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Setanaxib as a Potent Hypoxia-specific Therapeutic Agent Against Liver Cancer

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Abstract. *Background/Aim: Liver cancer has extremely poor prognosis. The cancerous tissues contain hypoxic regions, and the available drugs are poorly effective in hypoxic environments. NADPH oxidase 4 (NOX4), producing reactive oxygen species (ROS), may contribute to cancer malignancy under hypoxic conditions. However, its role in liver cancer has not been examined in detail. Our aim was to explore the effects of setanaxib, a recently developed selective NOX4 inhibitor, in liver cancer cells under hypoxic conditions. Materials and Methods: Liver cancer cell lines (HepG2, HLE and Alexander) were treated with hypoxia-mimetic agent cobalt chloride. Cytotoxicity assays, immunoblot analysis and ROS detection assay were performed to detect the effect of setanaxib under hypoxic conditions. Results: Setanaxib exhibited hypoxia-selective cytotoxicity and triggered apoptosis in cancer cells. Moreover, setanaxib caused mitochondrial ROS accumulation under hypoxic conditions. Treatment with antioxidants markedly attenuated setanaxib-induced cytotoxicity and apoptosis under hypoxic conditions. Conclusion: Setanaxib caused mitochondrial ROS accumulation in a hypoxia-selective manner and evoked cancer cell cytotoxicity by inducing apoptosis. Thus, setanaxib has a great potential as a novel anticancer compound under hypoxic conditions.*

Liver cancer is highly malignant, has an extremely poor prognosis, and is the second leading cause of cancer-related death worldwide, after lung cancer (1). Treatments for liver cancer include radiofrequency ablation, transarterial chemoembolization, liver transplantation and chemotherapy with drugs such as sorafenib and gemcitabine (2-4). Unfortunately, none of these treatments results in favourable outcomes.

Although the liver tissue is well-vascularized, aberrant proliferation of cancer cells may result in regions with poor blood circulation, as is the case with other tumours (5). In these regions, hypoxic conditions may establish. Surprisingly, the partial pressure of oxygen (pO2) in liver cancer tissue was reported to be 0.8%, indicating much more severe hypoxia compared to other types of cancer (6). Notably, hypoxic conditions markedly attenuate the cytotoxic effects of sorafenib and gemcitabine, contributing to the poor prognosis of liver cancer patients treated with these drugs (7-9). Hence, there is high demand for novel anticancer compounds capable of maintaining their efficacy under hypoxic conditions.

The transcription factor hypoxia-inducible factor-1 (HIF-1) plays an important role in hypoxic adaptation and controls the expression of many target genes involved in proliferation, survival, angiogenesis, and metabolism, thereby regulating hypoxic adaptation (10-13). However, these downstream targets of HIF-1 have not been characterized in detail.

The expression of the reactive oxygen species (ROS)-producing enzyme, NADPH oxidase 4 (NOX4), is induced in an HIF-1-dependent manner (14). It is known that ROS play an important role as a signalling molecules. Under hypoxic conditions, NOX4-derived ROS signalling promotes the acquisition of a malignant cancer phenotype, including epithelial mesenchymal transition (EMT), cell migration, and invasion (15, 16). However, the exact role of NOX4 in liver cancer cell viability has not been extensively investigated. Recently, setanaxib (GKT137831), a specific inhibitor of NOX4, has been discovered (17-19), and is being tested in clinical trials for various pathologies. Therefore, in this study, we aimed to examine the effect of setanaxib on cancer cell viability under hypoxic conditions and verify its efficacy as a candidate agent for the treatment of liver cancer.

Our results demonstrated that setanaxib exerted cancer cell cytotoxicity by inducing the production of mitochondrial

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Key Words: Cancer treatment, hypoxia, setanaxib, oxidative stress, liver cancer.

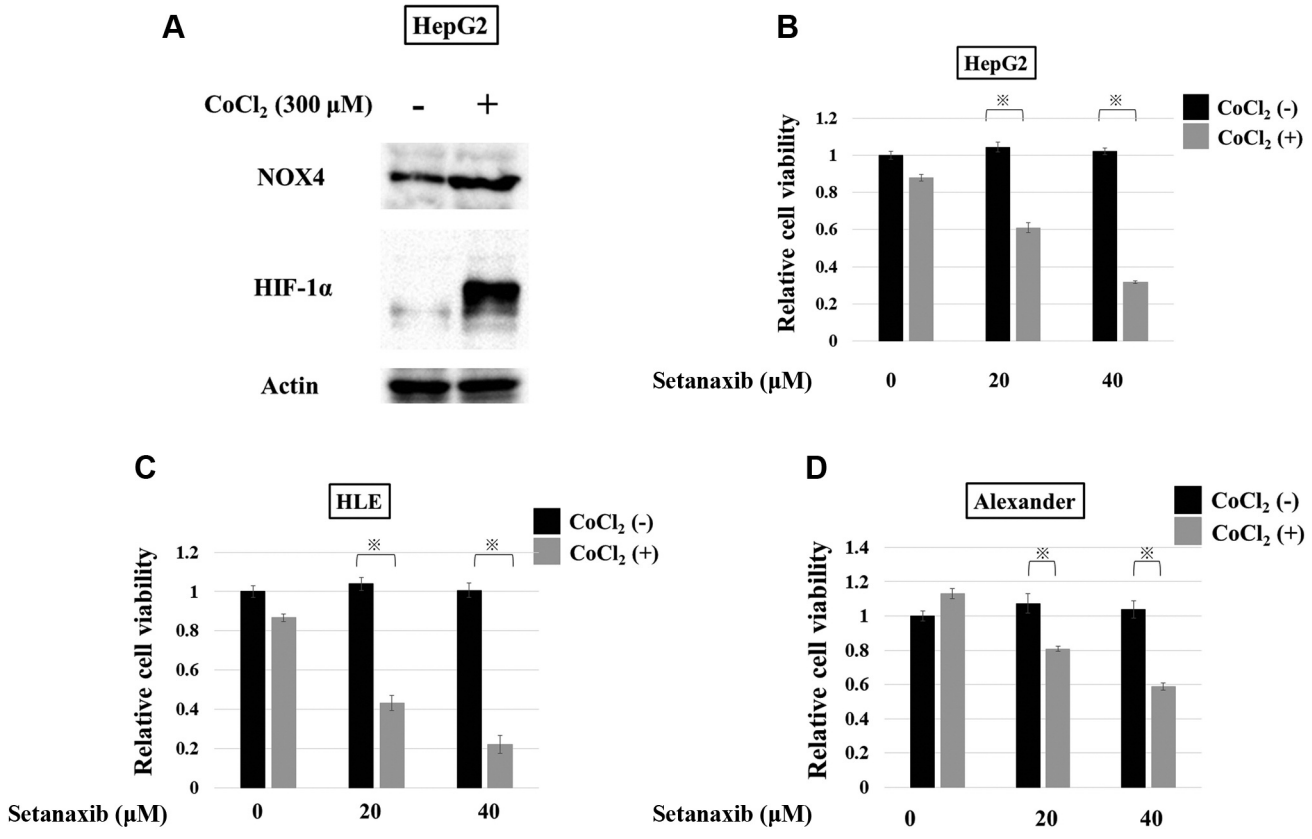

Figure 1. *Setanaxib induced selective cancer cell cytotoxicity under hypoxic conditions. (A) HepG2 cells were treated with 300 μM cobalt chloride for 48 h and the protein levels of HIF-1α, NOX4, and Actin were determined by immunoblot analysis. (B-D) A CCK8 assay was conducted to assess the viability of HepG2, HLE, and Alexander cells treated for 48 h with setanaxib under ordinary culture conditions or in the presence of 300 μM cobalt chloride (*p<0.05).*

ROS in a hypoxia-selective manner. We provide evidence that setanaxib has immense potential as an anticancer agent to be employed under hypoxic conditions.

Materials and Methods

Cell lines and culture conditions. The human liver cancer cell lines HepG2, HLE, and Alexander (PLC/PRF/5) were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). All cell lines were cultured in DMEM supplemented with 10% foetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and non-essential amino acids (Gibco BRL, Paisley, UK). The cells were cultured at 37˚C under 5% CO_2/95% air.

Reagents. Materials were obtained from the following sources: GKT137831 from Selleckchem (Huston, TX, USA); Z-VAD-FMK and PARP antibody from Cell Signaling Technologies (Beverly, MA, USA); Actin antibody and BES-H2O2-Ac from Wako Pure Chemical Industries (Osaka, Japan); NOX4 antibody from Proteintech (Rosemont, IL, USA); Hochest33342 from Calbiochem-Merck (Darmstadt, Germany); Dulbecco's modified Eagle's medium (DMEM), dimethylsulfoxide (DMSO), cobalt chloride, ascorbic acid, and MitoTEMPO from Sigma (St. Louis, MO, USA); HIF-1α antibody from GeneTex (Irvine, CA, USA); MitoSOXTM Red from Life Technologies (Paisley, UK).

Immunoblot analysis. Protein extraction and immunoblot analysis were performed as previously described (20). The antibody dilutions were performed in accordance with the manufacturers' instructions.

Cytotoxicity assay. The cells were seeded in 96-well plates at 5×10^3 cells/well. At 24 h after seeding, the medium was replaced by DMEM with or without 300 μM cobalt chloride, followed by treatment with serial dilutions of various agents. After 48 h of incubation, the cytotoxicity was evaluated by using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cell cytotoxicity was calculated relative to the vehicle control.

Plasmids and transfection. HepG2 cells were transfected with plasmids containing MISSION® short hairpin targeting human NOX4 (sh-NOX4 CCGGTCACCATCATTTCGGTCATAACTC GAGTTATGACCGAAATGATGGTGATTTTTG)- (Sigma), by using Lipofectamine LTX Reagent with PLUS Reagent (Thermo Fisher Scientific) for 48 h. The cells were then transferred to a medium containing 2 μg/ml puromycin (Sigma) for 2 weeks to obtain stable expression.

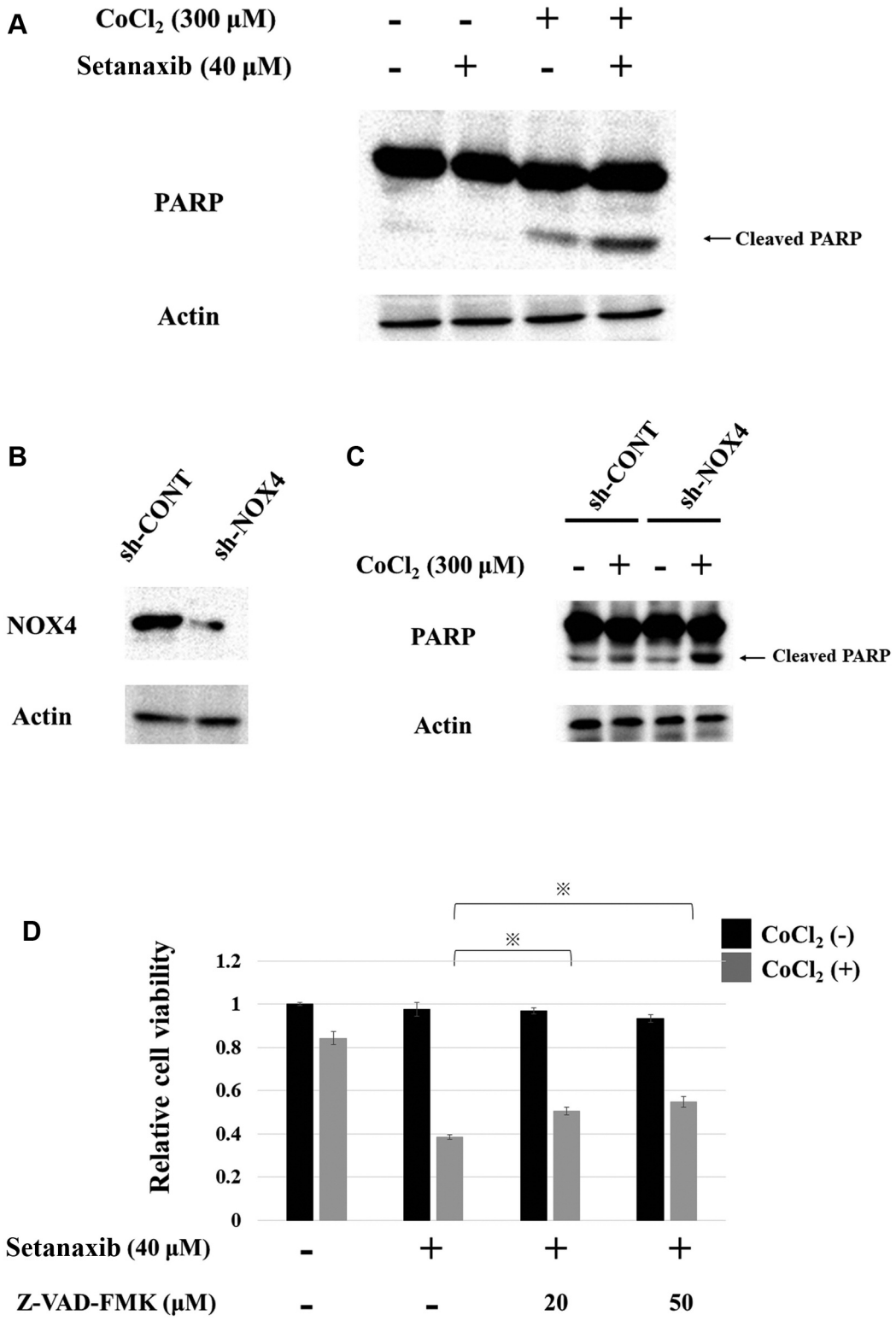

Figure 2. *Setanaxib induced apoptosis under hypoxic conditions. (A) The protein levels of PARP and Actin in HepG2 cells treated with setanaxib, with or without 300 μM cobalt chloride, for 48 h were determined by immunoblot analysis. (B) Immunoblot detection of NOX4 in HepG2 cells after transfection with NOX4 shRNA. (C) Immunoblotting analysis of HepG2 cells transfected with a non-speficic sh-CONT or sh-NOX4, in the absence or presence of 300 μM cobalt chloride for 48 h. (D) A CCK8 assay was performed to assess the viability of HepG2 cells treated with setanaxib, with or without Z-VAD-FMK, in the absence or presence of 300 μM cobalt chloride for 48 h (*p<0.05).*

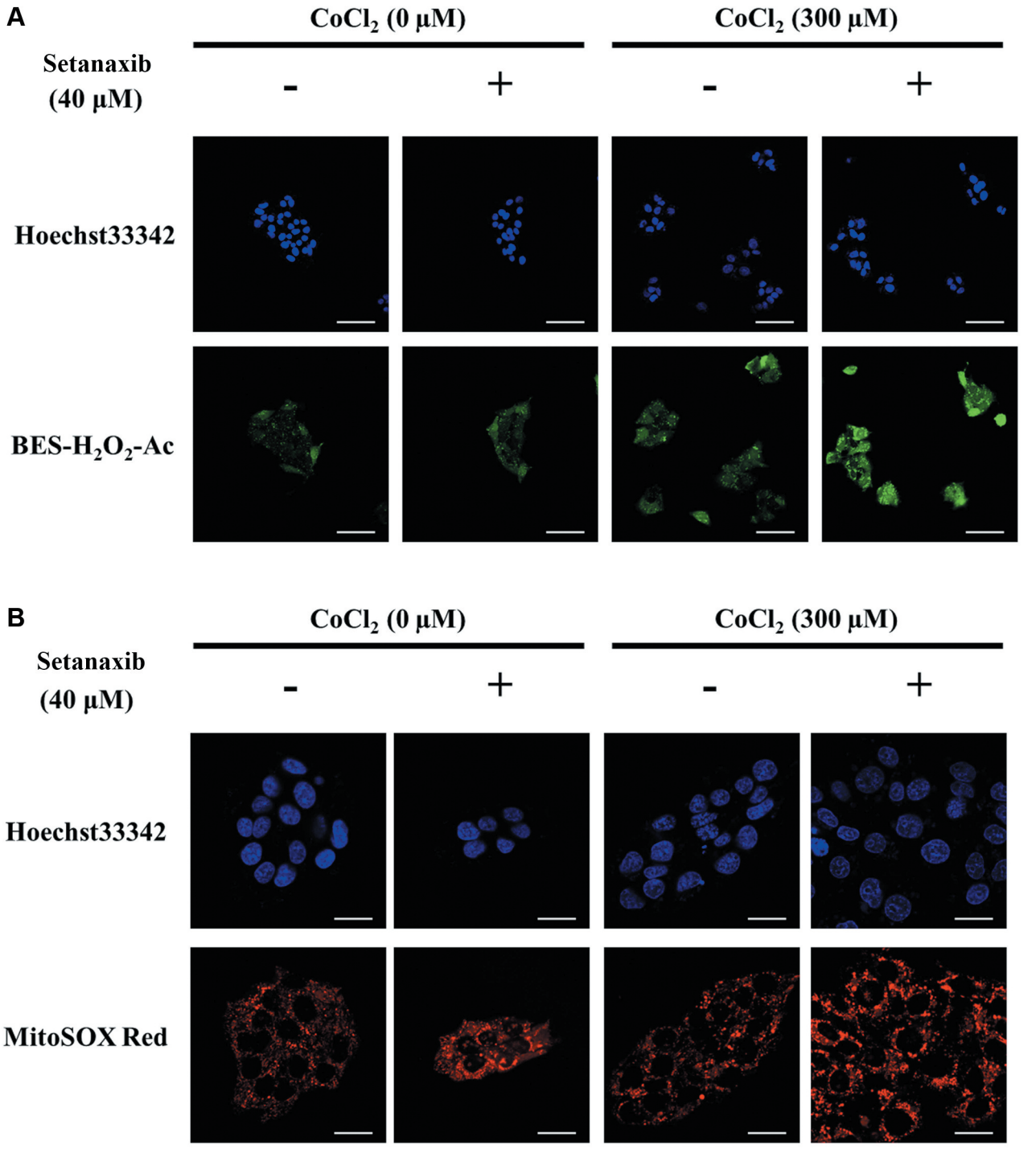

Figure 3. *Continued*

Confocal microscopy

MitoSOX red. Cell staining with Hoechst and MitoSox Red was detected by a confocal laser scanning microscope (LSM 880, Zeiss, Jena, Germany) using a 63×/1.2 W C-Apochromat water-immersion objective lens. Excitation of Hoechst and MitoSOX Red dyes was achieved by using 405- and 561-nm laser lines with the main beam splitters, MBS 405 and 488/633. The emission spectrum was measured in Lambda mode between 410 and 696 nm at 8.9-nm intervals to discriminate between specific fluorescence and autofluorescence.

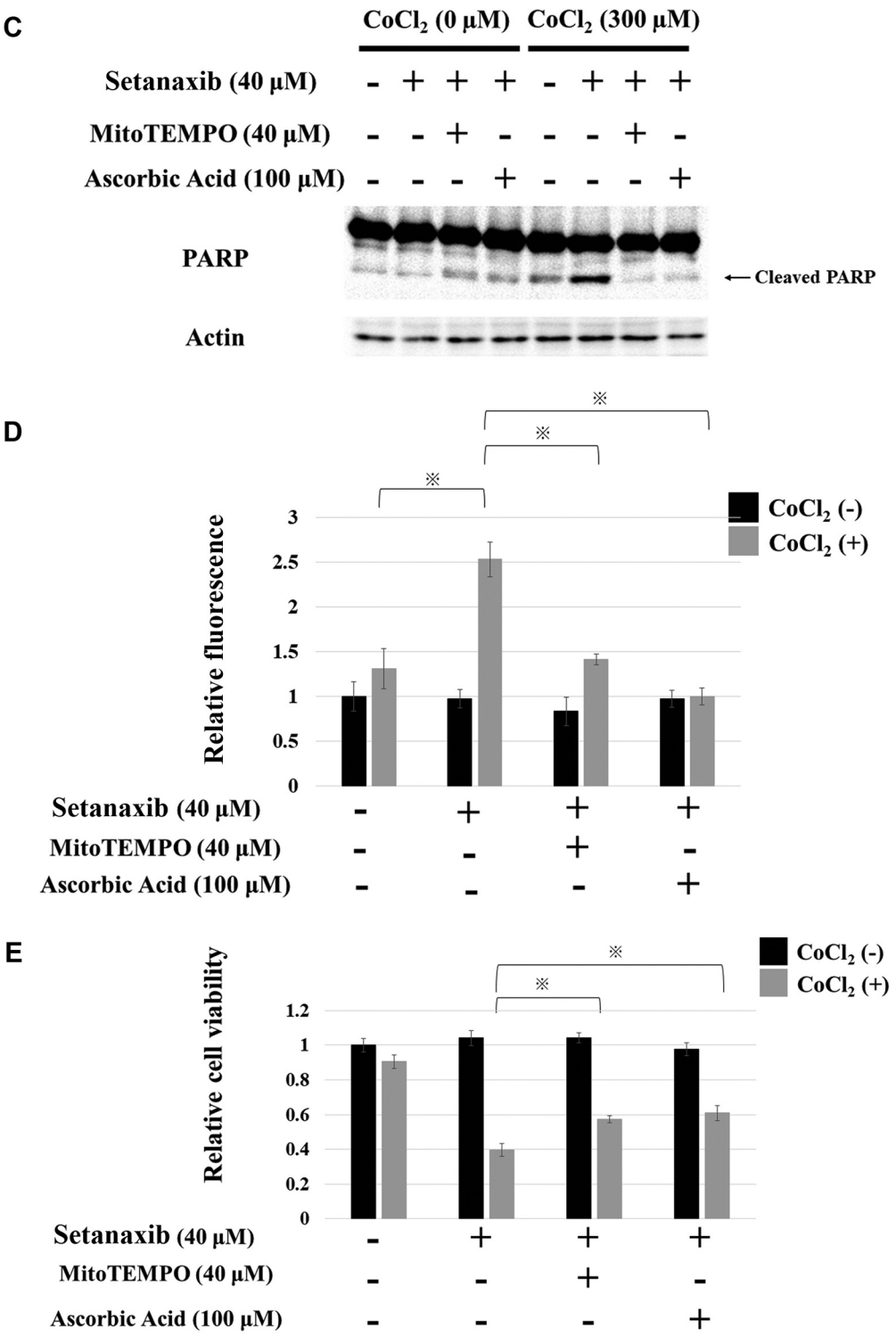

Figure 3. *Excessive production of mitochondrial ROS induced by setanaxib caused apoptosis. (A, B) HepG2 cells treated with setanaxib in the absence or presence of cobalt chloride (300 μM) were stained with Hoechst 33342 and BES-H_2O_2-Ac (5 μM) or with Hoechst 33342 and MitoSOX Red mitochondrial superoxide probes (10 μM). The insets indicate magnified images of the boxed areas. Bar, 20 μm. (B). HepG2 cells were treated with ascorbic acid (100 μM) or MitoTEMPO (40 μM) in the presence of setanaxib, with or without 300 μM cobalt chloride, for 48 h. (C) Immunoblot analysis of PARP and Actin. (D) MitoSOX Red fluorescence was assessed by using a plate reader. Fluorescence values were normalized to the cell viability in each treated sample, and calculated relative to the vehicle-treated control (*p<0.05). (E) CCK8 assay (*p<0.05).*

BES-H_2O_2-Ac. Images obtained after staining with Hoechst and BES-H_2O_2-Ac were examined by a LSM880 microscope with a 20×/0.8 objective (Plan-Apochromat) in Lambda scanning mode. The emission spectra of Hoechst and BES-H_2O_2-Ac was measured between 410 and 696 nm at 8.9-nm intervals, at the excitation wavelengths of 405 and 488 nm.

ROS plate reader assay. The cells were seeded at a concentration of 5×10^3 per well in 96-well plates (Sumitomo Bakelite, Tokyo, Japan), treated with DMEM, with or without 300 μM cobalt chloride, followed by treatment with serial dilutions of various agents for 48 h, and then 7.5 μM MitoSOX Red was added to the wells. MitoSOX florescence, reflecting cell activation, was measured after 1 h by using a fluorescence plate reader (excitation=510 nm, emission=580 nm).

Statistical analysis. Data were obtained from at least three independent experiments, and expressed as means±SD. The statistical significance of the differences between groups was determined by one-way analysis of variance (ANOVA), followed by Scheffe *post hoc* test or by two-way ANOVA followed by Scheffe post hoc test. *p*-Values of less than 0.05 were considered significant.

Results

Hypoxia-selective cytotoxicity of setanaxib. Cobalt chloride was used to mimic hypoxic conditions (21, 22). Treatment with cobalt chloride resulted in marked intracellular accumulation of HIF-1, a known marker of hypoxia, as well as in enhanced expression of NOX4 (Figure 1A). These results strongly suggested that NOX4 was inducible and NOX4 signalling activated under hypoxic conditions (23, 24).

The impact of setanaxib on cell viability was also investigated. Setanaxib did not affect cell viability under normal conditions but exhibited marked cytotoxicity under hypoxic conditions in HepG2 cells (Figure 1B). Furthermore, setanaxib showed hypoxia-specific cytotoxicity in HLE and Alexander cells (Figure 1C, D). These results strongly suggested that NOX4 was a regulator of liver cancer cell viability under hypoxic conditions, and that setanaxib exerted hypoxia-specific cytotoxicity in these cells.

Setanaxib cytotoxicity occurs via apoptosis. The role of NOX4 in cell viability is known to involve the regulation of apoptosis (25, 26). Therefore, we examined whether apoptosis was involved in the hypoxia-selective cytotoxicity of setanaxib. Under hypoxic conditions, setanaxib caused remarkable cleavage of the apoptosis marker PARP (Figure 2A). To determine the role of NOX4 under hypoxic conditions, we performed NOX4 knockdown in HepG2 cells (Figure 2B). Under hypoxic conditions, prominent PARP cleavage was observed in NOX4 knockdown cells (Figure 2C). In addition, setanaxib-induced cytotoxicity was largely prevented by cell treatment with the apoptosis inhibitor, Z-VAD-FMK (Figure 2D). These results demonstrated that NOX4 inhibited apoptosis under hypoxic conditions, and suggested that setanaxib induced cell death by blocking this pathway.

Setanaxib-induced apoptosis is caused by ROS. As reported by previous studies, cell apoptosis occurring under hypoxic conditions is due to excessive levels of mitochondrial ROS (27). Therefore, we investigated whether mitochondrial ROS were involved in setanaxib-induced apoptosis and cytotoxicity under hypoxic conditions. First, ROS intracellular levels were examined by using the BES-H_2O_2-Ac probe, a highly specific indicator of hydrogen peroxide (28); setanaxib treatment resulted in hypoxia-selective accumulation of ROS in HepG2 cells (Figure 3A). Moreover, cell staining with MitoSOX, a selective marker of mitochondrial ROS (29, 30), revealed that setanaxib treatment resulted in hypoxia-selective accumulation of mitochondrial ROS (Figure 3B).

Next, we examined how setanaxib-induced ROS production affected apoptosis. Treatment with ascorbic acid, an antioxidant (31), attenuated setanaxib-induced, hypoxia-selective PARP cleavage, and markedly reduced ROS levels. Moreover, MitoTEMPO (32), an antioxidant that selectively eliminates mitochondrial ROS, caused a marked attenuation of setanaxib-induced PARP cleavage and ROS accumulation (Figure 3C, D). The cytotoxicity of setanaxib was also markedly attenuated by treatment with MitoTEMPO (Figure 3E). These results strongly suggested that the hypoxia-selective cytotoxicity of setanaxib was caused by mitochondrial ROS-induced apoptosis.

Discussion

In the present study, we used liver cancer cell lines to explore the suitability of setanaxib as a hypoxia-specific anticancer drug, and to examine its mechanism of action in detail. We showed that setanaxib exerted hypoxia-selective cytotoxicity by promoting excessive ROS accumulation, which in turn caused apoptosis. Cobalt chloride was used to mimic hypoxic conditions. Additional studies employing other hypoxia-mimetic agents will be required to in-depth characterize the properties of setanaxib under hypoxic conditions.

We demonstrated that setanaxib exerted cytotoxicity only in hypoxic environments and that mitochondrial ROS were the cell death effectors. These results strongly suggested that NOX4-derived ROS signalling suppressed the mitochondrial ROS accumulation. We previously showed that autophagy, a mechanism of intracellular quality control, supports cell viability under hypoxic conditions (33). More specifically, the process involves mitophagy, the selective degradation of damaged mitochondria, which inhibits apoptosis and prevents excess mitochondrial ROS accumulation (27). NOX4-derived ROS signalling has been reported to activate autophagy (34). Therefore, hypoxia-selective setanaxib cytotoxicity may be due to the inhibition of NOX4-induced mitophagy, and to the subsequent accumulation of mitochondrial ROS. Notably, setanaxib did not affect cell viability under normoxic conditions. Conceivably, under these conditions, injured mitochondria are few and mitophagy is not required; as a

consequence, ROS do not accumulate and cytotoxicity is not triggered. Future studies are needed to examine the relationship between NOX4-derived ROS signalling and autophagy under hypoxic conditions.

The tumour microenvironment is characterized by nutrient deprivation in addition to hypoxia (35). Effective drugs targeting the tumour microenvironment must be directed against molecules that are essential for adaptation to hypoxic conditions and nutrient deprivation. As we have previously reported, NOX4 activates the PI3K/AKT signalling cascade, which is involved in cancer cell survival and proliferation under conditions of nutrient deprivation (36). Therefore, NOX4 is a candidate drug target not only under hypoxic conditions but also in nutrient-deprived tumour microenvironments. In addition, the expression of NOX4 is higher in many cancer tissues compared to normal tissues (37), and high NOX4 expression is reportedly associated with poor prognosis (38, 39). Moreover, NOX4 knockout does not affect the lifespan in mice (40). These findings suggest that NOX4-targeting therapies may be highly selective for cancer cells, possibly minimizing the risk of non-specific adverse effects.

The NADPH oxidase (NOX) family comprises a total of seven members in humans: five NOX (NOX1-5) and two DUOX (dual oxidase) isoforms (41). NOX4 inhibitors have low specificity and affect the activity of different NOX isoforms. Therefore, to date, the application of NOX4 inhibitors in clinical practice has been considered problematic (42, 43). In contrast, the recently developed setanaxib has extremely high specificity for NOX4. Moreover, a phase I clinical trial for setanaxib as a drug candidate for primary biliary cirrhosis has shown that the drug is well-tolerated and a phase II clinical trial has been undertaken (44).

In conclusion, setanaxib is a NOX4-specific novel potential anticancer agent. The effects of setanaxib against other tumours, in addition to liver cancer, will also have to be verified.

The present study is the first to demonstrate that setanaxib exerts remarkable hypoxia-selective cytotoxicity in liver cancer cell lines and that, therefore, is a promising candidate drug for liver cancer treatment.

Conflicts of Interest

The Authors declare that they have no conflicts of interest related to this study.

Authors' Contributions

Conception and design of the study, S.O., H.E.; Collection and assembly of data S.O., H.E., C.O., K.Y.; Analysis and interpretation of data S.O., H.E., C.O., K.Y., S.Y., M.T.; Drafting of the article S.O. All Authors reviewed and finally approved the manuscript.

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