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The Investigational New Drug XK469 Induces G₂-M Cell Cycle Arrest by p53-dependent and -independent Pathways¹

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Abstract

Purpose: XK469 {2-[4-(7-chloro-2-quinoxalinyloxy)phenoxypropionic acid}, a synthetic quinoxaline phenoxypropionic acid derivative, has broad activity against murine tumors and is entering Phase I clinical development as a topoisomerase II inhibitor. This study investigated the underlying molecular mechanism of XK469's effects on the cell cycle.

Experimental Design: Growth inhibition, cell cycle arrest, induction of p53 and p21 mRNA and protein, and cd2 phosphorylation and kinase activity were studied in treated cells from the H460 lung cancer line and p21 and p53 knockout cells of the HCT 116 colon cancer line.

Results: XK469 arrested H460 cells at G₂-M, which was associated with cd2 phosphorylation and decreased cd2 kinase activity. Moreover, XK469 stabilized p53 and subsequently increased p21WAF1/CIP1. Furthermore, HCT116 p21⁻/⁻ cells were less sensitive than wild-type cells to XK469-induced growth inhibition, but p53⁺/+ and p53⁻/⁻ cells were equally sensitive despite the absence of p21 induction in the p53⁻/⁻ cells.

Conclusions: When considered with published data, our study suggests a complex mechanism of XK469-mediated anticancer activity involving multiple pathways, including p53-dependent and -independent G₂-M arrest via inactivation of cd2-cyclin B1 kinase activity.

Introduction

A series of quinoxaline analogues of the herbicide Assure (DuPont) were evaluated for anticancer activity, and analogues with a halogen (F, Cl, Br) in the 7-position were found to possess anticancer activity, whereas Assure with a Cl in the 6-position does not (1). This suggests that a halogen at the 7-position is important for antitumor activity in this compound family. XK469{2-[4-(7-chloro-2-quinoxalinyloxy)phenoxypropionic acid} is a synthetic quinoxaline phenoxypropionic acid derivative (1–3) and a 7-chloro analogue of the herbicide Assure (Fig. 1A). In vitro, XK469 exhibits selective cytotoxicity for several murine solid tumors, including colorectal and mammary adenocarcinoma cell lines, as compared with both leukemia and normal epithelial cells (1, 3). In vivo, XK469 is active against a broad spectrum of murine tumors including pancreatic ductal carcinoma and colon and mammary adenocarcinomas (3). The R (+)- and S (−)-isomers of XK469 were similar in studies with animal tumor models (1). Importantly, XK469 was found to be highly active against multidrug-resistant tumors (1, 3). Moreover, XK469 has low toxicity in comparison with other anticancer agents, such as camptothecin (1, 3). XK469 has been approved to undergo Phase I clinical investigation by the National Cancer Institute.

The molecular mechanism of action of XK469 has not been determined (1). The mechanistic studies suggested that XK469 is a selective topoisomerase IIβ inhibitor (4, 5). However, it remains to be determined whether XK469 has effects on the cell cycle and apoptosis.

The cell cycle is regulated by the cyclins, CDKs, and CKIs (7). CDK activity is regulated through three distinct mechanisms, namely (a) by cyclin binding; (b) by positive and negative phosphorylation events; and (c) by interaction with CKIs (7). p21WAF1/CIP1 is one CKI that can induce a G₁ arrest and may also take part in a G₂-M arrest through its interactions with cyclin/CDK complexes (7). p21 is also transcriptionally up-regulated by the tumor suppressor gene p53 in stressful conditions (8). It has been well accepted that regulation of Rb phosphorylation is the critical event for G₁ progression (9). On the other hand, cyclin B1-cdc2 associated kinase activity is critical for the G₂-M transition, and the activity of the cdc2-cyclin B1 complex is regulated, at least in part, by the positive regulator cdc25c and two negative regulators Weel and Myt1 (10). The phosphatase cdc25c regulates cyclin B1-cdc2 activity by dephosphorylating cdc2 on Thr-14 and Tyr-15, leading to the activation of the cdc2-cyclin B1 complex (11, 12). On the other hand, the protein kinase Weel phosphorylates cdc2 on Tyr-15 (13, 14), and the kinase Myt 1 phosphorylates cdc2 on Thr-14 and Tyr-15 (15, 16). Both kinases are therefore capable of

¹The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; ActD, actinomycin D; CHX, cycloheximide.

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inactivating the cdc2-cyclin B1 complex, leading to a G2-M arrest (10).

In this study, we investigated the effect of XK469 on the cell cycle. We found that XK469 arrests cells in the G2-M phase of the cell cycle via inactivation of cdc2-cyclin B1 by phosphorylation of cdc2 on Tyr-15, which results in a decrease in cdc2 kinase activity. Moreover, we show that XK469 can stabilize the p53 protein and subsequently induce p21. Interestingly, although loss of p53 does not affect drug resistance, loss of p21 decreases sensitivity of the colon cancer cell line HCT116 to XK469. Thus, our results suggest that XK469-induced growth inhibition may be attributable to induction of p21 through a p53-independent mechanism and a decrease in the cdc2-cyclin B1 kinase activity, which explains the drug’s broad spectrum activity in tumor models.

Materials and Methods

Drugs. R (+) XK469 (free acid, NSC698215) was obtained from the National Cancer Institute Drug Synthesis Branch (Bethesda, MD) and dissolved in DMSO (Sigma Chemical Co.). XN472 [sodium 2-[4-(7-chloro-2-quinoxalinyloxy)phenoxy]propionate; Assure, (not shown) ethyl 2-[4-(6-chloro-2-quinoxalinyloxy)phenoxy]propionate. B and C, the effects of XK469 on growth inhibition. The human lung cancer H460 was treated with various concentrations of XK469 (○) or XN472 (□) for one day (B) or for 4 days (C), followed by assessment for cell survival as described in “Materials and Methods.” Bars, SD.

**Fig. 1** The differences of chemical structures and growth inhibition activity between XK469 and XN472. A, chemical structures of XK469 and its analogue XN472. XK469, sodium 2-[4-(7-chloro-2-quinoxalinyloxy)phenoxy]propionate; XN472; Assure, (not shown) ethyl 2-[4-(6-chloro-2-quinoxalinyloxy)phenoxy]propionate. B and C, the effects of XK469 on growth inhibition. The human lung cancer H460 was treated with various concentrations of XK469 (○) or XN472 (□) for one day (B) or for 4 days (C), followed by assessment for cell survival as described in “Materials and Methods.” Bars, SD.

**Cell Lines and Culture Conditions.** The human lung cancer cell line H460 was maintained in RPMI 1640 as described previously (17). The human colon cancer cell lines HCT116 wild-type (p53+/− p21+/−), HCT116 p53−/−, and HCT116 p21−/−, a generous gift from Bert Vogelstein (Johns Hopkins University, Baltimore, MD), were maintained in McCoy’s media as described previously (18, 19). These cells were supplemented with 10% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air. Cells were grown in the presence of varying concentrations of XK469 or XN472 for different time points.

**Growth Inhibition.** Cells were plated at a density of 5000/well (in triplicate) and treated with different concentrations of XK469 or XN472 as indicated in figure legends. After 1-day or 4-day treatment for H460 cells and a 4-day treatment for HCT116 cells, cells were stained with Crystal Violet. The resulting cells were destained with 10% acetic acid, followed by measuring absorbance at 600 nm. Growth inhibition was calculated as described previously (20, 21).

**Western Blot Analysis.** Whole cell lysates were prepared as described previously (20, 21), and protein concentration was determined using the Protein Assay kit (Bio-Rad, Hercules, CA). Cell lysates (40 μg) were electrophoresed through 7.5–12% denaturing polyacrylamide slab gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblooting. The blots were probed or reprobed with the antibodies, and then antibody binding was detected using Enhanced Chemiluminescence Reagent (Amersham Pharmacia, Biotech, Piscataway, NJ) according to the manufacturer’s protocol. The antihuman p53 monoclonal antibody Ab2, the antihuman p21/WAF1 mono-
clonal antibody and anti-actin (Ab-1) antibody were purchased from Calbiochem (Boston, MA). Mouse monoclonal anti-cyclin B1 antibody (GNS1), mouse monoclonal anti-Cdc2 antibody (17), rabbit polyclonal anti-Cdc25c (S-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antiphospho-cdc2 antibody specific for phospho Tyr-15 was purchased from Cell Signaling Technology (Beverly, MA).

**RNA Isolation and Northern Blot Analysis.** Total cellular RNA was purified using the Trizol method (Life Technologies, Inc.) according to the manufacturer’s instructions. Total RNA (20 μg) was separated in a 1.5% formaldehyde agarose gel and blotted to Hybond-N+ membrane (Amersham Pharmacia, Piscataway, NJ). The blots were hybridized either with random primed radiolabeled human p53 or p21 cDNA, a generous gift from Wafik El-Deiry (University of Pennsylvania, Philadelphia, PA), as described previously (8). Radioactive signals were analyzed by autoradiography.

**Cell Cycle Analysis.** Subconfluent cells were treated with or without XK469 or XN472 at 60 μg/ml for 4, 8, 12, and 24 h. Cells were then harvested and fixed with ice-cold 70% (v/v) ethanol for 24 h. After centrifugation at 200 × g for 5 min, the cell pellet was washed with PBS (pH 7.4) and resuspended in PBS containing propidium iodide (50 μg/ml), Triton X-100 (0.1%, v/v), and DNase-free RNase (1 μg/ml). Cells were then incubated at room temperature for 1 h, and DNA content was determined by flow cytometry using a FACSScan flow cytometer (Becton Dickinson, San Jose, CA).

**Cdc2 Kinase Activity Analysis.** A total of 1000 μg of H460 protein lysate was precleared with protein A-agarose beads (Santa Cruz Biotechnology) and subsequently incubated with 2 μg of mouse monoclonal anti-cdc2 antibody. Antibody was precipitated with protein A-agarose beads. The resulting precipitated complexes were washed with PBS and then resuspended in 20 μl of kinase buffer [100 mM Tris (pH7.5), 20 mM MgCl2, and 2 mM DTT]. Kinase assays were then performed for 30 min at 30°C by adding 5 μl of H1 substrate mixture (50 μM ATP and 10 μCi of [γ-32P]ATP; Amersham) and 10 μg of histone H1 (Roche Molecular Biochemicals). Reactions were stopped by the addition of SDS-PAGE loading buffer, and proteins were resolved on a SDS-10% polyacrylamide gel.

**Results**

**XK469 Effectively Inhibits Cancer Cell Growth in Vitro.** To investigate the inhibitory effect of XK469 on cell growth, the human lung cancer cell line H460 was treated with increasing concentrations of XK469 or the inactive analogue XN472 (DuPont) or DMSO alone for 1 day, followed by growth inhibition assessment. Fig. 1B shows that ~10% of XK469-treated cells survived, as compared with little effect of XN472 on proliferation, whereas DMSO had no effects on cell growth (data not shown). Extending the exposure period to 4 days increased the toxicity of XK469, but XN472 still did not affect cell viability (Fig. 1C). We also examined the effects of both compounds on the human colon cancer cell line HCT116 and similar results were obtained: XN472 did not inhibit cell proliferation over 4 days at 20 μg/ml (data not shown), whereas this same concentration of XK469 inhibited cell proliferation by ~80% (Fig. 4). Therefore, XN472 can serve as a structurally similar negative control. The inhibitory effect of cancer cell growth is XK469 specific and such activity may be attributed to chlorine in the 7-position of its structure because XN472 with the 6-chloro substituent does not have antiproliferative activity.

**XK469 Arrests Cells in G2-M Phase of the Cell Cycle.** Because XK469 can effectively inhibit cancer cell growth in vitro (Fig. 1B) and in vivo (1, 3), we reasoned that this inhibitory activity may be attributable to its ability to interfere with the cell cycle. To this end, we treated H460 cells with XK469 at 60 μg/ml for 4, 8, 12 and 24 h, followed by cell cycle analysis using flow cytometry. Fig. 2 shows that XK469-treated cells start to arrest in G2-M phase at 8 h, and continue to accumulate over the treatment periods. For example, at 8 h, 41% of the cell population was in the G2-M phase, and by 12 and 24 h treatment, the G2-M population was about 74% and 76%, respectively. In contrast, treatment with XN472 or DMSO alone did not change in the cell cycle distribution (data not shown). This G2-M block has also been observed in HCT116 cells (data not shown). Thus, these results suggested that the inhibitory effect of XK469 on cancer cells might be due to a G2-M arrest of the cell cycle.

**XK469 Regulates the Expression of p53.** We have shown that XK469 can arrest cells in the cell cycle and because p53 is able to induce cell cycle arrest, we tested the effect of XK469 on the levels of p53. As shown in Fig. 3A, p53 protein was increased after exposure of HCT116 cells to XK469. In contrast, such increase was not observed in XN472 and tested for the levels of p53 (Fig. 3A). As expected, HCT116 p53−/− cells failed to increase the expression of p53 (Fig. 3A). To investigate the mechanism underlying such increase in p53 by XK469, we treated HCT116 p53−/− with XK469 and tested for the levels of mRNA. As shown in Fig. 3B, p53 mRNA levels remain unchanged in XK469-treated cells, suggesting a posttranscriptional mechanism. As expected, p53 mRNA was not detected in HCT116 p53−/− cells with or without XK469 treatment (Fig.
To gain further insight into the regulation of p53 expression by XK469, the protein synthesis inhibitor CHX and the transcription inhibitor ActD were used. As shown in Fig. 3D (upper panel), an increase in p53 protein levels by XK469 was blocked in CHX-treated cells but not in ActD-treated cells, which is consistent with the previous studies showing that induction of p53 occurs without an increase in its mRNA but requires translation regulation (22–24). Thus, our results suggest that an XK469-mediated increase in p53 protein is through a posttranscriptional mechanism.

**XK469 Induces the Expression of p21.** It has been well documented that p53-mediated cell cycle arrest is accomplished primarily through the transcriptional activation of its downstream targets including p21, a CKI that plays an important role in the cell cycle (8). Because XK469 increased the expression of p53 protein, it was reasonable to assume that increased p53 protein can induce p21 expression. To test this possibility, we examined the expression of p21 after exposure of H460 and HCT116 cells to XK469 and found that p21 protein is increased in XK469-treated cells but not in XN472-treated H460 cells (Fig. 3A). Like the level of p53 protein, induction of p21 by XK469 was also time- and dose-dependent (Fig. 3, E and F).

Because induction of p21 can occur in a p53-dependent and independent manner (25), we next asked whether induction of p21 by XK469 is through a p53-dependent mechanism. To this end, we treated HCT116 p53+/+ and p53−/− cells with XK469 and then tested for the induction of p21. Fig. 3A shows that induction of p21 is only detected in HCT116 p53+/+ cells but not in HCT116 p53−/− cells, which suggests that induction of p21 is p53-dependent. We next investigated the transcriptional regulation of p21 by XK469 and found that unlike p53, the mRNA of which was not induced after exposure of cells to XK469, p21 mRNA levels were increased in XK469-treated cells (Fig. 3C), which suggested that regulation of p21 expression is through a transcriptional mechanism. The transcriptional regulation of p21 by XK469 was further supported by the results showing that ActD blocked p21 mRNA (Fig. 3C) and the subsequent protein levels (Fig. 3D). Furthermore, we found that CHX can block induction of p21 protein (Fig. 3D). Thus, our results indicate that XK469-induced expression of p21 is p53-dependent and that this induction is regulated by p53 at the transcriptional level.

**Role of p21 in XK469-induced Growth Inhibition.** Because p21 is known to arrest cells in both G1 and G2-M phases...
of the cell cycle, and its levels increase with XK469 treatment, we next investigated whether the p21 gene was required for chemosensitivity to XK469. To test this possibility, HCT116 wild-type, p21<sup>−/−</sup>, and p53<sup>−/−</sup> cells to XK469. HCT 116 cells were incubated in the presence or absence of various concentrations of XK469 for 4 days. Cells were stained as described in “Materials and Methods.” The % growth inhibition relative to untreated cells was determined after drug exposure as described in “Materials and Methods.”

The Effects of XK469 on G<sub>2</sub>-M Targets. We have shown that XK469 arrests cells in the G<sub>2</sub>-M phase of the cell cycle. To understand the mechanism underlying this arrest, we tested the effects of XK469 on the expression of select molecular targets that regulate the G<sub>2</sub>-M transition. We treated H460 cells with XK469 and tested for the protein levels of cyclin B1, cdc2, and cdc25c. As shown in Fig. 5A, the levels of cyclin B1 and cdc2 were slightly increased after exposure of H460 cells to the different concentrations of XK469, whereas the total protein levels of cdc25c remained unchanged. Similar results were obtained with XK469-treated HCT116 cells (data not shown). Slight increases in the levels of cyclin B1 and cdc2 were not surprising because of the increase in proportion of cells at the G<sub>2</sub>-M boundary. However, these increases in protein levels may not correlate with kinase activity. Because the cdc2, cyclin B1, and cdc25c levels do not correlate with a XK469-induced G<sub>2</sub>-M block, we thought that XK469 might alter the status of cdc2 phosphorylation. Our hypothesis was that if XK469 causes phosphorylation of cdc2 on Thr-14, or Tyr-15, or both, then cdc2 becomes inactivated, thus resulting in a G<sub>2</sub>-M arrest. Fig. 5B shows that phosphorylation of cdc2 on Tyr-15 was detected after a 4-h exposure to XK469, increased to a much higher degree at 8 h, and peaked at 12 h, although such an increase starts returning to normal around 24 h. Consistent with this change, cells started to arrest in G<sub>2</sub>-M at 8 h, and the majority of the cell population accumulated in G<sub>2</sub>-M at 12 h after XK469 treatment (Fig. 2). Consistent with its phosphorylation status, cdc2 kinase activity was decreased after a 12 h or 24 h exposure of H460 cells to XK469, although such change was not observed at 4 and 8 h treatments (Fig. 5C). To investigate the mechanism underlying this phosphorylation, we next went on to test for the expression of the negative regulator Wee1, a primary regulator for cdc2 phosphorylation at Tyr-15. We found that the levels of Wee1 do not increase in XK469-treated cells (data not shown), which suggests that Wee1 may not be responsible for cdc2 phosphorylation. Taken together, our results suggest that XK469 causes cdc2 phosphorylation on Tyr-15, thereby inactivating cdc2 and leading to a G<sub>2</sub>-M arrest.

Discussion

In this study, we demonstrated that XK469 could inhibit <i>in vitro</i> growth of the lung cancer cell line H460 and the colon cancer line HCT116. These results were consistent with the initial findings showing that XK469 is a potent agent against...
solid tumors (1–3). Although XK469 has been shown to be a potent inhibitor of cell growth and has been approved for Phase I clinical investigation, the molecular mechanism underlying its activity remains unclear. Previous studies have shown that XK469 is a selective topoisomerase IIβ poison (4, 5). Because topoisomerase IIβ plays an important role in orchestrating the ordered compaction of chromatin that is necessary for mitosis, inhibition of topoisomerase IIβ is believed to induce G2-M arrest (26). Consistent with the role of topoisomerase II in the G2-M transition, we found that XK469 can cause a G2-M arrest. However, we found G2-M block at high micromolar concentrations, well below the millimolar concentrations of XK469 required to affect topoisomerase IIβ (4). Therefore, proving that XK469 is a selective topoisomerase IIβ poison will require further investigation in different systems. Nevertheless, we have clearly shown that XK469 can induce a G2-M arrest, p53-dependent induction of p21, and an increase in cdc2 phosphorylation, providing possible mechanisms by which XK469 can affect the cell cycle.

p53 plays important roles in the cell cycle and in apoptosis in response to stressful stimuli, including DNA damage and chemotherapeutic agent treatment (27, 28). The ability of p53 to induce cell cycle arrest and apoptosis is an important aspect of its growth suppressive activities (27). We have shown that the level of p53 protein increases in cells exposed to XK469, suggesting that the p53 pathway might be activated by XK469. It is clear that induction of cell cycle arrest and apoptosis by p53 is, at least in part, carried out through the transcriptional activation of p53 targets (28). Our preliminary data showed that XK469 can cause activation of caspases and subsequent apoptosis (data not shown), but the mechanism of XK469-induced apoptosis and its involvement of p53 remain to be determined. Here, our results showed that p21 was induced by XK469 through a p53-dependent manner because p53−/− cells failed to do so (Fig. 3A). Thus, our results suggest that cell cycle arrest may be p53 mediated and such an arrest is partially attributable to p21 because of its role in the cell cycle. It is possible that other genes may also be involved in XK469-mediated growth inhibition, because several targets of p53, such as Gad4d54 and 14–3–3δ, have been implicated in the G2-M arrest (28), but this issue requires further investigation.

Although XK469-induced p53-dependent expression of p21, it is possible that such induction may not completely account for the growth inhibition caused by XK469. Because HCT116 p21−/− cells were less sensitive to XK469 than wild-type p21 cells, we argue that p21 plays a role in this inhibition. Because p21−/− cells were not completely resistant to XK469, we believe that there are other mechanisms underlying the cellular response to XK469. In contrast to p21−/− cells, p53−/− cells exhibited the same sensitivity to XK469 as wild-type p53 cells. This is not surprising, given the fact that loss of p53 is not always correlated with chemosensitivity (20) and that the role of p53 in chemosensitivity is believed to be drug dependent and cell-type dependent as observed in HCT116 cells (19) and other cell lines (20). In this regard, the relationship between p53 status and sensitivity of XK469 will need further investigation. Although the p53 status is not critical for XK469 growth inhibition, it is possible that p53 may attenuate G2-M progression in XK469 treated cells. On the other hand, induction of p21 by XK469 may be responsible at least partially for this G2-M arrest. We believed that such induction is through a p53-dependent mechanism because p21 is not induced in p53−/− cells (Fig. 3A). Thus, induction of p21 through a p53-dependent pathway may lead to G2-M arrest in XK469-treated cells.

The G2-M transition is controlled at least in part by cdc2-cyclin B1 activity through two opposite mechanisms (10). As a positive regulator of the G2-M transition, cdc25c has been shown to activate cdc2 by removing phosphates from Thr-14 and Tyr-15 (10). On the other hand, Wee1 and Myt1 phosphorylate cdc2 on Thr-14 and Tyr-15, thereby inactivating it (10). We have shown that XK469 causes a G2-M block of cancer cells. By investigating the expression levels of cdc2 and cyclin B1, we found that both proteins are slightly increased in XK469-treated cells (Fig. 5). But, we found no change in the levels of cdc25c; therefore, XK469 may not affect cdc25c expression. It is known that phosphorylation of cdc2 on Thr-14 and Tyr-15 by inhibitory kinases leads to inactivation of cdc2 and subsequent G2-M block (10). Consistent with this notion, a significant increase in phosphorylation of cdc2 on Thr-15 was observed in XK469-treated cells, which resulted in decreased kinase activity (Fig. 5C; Ref. 10) and, therefore, a G2-M arrest. Thus, increased phosphorylation of cdc2 on Tyr-15 may be one mechanism by which cells undergo G2-M arrest during XK469 exposure. Phosphorylation of cdc2 on Tyr-15 is carried out primarily by Wee1 (10), but preliminary studies found that the expression levels of Wee1 did not increase in XK469-treated cells, which suggested that Wee1 may not play an important role in response to XK469 and G2-M arrest. However, additional experiments are needed to determine the effects of XK469 on the Wee1 kinase activity.

In summary, we have found that XK469 can increase the expression of p53 and p21, and arrest human cancer cells at the G2-M phase of the cell cycle. Moreover, our results show that cdc2, a critical regulator in the G2-M transition, is phosphorylated on Tyr-15 after exposure to XK469 and a decrease in cdc2 kinase activity, providing a possible mechanism of XK469-mediated G2-M block. Finally, we found that p21−/− cells are less sensitive to XK469 than their counterpart p21+/+ cells, but not totally resistant, providing genetic evidence of the involvement of p21 in XK469-induced growth inhibition. Future experiments will focus on the pathway by which XK469 induces cdc2 phosphorylation and affects the subcellular localization of cdc2-cyclin B1. In addition, these findings provide molecular targets for clinical validation of mechanism in patients enrolled in the Phase I clinical trials.

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References


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