight decline in XK469-induced protein-DNA crosslinks in the continuing presence of XK469 in Fig. 2*B* may reflect preferential protein crosslinking to replication intermediates. SV40 DNA replication intermediates saturate with label in ≈15 min, but the label in completely replicated genomes continues to accumulate. Thus, label in completed genomes constitutes a larger fraction of the labeled SV40 DNA at longer labeling times. Addition of XK469 to cells that have already been lysed does not cause protein-DNA crosslinking (data not shown), suggesting that the drug-induced protein-DNA crosslinks require active enzymes.

Reversible drug-induced protein-DNA crosslinks suggest topoisomerase poisoning. To test the possible roles of cellular topoisomerases in XK469-induced protein-DNA crosslinking, we used CV-1 cells made resistant to specific topoisomerase poisons. CPTCV10c22 cells are resistant to 1.5 μM camptothecin (see *Materials and Methods*). In CPTCV10c22, topoisomerase I is reduced to 10% of normal as determined by Western blotting, and the catalytic activity of the remaining topoisomerase I (also ≈10% of wild-type CV-1 cell activity) is inhibited by camptothecin in a manner indistinguishable from wild-type enzyme (H.G., unpublished data). As expected, camptothecin-induced topoisomerase I-DNA crosslinks are greatly reduced in CPTCV10c22 cells in comparison to drug-sensitive parental CV-1 cells (Fig. 2*C*). The decreased camptothecin-induced topoisomerase I-DNA crosslinking reflects the decrease in cellular topoisomerase I. XK469-induced protein-DNA crosslinks in CPTCV10c22 cells and drug-sensitive parental cells are indistinguishable, indicating that the target of XK469 is not topoisomerase I.

Reduction of topoisomerase II expression is a common mechanism of resistance to topoisomerase II poisons, such as *m*-AMSA, that stabilize covalent topoisomerase II-DNA cleavable complexes (21, 22). In CV-1 cells selected for resistance to 3 μM *m*-AMSA, topoisomerase II-DNA crosslinks induced by *m*-AMSA were greatly reduced compared with the crosslinks induced by this drug in parental CV-1 cells (Fig. 2*E*). Western blot analysis (Fig. 3) shows that topoisomerase IIα (the p170 isozyme of topoisomerase II) is significantly reduced and the topoisomerase IIβ (p180 isozyme) is undetectable. Thus, the decrease in *m*-AMSA-induced topoisomerase II-DNA crosslinks in AMCV1 cells is consistent with the decreases in topoisomerase II isozymes in this cell line. XK469-induced protein-DNA crosslinks in these *m*-AMSA-resistant AMCV1 cells were also greatly reduced in comparison to those induced in parental cells by XK469 (Fig. 2*F*). This strongly suggests that the target of XK469 is a type II topoisomerase.

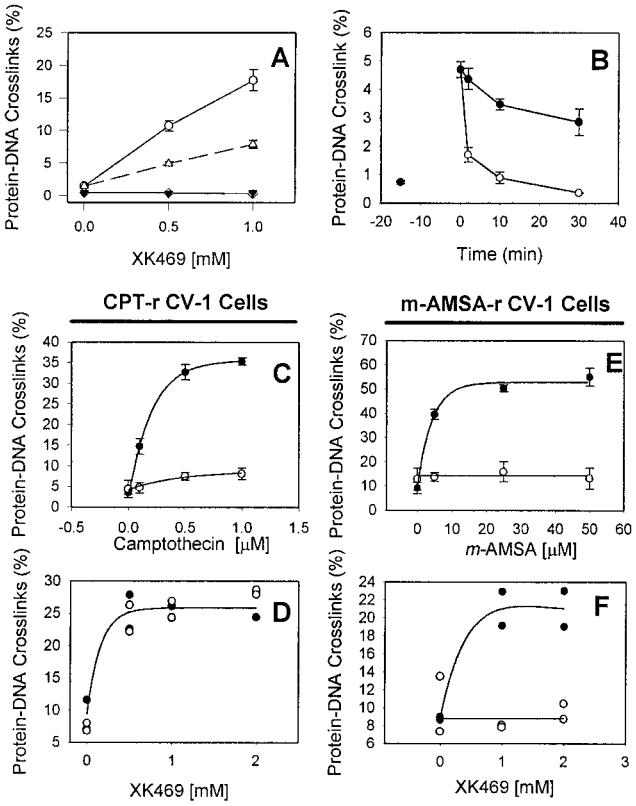

Fig. 2. XK469-induced protein-DNA crosslinks to cellular and SV40 DNA. (*A*) Replicating SV40 genomes were pulse-labeled with ^{3}H-dT for 30 min, and the drugs were added 15 min after the start of labeling. Labeling medium with drug was drawn off and the cells were lysed with Hirt lysing fluid. The Hirt supernatant, containing pulse-labeled SV40 DNA, was assayed for protein-SV40 DNA crosslinks. ○, R-isomer; △, S-isomer; ▼ R-isomer, proteinase K digested before assay; ◇, S-isomer, proteinase K digested before assay. Error bars are ±SD derived from four points. (*B*) Reversal of S(−)XK469-induced protein-DNA crosslinks upon removal of the drug. SV40-infected CV-1 cells were labeled with ^{3}H-dT beginning at $t = -30$ min. At $t = -15$ min, the labeling medium in all samples was made 1 mM in S(−)XK469. At $t = 0$, the labeling medium with XK469 in one set of samples was replaced with labeling medium without drug. At $t = 2$, 10, and 20 min, samples were harvested by removal of labeling medium and addition of Hirt lysing fluid. SV40 DNA was selectively extracted and measured for protein-DNA crosslinks (see *Materials and Methods*). (*C*) Camptothecin-induced topoisomerase I-cellular DNA crosslinks in drug-sensitive parental CV-1 cells (●) and in CPTCV10c22 cells (○) resistant to 1.5 μM camptothecin (CPT-r). (*D*) S(−)XK469-induced protein-DNA crosslinks in drug-sensitive parental CV-1 cells (●) and in CPTCV10c22 cells (○). (*E*) *m*-AMSA-induced topoisomerase II-DNA crosslinks in drug-sensitive parental CV-1 cells (●) and in AMCV1 cells (○) resistant to 3 μM *m*-AMSA. (*F*) S(−)XK469-induced protein-DNA crosslinks in parental CV-1 cells (●) and in AMCV1 (○) cells.

The *m*-AMSA-resistant cell line, AMCV1, described in this report is the fourth drug-resistant cell line lacking detectable topoisomerase IIβ to be reported. Human leukemia cells resistant to *m*-AMSA have no topoisomerase IIβ protein (23). Human small cell lung cancer cells resistant to mitoxantrone (24) and Chinese hamster cells resistant to 9-OH-ellipticine (25) also have been reported to lack detectable topoisomerase IIβ. Clearly, topoisomerase IIβ is not an essential enzyme in cultured cells but is an important target for topoisomerase II-targeting

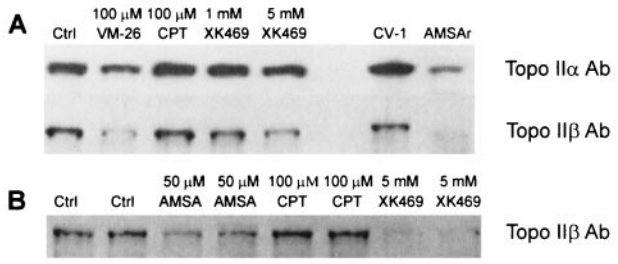

Fig. 3. Western blot of topoisomerase II isozymes in AMCV1 cells and band depletion assays for topoisomerase II isozymes in CV-1 cells. (*A* Right lanes) Western blot analysis of 3 μM *m*-AMSA-resistant AMCV1 cells (AMSAr). Extracts of drug-sensitive parental CV-1 cells and AMCV1 cells were subjected to acrylamide gel electrophoresis in SDS. The separated proteins were transferred to a poly(vinylidene difluoride) membrane and were probed with topoisomerase IIα- or topoisomerase IIβ-specific antibody (Ab). (Left lanes) Band depletion analysis of topoisomerase II isozymes in CV-1 cells treated with VM-26 and XK469. Cells were treated for 15 min with the indicated drugs before extraction. The control was treated with the drug solvent, DMSO. Camptothecin (CPT), a topoisomerase I poison, was included as an additional control. The S-isomer of XK469 was used in this experiment. (*B*) Band depletion for topoisomerase IIβ isozyme in CV-1 cells treated with *m*-AMSA, XK469, and camptothecin.

drugs. The reversibility of the XK469-induced protein-DNA crosslinks upon removal of the drug, the requirement for active enzymes, and the absence of XK469-induced protein-DNA crosslinks in *m*-AMSA-resistant cells all indicate that XK469 is a topoisomerase II poison and not a nonspecific protein-DNA crosslinking agent.

Band Depletion Assays. To determine whether one or both of the topoisomerase II isozymes were the intracellular target of XK469, we used a band depletion assay (26, 27). Stabilization of covalent topoisomerase-DNA crosslinks by topoisomerase poisons prevents the efficient extraction of the target topoisomerases, resulting in reduced topoisomerase bands as detected by Western blotting with specific antibodies, after acrylamide gel electrophoresis. Exposure to the topoisomerase II poisons VM-26 (Fig. 3*A*) and *m*-AMSA (Fig. 3*B*) resulted in depletion of the topoisomerase IIα band and the topoisomerase IIβ band relative to DMSO treated controls. VM-26 and *m*-AMSA are known to target both topoisomerase II isozymes (12, 14). Camptothecin, a specific topoisomerase I poison (26), had no effect on either topoisomerase II isozyme (Fig. 3 *A* and *B*). XK469 caused little or no depletion of topoisomerase IIα and a significant dose-dependent reduction of topoisomerase IIβ (Fig. 3 *A* and *B*). In five similar experiments, two of which are shown in Fig. 3, we have seen clear depletion of topoisomerase IIβ by XK469. In each case, reduction of topoisomerase IIα, if any, was marginal (<15% in the experiment shown). XK469 appears to have only marginal effect on topoisomerase IIα, and topoisomerase IIβ appears to be the drug's primary target.

XK469-Induced Protein-DNA Crosslinks Are Stable to Isopycnic DNA Banding by CsCl Ultracentrifugation. When DNA from DMSO-treated human fibroblasts was banded by CsCl gradient ultracentrifugation and was assayed for protein-DNA crosslinks, the value was <10% (Fig. 4). This value was only slightly reduced by proteinase K predigestion of the samples, indicating that it is mainly attributable to nonspecific DNA binding to the GF/C filters. The background binding of protein-free DNA in the protein-DNA crosslink assay is slightly higher for sheared cellular DNA, which is partially single stranded, than it is for nonsheared SV40 DNA (see Fig. 2). When DNA from XK469-treated human fibroblasts was banded by CsCl ultracentrifugation and was assayed for protein-DNA crosslinks, ≈30–40% of the DNA was found to be crosslinked to protein. Predigestion with proteinase K eliminated the filter binding and demonstrated that the signal in this assay was attributable to protein bound to the DNA. Exposure to either 1 mM XK469 or 10 μM camptothecin (a topoisomerase I poison) resulted in a significant amount of protein label banding with DNA in CsCl gradients. In DMSO-treated cells (solvent controls), there was only a small amount of protein-label with the DNA, and it was not removed by proteinase K digestion. By amino acid analysis (not shown), there is ≈0.5 μg of protein for every 25–30 μg of DNA from cells exposed to 1 mM XK469 and banded by CsCl density gradient ultracentrifugation.

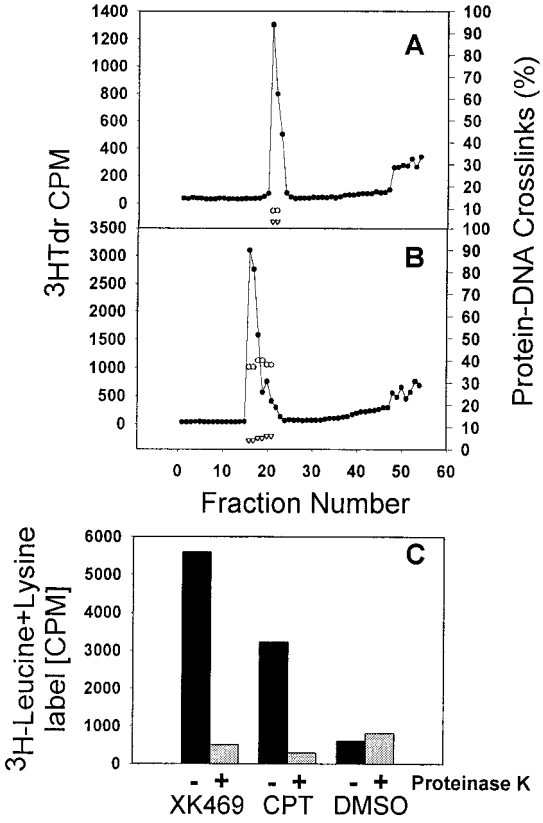

Fig. 4. XK469-induced protein-DNA crosslinks stable to DNA banding by cesium chloride ultracentrifugation. GM637d2 human fibroblasts were labeled with ^{3}H-dT (1 μCi/ml, 43 hr). The cells were then either treated with the solvent, DMSO (*A*), or with 1 mM (±)XK469, acid form (*B*) for 15 min. The labeling medium was drawn off, and the cells then were lysed by addition of 6 M GuHCl. DNA was sheared by vortexing the GuHCl cell lysate, and the samples then were layered onto CsCl step gradients (see *Materials and Methods*). After ultracentrifugation and elution of the gradient, 5 μl of each 200 μl fraction was removed for direct scintillation counting. DNA containing fractions from each experiment, identified by tritium incorporation, were dialyzed against TE buffer (10 mM Tris·HCl, pH 7.6/1 mM EDTA) to remove CsCl and then were assayed for protein-DNA crosslinks by the GF/C filter assay. Tritiated thymidine incorporation (DNA, ●) is indicated on the left axis of each graph, and protein-DNA crosslinks are indicated on the right axis either with (▽) or without (○) proteinase K predigestion. Fractions were pooled in pairs for the assays, and the value for the pair is indicated for each fraction. (*C*) Protein labeled with ^{3}H-leucine + lysine also bands with DNA by CsCl ultracentrifugation after exposure to either 1 mM (±)XK469 (acid form) or to 10 μM camptothecin (black bars). Predigestion with proteinase K (light bars) eliminates this protein label associated with the DNA.

Topoisomerase IIβ Is Crosslinked to DNA by Exposure of Cells to XK469. Exposure of subconfluent MCF-7 human breast cancer cells to 2 mM S(−)XK469 resulted in substantial crosslinking of

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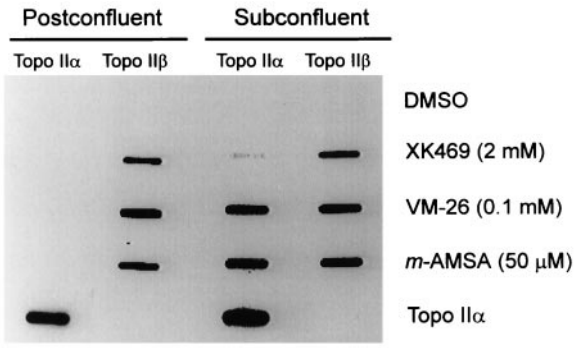

Fig. 5. Covalent binding of topoisomerase IIα and topoisomerase IIβ to cellular DNA after exposure of cells to topoisomerase poisons. Subconfluent and postconfluent human breast cancer cells (MCF-7) were treated with S(−)XK469, VM-26, *m*-AMSA, or the solvent, DMSO, for 15 min and then were lysed with GuHCl as described (see *Materials and Methods*). The cellular DNA was banded by CsCl gradient ultracentrifugation, and the CsCl then was removed by dialysis of the pooled DNA fractions. A 30-μg DNA aliquot was removed, and $MgCl_2$ was added to a final concentration of 5 mM. The DNA was digested with protease-free DNase I (Boehringer Mannheim, 0.1 units/ml, 37°C, 1 hr). The aliquot then was applied to a poly(vinylidene difluoride) membrane with a slot blot device. Purified human topoisomerase IIα (TopoGen) also was applied as a control. Blotted proteins were probed with antibodies to human topoisomerase IIα and human topoisomerase IIβ (indicated at the top of each column).

topoisomerase IIβ to cellular DNA, confirming that XK469 is a topoisomerase IIβ poison (Fig. 5). In agreement with the band depletion results, there was also slight covalent crosslinking of topoisomerase IIα to cellular DNA, indicating that XK469 has trace activity as a topoisomerase IIα poison. The DNA intercalating topoisomerase II poison *m*-AMSA and the nonintercalating topoisomerase II poison VM-26 caused substantial covalent attachment of both topoisomerase IIα and topoisomerase IIβ to cellular DNA. Several research groups have shown that topoisomerases IIα and IIβ are differentially regulated in cultured cells. As cells exit from exponential growth, with a high proportion of S-phase cells, and enter plateau phase, with a higher proportion of cells in G_1/G_0, topoisomerase IIα decreases while topoisomerase IIβ remains constant or even increases (8–10). In postconfluent cells, in which topoisomerase IIα is down-regulated, all three topoisomerase II poisons caused covalent attachment of topoisomerase IIβ to cellular DNA and no detectable crosslinking of topoisomerase IIα to DNA.

Inhibition of Superhelical DNA Relaxation. S(−)XK469 inhibited the ATP-dependent relaxation of superhelical pBR322 DNA by purified topoisomerase IIβ with an IC_{50} of 160 μM (Fig. 6). In contrast, topoisomerase IIα was very resistant to inhibition by S(−)XK469 (IC_{50} = 5 mM). The difference in inhibition of the two topoisomerase II isozymes is in good agreement with the differences seen in topoisomerase II poisoning by XK469.

Most topoisomerase IIα poisons also act as topoisomerase IIβ poisons (6, 12, 14, 24, 25). No highly topoisomerase IIβ-specific drug has previously been reported. A topoisomerase II poison highly specific for the p180 isozyme would be expected to have unique effects on treated cells if the two isozymes serve different cellular functions. Drugs highly specific for one or the other of the topoisomerase II isozymes would be valuable tools for investigating the roles of these isozymes in cellular metabolism. Although many drugs inhibit both topoisomerase IIα and IIβ, the relative contribution of each as an antineoplastic target is poorly understood. The fact that topoisomerase IIβ is less abundant than topoisomerase IIα does not mean that it is a less important target. The remarkable solid tumor specificity of

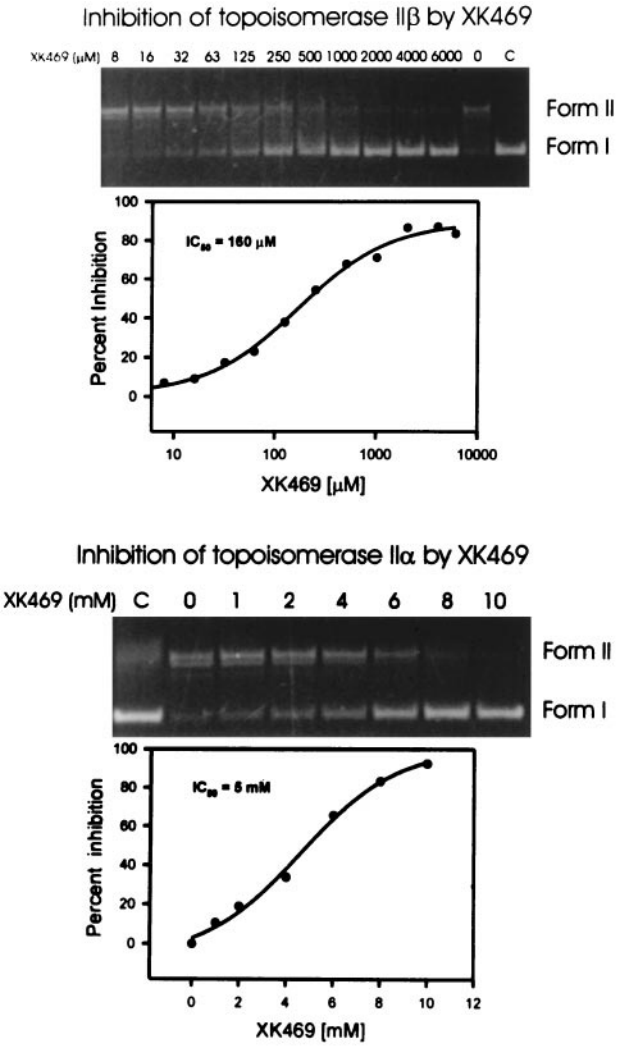

Fig. 6. Catalytic inhibition of topoisomerase II isozymes by S(−)XK469. ATP-dependent relaxation of superhelical pBR322 DNA is shown for purified human topoisomerase IIβ (*Upper*) and purified human topoisomerase IIα (*Lower*). Inhibition was measured by quantitation of the form I band in the ethidium stained gels. The graph from which the IC_{50} value was determined is shown below each gel. Controls (C) are pBR322 DNA without added enzyme.

XK469 suggests that topoisomerase IIβ may be a very important target for anticancer drugs. In addition to carrying out different roles in the cell, the two topoisomerase II isozymes may be sensed differently as DNA damage in the presence of topoisomerase poisons and may activate different signal transduction pathways and cell cycle checkpoints.

The marked differences in cell cycle regulation of topoisomerase IIα and topoisomerase IIβ may be the key to XK469's solid tumor selectivity. Acute leukemias have a large proportion of S-phase cells whereas solid tumors tend to develop large G_1/G_0 populations (28). The pronounced selectivity of XK469 for topoisomerase IIβ may allow selective targeting of solid tumors with significantly less toxicity for rapidly proliferating normal tissues and leukemias with high S-phase fractions and relatively high levels of topoisomerase IIα. Although both solid tumors and many normal tissues would be expected to have large G_1/G_0 populations, solid tumor cells might be more likely to have defects in cell cycle checkpoints that prevent replication in the presence of DNA damage caused by drug-stabilized topoisomerase IIβ-DNA cleavage complexes.

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