Abstract

The role of DNA topoisomerase (Topo) IIβ in cancer chemotherapy remains unclear, although this particular isoform has been implicated in drug resistance. In this study, we investigated Topo IIβ as a target for 2-[4-(7-chloro-2-quinoxalinyloxy)phenoxy]-propionic acid (XK469), a novel synthetic quinoxaline phenoxypropionic acid derivative, in a Waldenstrom’s macroglobulinemia (WM) model. In vitro, the WSU-WM cell line was exposed to 1.0, 2.0, 5.0, 8.0, and 10 μM XK469. Our results demonstrate a concentration-dependent cell growth inhibition with a concentration-independent inhibition of Topo IIβ, as determined by band depletion assay. The cell growth inhibition of cells correlated well with increase in Bax:Bcl-2 ratio and poly(ADP-ribose) polymerase (PARP) cleavage. We used our established WSU-WM severe combined immunodeficient mouse xenograft model to test the efficacy and effect of XK469 on Topo IIβ in vivo. Topo IIβ was inhibited equally using two different dose schedules (20 and 40 mg/kg, i.v., for a total of 120 and 240 mg/kg, respectively); however, there was no significant decrease in tumor weight. Western blot analysis of cells isolated from s.c. tumors showed no induction of the Bax protein and a very low Bax:Bcl-2 ratio of ~0.3 in correlation with minimum PARP cleavage. Our study shows that XK469 inhibits Topo IIβ in WSU-WM cells both in vitro and in vivo at or below the maximum tolerated dose in severe combined immunodeficient mice. However, there was no change of apoptosis-related molecules such as PARP, Bax, and Bcl-2 or reduction in tumor weight in association with Topo IIβ inhibition. We conclude that Topo IIβ inhibition by XK469 as a target is not sufficient for therapeutic effects in WSU-WM.

Introduction

There are two major Topos2 (Topo I and II) in eukaryotic cells, which catalyze changes in DNA topological conformation by introducing transient single- or double-stranded breaks (1). Type II DNA Topos are known in two isoforms, termed DNA Topo IIα and Topo IIβ (2). The human Topo IIα was the first isoform to be cloned and has been studied extensively, whereas the Topo IIβ isoform is relatively new, and less information is available on its functional role in cancer (3). However, it is known that Topo IIβ is more widespread and is found in nonproliferating as well as fully differentiated tissues and cells, contrary to Topo IIα, which is preferentially highly expressed in proliferating cells (2, 4). Expression of Topo IIβ remains relatively constant at all phases of the cell cycle, with most cells in G0 phase found to express only Topo IIα (2).

Topo II poisons increase cleavage complexes formed by Topo enzymes, stabilize DNA strand passing intermediates, and cause Topo II-mediated cleavage to become permanent double-stranded breaks, leading to accumulation of intermediates that ultimately result in cell death (1, 5). Hence DNA Topo II is fast becoming a therapeutic target for chemotherapy in malignant cells that highly express this enzyme. XK469 is a synthetic quinoxaline phenoxypropionic acid derivative found to possess antitumor activity in solid tumors (6). The complete mechanism behind the antitumor effect is not known; however, XK469 was found to target Topo IIβ in solid tumors at high concentrations of 1 μmol (7).

WM is an indolent B-cell malignancy due to neoplastic proliferation of differentiated B-lymphocytes (8, 9) and remains incurable with current treatment protocols (10). The WSU-WM cell line, which was established in our laboratory and propagates in liquid culture and SCID mice, was used as a preclinical model (11) to study the antitumor effect of XK469 agent.

In this study, we investigated inhibition of Topo IIβ by XK469 in our WM model, and we found that XK469 inhibits Topo IIβ in a dose-independent manner. However, Topo IIβ inhibition was not sufficient for therapeutic response in WSU-WM tumors.
Materials and Methods

Cell Line. The WSU-WM cell line established in our laboratory from a patient with WM has been described in detail in a previous publication (11). WSU-WM cells were grown and maintained as a suspension in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO₂.

XK469 and Antibodies. XK469 (racemic, sodium salt, NSC6656689) was provided by the National Cancer Institute Drug Synthesis Branch. For use in our studies, it was dissolved in 1% NaHCO₃.

Monoclonal antibodies against Topo IIα, PARP, Bax, and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and Topo IIβ p180 is an antibody obtained from PharMingen (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Cytotoxicity Assay. Cytotoxicity assays were performed using WSU-WM cells (2 x 10⁵ cells/ml) in 24-well titer plates in 1000 μl of culture medium. Cells were exposed to XK469 at final concentrations ranging of 0, 1, 2, 5, 8, and 10 μM for up to 72 h. The surviving fraction of viable cells after treatment with each concentration, compared with control cells, was calculated using the trypan blue (0.4%) exclusion method (Life Technologies, Inc., Grand Island, NY). Cell number versus drug concentration was plotted from an average of triplicate points for each treatment and representative of three independent experiments. The IC₅₀ value was calculated from dose-response curves as the concentration of drug that reduced the number of viable cells to 50% of control.

Western Blot Analysis. WSU-WM cells were seeded in complete or serum-free medium at 2 x 10⁵ cells/ml; exposed to 2, 5, and 8 μM XK469; and incubated for 24, 48, and 72 h. Control cells remained untreated in either complete or serum-free media. Proteins obtained from whole-cell extracts were quantified using the BCA protein assay kit (Pierce, Rockford, IL). Protein (100 μg) was subjected to 7.5% SDS-PAGE gel electrophoresis and probed with primary antibody for Topo IIβ (2 μg/ml in 5% nonfat dry milk containing TBST).

Protein (20–50 μg) was subjected to 10% or 12% SDS-PAGE gel electrophoresis and identified with primary monoclonal antibodies (1:1000 dilution; Santa Cruz Biotechnology) to PARP, Bax, Bcl-2, or rabbit polyclonal antisera against G3PDH (1:5000 dilution; Trevigen Inc., Gaithersburg, MD) to determine equal sample loading. After incubation, membranes were washed well in TBS and incubated with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; 1:1000 dilution in TBS) for 45 min to 1 h at 25°C. Specific proteins were visualized using an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were stripped between multiple probing by incubating membranes in stripping buffer [0.5 M NaCl and 0.5 M glacial acetic acid (pH 2.5)] for 5 min at 25°C and diluting with equal volume of 1 M Tris (pH 8.0) for 5 min at 25°C.

Band Depletion Assay for Topos. WSU-WM cells exposed to 2, 5, and 8 μM XK469 for 72 h and to 5 μM for 48 and 72 h, all in complete medium, were pelleted and lysed immediately with SDS-PAGE sample buffer [4% SDS, 2% 1-mercaptoethanol, 20% glycerol, 125 μM Tris-HCl (pH 6.8)]. Protein (100 μg protein/lane) was electrophoresed in 7.5% SDS-polyacrylamide gels and transferred to Hybond C-extra membranes (Amersham Life Science, Arlington Heights, IL; Ref. 5). Membranes were blocked in 10% milk in TBST for 1 h and incubated with primary antibody for Topo IIβ (2 μg/ml in 5% nonfat dry milk containing TBST) for 4 h at 37°C. Membranes were washed and/or stripped, blocked in 10% milk in TBST, and incubated with primary antibodies for Topo II and IIα, respectively (1:1000 in 5% nonfat dry milk containing TBST) for 1 h at 25°C. Topo bands were visualized after incubation with horseradish peroxidase-conjugated secondary antibody, (Santa Cruz Biotechnology; 1:1000 dilution in TBST) using an enhanced chemiluminescence assay. To ensure that total Topo IIβ contents within cells were constant during this assay, we preheated another set of samples to 65°C before lysing them under denaturing conditions (5). Western blot analysis with 7.5% SDS-PAGE and monoclonal antibody against Topo IIβ was used to check for baseline levels of Topo IIβ expression.

Xenografts. Four-week-old female Fox Chase C.B.17 SCID mice were obtained from Taconic Laboratory (Germantown, NY). The animals were adapted, and WSU-WM xenografts were developed as described previously (11). There were three experimental groups of 7 animals each including control. Experimental groups received six daily doses of 20 (i.v. injection) or 40 mg/kg (i.v. injection) XK469. Two animals per group were sacrificed 24 h after completing the injections, and tumors were removed and immediately assayed for Topo IIβ inhibition or apoptosis-related molecules. The remaining animals were observed for measurement of s.c. tumor, changes in weight, and toxicity to XK469. Animals were euthanized when their total tumor reached 2000 mg to avoid animal discomfort. All studies involving mice were performed under an institutional review board-approved protocol.

Western Blot Analysis and Band Depletion Assay for Xenografts. Two mice with bilateral tumors per experimental group described above were sacrificed 24 h after administration of the last dose. The tumors were dissected and mechanically dissociated into single-cell suspension in RPMI 1640. Cells were lysed as described previously for Western blot analysis and band depletion assay, and protein concentrations were quantified. Total cell lysate was then subjected to either Western blot analysis or band depletion assay as described above.

Results

XK469 Caused Cell Growth Inhibition in Vitro. Exposure of WSU-WM cells to 0, 1, 2, 5, 8, and 10 μM XK469 for 0, 24, 48, and 72 h resulted in a dose-dependent cell growth inhibition (Fig. 1). Compared with control, XK469 caused minimum growth inhibition when used at 1 μM for 72 h. However, WSU-WM cell growth was inhibited up to approximately 50% by 5 μM XK49 for 72 h and >90% by 10 μM XK49 for 72 h.
and 10.0 mg/kg/i.v. injection for a total dose of 120 and 240 mg/kg (Fig. 1). Administering daily doses of XK469 at 20 and 40 mg/kg/i.v., seen was effective in inhibiting Topo II activity at 48 or 72 h (Fig. 2). However, 5 μM XK469, and there was no down-regulation of the Bcl-2 protein. Exposure of the Topo enzyme, which results in reduced bands in a constitutive manner (Fig. 2). This is contrary to studies done on solid tumors that required millimolar concentrations of XK469 for inhibition of the Topo II enzyme (2,11). The inhibitory effect of Topo II poisons is concentration-independent on the amount of enzyme present in cells causing a complex stabilization (1,12). Band depletion assay indicated that XK469 inhibited Topo IIβ in a dose-independent but time-dependent manner (Fig. 2). This is contrary to studies done on solid tumors that required millimolar concentrations of XK469 for inhibition of the Topo II enzyme (7,12). One possible explanation for these differences may be due to the WM model, which is representative of a differentiated indolent disease that expresses higher levels of the Topo IIβ isozyme in WSU-WM cells (Fig. 2). Agents that target intracellular Topos prevent the extraction of the Topo enzyme, which results in reduced bands in a band depletion assay, as detected by specific monoclonal antibodies (5). Using the band depletion assay, we demonstrate that XK469 inhibited Topo IIβ in a dose-independent but time-dependent manner (Fig. 2). This is contrary to studies done on solid tumors that required millimolar concentrations of XK469 for inhibition of the Topo IIβ enzyme (7,12). One possible explanation for these differences may be due to the WM model, which is representative of a differentiated indolent disease that expresses higher levels of the Topo IIβ isozyme (2,11). The inhibitory effect of Topo II poisons is independent on the amount of enzyme present in cells causing a complex stabilization (1,12). Band depletion assay indicated that XK469 completely inhibited Topo IIβ at 2 μM, resulting in DNA breaks demonstrated by smear in a DNA cleavage assay (data not shown); however, low growth inhibition and a low Bax:Bcl-2 ratio was observed at this concentration (Figs. 1 and 2a). XK469 (at IC50) triggered a well-known cascade of events (13–17) for the induction of apoptosis in WSU-WM cells. This included up-regulation of Bax and down-regulation of Bcl-2 (Fig. 2b). Our data show that although a low concentration (2 μM) of XK469 inhibited Topo IIβ, there was no significant cleavage of the PARP protein (Fig. 4a). However, at higher concentrations (5 and 8 μM), XK469 demonstrated cleavage of PARP, which has been shown to result in apoptosis (Ref. 18; Fig. 4b). Our narrow choice of dose range was based on the high sensitivity of WSU-WM cells to XK469. XK469, at 2–8 μM, inhibited cell proliferation from 15% to >80%, respectively (Fig. 1). Despite the fact that equal inhibition of Topo IIβ was observed at the two concentrations, significant differences

In Vitro Topo IIβ Inhibition. In vitro, XK469 at 2, 5, and 8 μM but not 1 μM inhibited Topo IIβ in a concentration-independent manner (Fig. 2a). Rather, the inhibition was time-dependent because 5 μM XK469 showed decreasing bands from 48 to 72 h (Fig. 2b). Amsacrine (mAMSA), a Topo II poison known to target both Topo II isozymes, resulted in depletion of Topo IIβ relative to DMSO when used as an internal control for the band depletion assay. XK469 does not inhibit Topo IIα or Topo I (Fig. 2c). To ensure that depletion of Topo II bands detected in such an assay is not due to decreased levels of the enzyme, a heat treatment procedure is used. This procedure is known to inhibit the nicking but not religation activity of Topos, hence returning the Topo signal to baseline levels, reflecting nondegradation of protein (Fig. 2d).

In Vitro Bax and Bcl-2 Expression. Induction of the Bax protein was insignificant when cells were exposed to 2 μM XK469, and there was no down-regulation of the Bcl-2 protein at 48 or 72 h (Fig. 3a). However, 5 μM XK469 induced Bax protein expression by 48 and 72 h and caused a decrease in constitutive levels of Bcl-2 by 72 h compared with control untreated cells. The Bax:Bcl-2 ratio was >11-fold in favor of Bax with 5 μM XK469 (Fig. 3b).

In Vitro PARP Cleavage. The proteolytic activation of PARP resulting in its cleavage from 116 to 85 kDa is a well-documented feature of apoptosis. Exposure of WSU-WM cells to 2 μM XK469 showed minimal PARP cleavage at 72 h, compared with 5 and 8 μM concentrations (Fig. 4).

In Vivo Inhibition of Topo IIβ and Antitumor Activity of XK469. Administering daily doses of XK469 at 20 and 40 mg/kg/i.v. injection for a total dose of 120 and 240 mg/kg (#1 and #2) in two mice, respectively, demonstrated that both doses were effective in inhibiting Topo IIβ, as determined by band depletion assay (Fig. 5).

In Vivo Bax and Bcl-2 Expression and PARP Cleavage. We evaluated the levels of PARP and PARP cleavage and Bax and Bcl-2 protein expression in WSU-WM tumors excised and dissociated from WSU-WM SCID xenograft mice (Fig. 6). High-level protein expression of Bcl-2 with low expression of Bax was found in tumors from control mice, with PARP mostly in its uncleaved form. Tumors taken from duplicate mice of each treatment group showed approximately equal low expression levels of the Bax protein, whereas the Bcl-2 protein level remained relatively higher. The Bax:Bcl-2 ratio required for the induction of apoptosis was low at ~0.3. The tumors from these treated groups also exhibited low cleavage of the PARP protein compared with control.

Effect of XK469 on Tumor Weight of the SCID Xenografts. We determined the average tumor weight during the entire time of tumor growth after treatment of SCID xenografts with a total of 120 and 240 mg/kg of XK469, respectively (Fig. 7), after which most of the mice were euthanized. There was no significant difference in rate of tumor growth with SCID mice treated at 120 mg XK469 compared with untreated controls. However, at the MTD of 240 mg/kg, there was some delay in tumor growth and a decrease in the average tumor weight (median, 1000 mg) compared with controls (median, 1700 mg).

Discussion

DNA Topos are evolving as chemotherapeutic targets, although the role of Topo IIβ inhibition in cancer has yet to be fully elucidated (2). We have found that at low (micromolar) concentrations, XK469 specifically inhibits Topo IIβ but not Topo I or the Topo IIα isozyme in WSU-WM cells (Fig. 2). Agents that target intracellular Topos prevent the extraction of the Topo enzyme, which results in reduced bands in a band depletion assay, as detected by specific monoclonal antibodies (5). Using the band depletion assay, we demonstrate that XK469 inhibited Topo IIβ in a dose-independent but time-dependent manner (Fig. 2). This is contrary to studies done on solid tumors that required millimolar concentrations of XK469 for inhibition of the Topo IIβ enzyme (7,12). One possible explanation for these differences may be due to the WM model, which is representative of a differentiated indolent disease that expresses higher levels of the Topo IIβ isozyme (2,11). The inhibitory effect of Topo II poisons is independent on the amount of enzyme present in cells causing a complex stabilization (1,12). Band depletion assay indicated that XK469 completely inhibited Topo IIβ at 2 μM, resulting in DNA breaks demonstrated by smear in a DNA cleavage assay (data not shown); however, low growth inhibition and a low Bax:Bcl-2 ratio was observed at this concentration (Figs. 1 and 2a). XK469 (at IC50) triggered a well-known cascade of events (13–17) for the induction of apoptosis in WSU-WM cells. This included up-regulation of Bax and down-regulation of Bcl-2 (Fig. 2b). Our data show that although a low concentration (2 μM) of XK469 inhibited Topo IIβ, there was no significant cleavage of the PARP protein (Fig. 4a). However, at higher concentrations (5 and 8 μM), XK469 demonstrated cleavage of PARP, which has been shown to result in apoptosis (Ref. 18; Fig. 4b). Our narrow choice of dose range was based on the high sensitivity of WSU-WM cells to XK469. XK469, at 2–8 μM, inhibited cell proliferation from 15% to >80%, respectively (Fig. 1). Despite the fact that equal inhibition of Topo IIβ was observed at the two concentrations, significant differences
existed in the cytotoxicity and initiation of other pathways resulting in programmed cell death. 3

In vivo, we found that XK469 doses at MTD (240 mg/kg) or below MTD (120 mg/kg) in SCID mice resulted in equal inhibition of Topo IIα/H9252 (Fig. 5). However, this inhibition was not sufficient to induce Bax, down-regulate Bcl-2, or induce PARP cleavage (Ref. 19; Fig. 6). When tumor response was determined according to tumor weight with respect to time, no reduction in tumor size was observed at 120 mg/kg, although this concentration was sufficient to deplete Topo IIβ. However, at a higher concentration of 240 mg/kg, Topo IIβ was equally as depleted as at the former dose, but there was a slight reduction in tumor burden of the SCID xenografts. The in vivo concentration of XK469 necessary to

3 Unpublished data.
achieve therapeutic effect in solid tumors has been determined in regular mice to be between 350 and 600 mg/kg (20–22), about four times the concentration needed for Topo IIβ inhibition in our SCID model. We speculate that XK469 has multiple pathways for the induction of cell death, which may be associated with a target independent of Topo IIβ, and appears rather to be dose dependent. The role of Topo IIβ as a target for XK469 still remains unclear; however, our study supports data that Topo IIβ inhibition is not the primary cause of apoptotic events in WSU-WM cells.

Collectively, this study demonstrates that XK469 inhibits Topo IIβ in WSU-WM cells in vivo and in vitro at subtherapeutic doses and low micromolar concentrations. However, this inhibition does not result in a therapeutic response or induce apoptosis in either SCID xenografts or the cell line.

References


2-[4-(7-Chloro-2-quinoxalinyloxy)phenoxy]-propionic Acid (XK469) Inhibition of Topoisomerase II $\beta$ Is Not Sufficient for Therapeutic Response in Human Waldenstrom’s Macroglobulinemia Xenograft Model
