Cancer Chemother Pharmacol (2002) 49: 281–286
DOI 10.1007/s00280-002-0425-7

ORIGINAL ARTICLE

Hong Lin · Balanehru Subramanian · Alex Nakeff
Ben D. Chen

XK469, a novel antitumor agent, inhibits signaling by the MEK/MAPK signaling pathway

Received: 3 October 2001 / Accepted: 24 December 2001 / Published online: 24 January 2002
© Springer-Verlag 2002

Abstract *Purpose:* XK469 (NSC 697887) is a novel antitumor agent with broad activity against a variety of tumors including drug-resistant tumors. Previous studies have indicated that XK469 is an antiproliferative agent with a low cytotoxic effect in human H116 tumor cells. In this study, we sought to determine the signaling pathways involved in mediating its antiproliferative activity. *Methods:* The antiproliferative activity of XK469 was tested using human U-937 leukemia cells in culture. XK469-induced cell cycle arrest was determined using flow cytometric analysis. Phosphorylation/activation of MEK and MAPK was analyzed using immunoblot analyses with specific antibodies against p-MEK and p-MAPK. *Results:* Cell cycle analysis revealed that XK469 arrested U-937 cells at the G_2/M phase. Compared with conventional anticancer agents, XK469 showed very low, if any, cytotoxic or proapoptotic effect against U-937 cells. In contrast, treatment of U-937 cells with vinblastine, doxorubicin and *m*-AMSA resulted in extensive cell death through apoptotic pathways. XK469, but not other agents, potently inhibited the phosphorylation/activation of MEK in U-937 cells cultured in serum-containing medium. XK469 was also able to block the activation of MEK by serum addition in starved U-937 cells. Exposure of cells to XK469 for 1 h was sufficient to inhibit the activation of MEK and its downstream kinase, MAPK. The antiproliferative response to XK469 was correlated with a steady accumulation of cyclins B1 and A, which appeared to be a direct result of G_2/M arrest. *Conclusions:* Our findings suggest that the antiproliferative effect of XK469 is mediated by inhibiting the MEK/MAPK signaling pathways in U-937 human leukemia cells.

Keywords Antiproliferation · MEK · MAPK · XK469

H. Lin · B.D. Chen (✉)
Division of Hematology-Oncology,
516 Hudson Webber Cancer Research Center,
Barbara Ann Karmanos Cancer Institute,
4100 John R. Detroit, MI 48201, USA
E-mail: chenb@karmanos.org
Tel.: +1-313-9660641
Fax: +1-313-9667322

B. Subramanian · A. Nakeff
Josephine Ford Cancer Center, Henry Ford Health System,
1 Ford Place, Detroit, MI 48202, USA

Introduction

XK469 (NSC 697887), a synthetic quinoxaline phenoxypropionic acid derivative, has been found to have a broad range of activity against a variety of tumors, including MDR-expressing murine solid tumors [1, 2]. Gao et al. have shown that levels of XK469 in the millimolar range inhibit topoisomerase IIβ in various mammalian cell lines by inducing reversible protein-DNA crosslinks in a manner similar to *m*-AMSA [4′-(9-acridinylamino)methanesulfon-*m*-anisidide], a known topoisomerase II inhibitor [3]. Kessel and Horwitz, on the other hand, have reported that XK469 elicits an apoptotic response in murine L1210 cells, which correlates with the binding of the drug to the peripheral benzodiazepine receptor [4]. In contrast, we have recently shown that XK469 blocks human H116 cells in culture at the G_2/M phase with relatively low cytotoxic activity [5]. Additionally, we have shown that this effect is correlated with the inhibition of cyclin B1 ubiquitination, resulting in the accumulation of cyclin B1 in the G_2/M phase [6]. These findings suggest that XK469 may have multiple sites of action that are critically involved in growth regulation and survival.

Mitogen-activated protein kinases (MAPK/ERK) play a pivotal role in the mitogenic signal transduction pathway and are key components of the MAP kinase cascade, which includes MAPKK (MEK) and Raf-1 [7]. This cascade provides not only signal amplification but also additional regulatory intermediates that allow the kinetics, duration and amplitude of the activity to be precisely modified [8]. Disruption of the MAPK cascade may contribute to the molecular pathogenesis of

myeloid leukemias. Recent studies have shown that constitutive activation of MEK/MAPK pathways is associated with the neoplastic phenotype of a relatively large number of human tumor cells and myeloid leukemias [9, 10, 11]. Furthermore, Hayne et al. have shown that the MEK/MAPK pathway is essential for the G_2/M progression during cell cycle [12]. The involvement of MEK/MAPK pathways in regulating cell cycle progress suggests that they might be potential therapeutic targets in the treatment of myeloid leukemias.

In this study, we sought to determine the signaling pathways involved in the antiproliferative response to XK469 using the U-937 human leukemia cell model. Specifically, we asked whether XK469 might exert its antiproliferative effect by targeting MEK/MAPK signaling pathways. We now report that XK469 is an inhibitor of MEK and its downstream substrate, MAPK/ERK in U-937 leukemia cells. Our findings reveal that XK469 has a unique antiproliferative activity distinct from that of conventional antitumor and antileukemia agents.

Materials and methods

Cells

Cells of the human p53-null macrophage-like cell line, U-937 (ATCC CRL-1593.2), were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 2 m*M* glutamine. The U-937 cell line was derived originally from a patient with histiocytic lymphoma [13].

Reagents

XK469 (MW 326) was provided by Dr. Mark Wentland (Rensselaer Polytechnical Institute, Albany, N.Y.). Vinblastine, doxorubicin, and *m*-AMSA were purchased from Sigma (St. Louis, Mo.). Antibodies against cyclins A, B1, E and cdc2 were a gift from Pharmingen (San Diego, Calif.). Anti-MEK, p-MEK, MAPK and p-MAPK antibodies were obtained from Cell Signaling (Beverly, Mass.).

Cytotoxicity of XK469

Cells (2×10^5/well) were cultured in 96-well plates for 24 h in the presence of graded levels of drugs for various time periods. After drug treatment, cell viability was determined by trypan blue staining. Apoptotic cell staining was performed as described previously [14, 15]. In brief, after treatment cells were stained with acridine orange (10 µl of a 100-µg/ml solution into 100 µl cell suspension) in the dark. A minimum of 100 cells per sample were counted under a fluorescence microscope. Cells with condensed chromatin were counted as positive, whereas those with a normal chromatin pattern were counted as negative.

Flow cytometry

Cells were treated with XK469 for various times at 37°C in a humidified incubator in an atmosphere containing 7.5% CO_2. DNA cell cycle staining was done by resuspending washed U-937 cells (2×10^6 cells/ml) in 500 µl 1×PBS. An equal volume of ethanol (70%) was added drop-wise to cells and gently mixed, followed by incubation on ice for 30 min. After decanting the supernatant by centrifugation at 750 *g* for 5 min, the cells were stained with 1 ml chilled propidium iodide (PI) solution (20 µg/ml) containing RNase A (1000 U/ml) and Triton X-100 (0.1% v/v) at 37°C for 30 min [6]. Cells were then analyzed by flow cytometry with a FACScan (Becton Dickinson, San Jose, Calif.). Data were analyzed using Modfit software (v.5.01) for cell cycle profile.

Immunoblot analysis

Immunoblot analysis was performed as described previously [15]. Briefly, at the end of the treatment, cells were lysed and total cell lysates (4×10^5/sample) were boiled in SDS gel-loading buffer for 10 min. The samples were subjected to electrophoresis on 10% or 8% one-dimensional SDS-polyacrylamide gel. After electrophoresis proteins were transferred to a nitrocellulose membrane (pore size of 0.2 µm; Schleicher & Schuell, Keene, N.H.) at 4°C and 14 V overnight or 40 V for 2 h. Nonspecific binding sites on the nitrocellulose membrane were blocked by incubating in blocking buffer (5% nonfat milk) for 2 h at room temperature or overnight at 4°C. The blots were washed with Tris-buffered saline once and incubated for 2 h at room temperature with the primary antibody. After the removal of excess primary antibody with three washes, the blots were incubated with a secondary antibody (goat anti-mouse or goat anti-rabbit antibodies conjugated with horseradish peroxidase). The membrane was developed with the enhanced chemiluminescence (ECL) reagent and exposed to Hyperfilm-ECL (Amersham Life Science) for visualization.

Results

Cytotoxic effect of XK469 in vitro

U-937 cells were incubated with graded doses of XK469 and three widely used anticancer agents for various times. The cytotoxic effect of XK469 was determined in terms of trypan blue uptake. As shown in Fig. 1A, XK469 had a very low cytotoxic effect against U-937 cells compared with other anticancer agents. At the highest dose (100 µ*M*), XK469 had killed only about 15% of the cells after a 24-h treatment. Yet treatment of U-937 cells with vinblastine, doxorubicin and *m*-AMSA caused extensive cell killing in a concentration- and time-dependent manner. Cytotoxic responses to these agents were mediated through an apoptotic pathway as determined by morphological examination following acridine orange staining (Fig. 1B). XK469 did not induce apoptosis in U-937 cells.

XK469 is a G_2/M phase blocker

We studied the effect of XK469 on cell cycle progression in U-937 cells using a flow cytometric approach. U-937 cells were exposed to XK469 for 8, 16, 24 and 48 h, washed and stained with PI. Essentially no cells in the G_2/M phase were detected in control culture of U-937 cells (Fig. 2). Exposure of U-937 cells to XK469 resulted in a steady accumulation of cells in the G_2/M phase. The cell cycle profile indicated that over 55% of the cells were arrested in the G_2/M phase after treatment with 100 µ*M* XK469 for 24 h. The percentage of cells in the

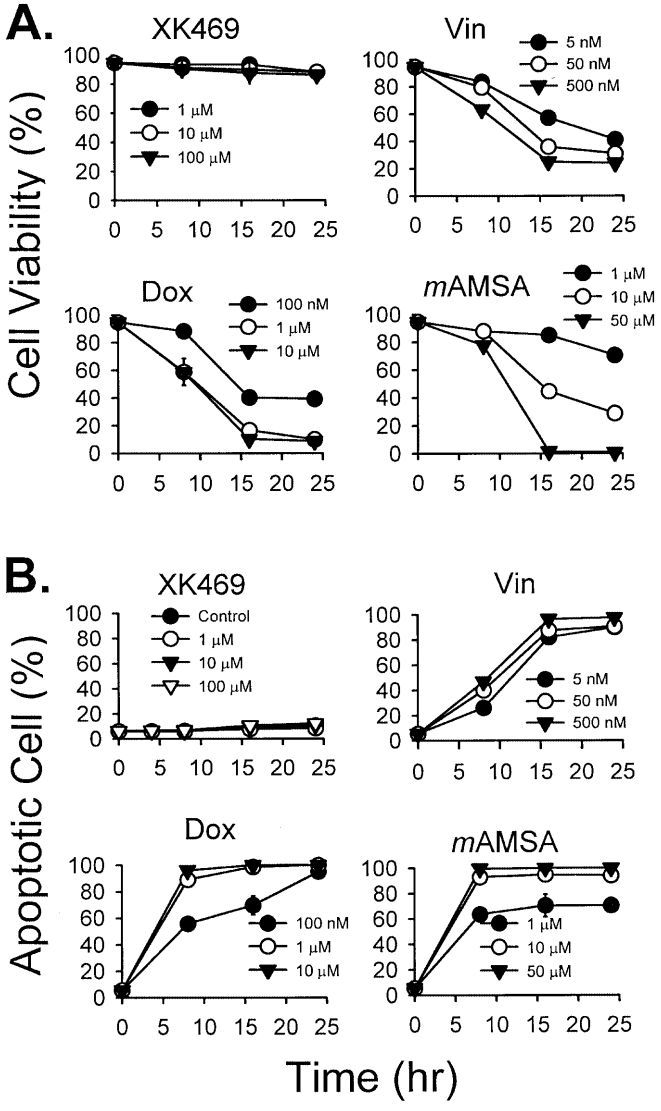


Fig. 1A, B Induction of apoptosis by vinblastine (*Vin*), doxorubicin (*Dox*) and *m*-AMSA but not XK469. **A** U-937 cells were incubated in the continuous presence of various doses of vinblastine, doxorubicin, *m*-AMSA and XK469 as indicated for 0, 8, 16 and 24 h. At the indicated times, cells were removed and stained with trypan blue to determine cell viability. Data are means ± SD from three experiments. **B** After treatment, U-937 cells were stained with acridine orange (10 μl of a 100-μg/ml solution into 100 μl cell suspension) for 10 min at 37°C, and examined for apoptotic cells by fluorescence microscopy. Data are means ± SD from duplicate experiments

G_2/M phase did not increase further after prolonged incubation with XK469 for 48 h.

XK469 inhibits the MEK/MAPK pathway

Since the MEK/MAPK pathway has been implicated in regulating the G_2/M transition [12], we assessed the possibility that XK469 might inhibit cell growth by targeting the MEK/MAPK signaling pathway in U-937 cells. The MEK activity in U-937 cells was determined by detecting its phosphorylated form (p-MEK) using an immunoblot analysis approach. Untreated control U-937 cells constitutively expressed high levels of activated MEK (Fig. 3A). Exposure of the cells to XK469 led to profound decreases in the levels of MEK activity. Inhibition of MEK activity was detected in U-937 cells 1 h after the addition of XK469 with a near complete inhibition of MEK activity within 4 h. In contrast, none of the anticancer agents (vinblastine, doxorubicin or *m*-AMSA) examined in this study inhibited MEK.

Additional experiments were carried out to assess the effect of XK469 on the activation of MEK in U-937 cells that had been starved in serum-free medium for 24 h. Starvation of U-937 cells resulted in a complete down-regulation of MEK activity, which was readily reactivated by the addition of fresh FBS to the culture medium (Fig. 3B). Activation of MEK was inhibited by prior treatment of starved U-937 cells with XK469, but not by vinblastine, doxorubicin or *m*-AMSA. As expected, inhibition of MEK activation by XK469 was associated with depressed activation of MAPK, a downstream kinase of MEK. Likewise, only XK469 was found to inhibit the activation of MAPK in U-937 cells.

XK469 induces the accumulation cyclin B1

The preceding experiments using flow cytometry showed that XK469 arrested U-937 cells at the G_2/M phase. Since the degradation of cyclins B1 and A is essential for the G_2/M transition during the cell cycle [16, 17, 18, 19], we sought to determine whether XK469 might induce the accumulation of cyclin B1 in U-937 cells. Cells were treated with XK469 for various times. Total cell lysates were subjected to immunoblot analyses with antibodies against cyclin A, cyclin B1, cdc2, p53, p21 and cyclin E. As shown in Fig. 4, treatment of U-937 cells with XK469 led to a steady accumulation of cyclin B1 and, to a lesser extent, cyclin A. In contrast, XK469 greatly decreased the levels of cyclin E. The level of cdc2, a cyclin B1-associated kinase, was not affected by XK469. As expected, we did not detect p53 protein in U-937 cells, nor did we detect p21, a downstream mediator of the p53 response, in either control or XK469-treated U-937 cells.

Discussion

In this study, we examined the effect of XK469 on the activation of MEK/MAPK signaling pathways. The study was prompted by our observation that treatment of U-937 cells with XK469 led to cell growth arrest at the G_2/M phase with low or no cytotoxic effect. Activation of MEK and MAPK was demonstrated in U-937 cells by immunoblot analyses with specific antibodies against the phosphorylated form of MEK and MAPK. We showed that XK469 was an inhibitor of MEK activation. This property was not shared with other

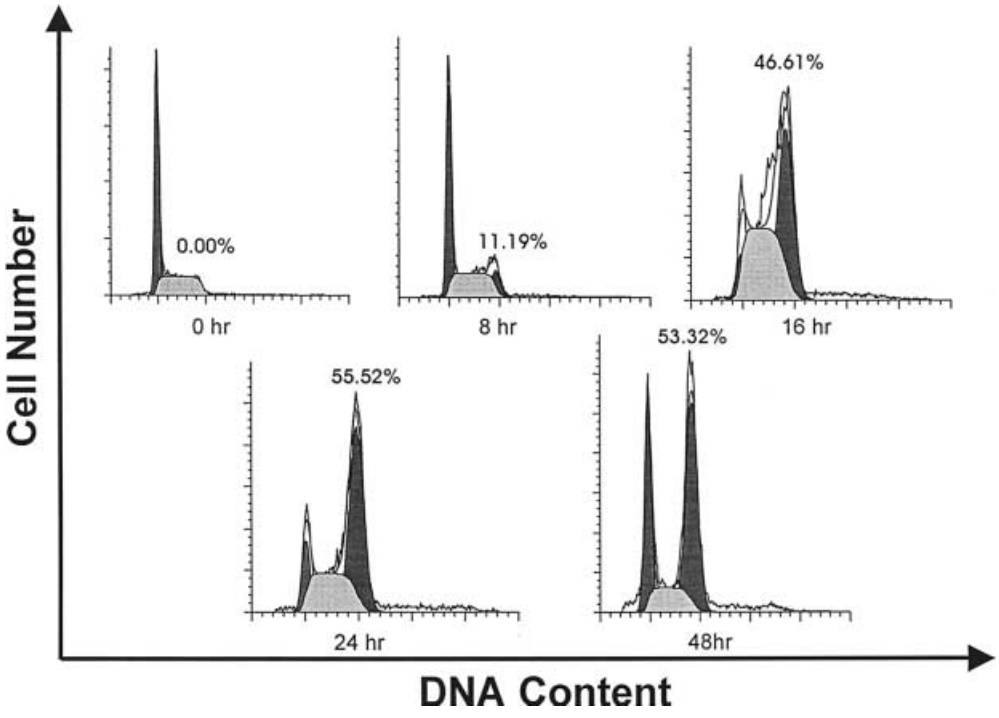
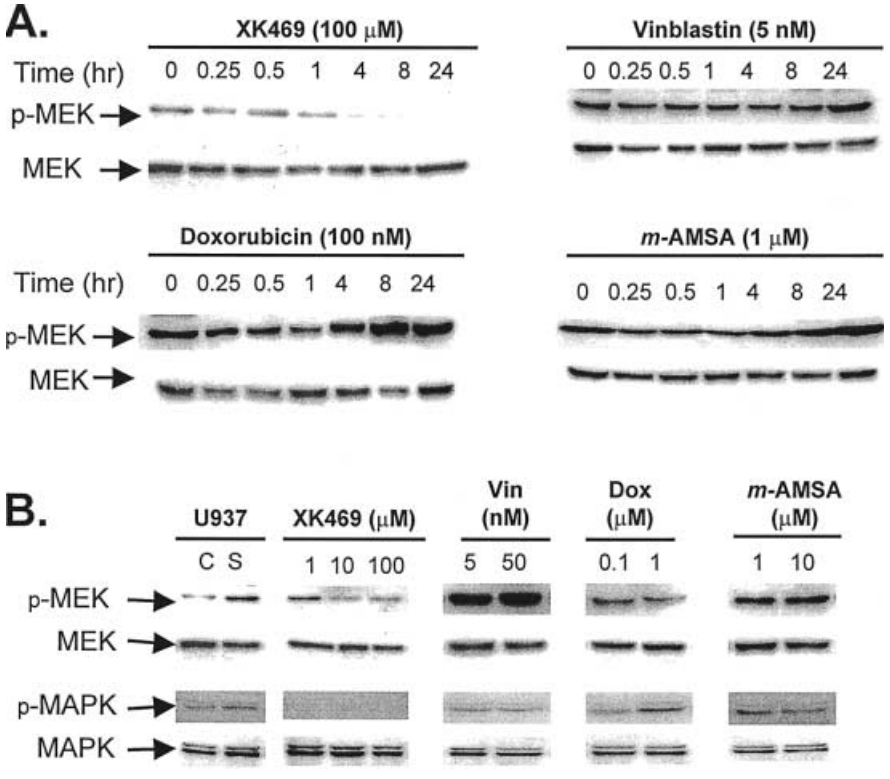


Fig. 2 Cell cycle analysis. Asynchronized U-937 cells were cultured in the presence of XK469 (100 μM) at 37ºC for 0, 8, 16, 24 and 48 h. At the end of treatment, cells were washed, resuspended in PBS and stained with PI for flow cytometric analysis as described in detail in Materials and methods. Data were analyzed using Modfit software (v.5.01)

commonly used anticancer agents including vinblastine, doxorubicin and *m*-AMSA. Our results suggest that XK469 has a unique mechanism of action distinct from that of conventional anticancer agents.

XK469 not only inhibited the ability of MEK to phosphorylated MAPK, but also inhibited the phosphorylation/activation of MEK itself (Fig. 3). The fact that MEK phosphorylation was inhibited by XK469 implies that this agent inhibits the activation of Raf under these conditions [7, 8]. Activation of MEK was achieved by starving U-937 cells in serum-free medium for 24 h, which resulted in nearly complete downregulation of MEK activity. They were then exposed to XK469, vinblastine, doxorubicin and *m*-AMSA for 1 h



prior to the addition of FBS to reactivate MEK. In a series of experiments, we found that only XK469 was able to prevent the activation of MEK and its downstream kinase, MAPK. This finding is consistent with the notion that the antiproliferative effect of XK469 is mediated by inhibiting the MEK/MAPK pathway and that XK469 is a G_2/M blocker (Fig. 2). In a recent study, Hayne et al. [12] have established that the MEK/MAPK pathway is necessary for G_2/M progression. The ability of XK469 to arrest cells in the G_2/M phase is also supported by the accumulation of cyclins B1 and A, but not cyclin E, in XK469-treated U-937 cells (Fig. 4). It has been established that degradation of cyclin B1 is essential for the G_2/M transition during the cell cycle [16, 17, 18, 19].

A recent study by Gao et al. has indicated that XK469 is a topoisomerase IIβ poison. This was deduced from the fact that XK469, like *m*-AMSA, can induce reversible protein-DNA crosslinks in mammalian cells [3]. However, the levels of XK469 used in the cell-free systems (millimolar range) may be much greater than the intracellular concentration of this agent. Using radioactive XK469 (^{3}H-XK469) as the probe, we have recently shown that the total intracellular intake of XK469 by H116 cells under optimal conditions is less than 2% of the total extracellular XK469 in the culture medium [6]. Furthermore, treatment of U-937 cells with *m*-AMSA, a potent apoptotic inducer of U-937 cells, could not block the activation of MEK and its downstream substrate, MAPK. Thus, it appears that XK469 is not a topoisomerase II poison in U-937 cells, in contrast to the previous report [3].

Growth arrest induced by XK469 is independent of p53 protein, a suppressor of tumor cell growth. Activation of the p53 protein has been detected in cells responding to DNA damage and cellular stress, which can affect cell fate by causing either growth arrest at the G_1/S and G_2/M phases or apoptotic cell death [20]. Like many other tumor cell lines, U-937 cells lack functional p53 protein. No expression of p21, a cyclin-dependent kinase inhibitor [21, 22], was detected in U-937 cells undergoing growth arrest (Fig. 4). This finding strongly suggests that the antiproliferative response to XK469 in U-937 cells is independent of p53.

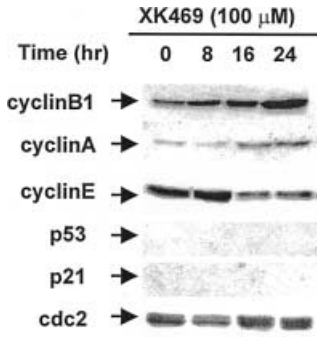


Fig. 4 Effect of XK469 on the expression of cell cycle regulators in U-937 cells. Asynchronized U-937 cells were cultured in the presence of XK469 (100 μ*M*) for various times as indicated. After treatment, cells were removed from culture, washed, counted and lysed with SDS sample buffer. Samples were subjected to SDS-PAGE and immunoblot analysis with antibodies against cyclin B1, cyclin A, cyclin E, p53, p21 and cdc2

In contrast to our results, Kessel and Horwitz [4] have recently reported that XK469 can induce an apoptotic response in murine L1210 leukemia cells in culture. The apoptotic response to XK469 was correlated with drug binding to the peripheral benzodiazepine receptor (PBR), suggesting that receptor binding may be a factor in drug-induced cytotoxicity in L1210 cells. Their finding indicates that XK469 may have multiple sites of action, and the responsiveness to XK469 may vary between cell types depending on the expression of these sites. It is pertinent to mention that the ability of U-937 to resist XK469-induced apoptosis is not due to lack of p53 expression in U-937 cells since L1210 leukemia cells, like U-937 cells, are p53-deficient [23]. Furthermore, we showed that vinblastine, doxorubicin and *m*-AMSA, three commonly used anticancer agents, were effectively able to induce apoptosis in U-937 cells.

Acknowledgement This work was supported by Public Health Service Grant CA 73212 awarded by the National Cancer Institute, U.S. Department of Health and Human Services.

◀

Fig. 3A, B Inhibition of XK469 in the phosphorylation of MEK and MAPK. **A** Asynchronized U-937 cells were cultured in the presence of XK469 (100 μ*M*), vinblastine (5 n*M*), doxorubicin (100 n*M*), or *m*-AMSA (1 μ*M*) for various times as indicated. After treatment, cells were removed from culture, washed, counted and lysed with SDS sample buffer. Samples were subjected to SDS-PAGE and immunoblot analyses with antibodies against phosphorylated MEK and MEK. **B** U-937 cells were starved in serum-free medium for 24 h, then exposed to XK469, vinblastine, doxorubicin or *m*-AMSA at the doses indicated for an additional l h. Activation of MEK was initiated by adding 20% FBS to the culture medium for 30 min at 37ºC. After treatment, the U-937 cells were harvested and lysed with SDS sample buffer. Samples were subjected to SDS-PAGE and immunoblot analysis with antibodies against p-MEK, MEK, p-MAPK and MAPK. XK469 prevented serum-activated phosphorylation of MEK and MAPK (*C* control, *S* addition of serum)

References

1. LoRusso PM, Parchment R, Demchik L, Knight J, Polin L, Dzubow J, Behrens C, Harrison B, Trainor G, Corbett TH (1998) Preclinical antitumor activity of XK469 (NSC 656889). Invest New Drugs 16:287
2. Corbett TH, LoRusso P, Demchick L, Simpson C, Pugh S, White K, Kushner J, Polin L, Meyer J, Czarnecki J, Heilbrun L, Horwitz JP, Gross JL, Behrens CH, Harrison BA, McRipley RJ, Trainor G (1998) Preclinical antitumor efficacy of analogs of XK469: sodium-(2-[4-(7- chloro-2-quinoxalinyloxy) phenoxy]propionate. Invest New Drugs 16:129
3. Gao H, Huang KC, Yamasaki EF, Chan KK, Chohan L, Snapka RM (1999) XK469, a selective topoisomerase IIbeta poison. Proc Natl Acad Sci U S A 96:12168
4. Kessel D, Horwitz JP (2001) Pro-apoptotic interactions between XK469 and the peripheral benzodiazepine receptor. Cancer Lett 168:141

5. Subramanian B, Nakeff A, Valeriote FV (1999) Mechanism of antitumor action of a novel quinoxaline, XK469. Proc Am Assoc Cancer Res 40:1
6. Lin H, Liu YX, Subramanian B, Nakeff A, Valeriote F, Chen BD (2002) Mitotic arrest induced by XK469, a novel antitumor agent, is correlated with the inhibition of cyclin B1 ubiquitination. Int J Cancer 97:121
7. Kolch W (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 351:289
8. Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9:180
9. Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M (1999) Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 18:813
10. Kim SC, Hahn JS, Min YH, Yoo NC, Ko YW, Lee WJ (1999) Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: combined role of activation of MEK, hyperexpression of extracellular signal-regulated kinase, and downregulation of a phosphatase, PAC1. Blood 93:3893
11. Morgan MA, Dolp O, Reuter CW (2001) Cell-cycle-dependent activation of mitogen-activated protein kinase kinase (MEK-1/2) in myeloid leukemia cell lines and induction of growth inhibition and apoptosis by inhibitors of RAS signaling. Blood 97:1823
12. Hayne C, Tzivion G, Luo Z (2000) Raf-1/MEK/MAPK pathway is necessary for the G_2/M transition induced by nocodazole. J Biol Chem 275:31876
13. Sundstrom C, Nilsson K (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 17:565
14. Chen C, Lin H, Karanes C, Pettit GR, Chen BD (2000) Human THP-1 monocytic leukemic cells induced to undergo monocytic differentiation by bryostatin 1 are refractory to proteasome inhibitor-induced apoptosis. Cancer Res 60:4377
15. Lin H, Chen C, Chen BD (2001) Resistance of bone marrow-derived macrophages to apoptosis is associated with the expression of X-linked inhibitor of apoptosis protein (XIAP) in primary cultures of bone marrow cells. Biochem J 353:299
16. Coleman TR, Dunphy WG (1994) Cdc2 regulatory factors. Curr Opin Cell Biol 6:877
17. Doree M, Galas S (1994) The cyclin-dependent protein kinases and the control of cell division. FASEB J 8:1114
18. Vorlaufer E, Peters JM (1998) Regulation of the cyclin B degradation system by an inhibitor of mitotic proteolysis. Mol Biol Cell 9:1817
19. Hershko A (1996) Mechanisms and regulation of ubiquitin-mediated cyclin degradation. Adv Exp Med Biol 389:221
20. Rolfe M, Chiu MI, Pagano M (1997) The ubiquitin-mediated proteolytic pathway as a therapeutic area. J Mol Med 75:5
21. Sielecki TM, Boylan JF, Benfield PA, Trainor GL (2000) Cyclin-dependent kinase inhibitors: useful targets in cell cycle regulation. J Med Chem 43:1
22. Fukuchi K, Tomoyasu S, Nakamaki T, Tsuruoka N, Gomi K (1998) DNA damage induces p21 protein expression by inhibiting ubiquitination in ML-1 cells. Biochim Biophys Acta 1404:405
23. He AW, Cory JG (1998) Effect of ionizing radiation on wild-type and mutant mouse leukemia L1210 cells. Oncol Res 10:455