Evaluation of Erastin as a Therapeutic Agent Under Hypoxic Conditions in Pancreatic Cancer Cells

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Abstract. *Background/Aim:* In pancreatic cancer tissues, hypoxic areas exist due to poor blood flow. Attenuation of the pharmacological efficacy of existing anticancer drugs in these hypoxic areas necessitates the search for novel anticancer compounds. We aimed to determine whether erastin exhibits anticancer effects in a hypoxic environment. *Materials and Methods:* Pancreatic cancer cell lines were subjected to cobalt chloride, a hypoxia-mimicking agent. Cell viability assay, measurement of reactive oxygen species, and western blotting analysis were conducted to investigate the efficacy of erastin under hypoxic environments. *Results:* Erastin exhibited remarkable cytotoxicity and induced apoptosis under hypoxic conditions. Furthermore, erastin triggered the intracellular accumulation of reactive oxygen species in a hypoxic environment. Subsequent treatment with N-acetylcysteine, an antioxidant, markedly attenuated cytotoxicity, and apoptosis. *Conclusion:* Erastin induces cell death by accumulation of intracellular reactive oxygen species and inducing apoptosis under hypoxic conditions, proving its potential for further development as a novel anticancer compound.

Pancreatic cancer is known to have a poor prognosis. It is estimated that by 2030, the number of deaths from pancreatic cancer will have further increased, making it the second leading cause of mortality worldwide (1, 2). Early detection of pancreatic cancer is difficult. Furthermore, patients with newly diagnosed pancreatic cancer often develop distant metastasis or local recurrence. Thus, many patients are treated with systemic chemotherapy or radiotherapy rather

than surgery. The use of chemotherapeutic agents such as single-agent gemcitabine, TS-1 (tegafur, gimeracil, and oteracil potassium), and FOLFILINOX (fluorouracil, leucovorin, irinotecan, and oxaliplatin) has resulted in a 5-year survival rate of $\leq 10\%$ (3).

Pancreatic cancer tissues develop immature and heterogeneous vascular networks owing to active cellular proliferation (4). Oxygen and nutrition are supplied to cancer tissues through the bloodstream; thus, pancreatic cancer tissues are exposed to a hypoxic environment (5). Indeed, the mean partial pressure of oxygen of pancreatic cancer tissues was reported to be 0.4%, which is significantly lower than that of normal pancreatic tissues (6.8%) (6).

The hypoxic environment of tumour tissues markedly attenuates the pharmacological efficacy of anticancer agents (7-10). Therefore, there is an urgent for novel anticancer compounds that specifically target the hypoxic environment of pancreatic cancer tissues.

Previously, we identified mechanisms that stimulated cytotoxicity in hypoxic environments. We reported that it was possible to induce hypoxia-selective cytotoxicity by inhibiting autophagy and nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) (11-13). Specifically, inhibition of NOX4 or autophagy led to the excessive accumulation of reactive oxygen species (ROS) in cells, which subsequently induced hypoxic environment-selective cell death. Therefore, analysing specific indicators associated with intracellular accumulation of ROS under hypoxic conditions may be an effective strategy for identifying the efficacy of novel anticancer compounds.

Erastin, a ferroptosis inducer, is a potent agent for pancreatic cancer treatment (14). Erastin-induced cytotoxicity is caused by the accumulation of intracellular ROS (15-17). Therefore, we hypothesised that this compound would be able to induce cytotoxicity even in hypoxic environments. Proving that erastin exerts anticancer effects in both normal and hypoxic environments may lead to the development of a novel chemotherapeutic strategy against pancreatic cancer. However, to our knowledge, no previous studies have examined the effect of erastin on cell death under hypoxic conditions.

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Key Words: Apoptosis, hypoxia, erastin, reactive oxygen species, pancreatic cancer.

In this study, we aimed to investigate the effect of erastin on cell death in a hypoxic environment and explore its potential as a novel drug against pancreatic cancer.

Materials and Methods

Cell lines and culture conditions. The human pancreatic cancer cell lines PANC-1 and BxPC-3 were obtained from the European Collection of Cell Cultures (Salisbury, UK). The human pancreatic cancer cell line MIA PaCa-2 was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum to a final concentration of 10% (v/v), 50 µg/ml streptomycin, 50 U/ml penicillin, and non-essential amino acids (Gibco BRL, Paisley, UK).

Reagents. Materials were obtained from the following sources: Erastin from Cayman Chemical (Ann Arbor, MI, USA); hypoxia-inducible factor 1 alpha (HIF1α) antibody from GeneTex (Irvine, CA, USA); actin antibody and 3'-O-acetyl-6'-O-pentafluorobenzenesulfonyl-2',7'-difluorofluorescein (BES-H₂O₂-Ac) from Wako Pure Chemical Industries (Osaka, Japan); Hoechst 33342 from Calbiochem-Merck (Darmstadt, Germany); Dulbecco's modified Eagle's medium, N-acetylcysteine (NAC), ferrostatin-1, and cobalt chloride (CoCl₂) from Sigma-Aldrich (St. Louis, MO, USA); benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) and poly (ADP-ribose) polymerase (PARP) antibody from Cell Signaling Technologies (Beverly, MA, USA); necrostatin-1, a necroptosis inhibitor, from Abcam (Cambridge, UK); CellROX Green Reagent from Life Technologies (Paisley, UK).

CoCl₂, NAC and Hoechst 33342 were dissolved in phosphate-buffered saline (PBS). Erastin, ferrostatin-1, Z-VAD-FMK and necrostatin-1 were dissolved in dimethyl sulfoxide (DMSO). PBS or DMSO was used as a vehicle control.

Establishment of hypoxic conditions. In all experiments, cells were seeded at 60-70% confluence for 24 h, and then were exposed to erastin in the absence (vehicle control PBS) or presence of 300 µM CoCl₂.

Cell viability assay. The cells were seeded in 96-well plates at a density of 3×10³ cells/well. After treatment with or without CoCl₂, the cells were treated for 48 h with the erastin (2 or 20 µM) alone or in combination with the following compounds: NAC (0.5 mM), ferrostatin-1 (10 µM), necrostatin-1 (20 µM), or Z-VAD-FMK (50 µM). Cytotoxicity was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cell viability values were normalised to those of the vehicle control.

Western blotting analysis. Protein extraction and western blotting analysis were performed as previously described (18, 19). Immunoblotting was performed using antibodies to HIF1α (1:1,000), PARP (1:1,000), and actin (1:10,000).

ROS detection assays. Confocal microscopy: Cellular ROS accumulation in BxPC-3 cells was detected with BES-H₂O₂-Ac and CellROX per the manufacturer's instructions. Briefly, BxPC-3 cells were prepared in 35 mm glass-bottom µ-Dish (ibidi GmbH, Munich, Germany). BxPC-3 cells were treated with different concentrations

of erastin in the absence or presence of 300 µM CoCl₂ for 24 h. BES-H₂O₂-Ac or CellROX was then added to the cell culture media at a final concentration of 5 µM. Finally, BxPC-3 cells were incubated for 45 min at 37°C. The samples were analysed using confocal microscopy (LSM 700; Zeiss, Jena, Germany).

Fluorescence plate reader: BxPC-3 cells were seeded at a density of 3×10³ cells/well in 96-well plates (Sumitomo Bakelite, Tokyo, Japan). BxPC-3 cells were treated with different concentrations of erastin in the absence or presence of 300 µM CoCl₂ for 24 h, and then 5 µM BES-H₂O₂-Ac was added to the wells. BES-H₂O₂-Ac fluorescence was measured after 30 min using a fluorescence plate reader (excitation=485 nm, emission=520 nm). The fluorescence values obtained from each well were normalised to the cell viability results of the same sample well by CCK-8 assay.

Statistical analysis. Each experiment was repeated at least three times. Data are expressed as the mean±standard deviation. Comparisons between multiple groups were performed by one-way analysis of variance followed by Scheffe *post hoc* test or two-way analysis of variance followed by Scheffe *post hoc* test. Differences with values of *p*<0.05 were considered statistically significant.

Results

Cytotoxicity of erastin under hypoxic conditions. CoCl₂ was used to mimic a hypoxic environment (20). Intracellular accumulation of HIF1α, a hypoxia marker, was observed upon CoCl₂ treatment of BxPC-3 cells (Figure 1A). We subsequently examined the effects of erastin on cell survival. Remarkable cytotoxicity was observed upon treatment with 20 µM erastin under normal conditions, which was consistent with the results of a previous study (16). Under hypoxic conditions, considerable cytotoxicity was observed upon administration of 2 µM erastin, which was maintained at a concentration of 20 µM (Figure 1B). Additionally, we analysed the responses of PANC-1 and MIA PaCa-2 cells to investigate whether this was a common phenomenon in pancreatic cancer cell lines and found that erastin induced marked cytotoxicity under hypoxic conditions in these cells (Figure 1C-F). These results strongly suggest that erastin may be an effective anticancer compound, even under hypoxic conditions.

Effect of ROS on the cytotoxicity of erastin under hypoxic conditions. Next, we explored the mechanism of erastin-induced cytotoxicity in hypoxic environments. Under normoxic conditions, the cytotoxicity of erastin is caused by ferroptosis, which is mediated by lipid ROS accumulation (21, 22). Therefore, we investigated whether erastin leads to intracellular ROS accumulation in a hypoxic environment. We used the CellROX (23) and BES-H₂O₂-Ac (24) probes to detect ROS. As previously reported, under normoxic conditions, marked intracellular accumulation of ROS was observed upon treatment with 20 µM erastin, consequently inducing cell death. Marked intracellular accumulation of ROS and consequent cell death was also observed under hypoxic conditions upon

Figure 1. Erastin-induced cytotoxicity under hypoxic conditions. A: BxPC-3 pancreatic cancer cells were exposed to 300 μ M CoCl₂ for 48 h, followed by the western blotting analysis of hypoxia-inducible factor 1 alpha (HIF1 α) and actin. B: BxPC-3 cells were subjected to different concentrations of erastin for 48 h in the absence or presence of 300 μ M CoCl₂ then Cell Counting Kit-8 analysis was used to measure BxPC-3 cell viability. Corresponding HIF1 α and protein expression and viability results for PANC-1 (C, D) and MIA PaCa-2 (E, F) pancreatic cancer cells. *Significantly different at $p < 0.05$; n.s.: not significantly different.

Figure 2. Continued

Figure 2. The role of reactive oxygen species in the effects induced by erastin under hypoxic conditions. BxPC-3 cells subjected to treatment with erastin with or without 300 μ M CoCl_2 for 24 h were stained with Hoechst 33342 and 5 μ M CellROX Green (A) or Hoechst 33342 and 5 μ M BES- H_2O_2 -Ac (B). Bar=10 μ m. BxPC-3 cells were also subjected to erastin treatment, with or without 300 μ M CoCl_2 in the presence of the scavenger N-acetylcysteine (NAC) and then assessed by CCK-8 assay after 48 h (C) and by BES- H_2O_2 -Ac fluorescence (D) after 24 h. *Significantly different at $p < 0.05$; n.s.: not significantly different.

treatment with 2 and 20 μ M erastin (Figure 2A and B). Furthermore, upon treatment with NAC (25), which can eliminate a wide range of ROS, erastin-induced cytotoxicity under hypoxic conditions was inhibited, accompanied by a reduction in intracellular ROS levels (Figure 2C and D).

These results indicate that erastin-induced cytotoxicity depends mainly on the intracellular accumulation of ROS under hypoxic conditions.

Ferroptosis-independent cytotoxicity in a hypoxic environment. Erastin induces ferroptosis *via* lipid ROS accumulation. Therefore, ferrostatin-1, which selectively eliminates lipid ROS and inhibits ferroptosis, was used to investigate whether erastin-induced cytotoxicity involved ferroptosis. As previously reported (16), under normal conditions, ferrostatin-1 reversed cytotoxicity induced by 20 μ M erastin. However, under hypoxic conditions, ferrostatin-1 was not able to reverse cytotoxicity at the concentrations tested in our study (Figure 3A). Therefore necrostatin, an inhibitor of necroptosis (26), was used to investigate the involvement of a cell death type other than ferroptosis. Treatment with necrostatin also did not attenuate erastin-induced cell death (Figure 3B). These results indicate that erastin-induced cytotoxicity under hypoxic conditions does not involve ferroptosis or necroptosis, unlike that under normal conditions.

Apoptosis-dependent cytotoxicity under a hypoxic environment. We investigated the involvement of apoptosis in order to

identify the specific mechanism of erastin-induced cytotoxicity in a hypoxic environment. We studied the cleavage of PARP, an indicator of apoptosis. The results strongly suggested that PARP cleavage was induced by erastin treatment under hypoxic conditions (Figure 4A). Consistent with the cell viability results, PARP cleavage was not restored by treatment with ferrostatin-1 but was with NAC. These findings suggest that the cleavage of PARP induced by erastin in a hypoxic environment was not caused by lipid ROS but by other ROS (Figure 4B).

In addition, Z-VAD-FMK, an inhibitor of apoptosis (27), was used to further demonstrate that the cell death observed under hypoxic conditions was due to apoptosis. We found that the cytotoxicity of erastin under hypoxic conditions was markedly attenuated by Z-VAD-FMK (Figure 4C). Collectively, our results indicate that in a hypoxic environment, erastin-induced cytotoxicity is mediated by apoptosis.

Discussion

In the present study, we explored the potency of erastin as a novel anticancer compound targeting the hypoxic environment in pancreatic cancer and investigated the potential mechanisms underlying erastin-induced cytotoxicity. Using CoCl_2 to mimic a hypoxic environment, we found that erastin exhibited cytotoxicity under a hypoxic environment. In-depth investigations need to be conducted in the future using other agents that mimic hypoxic conditions to better elucidate the roles and effects of erastin.

Figure 3. Erastin-induced cytotoxicity was independent of ferroptosis and necroptosis. BxPC-3 cells were subjected to treatment for 48 h with erastin in the presence ferrostatin-1 (A) or necrostatin-1 (B) with or without 300 μ M CoCl₂. *Significantly different at $p < 0.05$; n.s.: not significantly different.

In this study, the cytotoxicity induced by erastin was attenuated by NAC, which commonly eliminates a wide range of ROS in normal and hypoxic environments. This finding demonstrates the role of ROS in mediating erastin-induced cell death. Erastin is a known inducer of ferroptosis, which is an iron-dependent mechanism of cell death mediated by lipid ROS, as first reported by Stockwell *et al.* in 2012 (22). Ferroptosis is recognised as a form of cell death distinct from apoptosis and necroptosis, the two well-established and classical forms of cell death (28). In the present study, cytotoxicity induced by erastin under normoxic conditions was attenuated by ferrostatin-1, which was consistent with the findings of another report (16). Therefore, the cytotoxicity induced by erastin in normoxic environments may be associated with ferroptosis. In addition, we revealed that erastin induces cytotoxicity even under hypoxic conditions, thereby fulfilling the aim of this study.

Surprisingly, erastin-induced cytotoxicity in a hypoxic environment was not attenuated by ferrostatin-1. Apparently, ferroptosis, which is mediated by lipid ROS, was not involved in erastin-induced cell death under hypoxic conditions. Furthermore, we found that erastin increased the level of ROS, except for lipid ROS, thereby inducing apoptosis. To the best of our knowledge, this is the first report showing that erastin can induce apoptosis in a hypoxic environment. We previously reported that strategies to increase the production of mitochondria-derived ROS and apoptosis rate may be effective in inducing cytotoxicity in hypoxic environments (11). It was also reported that erastin inhibits mitochondrial voltage-dependent anion channels leading to accumulation of ROS (29, 30). In the future, the specific types of ROS that accumulate upon erastin treatment in hypoxic environments and the detailed mechanisms causing apoptosis should be investigated.

Figure 4. Erastin induced apoptosis under hypoxic conditions. A: The expression level of poly (ADP-ribose) polymerase (PARP) and actin in BxPC-3 cells treated for 24 h with erastin in the absence or presence of 300 μM CoCl₂. B: BxPC-3 cells were treated with N-acetylcysteine (NAC) or ferrostatin-1 in the presence of erastin, with or without 300 μM CoCl₂, for 24 h, followed by the western blotting analysis of PARP and actin. C: CCK-8 assay was used to determine viability of BxPC-3 cells treated for 48 h with erastin with or without the apoptosis inhibitor Z-VAD FMK in the presence of 300 μM CoCl₂. *Significantly different at $p < 0.05$; n.s.: not significantly different.

Erastin has many advantages as a therapeutic agent. It was reported that cancer cells take up more iron than normal cells (31, 32). Ferroptosis is an iron-dependent form of cell death. Therefore, the use of erastin to induce ferroptosis can selectively induce cytotoxicity in cancer cells. In addition, it was revealed that erastin induced apoptosis and demonstrated cytotoxicity even in hypoxic environments, wherein chemotherapeutic agents were ineffective (7-10). Proper control of apoptosis is also important for the survival of normal cells. Therefore, it is of great concern that strategies to induce apoptosis in hypoxic environments may also impair the functioning of normal cells. However, unlike normal tissues, hypoxia is a characteristic feature of solid tumours (33, 34). Therefore, erastin not only enables the induction of ferroptosis in normally oxygenated areas and apoptosis in hypoxic areas but also has the potential to become a revolutionary therapeutic compound with fewer side-effects. Many animal studies have shown that erastin is effective in reducing tumours and does not induce serious side-effects (35-37). In addition, the presence of hypoxic areas in cancer tissues is a common phenomenon in many tumours besides pancreatic cancer (6). Thus, the cytotoxic effect of erastin on

cancer cells in a hypoxic environment in other organs should also be investigated.

In conclusion, we revealed that erastin exerts cytotoxic effects in a hypoxic environment, indicating its utility as a candidate compound against pancreatic cancer.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Conception and design of the study, S.O. and H.E. Collection and assembly of data S.O., H.E., Y.S., T.K., H.F., and M.T. Analysis and interpretation of data S.O. and H.E. Drafting of the article S.O. All Authors reviewed and approved the final version of the article.

Acknowledgements

Confocal microscopy analysis was performed by the Medical Science College Office of Tokai University. The Authors thank Yuko Okamoto and Akiko Sakuyama from the Department of Preventive Medicine, Tokai University School of Medicine, for their excellent

secretarial support. The Authors would also like to thank Editage (www.editage.jp) for English language editing.

This study was supported in part by the 2017-2019 Research and Study Program of Tokai University Educational System General Research Organization (S.O.), 2021 Tokai University School of Medicine Research Aid (S.O.), and Grant-in-Aid for Scientific Research (21K15960 to S.O. and 21K19671 to H.E.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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Received August 20, 2021

Revised September 17, 2021

Accepted September 21, 2021