Preclinical Evaluation of 2-[4-(7-Chloro-2-quinoxalinyloxy)phenoxy]-propionic Acid as a Modulator of Etoposide in Human Waldenstrom’s Macroglobulinemia Xenograft Model

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ABSTRACT
We have previously reported that XK469 (2-[4-(7-chloro-2-quinoxalinyloxyphenoxy)]-propionic acid) enhances topo IIβ expression in WSU-WM cells in vitro [E. Mensah-Osman et al., Mol. Cancer Ther., 1: 1321–1326, 2002]. To test the hypothesis that XK469-induced expression of topo IIα sensitizes WSU-WM cells to the topo IIα inhibitor etoposide (VP-16), we investigated the antitumor effects of XK469 and VP-16 in vivo, using the WSU-WM SCID xenograft model. Individual dosages of XK469 at 20–60 mg/kg injection i.v. for a maximum-tolerated dose of 240 mg/kg were achievable in SCID mice. Simultaneous administration of a subtherapeutic dose of XK469 (20 mg/kg) and VP-16 at its maximum-tolerated dose of 15 mg/kg proved to be highly toxic and lethal. However, daily sequential treatment of XK469 given i.v. via tail vein at 20 mg/kg for a total of 120 mg/kg, followed 7 h later by VP-16 i.p. at 15 mg/kg for a total of 90 mg/kg, had no significant toxicity in SCID mice. The sequential treatment was associated with enhanced antitumor activity. Tumor growth inhibition T/C, tumor growth delay T-C, and log10 kill for XK469 alone were 61%, 3 days and 0.46; VP-16 alone 6%, 12 days and 1.83, respectively; whereas the sequential administration of both agents gave a T/C value of 0%, T-C value of 23 days and a log10 kill of 3.5. On the basis of these animal results, we conclude that the sequential treatment of WSU-WM tumors with XK469 and VP-16 was highly active. The study supports our in vitro observation that XK469 potentiates VP-16 activity. The sequential use of both agents resulted in clinically significant antitumor activity in the WM model.

INTRODUCTION
WM1 remains an incurable disease to date (1), despite high response rate to therapy for variable periods of time (2). Empirically designed chemotherapy regimes have failed to provide long-term remission or cure. There is need for development of more rational therapy based on mechanistic interaction between different therapeutic agents.

We have earlier established a permanent WM cell line, WSU-WM, which grows well as a xenograft in SCID mice and is the only preclinical model for this malignancy (3).

XK469 is an analogue of the herbicide Assure discovered in a screen for solid tumor-selective agents (4, 5). It is a synthetic quinoxaline phenoxypropionic acid derivative, having both the (r+) and (S)-isomer forms. It has a polycyclic group and an ionizable negatively charged carboxyl end. XK469 is available either as a sodium salt racemic compound, which is more water soluble and active, or the racemic-free acid form (4). XK469 has broad activity against murine solid tumors such as colon, pancreatic, and mammary glands and highly active against multidrug-resistant tumors (4). Exposure of human colon carcinoma cells to XK469 for 24 h resulted in 90% inhibition of DNA synthesis, increased RNA and protein synthesis, and a block at the G2-M boundary of the cell cycle in association with increased expression of cyclin B1 (6, 7).

VP-16 is a DNA damaging-topo II β inhibitor that kills cells by increasing levels of topo II-mediated DNA breaks (8). It is currently used as standard chemotherapy for solid tumors and relapsed diffuse lymphomas.

We previously reported the effect of XK469 on topo IIα-mediated cytotoxicity in WSU-WM cell line in vitro (9, 10). We presented evidence that XK469 up-regulates topo IIα and enhances sensitivity of the cells to topo IIα inhibitor, VP-16. On the basis of these results, we designed sequential treatment of tumor-bearing SCID mice using XK469 at subtherapeutic and nontoxic doses followed by VP-16 at its MTD. Our results indicate that XK469 enhanced activity of the drug (VP-16) against WM. These results suggest a new chemosensitizing strategy for indolent B-cell tumors that may have clinical use.

MATERIALS AND METHODS
Drugs. VP-16 was obtained from Sigma-Aldrich Co. (St. Louis, MO) and used at 15 mg/kg/injection i.p.

XK469 (racemic, sodium salt, NSC656889) was provided by the National Cancer Drug Synthesis Branch. It was dissolved in 1% NaHCO3 and used at a concentration of 10 mg/ml.

1 The abbreviations used are: WM, Waldenstrom’s macroglobulinemia; VP-16, etoposide; MTD, maximum-tolerated dose.
WSU-WM SCID Mouse Xenograft Model. The WSU-WM SCID mouse xenograft model established in our laboratory by injection of human WSU-WM cells (5 × 10^6) into flank areas of SCID mice as has been described in detail in a previous publication (3). Palpable s.c. tumors that develop in ~2 weeks were propagated serially by transplanting tumor fragments of ~20–30 mg into bilateral flanks of new animals using a 12-gauge trocar needle. Groups of five animals were randomly removed after the development of palpable tumors and assigned to different treatment groups including control. Animals were observed for measurements of s.c. tumors, changes in weight, and adverse effects of drugs. Mice were euthanized when total tumor burden reached 2000 mg to avoid discomfort. All studies involving mice were performed under the Institutional Review Board-approved protocol.

Experimental Design for MTD and Schedule-Dependent Toxicity. MTD of XK469 was determined from the following groups with five mice/group: group 1, untreated control; group 2, daily treatment at 50 mg/kg/injection for 4 days; and group 3, daily treatment at 60 mg/kg/injection for 3 days.

Schedule-dependent toxicity was divided into four groups and assessed with daily treatments as follows: group no. 1, 20 mg/kg/injection XK469 plus 15 mg/kg/injection VP-16; group no. 2, 40 mg/kg/injection XK469 plus 15 mg/kg/injection VP-16; group no. 3, 20 mg/kg XK469 for 7 and 24 h followed by 15 mg/kg VP-16; and group no. 4, 40 mg/kg XK469 for 7 and 24 h followed by 15 mg/kg VP-16.

Antitumor Effect of XK469 at MTD. Animals were divided into five groups of five mice each, with each bilateral s.c. tumors as described for MTD. Treatment was started with doses of 20 and 40 mg/kg/injection for 6 days for MTD of 240 mg; 50 mg/kg/injection for 4 days for MTD of 200 mg; and 60 mg/kg/injection for 3 days for MTD of 180 mg, i.v. via tail vein. Two untreated groups of five mice each were used as controls.

Efficacy Trial Design for Sequential Treatment with XK469 and VP-16. Seventy-two h after serial transplantation of tumor fragments, animals were randomly removed and assigned to various treatment groups: group no. 1, untreated control; group no. 2, received injections of 20 mg/kg/injection. XK469 via tail vein for 5 days; group no. 3, received injections of 15 mg/kg VP-16 i.v. daily for 5 days; and group no. 4, received XK469 at 20 mg/kg i.v. followed 7 h later by 15 mg/kg VP-16 in 3 i.p. daily for 5 days.

Assessment of Tumor Response. Assessment of antitumor activity was done according to standard procedures used in our laboratory (8). Tumor weight (mg) = (a × b^2)/2, where a and b are the tumor length and width in (mm), respectively. Tumor growth inhibition (T/C) is measured as the median tumor weight of the treated group (T) divided by the median tumor weight of the control group (C) at the time when the median tumor weight in the control group has reached ~700 mg. The drug evaluation branch of the division of cancer treatment, National Cancer Institute, considers a T/C value ≤42% as significant antitumor activity. Tumor growth delay (T-C) is assessed by the difference between the median time, in days, required for the treatment group tumor (T) to reach 700 mg and the median, in days, for the control tumor group (C) to reach the same weight. The log10 tumor cell kill (gross) = (T-C)/(3.32)(T d). Tumor doubling time (T d) is the time required in days for the tumor to double its weight during the exponential growth phase. For comparison of activity with the agents and comparisons of activity between tumors, the log10 kill values were converted to an arbitrary activity rating (11; Table 1).

RESULTS

MTD of XK469 in Mice Is Significantly Lower Than Optimum Therapeutic Dosages. Table 2 shows an individual dosage of XK469 given daily at 40 mg/kg i.v. for a total dose of 240 mg/kg. Animals at this dose experienced weight loss of <5% and had scruffy fur, however, with full recovery 48–72 h after completion of treatment. Fifty mg/kg i.v. could be injected daily for 4 days to reach an MTD of 200 mg/kg, however, a high mortality rate with death of mice on the sixth day after treatment was observed. An individual dose of 60 mg/kg i.v. was given daily for only 3 days for a total dose of 180 mg/kg. This was also poorly tolerated by animals producing substantial morbidity with weight loss of ~10–18%, gastrointestinal complications, poor appearance, and slow or no recovery because of acute toxicity. All animals died by the fifth day after treatment was completed.

Fig. 1, A and B, are the growth rate of WSU-WM xenografts measured over the course of treatments at their corresponding doses. The results show no significant tumor growth delay in response to the MTD of 40 mg/kg i.v. for a total dose of 240 mg/kg. At MTD of 200 and 180 mg, for 50 and 60 mg/kg, respectively, dose-dependent tumor growth delay were observed, however, doses were highly toxic and all animals died by the sixth day after treatment.

Dose-Schedule Toxicity of XK469 and VP-16. We found an unusual dose-schedule limitation with the combination of XK469 and topo II inhibitors. VP-16. Concurrent administration of XK469 at 40 mg/kg plus VP-16 at 15 mg/kg resulted in acute toxicity within 1–3 days during period of treatment. Animals showed signs of severe gastrointestinal complications, immobility, and scruffy fur, all of which was not associated with weight loss. On the other hand, pretreatment with XK469 (at 20 or 40 mg/kg) followed by VP-16 (at 15 mg/kg) were well tolerated within a period of 7–24 h for 6 days (Table 3).

Antitumor Activity of XK469 and VP-16 in WSU-WM-Bearing SCID Mice. Table 4 shows the antitumor activity of XK469 alone (at 20 mg/kg), VP-16 (at 15 mg/kg), or 7-h pretreat-
ment with XK469 followed by VP-16 against WSU-WM-bearing SCID mice. When tumor response is assessed by T/C value, VP-16 alone (T/C = 6%) and its sequential combination with XK469 (T/C = 0%) are considered active against this tumor. However, if log_{10} kill values are added as a criterion, only the sequential combination had a clinically significant activity (++++). A score of ++++ (highly active) is needed to effect partial or complete tumor regression, and 7-h pretreatment with XK469 followed by VP16 was the only group that met this criterion. Two of the seven mice in this group achieved total cure because the bilateral palpable tumors disappeared completely, and mice were alive and well without tumors >150 days from treatment.

**DISCUSSION**

Preclinical toxicity evaluation of individual dosages of XK469 in SCID mice showed an MTD significantly lower than the suggested optimum therapeutic dose in regular mice (4, 5), with no substantial effect on the tumor growth rate (Fig. 1A). The MTD seemed to depend on single dose peaks as slightly higher individual doses produced substantial toxicity with slow or no recovery in SCID mice. This created an obstacle for a preclinical study to evaluate the antitumor effect of XK469 in our WSU-WM SCID xenografts. However, based on an *in vitro*-delineated mechanistic study where XK469 modulated

**Table 3** In vivo schedule of concurrent or sequential dosing for WSU-WM-bearing SCID mice

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Agent/s</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>Duration (days)</th>
<th>MTD</th>
<th>Toxicity</th>
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<tbody>
<tr>
<td>1</td>
<td>XK469</td>
<td>20 Concurrent</td>
<td>5</td>
<td>100</td>
<td>Acute</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP-16</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>XK469</td>
<td>40 Concurrent</td>
<td>3</td>
<td>120</td>
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<tr>
<td>3</td>
<td>XK469</td>
<td>20 Sequential</td>
<td>6</td>
<td>120</td>
<td>Nontoxic</td>
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<tr>
<td></td>
<td>VP-16</td>
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<tr>
<td>4</td>
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<td>VP-16</td>
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**Table 4** Antitumor activity of XK469 and VP-16 alone and in sequential combination in WSU-WM-bearing SCID mice

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>No. of T/C (%)</th>
<th>T-C (days)</th>
<th>Log_{10} kill (gross)</th>
<th>Antitumor activity</th>
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<tr>
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<td>61</td>
<td>3 0.46</td>
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<tr>
<td>VP-16</td>
<td>15</td>
<td>50</td>
<td>66</td>
<td>12 1.83</td>
<td>++</td>
</tr>
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</table>

"XK469 was injected i.v. 7 h before the injection of VP-16. T/C is tumor growth inhibition, T-C is tumor growth delay; log_{10} kill gross = (T-C)/(T_d) where T_d is the time (in days) required for tumor to double its weight. Rating score of (+++, active) or (+++, highly active) is needed to effect partial or complete tumor regressions; (+, marginal activity) and (+) is not active."
expression of topo IIα and increased topo IIα-mediated cytotoxicity (7), we proceeded to address the in vivo effect of XK469, as a sensitizer of WSU-WM xenograft tumors to the clinically active topo IIα inhibitor, VP-16.

The profound toxicity observed upon concurrent administration of XK469 and VP-16 (Table 3) has important clinical implications. Inhibition of both enzymes, topo IIα and β, by these two agents should not be scheduled concurrently. Ironically, other single agents that inhibit both topo IIα and β have proved not to be as lethal. We speculate that single agents that inhibit both topo IIα and β enzymes by mechanism of stabilizing DNA-topo complexes have the ability to trap only one enzyme at a time. Because these agents are not able to inhibit both enzymes simultaneously, they may allow for compensation of one isofrom toward the other. Inhibiting both topo II isoforms simultaneously with two separate agents allows for little or no topo II activity to take place and proves to be lethal. We have not come across any two of such inhibitors that are currently used in clinic. Interestingly, however, topo I and topo II inhibitors have been used in clinical trials without report of significant toxicities, probably because of their different functions.

We have previously reported that XK469 up-regulates topo IIα and enhanced topo IIα-mediated cytotoxicity by VP-16 in vitro (9, 10). This study was primarily designed to extend our in vivo findings using in vivo model. One of the challenges we faced in our efficacy trial was to create an indolent tumor scenario in SCID mice. As described earlier, the WSU-WM-SCID xenografts have attained aggressive features of the disease, and we speculate that this may be because of an additional myc translocation (3). The xenograft tumors proliferate rapidly with a relatively short doubling time and express baseline levels of topo IIα enzyme (3). It was difficult to provide direct evidence showing up-regulation of topo IIα in vivo by XK469 as we did in vitro under serum-free condition (9). We speculate that WSU-WM tumors express low levels of topo IIα during initial period of lag phase of growth after transplantation because topo IIα expression is proliferation dependent. However, we could not obtain enough tumor tissue to determine topo IIα expression given the small size of tumors during that lag-phase period. However, our observations show that administering VP-16 within 7–24 h after XK469 resulted in high antitumor activity with a better survival rate. The end points of this study were tumor growth inhibition (T/C), tumor growth delay (T-C), and log10 kill. XK469 by itself showed no activity based on all end points. When tumor responses are determined by the T/C value, VP-16 is considered active, probably because of high topo IIα baseline levels. However, taking the T-C value, which is time (in days) required for the treatment group and controls to reach the predetermined size of 700 mg, which allows for the quantification of tumor cell kill, then the antitumor effect of both agents given sequentially is significantly higher. Moreover, the log10 kill (gross) values for the sequential therapy (as opposed to VP-16 alone) was considered highly active by the usual clinical criteria (11, 12).

Seven h was determined to be the minimum time interval between administration of XK469 and VP-16 that did not produce high morbidity and mortality. When XK469 was injected 24 h before VP16 administration, a high antitumor effect was achieved, however, tumors reappeared after a few days and started to proliferate more rapidly (data not shown). Our hypothesis was that by 24 h, topo IIα levels were very high, and some cells had developed a better ability to escape VP-16 toxicity, probably because of a much efficient repair mechanism based on the topo IIα enzyme (13). We speculated that a shorter time interval allowed for elevated topo IIα levels that had not yet been fully incorporated into the repair process for resistance to VP-16.

Our study clearly illustrates that subtherapeutic concentration of XK469 increased the responsiveness of xenografts to subsequent administration of VP-16. These results provide basis for combination chemotherapy to use these two agents in clinical trials.

REFERENCES