ORIGINAL ARTICLE

XLN306 induces apoptosis in human breast carcinoma MX-1 cells

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Abstract XLN306 is a novel synthetic quinazoline derivative with potentially useful anticancer activity. In previous research, we showed that XLN306 is highly cytotoxic to many tumor cell lines. This paper reports an investigation of this cytotoxicity in a number of human carcinoma cell lines. The results show that human breast carcinoma MX-1 cells are extremely sensitive to XLN306 and that the cytotoxicity is due to dose- and time-dependent apoptosis as confirmed by DAPI stain and DNA fragmentation analysis. Both extrinsic and intrinsic pathways are involved in the apoptosis process. The findings indicate that XLN306 has apoptotic induction activity and may be useful for the management of various cancers, especially breast carcinoma.

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1. Introduction

Cancer, characterized by uncontrolled growth of abnormal cells, is undoubtedly one of the most serious and potentially life-threatening human diseases. In the past, the scientific community has produced remarkable advances in our understanding of cancer biology, including the realization that apoptosis (programmed cell death) of cancer cells is tightly regulated and critical for the development of the disease. Currently, most cancer chemotherapeutic agents exert their antitumor effects by triggering apoptosis in cancer cells.

Apoptosis is an evolutionary conserved process that occurs in various physiological and pathological situations. It is characterized by a number of morphological and biochemical features, including cell shrinkage, nuclear DNA fragmentation and membrane blebbing. The two main routes of apoptosis are the extrinsic and intrinsic (mitochondrial) pathways, both of which involve caspase activation.

XLN306 is a new cytotoxic compound with potential as an anticancer drug. In this paper, we report the effects of XLN306 on different human cancer cell lines and show that the human breast cancer cell line, MX-1, is the most sensitive to XLN306. In terms of the mechanism of the cytotoxic action, XLN306 was shown to cause apoptosis in MX-1 cells as determined by cell morphology, DNA ladder assay and protein expression.

2. Materials and methods

2.1. Chemicals

XLN306 was synthesized and supplied as a yellow solid by the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Fig. 1). The compound was dissolved in DMSO to give a 50 mmol/L stock solution, which was stored at 4°C when not in use. The purity of XLN306 was >95% as shown by 1H NMR spectroscopy.

2.2. Cell culture

The following human carcinoma cell lines were obtained from ATCC: breast MX-1 and MCF7 cells; pancreatic Bxpc3 cells; hepatocellular Bel7402 and HepG2 cells; lung A549 cells; colon HT29 and HCT8 cells; and ovarian A2780 cells. All cell lines were maintained in DMEM medium (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated newborn calf serum (PAA) at 37°C under 5% CO2.

2.3. Cytotoxicity

Cytotoxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded in 96-well microplates at a density of 4 x 10^3–1.2 x 10^4 cells/well and cultured for 24 h. They were then treated with XLN306 for 12, 24, 48, 72 or 96 h after which MTT (0.5 mg/mL) was added and the plates were incubated for 4 h in an incubator. The medium was removed and any formazan produced dissolved in DMSO. The optical density was then measured at 570 nm using a spectrophotometer (Thermo Multiskan MK3, Germany).

2.4. Flow cytometry

Cells treated with or without XLN306 at various concentrations were collected by trypsinization, washed twice with phosphate-buffered saline (PBS) and fixed in ice-cold 70% (v/v) ethanol at −20°C for 24 h. After centrifugation, the cell pellets were resuspended in 1 mL propidium iodide (PI) solution (50 mg/mL PI, 50 mg/mL RNase A, 0.03% Triton X-100 and 0.01% sodium citrate in PBS) and incubated for 30 min at 37°C. DNA histograms were obtained by fluorescence activated cell sorting (FACS) analysis.

2.5. Electrophoresis of DNA fragments

MX-1 cells (1 x 10^6) pretreated with or without XLN306 for 24 h were lysed in 200 µL lysis buffer (10 mmol/L EDTA; 50 mmol/L Tris–HCl pH 8.0; 0.5% sodium lauryl sulfate; 100 mg/mL proteinase K) at 37°C for 12 h and subsequently incubated with RNase (50 mg/mL) at 37°C for an additional 1 h. DNA in the lysate was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and then with chloroform. DNA was precipitated with two volumes of ethanol in the presence of 0.3 mol/L sodium acetate. After centrifugation at 13,000 rpm for 15 min, the DNA pellets were washed with 70% ethanol, air dried and resuspended in 20 mL TE buffer (10 mmol/L Tris–HCl 1 mol/mL EDTA pH 8.0). DNA was separated on 1% agarose gel containing 0.5 mg/mL ethidium bromide and photographed using a UV light sequence detection system.

2.6. DAPI staining

After incubating MX-1 cells with or without XLN306 for 24 h, the cells were stained with DAPI (10 µg/mL in PBS) for 30 min and then fixed with 4% paraformaldehyde for 15 min in the dark. After washing with PBS, cell morphology was observed using a fluorescence microscope (Olympus BX51, Japan).

2.7. Protein expression by Western blot analysis

Total cellular protein was extracted using lysis buffer and protein concentration measured using the Bio-Rad protein assay. An equal amount of protein was separated using 10% and 12% SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Bucks, UK). The membranes were blocked with 5% skimmed milk in PBS and incubated overnight with primary antibodies, followed by horseradish-peroxidase-conjugated antibodies.
at room temperature. β-Actin was used as internal positive control. Primary antibodies included Fas, FasL, Bcl-2, Bax, cytochrome C, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-AKT (Ser473), AKT, caspase-3 and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA). Signals were visualized using an enhanced chemiluminescence system (Amersham).

3. Results

3.1. Cytotoxicity of XLN306

Based on previous studies, a small panel of human cancer cell lines was used to investigate the cytotoxicity of XLN306. As shown in Table 1, the human breast cancer cell line, MX-1, was the most sensitive to XLN306 with an IC50 of 2.25 μmol/L at 96 h. To further investigate the cytotoxicity of XLN306, MX-1 cells were treated with a range of concentrations of XLN306 for 12, 24, 48 and 72 h. XLN306 showed dose- and time-dependent cytotoxicity to MX-1 cells with IC50 values of 13.12, 10.72, 5.6 and 4.99 μmol/L at 12, 24, 48 and 72 h, respectively.

3.2. Mechanism of XLN306 cytotoxicity

To gain an insight into the mechanism of XLN306 cytotoxicity, MX-1 cells were examined by flow cytometry following

![Flow cytometric analysis of propidium iodide stained DNA of MX-1 cells following XLN306 treatment. A: MX-1 cells were treated with different concentrations of XLN306 for 24 h; B: MX-1 cells were treated with 25 μmol/L XLN306 for different time; C: Increase in the sub-G1 fraction of XLN306-treated cells provides an estimate of the number of apoptotic cells (data are mean ± SD, n= 3).](image)
PI staining. As shown in Fig. 2, XLN306 treatment resulted in a dose- and time-dependent accumulation of MX-1 cells in the sub-G1 phase, which suggests that it causes cell death by the induction of apoptosis.

To further investigate the apoptotic effect, morphological analysis by fluorescence microscopy after DAPI staining was employed. After 24 h exposure to XLN306 at concentrations of 5, 10 and 20 μmol/L, MX-1 cells showed typical apoptotic characteristics including cell shrinkage, chromatin condensation and fragmentation. In contrast, cells in the untreated group showed normal morphology with blue nuclei (Fig. 3).

In addition, analysis of DNA fragmentation showed that treatment of MX-1 cells with XLN306 resulted in a dose-dependent and statistically significant increase in DNA fragmentation compared to the control (Fig. 4). All these results indicate that the growth inhibition of MX-1 cells by XLN306 is due to the induction of apoptosis.

3.3. Protein expression

It is well known that cellular apoptosis is mainly associated with alterations in two signaling pathways, namely the extrinsic and intrinsic pathways. To investigate the mechanism of apoptosis induction by XLN306, the protein expression of the two pathways was investigated.

The extrinsic pathway is also known as the death receptor pathway. Death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily, which consists of more than 20 proteins. Of these, Fas (CD95) is the most famous with FasL (CD95L) as its corresponding ligand7. As shown in Fig. 5, the expression of FasL in MX-1 cells treated with XLN306 (5–20 μmol/L for 12 h) exhibits a significant dose-dependent increase while the expression of Fas increases only at the highest dose of XLN306 (20 μmol/L).

The intrinsic pathway is also known as the mitochondrial pathway. As shown in Fig. 6, Bcl-2 exhibits a dose-dependent decrease in MX-1 cells treated with XLN306 (5–20 μmol/L for 12 h) while Bax, one of the proapoptotic members of the Bcl-2 family8, increased after treatment with 5 and 10 μmol/L XLN306 compared to the control. Total cytochrome c and cleaved caspase-3 (activated caspase-3) also exhibited dose-dependent increases on treatment with XLN306.

Furthermore, accumulated evidence shows that survival factor AKT exerts an anti-apoptotic action by regulation of molecules at both pre- and post-mitochondrial levels9–11. Our results (Fig. 6) show that the expression of p-AKT (Ser473) in MX-1

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Furthermore, accumulated evidence shows that survival factor AKT exerts an anti-apoptotic action by regulation of molecules at both pre- and post-mitochondrial levels9–11. Our results (Fig. 6) show that the expression of p-AKT (Ser473) in MX-1
protein, located on the outer mitochondrial membrane, is
membrane by proapoptotic members of the Bcl family16. Bcl-2
is closely linked to permeabilization of the outer mitochondrial
membrane. This indicates that the extrinsic pathway is involved
in the induction of apoptosis by XLN306. Furthermore, 20 μmol/L XLN306
decreased the expression of p-AKT (Ser473), which demonstrates
that induction of apoptosis by XLN306 involves multiple targets.

In conclusion, XLN306 is a promising drug for the management
of various human cancers whose mechanism of action involves
the induction of apoptosis.

4. Discussion

XLN306 is a novel synthetic compound that exerts a specific
activity against many human cancer cell lines, especially
MX-1. In order to illuminate its mechanism of action, flow
cytometric analysis was carried out. The results show that
XLN306 induces cell apoptosis in a dose- and time-dependent
manner and produces apoptotic bodies and DNA ladders.

To investigate the apoptosis signaling pathway induced by
XLN306, the expression of proteins related to the extrinsic and
intrinsic pathways was investigated. The Fas/FasL system
has been implicated in chemotherapy-induced tumor cell death
in a number of studies. Treatment with anticancer drugs
including doxorubicin, etoposide and cisplatin can trigger an
increase in FasL expression, which stimulates the receptor
pathway in an autocrine or paracrine manner by binding to
Fas12–15. In our study, we observed increases in the expression
of Fas and more particularly FasL, the latter being dose-
dependent. This indicates that the extrinsic pathway is
involved in the induction of apoptosis by XLN306.

Interestingly, changes in the expression of membrane-
bound and soluble FasL were not identical. Membrane-bound
FasL increased on treatment with 5 and 10 μmol/L XLN306
but decreased on treatment with 20 μmol/L XLN306. In
contrast, soluble FasL was relatively unaltered on treatment
with 5 and 10 μmol/L XLN306 but increased dramatically on
 treatment with 20 μmol/L XLN306. This indicates that
XLN306 induces FasL by different mechanisms at different
concentrations, a result that requires further research.

In the intrinsic pathway of apoptosis, caspase activation is
closely linked to permeabilization of the outer mitochondrial
membrane by proapoptotic members of the Bcl family16. Bcl-2
protein, located on the outer mitochondrial membrane, is
important for suppression of mitochondrial manifestations of
apoptosis17,18. The release of cytochrome c from mitochondria
directly triggers caspase-3 activation19. In this study, treatment
with XLN306 led to a decrease in Bcl-2, an increase in the release
of cytochrome c and finally caspase-3 activation. This suggests
that the intrinsic pathway is also involved in the induction of
apoptosis by XLN306. Furthermore, 20 μmol/L XLN306
decreased the expression of p-AKT (Ser473), which demonstrates
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In conclusion, XLN306 is a promising drug for the management
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