2-[4-(7-Chloro-2-quinoxalinyloxyphenoxy)-propionic Acid (XK469), an Inhibitor of Topoisomerase (Topo) IIβ, Up-Regulates Topo IIα and Enhances Topo IIα-mediated Cytotoxicity

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Abstract

Topoisomerase (Topo) IIα has proven to be an adequate anticancer target for tumors expressing this enzyme. In this study, we elucidated the effect of 2-[4-(7-chloro-2-quinoxalinyloxyphenoxy)-propionic acid (XK469; a new Topo IIβ inhibitor) in the modulation of Topo IIα levels and sensitivity to Topo IIα poisons. We demonstrate by Western blot analysis that indolent B-cell tumors express undetectable levels of this enzyme and are refractory to the effects of Topo IIα poisons such as VP16. Using the Waldenstrom's macroglobulinemia (WM) cell line WSU-WM, we show that XK469 induced the expression of Topo IIα protein by 24 h compared with control. Immunofluorescence studies by confocal microscopy using a specific monoclonal antibody against Topo IIα supported the immuno blot findings with high intensity staining in XK469-exposed cells. To determine the effect of up-regulating Topo IIα on sensitivity of Topo IIα-directed inhibitors, WSU-WM cells were exposed to simultaneous, sequential, and reverse order XK469 and VP16. We demonstrate that 24 h of exposure to XK469 before VP16 resulted in a maximum synergistic response. In contrast, simultaneous or reverse order exposure resulted in an antagonistic effect. A similar trend was observed with cells obtained from chronic lymphocytic leukemia patients, but not in normal lymphocytes. This increase in VP16 sensitivity after 24 h of XK469 exposure was associated with VP16-dependent DNA cleavage, as demonstrated by formation of a smeared DNA band in a SDS-KCL DNA cleavage assay. From this study, we concluded that XK469 up-regulates Topo IIα levels and consequently sensitizes indolent malignant B cells to the cytotoxic effect of VP16 in a schedule-dependent manner.

Introduction

Indolent B-cell malignancies are categorized as low-grade lymphoid tumors that remain incurable with available standard chemotherapy (1). These malignancies include CLL (2), follicular lymphoma, multiple myeloma, and WM (2). Some presently used agents for indolent malignancies include nucleoside analogues, alkylating agents, and prednisone, all of which cause an initial response but no cure; median survival is 5 years (3). Among some newer treatment modalities are the use of biological agents alone or in combination with standard chemotherapy (4) and immunotherapy using mouse-human chimeric anti-CD20 monoclonal antibody (Rituximab; Ref. 5). Although they appear promising, these developments have not achieved a cure, and there is a need for more innovative approaches that may include new strategic targets (6).

Topos are fast becoming targets for cancer therapy but have not been used in the treatment of indolent B-cell malignancy (7). Topo IIα has proven to be an adequate anticancer target for a number of solid tumors and certain hematological malignancies (8, 9). VP16 is a standard chemotherapeutic agent used for the treatment of actively proliferating tumors (10); however, this agent is not the choice for indolent low-grade B-cell malignancies. This is due to the fact that the target, Topo IIα, is present at very low levels in these indolent malignant cells (11). It is well documented that Topo IIα drops sharply to significantly low levels as cells get into nonproliferative and quiescent phase (12). The enzyme expression is also lost as cells become terminally differentiated (13, 14). Indolent B-cell tumors are generally well differentiated and in a quiescent nonproliferative state, representing cells with low Topo IIα expression (15). Early work demonstrated a low or even absent level of Topo IIα mRNA in a number of CLL patients (16). Another independent study looked at exposure of CLL to a number of Topo II inhibitors to assess detectable DNA cleavage. Resistance of human leukemic cells and normal lymphocytes to the effect of VP16 was attributed to the significantly low levels of Topo IIα in these nonproliferating cells (15). Previous work has demonstrated that a Topo I inhibitor (topotecan) was able to increase the levels of Topo IIα and the sensitivity of colon

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3 The abbreviations used are: CLL, chronic lymphocytic leukemia; Topo, topoisomerase; WM, Waldenstrom’s macroglobulinemia; PTN, PBS with 1% bovine serum albumin plus 0.1% Tween 20; 2CdA, 2-chloro-2’-deoxyadenosine; FBS, fetal bovine serum.
cancer xenografts to Topo IIα-directed poison VP16 (17). Studies cited above and numerous others over the last decade have strongly correlated the expression of Topo IIα with sensitivity to VP16 and other Topo IIα-targeting agents.

XK469 is a synthetic quinoxaline phenoxypropionic acid derivative found previously to target Topo IIα (18). Previous work has described Topo IIβ as highly expressed in indolent B-cell tumors, in which Topo IIβ remains elevated in both treated and untreated disease states (19). In this study, we report on the effects of Topo IIα induction by XK469 and sensitivity of indolent cells to VP16. This finding provides the theoretical basis for the combined use of Topo IIα and Topo IIβ poisons as a synergistic treatment of indolent B-cell malignancy.

Materials and Methods

Patient Samples. Lymphocytes from whole blood of three CLL patients and two normal donors were separated using Ficoll-Hypaque for isolation of mononuclear cells. Lymphocytes were cultured during the experimental period as a suspension in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO2.

Immunofluorescence Studies of DNA Topo II. Immunocytochemistry for the detection of Topo IIα was done according to the method described by Oloumi et al. (20). Briefly, cells were seeded at 2 x 10^5 cells/ml and exposed to 5 μM XK469. Cells (2 x 10^5) in ice-cold PBS were deposited by cytopinning (Cytospin II; Shandon, Pittsburgh, PA) onto cleaned glass slides at 800 rpm for 8 min. Slides containing cells were air dried for 10 min and immersed in cold 1% paraformaldehyde (Sigma) in PBS for 30 min at 4°C. They were then rinsed in PBS and immersed for 30 s in acetone at room temperature. The slides were again rinsed two times in PBS and immersed in PTN for 20 min at room temperature. Cells were stained and placed in a humidified chamber, into which 25 μl of 1:100 anti-Topo IIα antibody (TopoGen, Columbus, OH) in PTN were deposited, covered with parafilm strips, and placed overnight at 4°C. Slides were then removed from the chamber, rinsed two times for 5 min in PBS, rinsed once for 5 min in PTN, and incubated with 25 μl of 1:150 secondary antibody in PTN, Alexa Fluor 488 F(ab')2 fragment of goat antirabbit IgG (H+L), away from light at room temperature for 2 h. Cells on slides were rinsed in PBS, immersed in 0.5 μg/ml 4′,6-diamidino-2-phenylindole in PBS for 5 min, and rinsed again for 5 min in PBS. The washed cells were then drained, mounted in SlowFade mounting medium, sealed, and examined using a Zeiss LSM 310 confocal microscope.

Topo IIα-mediated DNA Cleavage. WSU-WM cells were seeded in serum-free medium at 2 x 10^5 cells/ml, exposed to 5 μM XK469 and 1 μM VP16 as single agents and either in combination or sequentially, and incubated for 48 h. Cells (2 x 10^5 cells/ml) were pelleted, lysed with lysis buffer (1% SDS, 25 mM EDTA, and 100 mM KCl), and incubated with 1.5 μg of DNase-free RNase (Roche Diagnostics Corp., Indianapolis, IN) per 250-μl reaction mixture for 1 h at 37°C. One hundred and sixty μg from a 20 mg/ml stock proteinase K per reaction mixture were added and incubated at 55°C for 2 h. Genomic DNA was extracted with buffer-saturated phenol followed by phenol:chloroform:isoamyl alcohol. After the addition of 10% 3 M sodium acetate, DNA was precipitated with 95% ice-cold ethanol in a dry ice bath (dry ice plus 95% ethanol) for 15 min. DNA samples were loaded onto a 10% agarose gel in Tris-borate EDTA (FisherBiotech, Fair Lawn, NJ), run at 100 V, and stained in 0.5% etidium bromide solution before analyzing with Eagle Eye II (Stratagene).

Drugs. VP16 was obtained from Sigma-Aldrich Co. and used at concentrations of either 10 nm or 1 μM. 2CdA was obtained from Orthobiotech Inc. (Raritan, NJ) and used at a concentration of 100 nm.

XK469 is described in detail in Ref. 21. WSU-WM cell line was maintained and cytotoxicity assay, Western blotting, and band depletion assay are explained in detail in Ref. 21.

Results

Topo IIα Levels in Indolent B-Cell Tumors. Our results show that cultured WSU-WM cells in serum-starved media in which serum concentration is reduced from 10% to 0% had a significant decrease in the level of DNA Topo IIα compared with cells cultured in complete (10% serum-containing) media (Fig. 1). Lymphocytes from CLL patients and normal lymphocytes showed no detectable Topo IIα expression. In contrast to the Topo IIα result, Topo IIβ level was elevated upon serum starvation and was present in the CLL sample, but not in normal lymphocytes.

XK469 Induces Topo IIα in WSU-WM Cells. In the presence of 10% FBS, there was an increase in the expression levels of Topo IIα in WSU-WM cells after 24-, 48-, and 72-h exposures of WSU-WM cells to 5 μM XK469 compared with the untreated control cells (Fig. 2a, Lanes 4–6). In contrast, exposure of WSU-WM cells to Amsacrine (m-AMSA), a known Topo IIα poison, resulted in depletion of Topo IIα relative to control (Fig. 2a, Lane 3). Whereas the Topo IIα level remain high in WSU-WM cells cultured in complete media (10% FBS), after serum deprivation for 48 h, the level of Topo IIα decreased and became almost undetectable (Fig. 2b). Interestingly enough, exposure of WSU-WM cells to XK469 in serum-free media for 48 h caused up-regulation of Topo IIα expression (Fig. 2b, Lane 3). The level of Topo IIα induc-
Induction of Topo IIα by XK469 in the WSU-WM cell line. Band depletion assay shows Topo IIα levels in WSU-WM untreated cells (Ctr) and in cells treated with 5 μM XK469 for 24, 48, and 72 h, respectively. In complete media DMSO (solvent) and m-AMSA (Topo II poison) used as control (a). b shows a Western blot depicting relative levels of Topo IIα in cells cultured with complete media (Ctr), serum-starved for 48 h (sf-Ctr), and exposed to XK469 in serum-free conditions for 48 h. The corresponding bottom panels are glycolysis-specific enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PDH), which was used as an equal protein loading control. c shows dose-dependent up-regulation of Topo IIα by XK469 in WSU-WM cells cultured in serum-free media.

Immunofluorescence Evidence of Topo IIα Induction in XK469-treated WSU-WM Cells. The observation that the level of Topo IIα is up-regulated by XK469 was further supported by immunofluorescence staining of intracellular Topo IIα (Fig. 3). WSU-WM cells exposed to XK469 in serum-free media showed strong immunofluorescence staining, in contrast to untreated controls. Secondary antibody alone under the same conditions of illumination showed no significant fluorescence (data not shown).

Cytotoxic Effect of VP16, a Known Topo IIα Poison. The increase in the Topo IIα level in XK469-treated WSU-WM cells prompted us to investigate the potential VP16-mediated cytotoxicity upon increase of Topo IIα, the known cellular target of VP16 (17). The cytotoxic effects of various combination exposures of WSU-WM cells to XK469 and VP16 were studied (Fig. 4b). Statistical analysis showed that cells were most sensitive to cytotoxic effects when VP16 was given 24 h after preexposure to XK469. It was important to note that when the two agents were given in the reverse order (i.e., VP16 and then XK469) or simultaneously, the cytotoxic effect was significantly reduced.

CLL lymphocytes taken from a patient exposed to similar dose and schedules of XK469 and VP16 showed trends similar to those observed in WSU-WM cells (Fig. 4b). Twenty-four h exposure to XK469 before VP16 exerted the most cytotoxicity in CLL lymphocytes. Strikingly, neither peripheral blood lymphocytes from normal donor nor the addition of toxic doses of the nucleoside analogue 2CdA in that schedule showed cytotoxicity (Fig. 4, c and d). Rather, the 24-h exposure to XK469 before 2CdA made the cells refractory to the cytotoxic effect of 2CdA (Fig. 4d).

**XK469 Enhances Topo IIα-mediated DNA Cleavage Induced by VP16 in a Schedule-dependent Manner.** It has been well documented that the formation of cleavable complexes is the basis of the cytotoxic effect of Topo II poisons and is directly dependent on the availability of target, in this case, Topo IIα for VP16 (7, 9, 22). To explain the cause of cytotoxicity found in the specific XK469-VP16 treatment schedule, we evaluated the effect of XK469 modulation of Topo IIα on the ability of intracellular Topo IIα to form cleavable complexes after exposure to VP16, as happened when VP16 was given 24 h after preexposure to XK469. Whereas it was not detectable in the other schedules (Fig. 5, Lanes 1–4 and 6), the DNA smear was clear in WSU-WM cells preexposed for 24 h to XK469 followed by VP16 (Fig. 5, Lane 5). The observed DNA smear is a typical characteristic of DNA cleavage due to the topo-drug-DNA ternary complex arrested upon the strong protein denaturant, SDS, treatment as part of the experimental procedures (11, 15). Hence, the result can be best explained by the VP16-Topo IIα-DNA ternary complex-mediated cleavage activity under the specific drug treatment schedule. The cytotoxicity associated with the particular drug treatment schedule is most likely due to VP16-mediated poison. This is consistent with the finding that elevation of Topo IIα, the VP16 target, is observed when WSU-WM cells or CLL lymphocytes were exposed to XK469 for 48 h (Fig. 2b). It is also important to point out that consistent with the cytotoxic response, cells pretreated with VP16 for 24 h before XK469 were resistant with no formation of cleavage complexes.

**Discussion**

In Ref. 21, we have shown that XK469 inhibits Topo IIβ in WSU-WM cells in vivo and in vitro. However, this inhibition...
did not result in a therapeutic response in SCID xenografts, nor did it induce apoptosis in the cell line. In this study, we demonstrated that XK469 induced the expression of Topo IIα, suggesting a direct modulation of Topo IIα by XK469. Previous studies have determined the lack of efficacy of Topo IIα poisons in the treatment of malignancies with predominance in nonproliferating cells as a result of their low Topo II levels (10). Consistent with the literature, we found that patient-derived CLL cells and normal lymphocytes had nondetectable levels of Topo IIα (Fig. 1). We observed stronger induction of Topo IIα and greater cytotoxicity in nonproliferating cells exposed to XK469. Topo IIα has been shown to peak at G2-M phase of the cell cycle (23), whereas XK469 is known to cause G2-M arrest in cells. Upon serum starvation with cells in G0-G1 phase, Topo IIα is down-regulated (Fig. 2b). Exposure to XK469 traps cells in G2-M, resulting in increased expression of Topo IIα in XK469-treated cells compared with control. However, we have observed in vivo up-regulation of Topo IIα, associated with increased proliferation of tumors treated with XK469 at low concentrations. In addition, flow cytometric analysis of cells exposed to XK469 showed maximum G2-M arrest at 48 h, after that, it begins to decline; the G2-M arrest by XK469 is therefore a transient phenomenon. Studies have demonstrated that exponentially growing cells induced to enter G0 phase by serum starvation caused a parallel decrease in the expression level of Topo IIα. When stimulated to enter the cell cycle, Topo IIα peaked at G2-M (23). Under certain conditions, quiescent cells may be stimulated to resume proliferation via up-regulation of Topo IIα (24). The increase in Topo IIα expression levels by XK469 in WSU-WM cells may be a physiological response to compensate for XK469-mediated Topo IIα inhibition rather than an indirect effect of G2-M arrest. It has been reported that tumor cells induced to express high levels of Topo IIα were transformed from highly resistant to a state of increased sensitivity to the effects of VP16 and other Topo IIα-targeting agents (17). Hence we hypothesized that induction of Topo IIα in indolent B-cell tumors would sensitize them to VP16.

Because Topo IIα is not an intracellular target of XK469, there was no stabilization of the covalent Topo-DNA cross-links (Ref. 22; Fig. 2a). Our data suggest the induction of Topo IIα in WSU-WM cells to be within 24 h of exposure.
exposure; thereafter, the expression is maintained at constant levels (data not shown). Due to the limited number of cells obtained from samples, we were unable to expose these cells to XK469 to determine induction of Topo II. However, the cytotoxic effect of XK469 before VP16 on these fresh cells demonstrated a pattern similar to that seen in the WSU-WM cell line (Fig. 3b). It was interesting to note that up-regulation of the α-isozyme in WSU-WM cells was achieved at low doses similar to the required dose for targeting the β-isozyme, with very low cytotoxic effect. XK469 is now in Phase I trial, and the toxicity has yet to be determined; however, in our hands, severe combined immunodeficient mice exposed to XK469 demonstrated high toxicity without reaching the optimal therapeutic dose. Western blot analysis of s.c. tumors obtained from mice treated with XK469 demonstrated an up-regulation of Topo IIα in s.c. tumors, similar to WSU-WM cells in culture (data not shown). The ability of XK469 to modulate Topo IIα in vivo was achieved at a level below the maximum tolerated dose where toxicity was almost insignificant in an efficacy trial.

Immunofluorescence staining of Topo IIα in WSU-WM cells exposed to XK469 in vitro demonstrated a higher number of positive cells compared with untreated controls (Fig. 3), in agreement with the levels of Topo IIα observed by immunoblot analysis.

Studies have shown that high levels of Topo IIα were associated with increased sensitivity and better response to the Topo IIα poisons such as VP16 (17). Indolent malignant B cells or serum-starved cell lines express significantly low levels of Topo IIα. We proceeded to determine whether the induction of Topo IIα in serum-starved cells by XK469 would render them more sensitive to VP16. Indeed, a synergistic effect on growth inhibition was observed when cells were exposed to XK469 24 h before etoposide. Cells maintained in complete media, with already high baseline expression of Topo IIα, did not demonstrate a specific schedule-dependent effect of the two agents combined. We speculated that lack of proliferation or inhibition of both Topo II isoforms led to enhanced cytotoxicity; however, this was quickly ruled out because the concurrent addition of the two agents or addition of the agents in reverse sequence did not show any synergistic or even additive cytotoxicity. Although the reduction in the number of cells exposed to XK469 24 h before VP16 was not dramatic, we observed that increased cytotoxicity was seen with serum-starved cells and dependent on an initial increase of Topo IIα, hence making it schedule specific (Fig. 4a). Induction of sensitivity and enhanced response to VP16 were consistent with the immunoblot analysis as well as the immunofluorescence stain and could be attributed to increases in the Topo IIα expression levels observed. Because we used our serum-starved cells as a model of indolent B-cell malignancy, we compared the schedule-dependent cytotoxicity of both agents on patient samples. Similar findings were observed when fresh CLL cells were exposed to similar concentrations and schedules of XK469 and VP16 (Fig. 4b). On the contrary, peripheral blood lymphocytes from a normal donor (with nondetectable expression level of both Topo II isoforms) were refractory to the effects of either agent alone or in combination (Fig. 4c).

We did not proceed to show latter time points beyond 72 h for the cytotoxicity assay because our control cells were showing poor viability due to either depleted serum in the case of cell line or life span for regular lymphocytes. However, selectivity of the cytotoxic effect to the malignant and not normal lymphocyte was attributed to the undetectable level of both Topo II isoforms as well as failure of XK469 to cause induction of Topo IIα in normal lymphocytes.

It was interesting to note that 24 h exposure of WSU-WM cells cultured in complete media to XK469 before 2CdA resulted in abrogation of the cytotoxic effect of 2CdA, even though 2CdA alone or given concurrently or in reverse sequence combination with XK469 resulted in a significant cytotoxic effect. This may be due to high Topo IIα levels implicated in the complex mechanism of resistance and repair, as shown in WSU-WM CLL to the alkylating agent melphalan (25). Serum-starved cells exposed for 24 h to XK469 before 2CdA did not demonstrate schedule-dependent cytotoxicity as with VP16 (data not shown).

Our findings are consistent with these reports in the literature and strongly suggest that up-regulation of Topo IIα can be a mechanism of resistance to XK469 by changing DNA topology and facilitating repair, rendering cells refractory to other cytotoxic agents besides Topo IIα inhibitors. Up-regulation of Topo IIα rendered WSU-WM cells refractory to the cytotoxic effect of 2CdA and was dependent on the time required for XK469 to induce Topo IIα expression.

The cytotoxic effect of Topo IIα-directed poisons such as VP16 involves interfering with the breakage/reunion reaction of Topo II, resulting in cleavage of DNA (12). Increases in the levels of the target enzyme for Topo IIα poisons render the cell hypersensitive and increase the cleavage of DNA via cleavable complex formation (26). Fig. 5 showed that the most cleavage of genomic DNA was obtained when WSU-WM cells were exposed for 24 h to XK469 before VP16, which was characterized by a smeared DNA.

Taken together, our data suggest that the initial increase in Topo IIα levels by XK469 leads directly and selectively to an increase in sensitivity of these cells to Topo IIα poisons such as etoposide. Previous findings have suggested a compensatory effect with up-regulation of Topo II when Topo I was inhibited (17). However, no such relationship is known to exist between the two Topo II isoforms. Our findings provide new information on the activities and importance of these two enzymes and their synergistic effect in therapeutic targeting of B-cell lymphoma. We conclude that XK469 modulates Topo IIα levels by increasing expression and renders WSU-WM cells more sensitive to Topo IIα poison such as VP16.

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References


