Indirubin-3-monoxime Prevents Tumorigenesis in Breast Cancer through Inhibition of JNK1 Activity

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c-Jun N-terminal kinases (JNKs) have a Janus face, regulating both cell apoptosis and survival. The present study focused on understanding the function of JNK in tumor development and the chemoresistance underlying JNK-mediated cancer cell survival. We identified an inhibitor of JNK1, an important regulator of cancer cell survival. Kinase assay data showed that JNK1-dependent c-Jun phosphorylation was inhibited by indirubin derivatives. In particular, indirubin-3-monoxime (I3M) directly inhibited the phosphorylation of c-Jun *in vitro*, with a half inhibition dose (IC₅₀) of 10 nM. I3M had a significant inhibitory effect on JNK1 activity. Furthermore, we carried out assays to determine the viability, migration, and proliferation of breast cancer cells. Our results demonstrated that cell growth, scratched wound healing, and colony forming abilities were inhibited by the JNK inhibitor SP600125 and I3M. The combination of SP600125 and I3M significantly decreased cancer cell proliferation, compared with either SP600125 or I3M alone. Our studies may provide further support for JNK1-targeting cancer therapy using the indirubin derivative I3M in breast cancer.

Key Words: Cancer proliferation, Indirubin-3-monoxime, c-Jun N-terminal kinase, Phosphorylation inhibition, Triple negative breast cancer, Tumorigenesis

INTRODUCTION

c-Jun N-terminal protein kinases (JNKs) are involved in various physiological processes (Mehan et al., 2011). JNKs are a cellular response activated through environmental stresses such as osmotic stress, UV irradiation, metabolic inhibitors, and heat shock (Cargnello and Roux, 2011). The JNK signaling cascade performs a function in a important of physiological processes, regarding cell survival, apoptosis, cell proliferation, and gene expression (Lin and Dibling, 2002; Zhao et al., 2015), and responds to cytokines (Verrecchia et

al., 2003) and growth factors. The JNK kinase family comprise JNK1-3; of these, JNK1, 2 are ubiquitously expressed, whereas JNK3 expression is limited to the testis, brain, and heart (Bogoyevitch and Kobe, 2006).

c-Jun activation is triggered by the JNK-induced phosphorylation of serine 63 and 73. It composes homo- or heterodimers with the ATF (activating transcription factor), MAF (musculoaponeurotic fibrosarcoma), and FOS to comprise the transcription factor activator protein-1 (AP-1). The JNK signaling pathway connected with neuronal cell death has been widely studied (Guan et al., 2005; Hui et al., 2005). Recent evidence indicates that JNK is connected

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with cancer cell survival (Park et al., 2019), and that the crosstalk between JNK and other pathways is important in cancer development. JNK activation is associated with a poor prognosis in breast cancer and is important for tumor initiation and metastasis in mouse models of breast cancer (Insua-Rodriguez et al., 2018).

Triple negative breast cancers (TNBC) have a poor clinical effect contrasted to other breast cancer subtypes. TNBCs do not have the typical human epidermal growth factor receptor 2 (EGFR2), progesterone receptor (PR), and estrogen receptor (ER) that are commonly found in breast cancer (Lehmann et al., 2011). Several TNBCs overexpress human EGFR, which correlates with poor prognosis (Nielsen et al., 2004). Previous reports determined that as amount of phospho-JNK expression increase, tumor node metastasis (TNM) stage also increase and that phospho-JNK expression is correlated with positivity expression for CK5/6, basal-like, EGFR, and triple-negative phenotype of breast cancer (Wang et al., 2010). Based on these findings, inhibition of JNK activity might be a key target for cancer therapy. Several JNK pathway inhibitors have been developed; however, problems with substrate specificity and side effects remain.

Indirubin was known as an active component of Danggui Longhui Wan, a Chinese herb used in the treatment, in the therapy of a variety of illness, as well as chronic myelocytic leukemia (Xiao et al., 2002). Indirubin and its derivatives have been verified as distinguished inhibitors of fibroblast growth factor receptor 1 (FGFR1), glycogen synthase kinase-3 β (GSK-3 β), JNK, cyclin-dependent kinases (CDKs), Src kinase, muscle glycogen phosphorylase β , and the aryl hydrocarbon receptor (Bain et al., 2003; Zhen et al., 2007). Indirubin inhibits CDK kinase activity by competing with ATP which binding to the catalytic site of CDK kinase (Nam et al., 2005). Indirubin and its derivatives have been inhibited the growth of cultured cell types through a arrest of the G1/S or G2/M phase of the cell cycle (Hoessel et al., 1999; Marko et al., 2001). Compared with its derivatives, indirubin itself was known a significant gastrointestinal toxicity, low absorption rate, and poor solubility and indirubin-3-monoxime (I3M) was known reduced toxicity and better pharmacological properties (Lo and Chang, 2013). I3M has also been reported to induce mitochondrial dysfunction and antipro-

liferative effects in vascular smooth muscle cells and trigger cell cycle arrest and growth inhibition in human neuroblastoma cells (Bain et al., 2003; Schwaibergger et al., 2010; Liao and Leung, 2013). In conclusion, I3M is one of the most important compound for the treatment of cancer.

In this study, we identified that I3M can regulate the JNK1 signaling pathway. Conclusionally, our studies indicate that I3M acts as a negative regulator of the JNK1 signaling pathway, and it may serve as a latent therapeutic agent for the cure of breast cancer.

MATERIALS AND METHODS

Cell culture plasmids and transfection

Human embryonic kidney 293 (HEK293) and MDA-MB 231 cell lines were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA), in a humidified incubator with an atmosphere of 95% air and 5% CO₂. A full-length *JNK1* gene was constructed via PCR and inserted into the mammalian expression vector pcDNA3. For plasmid DNA transfection, cells were plated at a density of 50~60% confluence, grown overnight, and transfected with appropriate expression vectors in the presence of the indicated combinations of plasmid DNA (2 μ g per well), using the Lipofectamine[®]2000 reagent (Invitrogen, USA).

Kinase assay

Cultured cells were harvested and lysed in buffer A, containing 50 mM Tris-HCl (pH 7.5, LPS solution, Korea), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, USA), 2 μ g/mL of leupeptin (Sigma-Aldrich, USA), 2 μ g/mL of aprotinin (Sigma-Aldrich, USA), 25 mM glycerophosphate (Sigma-Aldrich, USA), 0.1 mM sodium orthovanadate (Sigma-Aldrich, USA), 1 mM sodium fluoride (Sigma-Aldrich, USA), 1% NP-40 (VWR Life Science, USA), 0.5% deoxycholate (Sigma-Aldrich, USA), and 0.1% SDS (Sigma-Aldrich, USA), for 30 min at 4°C. The cell lysates were then subjected to centrifugation at 12,000 $\times$ g for 20 min at 4°C. The soluble fraction was incubated for 3 h with the appropriate antibodies against the indicated protein

kinase at 4°C. The immunocomplexes were then coupled to protein A-agarose during an additional hour of incubation at 4°C, after which they were pelleted via centrifugation. The immunopellets were rinsed thrice with lysis buffer and then twice with 20 mM HEPES (pH 7.4, Sigma-Aldrich, USA). The immunocomplex kinase assays were conducted by incubating the immunopellets for 30 min at 30°C, with 2 µg of GST-c-Jun as substrate protein in 20 µL of reaction buffer, which contained 0.2 mM sodium orthovanadate (Sigma-Aldrich, USA), 10 mM MgCl₂ (VWR Life Science, USA), 2 µCi [³²P] ATP (PerkinElmer, USA), and 20 mM HEPES (pH 7.4, Sigma-Aldrich, USA). The phosphorylated substrates were separated by SDS-PAGE and quantified using the Fuji FLA7000 phosphoimager (Fuji, Japan). The GST fusion proteins that were used as substrates were expressed in *E. coli* using pGEX-4T (Pharmacia, USA), and purified using glutathione-Sepharose (GE Healthcare Bio-Sciences AB, USA), as described previously. The protein concentrations were determined using the Bradford method.

Synthesis of indirubin and indirubin derivatives

Indirubin derivatives were synthesized and characterized via high-resolution mass spectrometry and ¹H NMR to verify their identity and purity (Moon et al., 2006). In brief, 176 mg (1 mmol) of indoxyl acetate and 1 mmol of isatin analogue in 5 mL of methanol were added to 256 mg (2.5 mmol) of Na₂CO₃ under a nitrogen atmosphere, and the mixture was stirred for 2~3 h at room temperature (25°C). The dark violet precipitate was filtered and washed twice with methanol and several times with cold water, and dried under reduced pressure (yield 50~60%). Subsequently, 6 mmol of hydroxylamine hydrochloride was added to a solution of indirubin analogues (1 mmol) in 10 mL of pyridine and the mixture was refluxed for 2~3 h at 120°C. After cooling, the product was neutralized with 1 N HCl and the precipitate was filtered and washed with water (Lee et al., 2008).

MTT assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, USA) was prepared as a stock solution of 5 mg/mL in phosphate-buffered saline (PBS, pH

7.2) and filtered. After the end of reaction time (60 h), 20 µL of MTT solution was added to each well. After incubation for 4 h at 37°C, the medium with the MTT reagent was removed and 150 µL of DMSO was added to dissolve the formazan crystals. The 96-well plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm for absorbance density values to determine the cell viability. All measurements were carried out in triplicate. Percentage of viable cells was calculated using the following formula: (%) = [100 × (sample abs) / (control abs)].

Wound healing assay and colony forming assay

MDA-MB 231 cells were cultured to confluence in 12-well plates, then scratched with a P200 micropipette tip. The wound healing area was monitored at 0, 24, 32, and 48 h using a microscope. Wound healing area measurements were performed using the ImageJ software. For the colony forming assay, MDA-MB 231 cells were seeded at 100 cells per 24-well plate, then incubated at 37°C and 5% CO₂ for 10~12 days until colony formation was observed. The medium was replaced every 3 days. After incubation, colonies were fixed with 100% methanol and stained with 0.05% crystal violet for 20 min at room temperature. Colony counting and measurements were performed using the ImageJ software.

Statistical analysis

All experiments were performed at least three times. Data are presented as mean ± standard deviation (SD) values. Student's *t*-test (two-tailed) was used to analyze differences between treated and untreated groups. Data were analyzed using the Sigma-Plot software program. *P*-values < 0.05 were considered statistically significant.

RESULTS

I3M negatively regulates JNK1 kinase activity

Previous studies have reported that I3M directly inhibits the activities of recombinant active JNK1, JNK2, and JNK3 (Xie et al., 2004). To verify the possible function of indirubin derivatives in regulation of the JNK1 signaling pathway, an *in vitro* kinase assay was performed. We used a

#	ST	MW	#	ST	MW	#	ST	MW	#	ST	MW	#	ST	MW	#	ST	MW
1		262. 2703	11		295. 2754	21		386. 1639	31		402. 4347	41		446. 4695	51		426. 4355
2		277. 28497	12		291. 31206	22		401. 1785	32		390. 4456	42		441. 4065	52		422. 4472
3		346. 26808	13		311. 73	23		276. 2974	33		430. 854	43		410. 4361	53		486. 404
4		361. 28275	14		403. 1814	24		296. 7153	34		386. 3701	44		464. 4074	54		441. 4065
5		276. 30024	15		305. 33915	25		388. 1667	35		430. 854	45		376. 4186	55		373. 2939
6		304. 30794	16		325. 75709	26		290. 3245	36		360. 3755	46		460. 8805	56		350. 3367
7		388. 30572	17		356. 181	27		310. 7424	37		402. 4568	47		460. 8805	57		424. 4632
8		309. 28377	18		367. 33824	28		277. 285	38		374. 4026	48		362. 3915	58		440. 4626
9		280. 26073	19		357. 34717	29		376. 4186	39		414. 3994	49		486. 5348			
10		324. 29844	20		341. 16633	30		362. 3915	40		414. 3994	50		412. 2457			

Fig. 1. Chemical structure of indirubin derivatives.

Fig. 2. Indirubin derivatives regulate JNK1 activity. After transiently transfected with plasmids expressing pcDNA3-HA-JNK1 at HEK 293 cells and then treated for 4 h with indirubin derivatives. The cells were lysed and then immunoprecipitated using anti-HA antibody, after 48 h of transfection. The immunoprecipitates were incubated with purified GST-c-Jun, then assayed for JNK1 activity.

Fuji FLA7000 phosphorimager to quantify the phosphorylation of c-Jun. Fig. 1 shows the chemical structure of indirubin compound. As shown in Fig. 2, indirubin compound markedly inhibited the *in vitro* phosphorylation of c-Jun by JNK1. Among the indirubin compound, I3M significantly blocked JNK1 kinase activity. To confirm the

JNK1 inhibitory effect of I3M, we performed an *in vitro* kinase assay, with JNK1 as the kinase and GST-c-Jun as the substrate, in a concentration-dependent manner (Fig. 3B). I3M markedly inhibited the *in vitro* phosphorylation of c-Jun by JNK1. These results suggest that I3M blocks JNK1 activity directly.

Fig. 3. I3M suppresses JNK1 activity. (A) Chemical structure of I3M. (B) After transiently transfected with plasmids expressing pcDNA3-HA-JNK1 at HEK 293 cells and then treated with various concentrations (5, 10, 100, 500, 10,000, and 100,000 nM) of I3M for 4 h. The cells were lysed and then immunoprecipitated using anti-HA antibody, after 48 h of transfection. The immunoprecipitates were incubated with purified GST-c-Jun, then assayed for JNK1 activity.

JNK1 signaling regulated by I3M inhibits cancer cell growth

To further comprehend the role of I3M in regulating JNK activity, we examined its effect on cancer cell growth using the MDA-MB-231 cell line. SP600125 is the most common JNK inhibitor; therefore, we investigated the effect of inhibition by I3M on the growth of cells compared with SP600125. Cell viability was assessed based on the MTT assay. The DMSO-treated MDA-MB-231 cells grew steadily over 60 h, while treatment with either SP600125 or I3M inhibited the viability of cells (Fig. 4A). SP600125 and I3M synergized to cause apoptosis. To confirm the effect of cell proliferation by I3M, we performed a scratched wound healing and colony forming analyses. We confirmed that the cellular proliferation delay caused by I3M could be increased by the JNK inhibitor SP600125 (Fig. 4B, C). As shown in

Fig. 4. Inhibition of JNK1 negatively regulates cancer cell growth. (A-C) MDA-MB 231 cells were treated for various time periods with DMSO, SP600125 (20 μ M), or I3M (10 μ M). (A) Cell viability was analyzed with the MTT assay. (B) Cell migration was analyzed with scratched wound healing assay. The data were quantified by measuring scratch area intensity using the ImageJ software. (C) Cell proliferation was analyzed with a colony forming assay. Cells were stained with crystal violet. Data represent the mean $\pm$ standard deviation (SD) values of triplicates (* $P \leq 0.001$). A-C All data were obtained from three independent experiments.

Fig. 5. Model for the role of I3M in the regulation of cancer proliferation. I3M directly inhibits JNK1 activity. I3M-reduced JNK1 activity decreases c-Jun phosphorylation. Decreased c-Jun phosphorylation inhibits cell proliferation.

Fig. 4B, the combination of SP600125 and I3M significantly decreased the migration distance, compared with either SP600125 or I3M. We also observed significant inhibition of colony formation ability in the cells treated with a combination of SP600125 and I3M (Fig. 4C). Cell growth, scratch wound healing, and colony forming ability were markedly blocked with the combination treatment than with the control or either SP600125 or I3M individually.

DISCUSSION

In a previous study, Xie et al. showed that I3M inhibits recombinant active JNK activity *in vitro* (Xie et al., 2004). The JNK signaling is mediated in the pathogenesis of various conditions such as ischemic stroke, Parkinson's disease (PD), as well as tauopathies, including Alzheimer's disease (AD) (Bode and Dong, 2007; Seki et al., 2012; Zeke et al., 2016; Hammouda et al., 2020). Additionally, the JNK pathway has important functions in both cancer cell survival and cell death processes (Kukekov et al., 2006). Therefore, it is unsurprising that JNK signaling is a key target for drug development. In most JNK function studies have used pharmacological inhibition of the signaling pathway by SP600125. However, SP600125 has been reported to inhibit 13 other protein kinases, including AMPK, CDK2, and SGK, as well (Bain et al., 2003).

Previous studies have revealed that the primary function of I3M is the inhibition of CDKs, which are important mediators of cell cycle progression, and GSK-3 β which is mediated in neurodegenerative disorders. I3M has a broad spectrum of anti-tumorigenic activities in various kinds of human cancer cells (Schwaiberger et al., 2010). The main

anticancer mechanism of I3M has been proposed to be the inhibition of CDKs (Hoessel et al., 1999; Leclerc et al., 2001) and GSK-3 β (Bain et al., 2003). Lo et al. identified that I3M inhibits survivin expression, which is important mediator of oral cell survival (Lo and Chang, 2013). However, the specific mechanism of I3M anticancer function is still unknown. Of note, the unexpected mechanism by which I3M could inhibit JNK activity has recently been highlighted in cerebellar granule neurons (Xie et al., 2004).

Previous reports determined that as amount of phospho-JNK expression increase, tumor node metastasis (TNM) stage also increase and that phospho-JNK expression is correlated with positivity expression for CK5/6, basal-like, EGFR, and triple-negative phenotype of breast cancer (Wang et al., 2010). Based on previous study, activated JNK may be a key regulator for cancer therapy. Several kinds of JNK signaling inhibitors have been developed; however, factors such as substrate specificity and potential side effects pose limitations to their use.

In this studies, we identified the inhibition of JNK1 pathway by indirubin derivatives (Fig. 2). We also identified that I3M caused potent JNK1 inhibition activity (Fig. 3). Our results showed that phosphorylation of c-Jun was decreased by I3M. To determine the role of I3M in inhibiting JNK kinase activity in breast cancer, we examined its effect on breast cancer cell growth. As expected, breast cancer cell growth was inhibited in treated cells with either SP600125 or I3M (Fig. 4A). Similar results were acquired with the colony forming assay and scratched wound healing assay (Fig. 4B, C). Cell growth, colony forming abilities, and scratch wound healing were significantly inhibited in response to the combination treatment than to the control or

either SP600125 or I3M alone. Our data strongly suggest that the JNK inhibitor I3M prevents breast cancer cell growth. It has been reported that SP600125 inhibits MCF-7 cell proliferation and cell cycle progression. Our data similarly support a role for JNK signaling inhibition by SP600125 in MCF-7 cell proliferation and cell cycle progression (Wood et al., 2018). These findings provide evidence of another mechanism for I3M anticancer activity, in addition to inhibition of CDK and GSK3 β .

In conclusion, our study found I3M to involve a key role in cancer cell proliferation through inhibiting JNK1 activity. Therefore, I3M, which inhibits JNK activity, could be a potential target for cancer therapy (Fig. 5).

Abbreviations

AP-1; activator protein-1, ATF; activating transcription factor, ER; estrogen receptor, EGFR2; epidermal growth factor receptor 2, I3M; indirubin-3-monoxime, JNK; c-Jun N-terminal kinase, MAF; musculoaponeurotic fibrosarcoma, PR; progesterone receptor, TNBC; triple negative breast cancer, TNM; Tumor-node-metastasis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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