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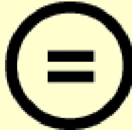

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삼중음성유방암 세포주에서 Src과 tubulin을 동시에 억제하는 KX-01의 항종양효과 연구

Antitumor effect of KX-01, a novel Src and tubulin inhibitor, in triple negative breast cancer cells

2014년 8월

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Antitumor effect of KX-01, a novel Src and tubulin inhibitor, in triple negative breast cancer cells

by

Seongyeong Kim

(Directed by Seock-Ah Im, M.D., Ph.D.)

A Thesis Submitted to the Interdisciplinary Graduate Program in Partial Fulfillment of the Requirements for the Degree of Master of Science in Cancer Biology at the Seoul National University, Seoul, Korea

July, 2014

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ABSTRACT

Antitumor effect of KX-01, a novel Src and tubulin inhibitor, in triple negative breast cancer cells

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Background: Src kinases have been involved in cell proliferation, invasion, and metastasis and is one of the highly expressed oncogenes in breast cancer. Therefore, it could be a reasonable target for therapeutic strategy. Although several Src inhibitors, targeting the ATP binding site, were developed, none of them have shown remarkable responses as monotherapeutic agents in breast cancer clin-

ical trials. KX-01 (KX2-391) is a novel peptidomimetic agent, and a non ATP-competitive Src inhibitor, which has potent efficacy for inhibiting both Src as well as tubulin polymerization. Its dual inhibition effects could potentially overcome the limitations of the Src inhibitors that have been evaluated previously.

Materials & Methods: To determine the antitumor effect of KX-01, MTT assay was performed to test cytotoxic effect of KX-01 on breast cancer cell lines. Then, through flow cytometry assay, cell cycle changes and aneuploidy population changes were examined after KX-01 treatment. Migration inhibitory effect was examined by wound healing assay and expression of protein molecular level changes by KX-01 treatment was verified by western blotting. The microtubule polymerization inhibitory effect was checked by immunofluorescence assay. Mouse xenograft model bearing MDA-MB-231 tumor was used to demonstrate *in vivo* effect of KX-01.

Results: KX-01 effectively inhibited cell growth in most breast cancer cells including ER/PR/HER2 negative breast cancer cells. KX-01 down-regulates the phospho-Src expre-ssion. Phosphorylated FAK, ERK, AKT and STAT3 were also down-regulated by KX-01 treatment in MDA-MB-231, -468 and BT-549 cells. By wound healing assay, migration inhibitory effect of KX-01 was confirmed in BT-549 cells.

Increase of G2/M cell cycle arrest was observed in KX-01 sensitive cell lines by dose dependent manner. In addition, increased multi-nucleated cells were observed along with the elevated aneuploidy levels in KX-01 sensitive cell lines indicating that mitotic catastrophes could be induced by KX-01 treatment. The *in vivo* data also showed that delay of tumor growth in MDA-MB-231 mouse xenograft model.

Conclusion: Inhibition of cell growth and migration, as well as an induction of mitotic catastrophe, were observed in triple negative breast cancer cell lines treated with KX-01. Moreover, using mouse xenograft model, I confirm antitumor effect of KX-01 *in vivo*. Our data strongly supports utilizing KX-01 as a new therapeutic agents for treating triple negative breast cancer.

Keywords: Src inhibitor, Tubulin inhibitor, Triple negative breast cancer, Mitotic catastrophe

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INTRODUCTION

Breast cancer is one of the most common cancer in women. It is classified into several subtype according to their molecular marker expressions. The status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) has been used as prognostic marker for identifying a high risk phenotype and predictive markers for selection of the most efficient therapies. Some cases of breast cancer do not express ER, PR, and HER2 and classified as a triple negative breast cancer (TNBC). This type of cancer is normally carrying a poor prognosis [1]. Unlike other subtype, TNBC does not have druggable target, chemotherapeutic agents were mainstream of the treatment of these patients group. Unfortunately, chemo-resistance could be easily occurred during process of treatments, so new therapeutic strategy for TNBC is needed.

Src family kinases (SFKs) are non-receptor tyrosine kinases and its activity is greatly increased among various solid tumor including breast cancer [2-5]. Src signaling activation can induce cell proliferation, invasion, and metastasis which makes tumor more aggressive [4]. Therefore, targeting Src signaling in breast cancer can be a reasonable strategy to treat breast cancer, especially TNBC

which has no available targeted therapy. For this reason, several Src inhibitors were developed in market, but none of them have shown remarkable responses as mono-therapeutic agents [6].

Mitotic catastrophe is a type of cell death that occurs during mitosis [7-11]. This phenomenon could occurred by aberrant mitosis or accumulation of damaged chromosomes [7]. Tumor cells are usually composed of high population of tetraploid cells which make them more prone to mitotic aberrations and could be more sensitive to mitotic catastrophe inducing agent [11]. Moreover, mitotic catastrophe can be exploited for elimination of apoptosis-resistance cancer cells [9]. Therefore, induction of mitotic catastrophe can be an alternative of ways to overcome chemo-resistance which are induced by apoptosis-resistance cells. KX-01 is a novel non ATP-competitive Src inhibitor which has a potent to inhibit tubulin polymerization [6, 12-15]. The effect of KX-01 was tested on an *in vivo* xenograft model using TNBC cancer cell line and showed promising effect [6]. However, *in vitro* effect of KX-01 is still needed more study to fully understand working mechanism of KX-01. In this study, Src inhibitory effect of KX-01 was confirmed *in vitro* using western blotting assay. I also confirmed tubulin inhibitory effect of KX-01 through cell cycle analysis and immunofluorescence assay. Interestingly, KX-01 treatment led to increase the population of

aneuploidy cells induced by mitotic catastrophe. *In vivo* study also verified KX-01 effectively inhibit tumor growth in mouse model. Based on our results, the working mechanism of KX-01 could be better understand and utilize our data to apply a new therapeutic strategy for breast cancer treatment.

MATERIALS AND METHODS

1. Antibodies and Reagents

KX-01 were kindly provided by Kinex Pharmaceutical (Buffalo, NY, USA). The compound was initially dissolved in dimethyl sulfoxide (DMSO) and stored at -80℃. Paclitaxel was obtained from Samyang Co., Ltd. (Seoul, Korea). Antibodies against p-Src (y416), FAK, p-p130cas, P-ERK (t202/y204), ERK (p44/p42), p-AKT (s473), AKT, p-STAT3 (y705), and STAT3 were purchased from Cell signaling Technology (Danvers, MA, USA). Antibodies against p-FAK (y397) and p130cas were purchased from BD Biosciences (San Jose, CA, USA). Antibodies against Src and p-FAK (y861) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against Actin was purchased from Sigma Aldrich (St. Louis, MO, USA).

2. Cell lines and cell culture

The Human Breast cancer cell lines (BT-474, -549, HCC1937, Hs578T, MCF7, MDA-MB-231, -468, SK-BR-3) were purchased from the American Culture Collection (ATCC; Manassas, VA, USA). Among these cell lines, BT-549, HCC1937, Hs578T, MDA-MB-231, and −468 cell lines lack expression of ER and PR and do not exhibit amplification

/overexpression of HER2 [16]. MCF7 and T47D cells are ER-positive breast cancer cell lines [17]. BT-474 and SK-BR-3 cells are HER2 overexpressing breast cancer cell lines [18]. The cells were cultured in RPMI 1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Life Technologise; Carlsbad, CA, USA) and 10㎍/mL gentamicin (Cellgro; Manassas,VA, USA) at 37℃ in a 5% CO_2 atmosphere.

3. Cell growth inhibitory assay

Cells (6-10 x 10^3 in 100㎕/well) were seeded in 96-well plates and incubated overnight at 37℃ in 5% CO_2. The cells were exposed to increasing concentrations of KX-01 (doses ranged from0-5 ^{3} μmol/L) or paclitaxel (doses ranged from 0-1 μmol/L) for 3 days. After drug treatment, 50μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide solution (Sigma Aldrich) was added to each well and the plates were incubated for 4 hours at 37℃ before the media was removed. After dissolving the formazan crystals with 150μL of DMSO, the absorbance of each well was measured at 540 nm with a VersaMax^{TM} microplate reader (Molecular Devices; Sunnyvale, CA, USA). The absorbance and IC_{50} of KX-01 were analyzed using Sigma Plot software (Statistical Package for the Social Sciences, Inc. (SPSs); Chicago, IL, USA). Six replicate wells were included in each

analysis and at least three independent experiments were conducted.

4. Western bolt analysis

Cells were collected drug treatment, washed with ice-cold PBS, and incubated in extraction buffer [50mM Tris-HCl (pH 7.4), 150mM Nacl, 1% NP40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM sodium fluoride, 1mM sodium pyrophorphate, 2mM phenylmethylsulfonyl fluoride, 1mg/mL pepstatin A, 0.2mM leupeptin, 10㎍/mL aprotinin, 1mM sodium vanadate, 1mM nitrophenylphosphate, and 5mM benzamidine] on ice for 30min. The lysates were cleared by centrifugation at 13,000 rpm for 20 min. Equal amounts of proteins were separated on an 8%-15% SDS-polyacrylamide gel. The resolved proteins were transferred onto nitrocellulose membranes, and the blots were probed with primary antibodies overnight at 4℃. Antibody binding was detected using an enhanced chemiluminescence system according to the manufacturer' s protocol (Amersham Biosciences; Piscataway, NJ, USA) [19].

5. Wound healing assay

Cells were seeded in 6-well plates and incubated overnight at 37℃ in 5% CO_2. Cells were scratched with blue

tip and washed with PBS. Cells were incubated with medium alone or medium containing 20nmol/L of KX-01. After 48hours, the plate were examined by light microscopy to monitor resealing of the cell monolayer [20].

6. Cell cycle analysis

Cells treated with KX-01 were harvested, fixed with cold 70% Ethanol, and then stored at -20℃ for at least 24 hours. The cells were washed in PBS and incubated with 10 ㎍/mL RNase A (Sigma Aldrich) at 37℃ for 20min. Next, the cells were stained with 20㎍/mL propidium iodide (Sigma Aldrich) and the DNA contents of cells (10,000 cells per experimental group) were quantified using a FACS Calibur flow cytometer (BD Biosciences).

7. Immunofluorescence assay

Cells were plated on 0.01% poly-L-Lysine (Sigma Aldrich)-coated coverslips, and treated with DMSO as a control or 100nmol/L of KX-01. After 24 hours, the coverslips were rinsed once in PBS (37℃), fixed in 3.7% paraformaldehyde for 10min, permeabilized with PBS-T (0.5% Triton X-100 in PBS) for 5min, and incubated with primary antibody for 24 hours at 4℃. The coverslips were rinsed three times for 10 min in PBS followed by incubation with the

appropriate fluorophore-conjugated secondary antibody (Invitrogen; Carlsbad, CA, USA). The cells were counter-stained with DAPI (300nM; Invitrogen) and the coverslips were mounted on slides using Faramount aqueous mounting medium (DAKO; Denmark). Immunofluorescence was visualized using a Zeiss LSM 510 laser scanning microscope.

8. *In vivo* studies

Animal experiments were carried out in the animal facility of the Seoul National University (Seoul, Republic of Korea) in accordance with institutional guidelines. To measure the *in vivo* activity of KX-01, 5-week-old female BALB/c athymic nude mice were purchased from Central Lab Animal, Inc. (Seoul, South Korea). The mice were allowed to acclimatize to conditions in the animal facility for 1 week before being injected with cancer cells. Mice were injected subcutaneously with MDA-MB-231 cells in 200 μL of PBS. When the tumor reached a volume of 150mm^3, mice were randomly divided into treatment groups and received vehicle control. KX-01 was administered via oral gavage once daily at a concentration of 5mg/kg for 2 weeks and twice daily at a concentration of 5mg/kg for extra 2 weeks. The tumor was measured every other day using calipers and the volume was calculated with the following formula: $[(width)^2 x (height)]/2$. At the end of measurement

period (day28), the mice were sacrificed. The tumors were excised and fixed in neutral-buffered formalin for routine histologic examination and immunohistochemical staining. At the same time, total proteins were extracted from fresh tissue to detect protein expression and activity of Src and FAK [19].

9. Immunohistochemistry

The histologic section from individual paraffin-embedded xenograft tumor tissues were deparaffinized and degydrated. IHC detection of proliferating cells was conducted using the antibody against Ki-67 (GeneTex; Irvine, CA, USA) at a dilution of 1:100. Terminal deoxynucletidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was conducted for IHC detection of apoptosis using ApopTag *In situ* Apoptosis Detection Kit (Chemicon International; Temecula, CA, USA), in accordance with manufacturer' s protocol.

10. Statistical analysis

Statistical analyses were conducted using SigmaPlot version 9.0. A two-sided Student *t* test was used when appropriate. The results are expressed as the mean ±SD or SE. A *P* value less than 0.05 was considered to be

statistically significant. All experiments were conducted in duplicate or triplicate and repeated at least twice.

RESULTS

KX-01 effectively inhibits cell growth of breast cancer cells and regulates phosphorylation of Src family kinase depends on the exposure time to KX-01

First, I examined the inhibitory effect of KX-01 on the growth of 9 breast cancer cell lines (MDA-MB-231, -468, BT-474, -549, Hs578T, SK-BR-3, MCF7, T47D, and HCC1937). Among 9 of them, 7 cell lines were sensitive to KX-01 including MDM-MB-231, -468, and BT-549 cells. These triple negative breast cancer (TNBC) cells were sensitive to KX-01 at IC_{50} values lower than 0.1 μmol/L (Fig. 1, Table 1). Unlike these sensitive three cell lines, Hs578T cell, shares a same subtype with sensitive three cell lines, Hs578T cell, which is also TNBC cell, was less sensitive to KX-01.

Next, I examined the *in vitro* action mechanism of KX-01 in sensitive and less sensitive cell lines. To determine whether KX-01 directly inhibits activity of Src and FAK, western blotting was performed to measure the total and phosphorylated levels of these proteins following by KX-01 treatment. The phosphorylation of Src, FAK, and p130cas in BT-549 cell was decreased in short time exposure by KX-01. Other sensitive cell lines, MDA-MB-231, and −468 cells, also showed similar responses with BT-549 cell (Fig. 2).

Inhibitory effect on cell migration was also checked after cells were treated with KX-01. Consistent with the western blot results, cell migration was inhibited in BT-549 cell, which is sensitive to KX-01. Compare with BT-549 cell, Hs578T cell, which is less sensitive to KX-01, did not show any inhibitory effect on cell migration (Fig. 3). I assumed cell migration inhibitory effect was a consequence of down-regulation of phosphorylated Src, so I checked molecular level changes with long term exposure of KX-01. Interestingly, after 24 hours treatment of KX-01, the inhibitory effect of phosphorylated Src is restored (data not shown). However, survival and proliferation related signals are still down-regulated by KX-01 treatment (Fig. 4). In KX-01 sensitive cell lines, I could observed down regulation of active form of ERK, AKT, and STAT3. Since KX-01 was developed to inhibit transphosphorylation from Src to other interaction molecules as well as inhibit Src auto phosphrylation, cell survival and proliferation pathways were still can be inhibited from activation by KX-01 treatment. Therefore, downstream molecules still can be regulated by KX-01 although levels of phosphorylated Src is restored after long term exposure of KX-01.

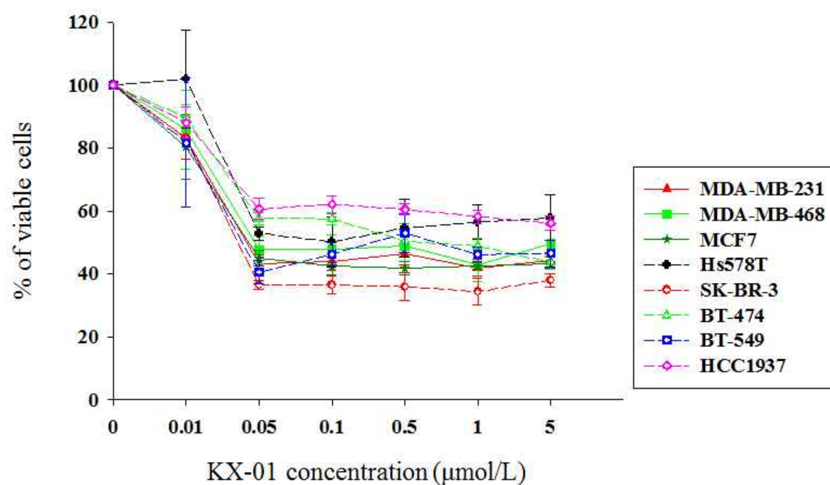


Figure 1. The growth inhibitory effect of KX-01 in breast cancer cell lines.

Cells were incubated with KX-01 for 72 hours as indicated KX-01 concentrations. Growth inhibition was analyzed by the MTT assay. The results are presented as percentages of the vehicle control.

Table 1. Growth inhibitory effect of KX-01.

Cell line	Subtype	KX-01 IC_{50} (μmol/L, means ± SD)
MDA-MB-231	Triple negative	0.0446±0.0009
MDA-MB-468	Triple negative	0.0613±0.0017
BT-549	Triple negative	0.0467±0.0019
Hs578T	Triple negative	>5
SK-BR-3	HER2	0.0338±0.0010
BT-474	HER2	0.1286±0.0076
MCF7	Luminal (ER +)	0.0418±0.0010
T47D	Luminal (ER +/PR +)	0.0435±0.0423
HCC1937	Triple negative	>5

The IC50 values of KX-01 determined by MTT assay as described in Materials and Method.

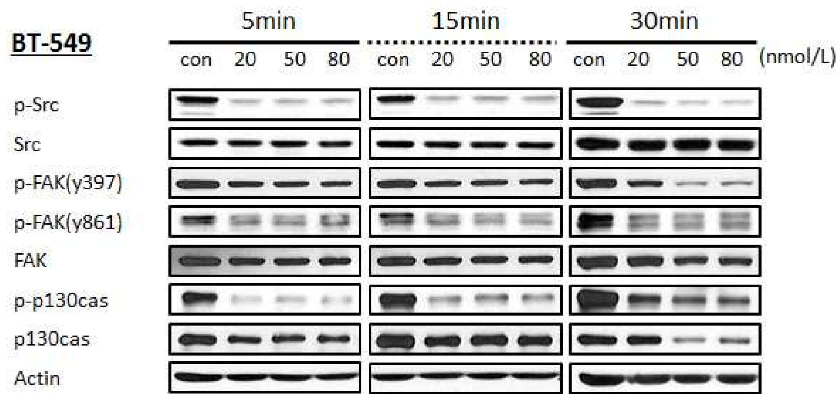


Figure 2. KX-01 down-regulates phospho-Src levels in KX-01 sensitive cell lines.

(A) Western blot results show molecular expression changes related following by KX-01 treatment. BT-549 cell was treated with indicated concentration of KX-01. Cells were exposed to KX-01 with indicated time conditions. Active form of Src, FAK, and p130cas levels were down-regulated by KX-01 treatment

(Figure 2, continued)

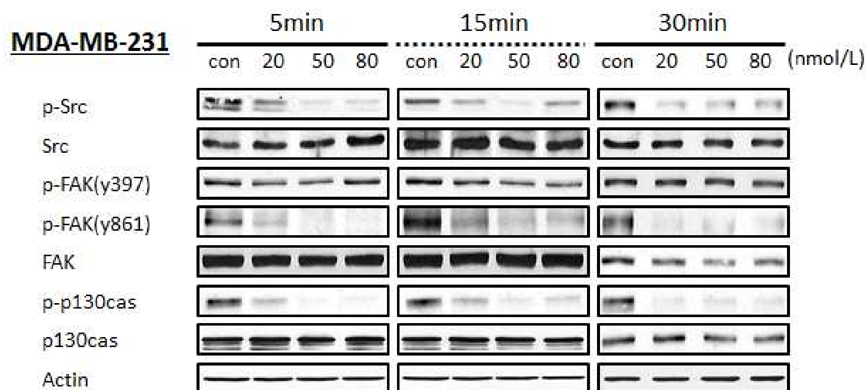


Figure 2. KX-01 down-regulates phospho-Src levels in KX-01 sensitive cell lines.

(B) Western blot results show molecular expression changes related following by KX-01 treatment. MDA-MB-231 cell was treated with indicated concentration of KX-01. This western blotting result also showed same responses with the result of BT-549 cell. phospho-Src, FAK, and p130cas levels were decreased by KX-01 treatment.

(Figure 2, continued)

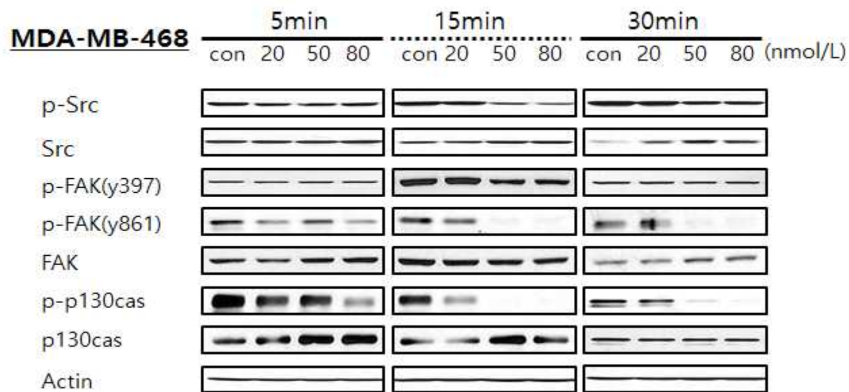


Figure 2. KX-01 down-regulates phospho-Src levels in KX-01 sensitive cell lines.

(C) Same as other KX-01 sensitive cell lines results, western blotting result of MDA-MB-468 cell was also down-regulated phospho-Src, FAK, and p130cas levels followed by KX-01 treatment. This phenomenon was significantly observed in short time exposure of KX-01.

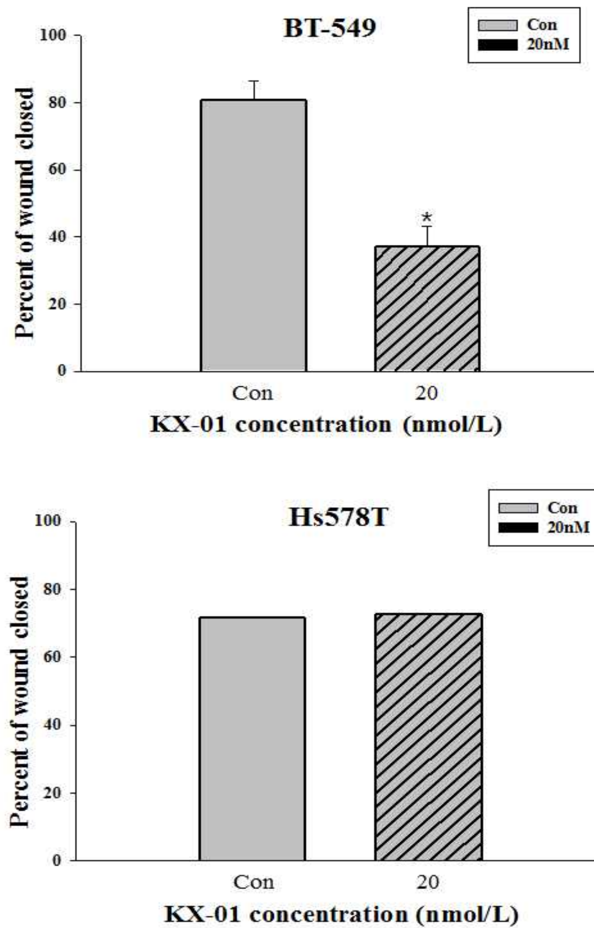


Figure 3. KX-01 inhibits cell migration in BT-549 cell.

Wound healing assay results demonstrate migration inhibitory effect of KX-01. BT-549 and Hs578T cells were seeded on 6-well plate. Cells were incubated with KX-01 for 48 hours with indicated KX-01 concentration. After 48 hours, plate was examined with microscopy and the length of gap filling was analyzed using Image J (NIH) software.

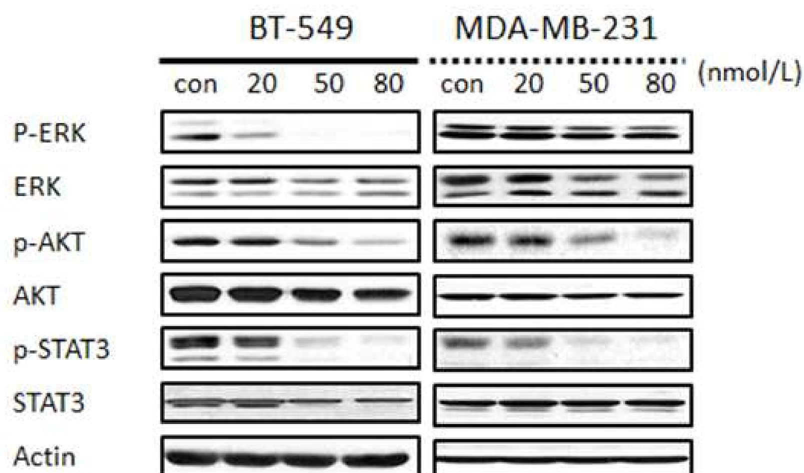


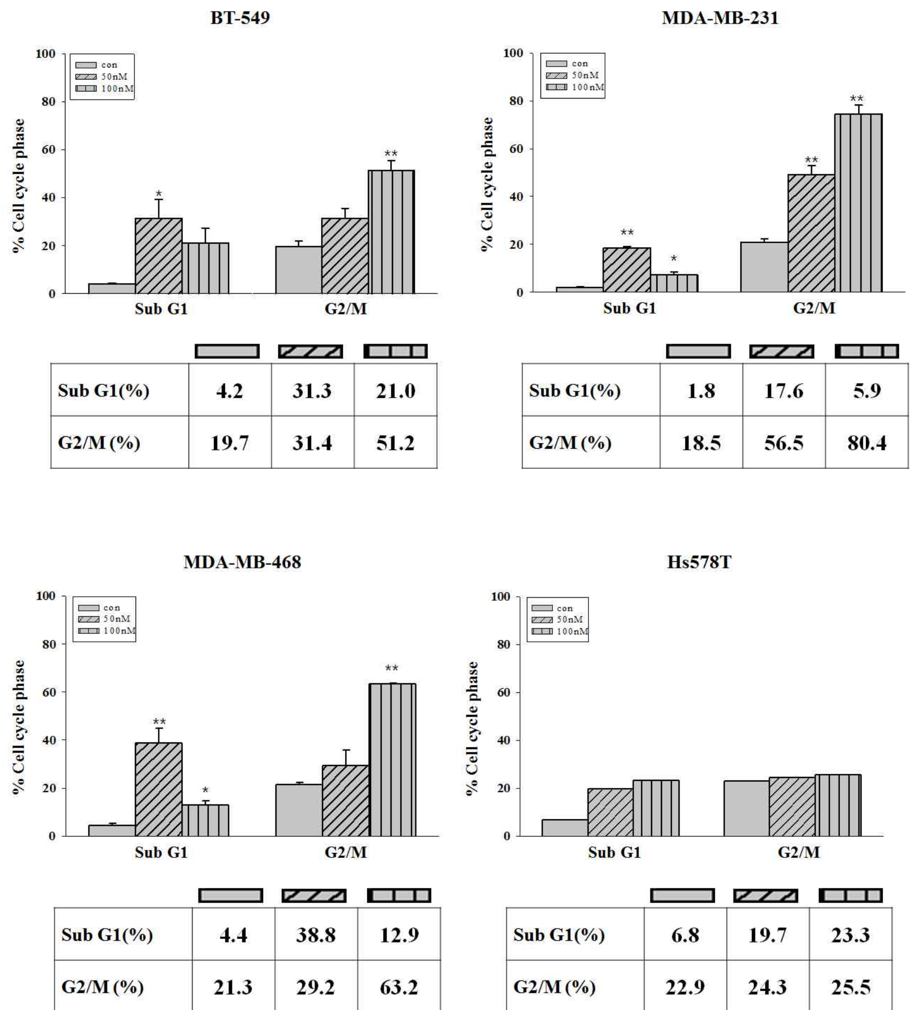
Figure 4. KX-01 down-regulated cell survival and proliferation signals.

Western blot results show molecular expression changes which are related with Src signaling. Cells were incubated with KX-01 for 24 hours as an indicated drug concentrations.

KX-01 cause cell death and G2/M cell cycle arrest

Next, I investigated the effects of KX-01 on cell cycle progression. Cell cycle analysis was conducted using flow cytometry in BT-549, MDA-MB-231, -468 and Hs578T cells. KX-01 induced apoptosis and G2/M cell phase arrest in KX-01 sensitive cell lines with KX-01 treatment (Fig. 5). Interestingly, the population of apoptosis was much higher when KX-01 was treated with 50nmol/L compare with 100nmol/L treatment. On the other hands, cell cycle arrest at the G2/M phase was induced as dose dependent manner.

The maximum increase point of cell cycle arrest is different between Sub G1 and G2/M arrest. The reason of this phenomenon could be explained by characteristic of KX-01. KX-01 could lead different responses according to drug concentration. KX-01 is known that about 20nmol/L of KX-01 could inhibit Src phosphorylation as 80nmol/L of KX-01 could inhibit tubulin polymerization [6]. Thus, I presume when cells were treated with 50nmol/L of KX-01, only Src phosphorylation is inhibited so Sub G1 population is highest. When KX-01 concentration passes 80nmol/L, tubulin polymerization is interrupted and occurring G2/M arrest is take a front rather than induction of Sub G1 population.



BT-549

Sub G1(%)	4.2	31.3	21.0
G2/M (%)	19.7	31.4	51.2

MDA-MB-231

Sub G1(%)	1.8	17.6	5.9
G2/M (%)	18.5	56.5	80.4

MDA-MB-468

Sub G1(%)	4.4	38.8	12.9
G2/M (%)	21.3	29.2	63.2

Hs578T

Sub G1(%)	6.8	19.7	23.3
G2/M (%)	22.9	24.3	25.5

Figure 5. G2/M arrest is occurred by KX-01 treatment. BT-549, MDA-MB-231, -468, and Hs578T cells were treated with indicated concentration of KX-01 for 48 hours. The percentages of cells in the G2/M or sub G1 phase were determined by cytometry analysis. The columns represent the mean of 3 independent experiments and are shown with error bars (±SE). *, $p<0.05$; **, $p<0.005$.

KX-01 increase aneuploidy and induces mitotic catastrophe

KX-01 has a potency to depolymerize microtubule and it might led an induction of G2/M arrest. To further investigation, I examined that KX-01 can induce aneuploidy. First, I categorize the population which has DNA contents more than 6N is aneuploidy population. Then, the population of aneuploidy in each cell lines was analyzed by flow cytometry analysis and normalized its control values. The cells, sensitive to KX-01, showed increases of aneuploidy population after exposed to KX-01. However, less sensitive cell line Hs578T did not show any significant changes (Fig. 6).

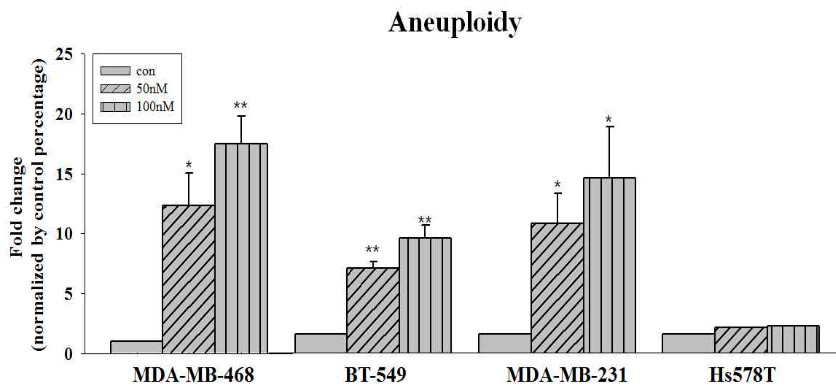
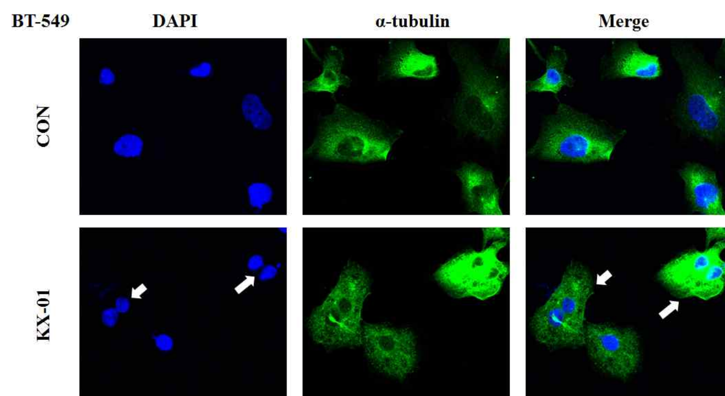


Figure 6. KX-01 could increase aneuploidy in KX-01 sensitive cell lines.

BT-549, MDA-MB-231, -468, and Hs578T cells were treated with indicated concentration of KX-01 for 48 hours. The percentages of cells which contained more than 6N were determined by flow cytometry analysis and normalized by each control values. Each columns are shown with error bars (±SE). *, $p<0.05$; **, $p<0.005$.

To investigate the induction of aneuploidy is due to mitotic error, I also checked induction of mitotic catastrophe, another type of cell death during mitosis. Induction of multi-nucleated cells are indicator of mitotic catastrophe, so I examined KX-01 could increase multi-nucleated cells to verify increase of mitotic catastrophe. In KX-01 sensitive cell lines, I could observe increase of multi-nucleated cell populations. Unlike KX-01 sensitive cell lines, less sensitive cell line, Hs578T did not showed significant changes (Fig. 7). Moreover, through metaphase spread, I could confirm that actual chromosome number increases followed by KX-01 treatment (data not shown). To conclude these results, KX-01 could induce mitotic catastrophe by inhibition of microtubule polymerization.

Figure 7. (A)



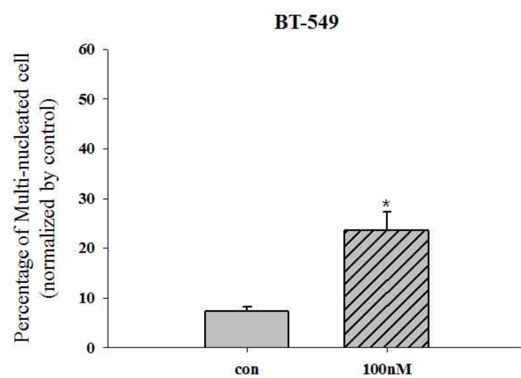
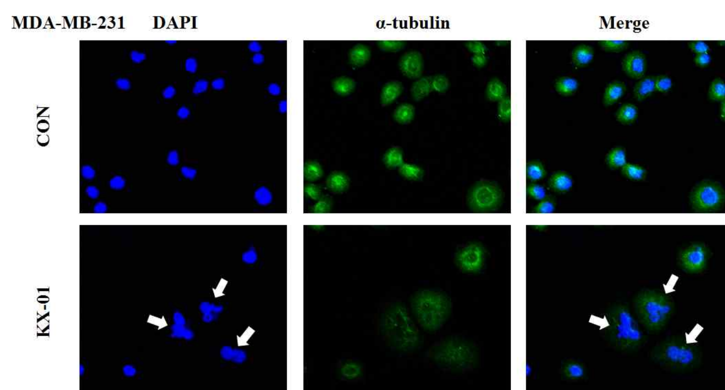


Figure 7. (B)



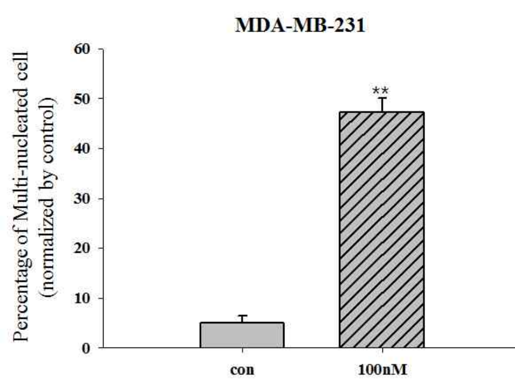
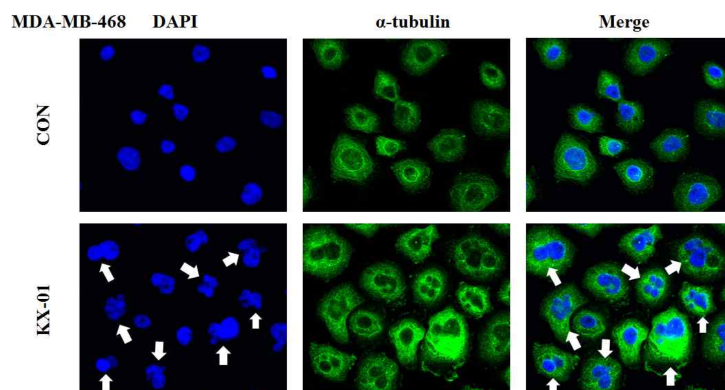


Figure 7. (C)



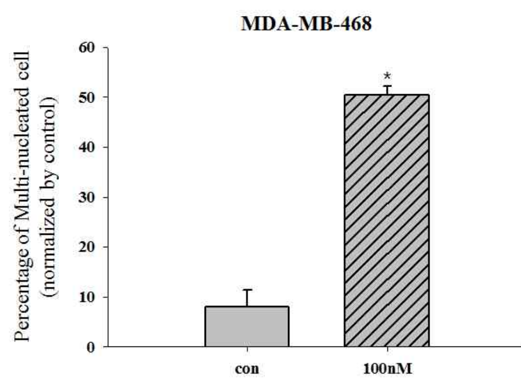


Figure 7. (D)

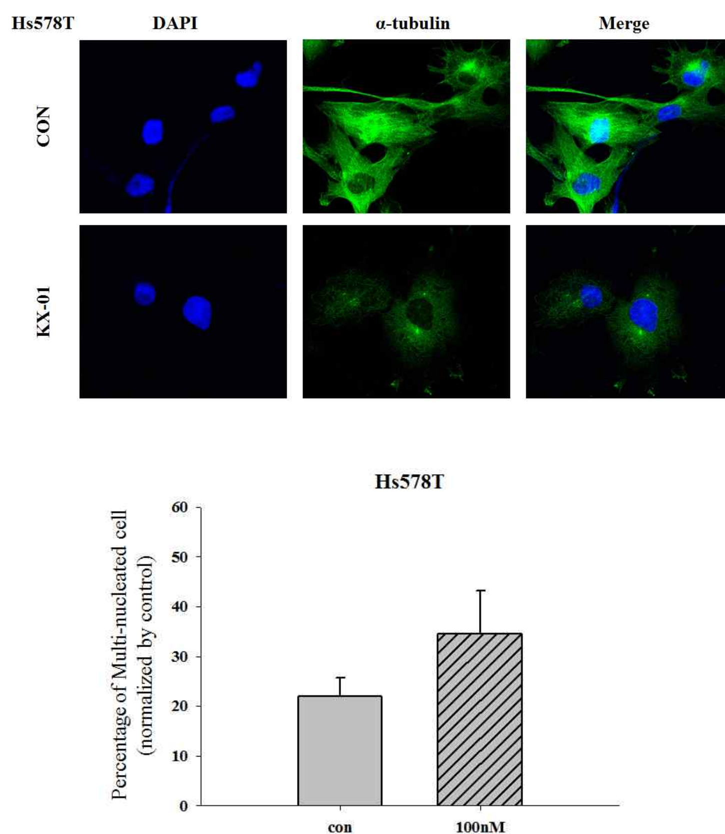


Figure 7. The number of multi-nucleated cells are increased by KX-01 treatment.

(Up) BT549(A), MDA-MB-231(B), -468(C), and Hs578T(D) cells were incubated with 100nmol/L of KX-01 or control for 24 hours. confocal microscopy was used to observe the signal corresponding to alpha-tubulin (green) and DNA was counterstained with DAPI (blue). White arrows were marked on multi-nucleated cells. (Down) The number of multi-nucleated cells were counted and normalized by each control values.

Each columns represent the mean of 3 independent experiments and are shown with error bare (±SE). *, $p<0.05$; **, $p<0.005$.

KX-01 inhibits the tumor growth *in vivo* mouse model

To confirm the antitumor effect of KX-01, which was examined *in vitro* assays, I test antitumor effect of KX-01 with *in vivo* mouse model established with MDA-MB-231 cells, which is sensitive to KX-01. Ten mice (five per group) were injected with 5 X 107 MDA-MB-231 TNBC cells and randomly received vehicle alone or 5mg/kg of KX-01.

As a result, KX-01 significantly delayed tumor growth during treatment (Figure 8). There were no significant weight changes in mice while they were treated with KX-01 (Figure 9). It would indicate that KX-01 did not shown toxicity on mice during KX-01 treatment. Tumor tissues from mice treated with KX-01 showed lower Ki-67 expression compare with vehicle control tissue (Figure 10). Since Ki-67 used as a proliferation marker [19, 20], this result suggests that KX-01 could lower proliferation ability compare with vehicle control. TUNEL assay was used to measure apoptotic cells, and tumor tissues from KX-01 treatment group showed significant increment of apoptotic cells comparing with vehicle control (Figure 10). In conclusion, these *in vivo* data demonstrated the antitumor effect of KX-01 in MDA-MB-231 TNBC xenograft model.

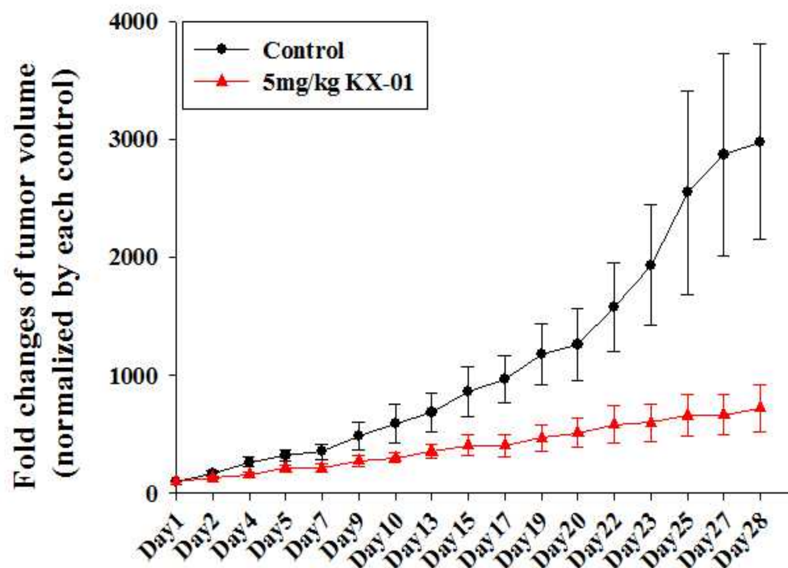


Figure 8. KX-01 inhibited growth in a xenograft model of MDA-MB-231 human breast cancer.

Balb/c nude mice were injected with 5 x 10^7 MDA-MB-231 cells. The mice were separated into 2 treatment groups randomly (n=5 tumors per group). The vehicle group received (2-Hydroxypropyl)-β-cyclodextrin solution, 10% solution in water and the other group was treated with oral gavage once daily at a concentration of 5mg/kg of KX-01 for 14 days and twice daily for 10 days. Tumor volumes were recorded as mm^3 and normalized by each control values. Each mean values are shown with ± SE.

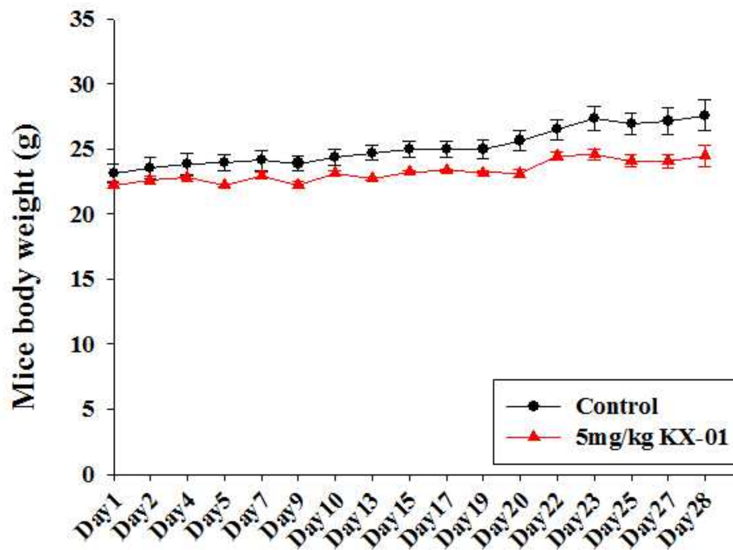


Figure 9. Effect of KX-01 on mice body weight.

Balb/c nude mice weight changes during treatment of KX-01 or (2-Hydroxypropyl)-β-cyclodextrin solution, 10% solution in water were demonstrated with graph. Each dot indicated with mean values of mice weight. Mice weights were measured three times weekly. No significant difference of body weight was detected.

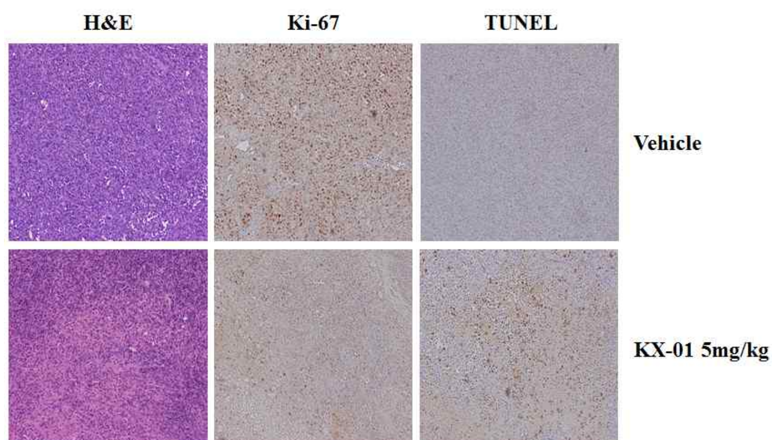


Figure 10. KX-01 reduced proliferation and increased apoptosis in MDA-MB-231 Xenogratf model.

The tumors were removed from the mice after drug treatment ended, and pathologic examination was done using H&E slides (200X). Immunohistochemical stain for Ki-67 and TUNEL assay showed decreased Ki-67 with increased apoptosis in KX-01 treatment tumors.

DISCUSSION

KX-01 is a small molecule inhibitor which can inhibit both Src and tubulin and underway phase II clinical trials [12, 13]. In previous study, KX-01 showed promising responses *in vivo* using breast cancer cell lines [6]. However, underlying mechanism of KX-01 antitumor activity is still needed to discover. In this study, I attempt to explore the mechanism of KX-01 antitumor activity *in vitro* using triple negative breast cancer cell lines. Through exposure time management, I identify KX-01 can down-regulate Src phosphorylation in short time period. When the exposure time became longer, regulation effect of phosphorylated Src was decreased. In long term exposure, Src signaling related molecules are still down-regulated despite of phosphorylated Src levels were restored. The reason of different inhibitory responses depend on each exposure time of KX-01 is due to the characteristic of KX-01. Unlike other Src inhibitors, KX-01 did not bind to ATP pocket of Src [6, 12, 15]. Thus, I think that there is a possibility of that phosphorylated Src level could be restored. Src signaling inhibitory effect in long term exposure could be explained by trans-phosphorylation inhibitory effect of KX-01. KX-01 has a potency to inhibit a trans-phosphorylation ability of Src thus even though phosphorylated Src level is restored, its downstream could be remain inactive forms. Through this mechanisms,

KX-01 can effectively inhibit Src signaling and this effect drew a result of cell growth and migration inhibition.

Another characteristic of KX-01 is inhibitory effect of microtubule polymerization [6, 12, 15]. Microtubule polymerization inhibitory effect was observed when KX-01 concentration was elevated more than 80nmol/L. In this study, I could also detect increment of G2/M phase arrest and induction of aneuploidy when I treated 100nmol/L of KX-01. Significant increasing of multi-nucleated cell population and chromosome verify that KX-01 is mitotic catastrophe inducing drug *in vitro*. This phenomenon can be identified by induction of co-localization of phospho-histone H3 and caspase3, which is indicator of mitotic catastrophe [7]. Therefore, futher study is needed to verify KX-01 as a mitotic catastrophe inducing drug.

In conclusion, antitumor effect of KX-01 is occurred by dual inhibitory effect, Src inhibition and microtubule polymerization inhibition.

Various Src inhibitors were developed which are targeting ATP binding pocket, however their outcome of clinical trial was not remarkable [6]. Nonetheless, Src is still an attractive target for treating breast cancer patients. Thus, inhibition of Src signaling through KX-01 treatment could be an alternative option to breast cancer treatment.

The relationship between Fyn and microtubule polymerization was reported in neuronal cell development [21]. Moreover recruiting tyrosine phosphorylated molecules during microtubule polymerization region was also discovered [22] and recently, several papers were published which explained possibility of involvement of src and FAK during mitosis [23-26]. Therefore, I strongly assume that Src activity could influenced on microtubule polymerization. Further study is needed to better understand working mechanism of KX-01.

In summary, this report demonstrates Src inhibitory effect of KX-01 in vitro for the first time. Moreover, this report verified induction of mitotic catastrophy in TNBC cells by KX-01 treatment. These dual effects would be key points differentiating KX-01 from all the other Src inhibitors which grant a higher opportunity for KX-01 to stand out as a promising TNBC treatment.

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국문초록

Src kinase는 유방암에서 높게 발현되는 발암 유전자중 하나로서, 세포의 증식, 침윤 및 전이 과정에 관여하는 발암 유전자로 알려져 있다. 그러므로 유방암을 치료하는데 사용할 수 있는 표적으로서 Src kinase를 활용할 수 있을 것이라는 가능성이 제시되어 오고 있다. Src kinase의 ATP binding site를 표적으로 하는 Src 억제제가 다수 개발되었지만 임상적으로 유의미한 효과를 거두지는 못한 실정이다. 본 연구에서 사용한 KX-01 (KX2-391) 항암제는 새로운 peptidomimic 항암제로 ATP binding site를 표적으로 하지 않는다는 특징을 가지고 있다. 또한 기존의 Src 억제제와는 다르게 KX-01은 microtubule의 polymerization을 억제하는 효과도 가지고 있다. KX-01의 Src과 tubulin을 동시에 억제하는 효과가 기존의 Src 억제제가 가지고 있는 한계점을 극복할 수 있는 가능성을 시사하고 있다고 생각한다. 따라서 본 실험에서는 삼중음성유방암 세포주를 이용하여 KX-01의 항종양효과를 *in vitro* level에서 증명하고, *in vivo* model에서 효과적으로 작용하는지를 보여주고자 하였다.

MTT assay를 이용하여 KX-01의 세포 성장 억제 효과를 확인한 결과, 대부분의 유방암 세포주에서 세포 성장이 저해되는 것을 확인할 수 있었다. KX-01에 감수성을 보이는 삼중음성유방암 세포주를 이용해 Src의 level 변화를 확인해 본 결과, KX-01을 30분 이내의 짧은 시간동안 처리한 결과, phospho-Src의 level이 감소하는 것을 확인하였으며, 동시에 Src에 의해 활성화되는 phospho-FAK, p130cas의 level도 같이 감소하는 것을 확인할 수

있었다. 이러한 효과는 BT549, MDA-MB-231, -468 세포주에서 동일하게 확인할 수 있었다. 또한 KX-01을 처리하였을 때, 세포의 이동성이 현저하게 감소하는 것을 wound healing assay를 통하여 확인할 수 있었다. KX-01을 24시간 이상 처리하였을 경우에도 ERK, AKT, STAT3와 같이 세포 성장과 전이에 관련된 molecule 들의 활성화가 지속적으로 저해되는 것을 확인할 수 있었다.

KX-01을 처리하였을 때, KX-01의 용량이 증가함에 따라 G2/M 기에 멈춰있는 세포의 수가 증가하는 것을 FACS를 이용한 cell cycle analysis를 통하여 관찰할 수 있었다. 뿐만 아니라, DNA의 양이 6N 이상을 가지는 aneuploidy 세포의 비율 또한 KX-01의 용량이 증가함에 따라 현저하게 증가하고 있음을 확인할 수 있었다. Aneuploidy의 증가는 mitotic catastrophe가 발생하였을 때 나타나는 하나의 현상이므로, 면역 형광법을 이용하여 mitotic catastrophe의 또 다른 지표인 multi-nucleated 세포의 증가를 확인 해 보았다. 그 결과 KX-01에 감수성을 보이는 세포주인 BT-549, MDA-MB-231, -468 세포주에서 KX-01을 처리하였을 때, multi-nucleated 세포의 수가 눈에 띄게 증가한 것을 확인할 수 있었다. MDA-MB-231을 이용해 구축한 in vivo 실험에서도 KX-01의 처리가 종양의 성장을 효과적으로 저해하고 있는 것을 확인할 수 있었다.

본 연구에서 KX-01에 감수성을 보이는 삼중음성유방암 세포주의 경우, KX-01을 처리하였을 때 세포의 성장이 감소하고, 세포 이동능력이 저해될 뿐 아니라 mitotic catastrophe라는 현상이 유도되는 것을 확인할 수 있었다. 또한 마우스 생체 이종이식 종양을 이용한 동물 실험에서도 KX-01 투여로 종양의 성장을 저해하

는 것을 확인할 수 있었다. 이러한 결과들은 KX-01이 효과적인 항암제로서 역할을 할 수 있다는 가능성을 보여주고 있으며, 차후 분자표적치료가 어려운 삼중음성유방암을 치료하는데 있어서 활용될 수 있는 근거로 작용할 수 있을 것이라 생각한다.

주요어: Src 억제제, Tubulin 억제제, 삼중음성유방암,

Mitotic catastrophe

학번: 2012-23648