BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 30(2), 184-190 (2022)

Extracellular Concentration of L-Cystine Determines the Sensitivity to System x_c^- Inhibitors

Md Abdullah and Seung Jin Lee*

College of Pharmacy, Chungnam National University, Daejeon 34134, Republic of Korea

Abstract

Targeting the cystine/glutamate exchange transporter, system x_c^-, is a promising anticancer strategy that induces ferroptosis, which is a distinct form of cell death mediated by iron-dependent lipid peroxidation. The concentration of L-cystine in culture medium is higher than the physiological level. This study was aimed to evaluate the effects of L-cystine concentration on the efficacy of ferroptosis inducers in hepatocellular carcinoma cells. This study showed that treatment with sulfasalazine or erastin, a system x_c^- inhibitor, decreased the viability of Huh6 and Huh7 cells in a dose-dependent manner, and the degree of growth inhibition was greater in medium containing a physiological L-cystine concentration of 83 μM than in commercial medium with a concentration of 200 μM L-cystine. However, RSL3, a glutathione peroxidase 4 inhibitor, decreased cell viability to a similar extent in media containing both L-cystine concentrations. Sulfasalazine and erastin significantly increased the percentages of propidium iodide-positive cells in media with 83 μM L-cystine, but not in media with 200 μM L-cystine. Sulfasalazine- or erastin-induced accumulation of lipid peroxidation as monitored by C11-BODIPY probe was higher in media with 83 μM L-cystine than in media with 200 μM L-cystine. In contrast, the changes in the percentages of propidium iodide-positive cells and lipid peroxidation by RSL3 were similar in both media. These results showed that sulfasalazine and erastin, but not RSL3, were efficacious under conditions of physiological L-cystine concentration, suggesting that medium conditions would be crucial for the design of a bioassay for system x_c^- inhibitors.

Key Words: L-Cystine, Sulfasalazine, Erastin, Ferroptosis, System x_c^-, Glutathione peroxidase 4

INTRODUCTION

Ferroptosis is a caspase-independent, non-apoptotic form of cell death that is mediated by iron-dependent lipid peroxidation (Dixon *et al*., 2012). Accumulation of lipid-structured free radicals leads to membrane permeabilization if the glutathione-dependent lipid peroxide repair system is not functioning properly (Hassannia *et al*., 2019). Glutathione peroxidase 4 (GPX4) activity is essential to prevent the accumulation of toxic lipid reactive oxygen species (ROS) by reduction of lipid peroxides to lipid alcohols with the use of glutathione (GSH) (Hassannia *et al*., 2019). GPX4 is unique compared to other GPX family members in that it can act as a phospholipid hydroperoxidase (Seiler *et al*., 2008). 1S, 3R-RSL3 (RAS synthetic lethal3) triggers ferroptosis by covalently binding to selenocysteine at the active site of GPX4 (Yang *et al*., 2014). Inhibitors of squalene synthase and HMG-CoA reductase suppress the generation of metabolites, such as lipid-soluble antioxidant coenzyme Q_{10} and Sec-tRNA, therefore depleting GPX4 and promoting ferroptosis (Hassannia *et al*., 2019).

GPX4 can be inactivated upon the depletion of intracellular GSH (Seiler *et al*., 2008). L-Cysteine, one of the components of GSH, can be either synthesized *de novo* through transsulfuration pathway or supplied by the reduction of L-cystine imported from the extracellular space via system x_c^- (Parkhitko *et al*., 2019; Zhu *et al*., 2019). Some populations of cancer cells show epigenetic silencing or defects in the transsulfuration pathway, which render them exclusively dependent on L-cystine uptake through system x_c^- (Dixon *et al*., 2012; Zhu *et al*., 2019). Therefore, system x_c^- has been suggested to be a potential therapeutic anticancer target for the induction of ferroptosis (Guo *et al*., 2011; Dixon *et al*., 2012; Hassannia *et al*., 2019). System x_c^- is composed of a disulfide-linked heterodimer of SLC7A11 (xCT) and SLC3A2 (4F2hc) (Kim *et al*., 2001). Erastin can bind SLC7A5 or the SLC7A5/SLC3A2 complex and interfere with L-cystine uptake of system x_c^- *in*

Open Access **https://doi.org/10.4062/biomolther.2021.105**

Received Jun 25, 2021 **Revised** Sep 10, 2021 **Accepted** Sep 16, 2021
Published Online Oct 19, 2021

***Corresponding Author**
E-mail: s.j.lee@cnu.ac.kr
Tel: +82-42-821-5940, Fax: +82-42-822-5343

trans (Yagoda *et al*., 2007; Dixon *et al*., 2012). Sulfasalazine, an FDA-approved immunosuppressant used to treat rheumatoid arthritis, ulcerative colitis, and Crohn's disease, can suppress system x_c^- and induce ferroptosis in experimental cancer models (Gout *et al*., 2001; Bridges *et al*., 2012). Sorafenib, a clinically approved multi-kinase inhibitor, also inhibits system x_c^- and triggers ferroptosis (Dixon *et al*., 2014).

Optimization of experimental conditions is essential for efficient *in vitro* drug evaluation. The level of cyst(e)ine in human plasma is about 79.4 ± 7.6 μM (Chawla *et al*., 1984), whereas the concentration of L-cystine in Roswell Park Memorial Institute (RPMI) medium or Dulbecco's modified essential medium (DMEM), commonly used media for cell culture, is 200 μM. However, it has not been determined whether the concentration of L-cystine in the medium can act as a determinant of the sensitivity for ferroptosis inducers. This study investigated the effects of two concentrations of L-cystine representing physiological condition and routine culture medium on ferroptosis induced by sulfasalazine, erastin, and RSL3. Ferroptosis was determined by cell viability, membrane permeabilization, and lipid peroxidation in Huh6, Huh7, and SK-HEP-1 hepatocellular carcinoma cell lines which were susceptible to ferroptotic cell death in our previous study (Kim *et al*., 2019; Yuk *et al*., 2021).

MATERIALS AND METHODS

Materials

Huh6, Huh7, and SK-HEP-1 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). DMEM with no glutamine, methionine, RPMI with no glutamine, methionine, and L-cystine, fetal bovine serum, antibiotics, and L-glutamine were obtained from Thermo Fisher Scientific (Waltham, MA, USA). L-Methionine, L-cystine, sulfasalazine, and propidium iodide (PI) were purchased from Merck (Darmstadt, Germany). Erastin and RSL3 were supplied by Selleckchem (Houston, TX, USA).

Cell culture and media

Huh6 and Huh7 cells were routinely maintained in DMEM (Thermo Fisher Scientific, cat. 11995-065) and SK-HEP-1 cells were maintained in RPMI-1640 (Thermo Fisher Scientific, cat. 31800022) supplied with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 incubator as described previously (Kim *et al*., 2019). The DMEM media for cell maintenance contains 200 μM L-cystine, 200 μM L-methionine and 3.97 mM L-glutamine, whereas RPMI-1640 media contains 200 μM L-cystine, 100 μM L-methionine and 2 mM L-glutamine.

The media with 83 μM or 200 μM L-cystine was prepared by adding L-cystine to the DMEM or RPMI-1640 with no glutamine, no methionine, no cystine (Thermo Fisher Scientific, cat. 21013-024; Merck, cat. R7513). The concentration of L-methionine and L-glutamine was adjusted to the concentration for maintenance media. Cells were regularly checked for the absence of mycoplasma contamination.

Cell viability

Cells were seeded in fresh maintenance medium in 96-well plates at a density of 2,000 cells per well (Kim *et al*., 2020). At 24 h after seeding, media was replaced by the media containing 200 μM or 83 μM L-cystine with each indicated drug. Cells were exposed to sulfasalazine for 72 h (Huh6 cells) or for 24 h (Huh7 cells). Erastin or RSL3 were treated for 72 h in these cells. Paired control cultures were treated with the vehicle dimethyl sulfoxide (DMSO) alone. Cell viability was measured using the CellTiter Glo® assay in accordance with the manufacturer's instructions (Promega, Madison, WI, USA) using an EnVision® multimode plate reader (Perkin Elmer, Waltham, MA, USA). Independent experiments were performed in triplicate. Cell viability was expressed as a percentage of the viability for drug-treated group at each concentration in compared to the viability for vehicle-treated group. GI_{50} was defined as the dose that decreased cell viability by 50%.

PI staining

After treatment as indicated, the cells were washed with phosphate-buffered saline (PBS) and incubated with 1 μg/mL PI for 30 min in the dark (Kim *et al*., 2020). Bright field and fluorescence images with excitation/emission at 535/617 nm were acquired using the EVOS™ FL Auto Imaging System (Thermo Fisher Scientific). Three random images of each treatment group from independent experiments were captured to calculate the percentage of PI-positive cells.

Detection of lipid peroxidation

Lipid peroxide was detected using the Image-iT™ Lipid Peroxidation Kit, which is based on the lipophilic BODIPY®581⁄591 C11 probe (Thermo Fisher Scientific) (Kim *et al*., 2020). After treatment as indicated, 1 μM probe was added and incubated for a further 30 min at 37°C. Cells were collected by trypsinization and the fluorescence with excitation/emission at 488/525 nm was measured from 5,000 cells using a Guava® easyCyte flow cytometer and analyzed with InCyte2.6 software (Luminex, TX, USA). Mean fluorescence intensity of C11-BODIPY were calculated from independent experiments.

Statistical analyses

Data are expressed as the mean ± standard deviation (SD), with n indicating the number of independent *in vitro* experiments for a particular set of experiments. Statistical analyses were performed using one-way analysis of variance, followed by Tukey's multiple-comparison test. All statistical analyses were performed using SPSS statistical software for Windows (version 26; SPSS Inc., Chicago, IL, USA). In all analyses, $p<0.05$ was taken to indicate statistical significance.

RESULTS

Effects of extracellular L-cystine concentration on the changes in cell viability induced by system x_c^- inhibitors

We compared the effects of two concentrations of extracellular L-cystine: 200 μM for commercial medium condition and 83 μM for physiological condition (Gout *et al*., 2001). Sulfasalazine decreased Huh6 cell viability with the GI_{50} values of 345 μM in medium with 83 μM L-cystine, whereas fail to do in medium with 200 μM L-cystine (Fig. 1A). Sulfasalazine-induced growth inhibition was greater in medium with 83 μM L-cystine than 200 μM L-cystine in Huh7 cells, with the GI_{50} values of 209 μM and 371 μM, respectively. The efficacy of erastin to decrease cell viability was also higher in medium containing 83 μM L-cystine than in medium containing 200 μM

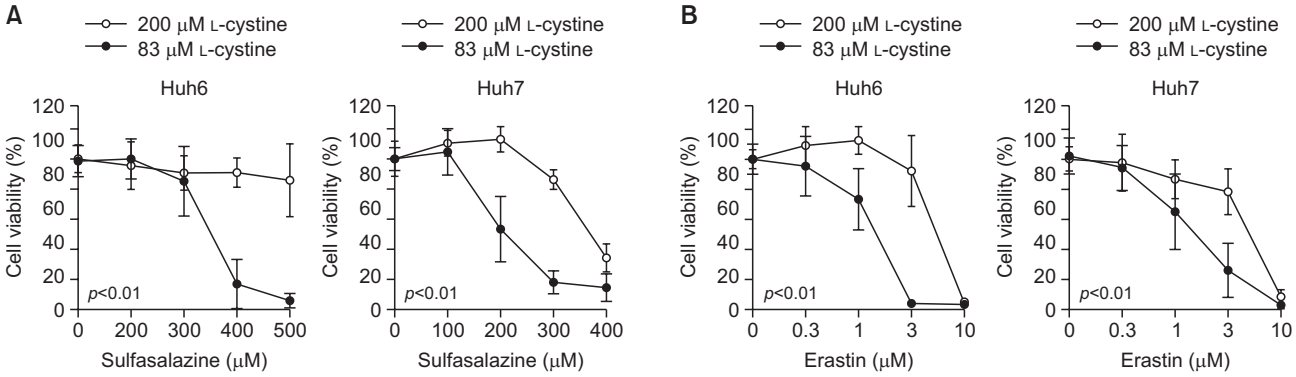

Fig. 1. Physiological level of L-cystine in medium potentiated growth inhibition by sulfasalazine and erastin in Huh6 and Huh7 cells. (A) Huh6 cells were treated in medium containing L-cystine at a concentration of 200 μM (white circles) or 83 μM (black circles) with sulfasalazine for 72 h (n=3 for 200 μM and n=8 for 83 μM). Huh7 cells were exposed to media of 200 μM or 83 μM L-cystine with sulfasalazine for 24 h (n=3 for 200 μM and n=6 for 83 μM). (B) Erastin was treated in Huh6 and Huh7 cells in medium containing L-cystine of 200 μM (n=5) or 83 μM (n=8) for 72 h. Viability was measured using CellTiter-Glo® assay. All data are expressed as the mean ± SD; p<0.01, 200 μM versus 83 μM L-cystine in each media.

L-cystine (Fig. 1B). Erastin at the dose of 3 μM induced 10 ± 12% and 26 ± 18% of cell viability under medium containing 83 μM L-cystine in Huh6 and Huh7 cells, respectively, whereas 85 ± 23% and 78 ± 15% of cell viability under medium containing 200 μM L-cystine.

As cell viability measured by CellTiter Glo® assay is based on intracellular ATP level indicative for metabolic activity, cell death was confirmed with PI staining which reflects the increase of membrane permeability, a characteristics of ferroptotic cell death. Treatment of Huh6 cells with 400 μM sulfasalazine or 10 μM erastin for 72 h significantly increased the PI-positive cell fraction in medium containing 83 μM L-cystine by 74.1 ± 20.3% and 93.8 ± 5.8%. However, the increase was minimal in medium containing 200 μM L-cystine (Fig. 2A). Similarly, the PI-positive cell fraction following treatment with 300 μM sulfasalazine for 24 h or with 10 μM erastin for 72 h in Huh7 cells was significantly greater in media containing 83 μM L-cystine than 200 μM L-cystine (Fig. 2B). The effect of extracellular L-cystine on the sensitivity to sulfasalazine and erastin was also tested in SK-HEP-1 cells which are sensitive to ferroptosis (Yuk *et al*., 2021). SK-HEP-1 cells showed significantly higher percentage of PI-positive cell fraction in media containing 83 μM L-cystine than 200 μM L-cystine.

Effects of extracellular L-cystine concentration on RSL3-induced changes in cell viability

RSL3 decreased Huh6 and Huh7 cell viability in a dose-dependent manner under both conditions (Fig. 3). RSL3-induced growth inhibition showed no significant differences according to the extracellular L-cystine concentration in either cell line. Membrane permeability as shown by PI staining was increased by treatment of 0.3 μM RSL3 for 24 hours both in media containing in Huh6 and Huh7 cells (Fig. 4). SK-HEP-1 cells showed no significant difference in the percentage of PI-positive cell fraction in media containing 83 μM L-cystine and in media containing 200 μM L-cystine.

Changes in lipid peroxidation by ferroptosis inducers in low-L-cystine medium

As the extracellular concentration of L-cystine determines the efficacy of sulfasalazine and erastin, but not RSL3, next, we examined whether sulfasalazine- or erastin-induced lipid peroxidation was dependent on L-cystine concentration in the medium. Treatment in the presence of 83 μM L-cystine significantly increased lipid peroxidation by 2.0-fold in Huh6 cells and by 3.9-fold in Huh7 cells, whereas treatment with sulfasalazine in the presence of 200 μM L-cystine did not change the level of lipid peroxidation as determined by C11-BODIPY probe (Fig. 5A). Erastin induced lipid peroxidation by 1.5-fold and 2.6-fold in media with 200 μM and 83 μM L-cystine, respectively, in Huh6 cells and by 1.0-fold and 1.7-fold, respectively, in Huh7 cells (Fig. 5B). The level of lipid peroxidation was significantly different between 83 μM and 200 μM L-cystine upon the exposure of sulfasalazine or erastin. However, lipid peroxidation by the treatment of 0.3 μM RSL3 was increased to the similar levels in both media containing 83 μM and 200 μM L-cystine (Fig. 5C).

DISCUSSION

We previously demonstrated that sulfasalazine and erastin increase ferroptotic cell death in Huh6 and Huh7 cells, which is prevented by pretreatment with ferrostatin-1 (Kim *et al*., 2019). In this study, we demonstrated that extracellular L-cystine determines the efficacy of system x_c^- inhibitors for ferroptotic cell death in these cell models. The degree of growth inhibition by sulfasalazine or erastin was greater in medium containing a physiological level of 83 μM L-cystine than in medium containing 200 μM L-cystine. The increase in membrane permeability and lipid peroxidation were greater in low-L-cystine media than in standard media. The GI_{50} of sulfasalazine in physiological L-cystine concentration in Huh7 cells (209 μM) is likely to be clinically achievable, as the therapeutic level of sulfasalazine in ulcerative colitis is 80-200 μM (Gout *et al*., 2001). However, the inhibitory effects of RSL3, a GPX4 inhibitor, on cell viability were not dependent on the L-cystine concentration in the medium.

L-Cystine is the predominant form of cyst(e)ine in plasma and extracellular body fluids (Parkhitko *et al*., 2019; Zhu *et al*., 2019). L-Cysteine is the one of the least abundant amino acid in the cell and supplied by the reduction of L-cystine which

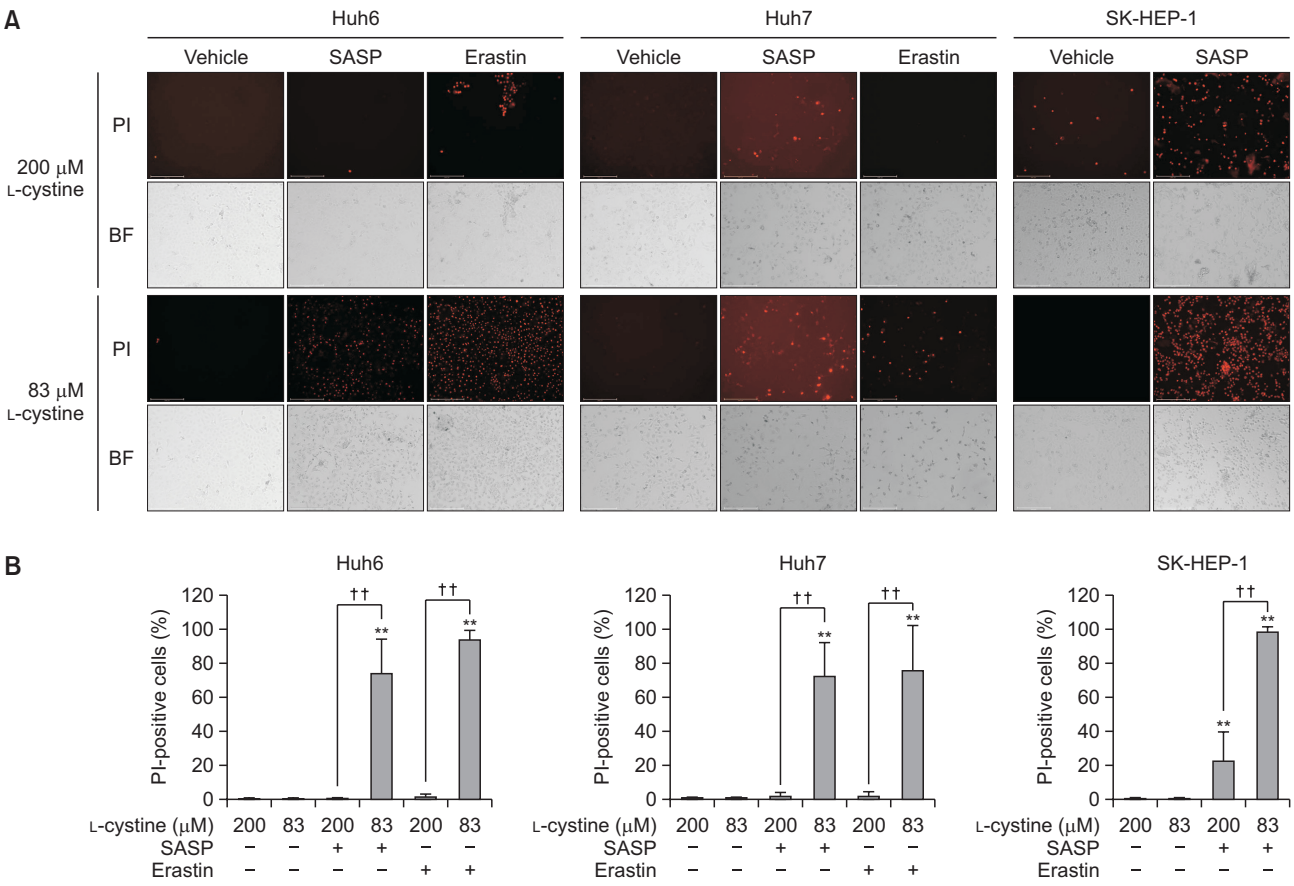

Fig. 2. Sulfasalazine- or erastin-induced membrane permeabilization was greater in medium containing a physiological level of L-cystine than normal medium. Sulfasalazine (SASP) in media containing 200 μM or 83 μM L-cystine was exposed at the dose of 400 μM for 72 h for Huh6 cells, at the dose of 300 μM for 24 h for Huh7 cells (n=3 for 200 μM and n=6 for 83 μM) or at the dose of 300 μM for 14 h for SK-HEP-1 cells (n=3). Erastin was treated in Huh6 and Huh7 cells in medium containing 200 μM or 83 μM L-cystine for 72 h (n=3). Cells were stained with 2 μg/mL propidium iodide (PI) for 20 min. Three bright field (BF) and fluorescent field images of each group were randomly captured to quantify the percentage of PI-positive cells with the EVOS® cell imaging system. All data are expressed as the mean ± SD. N.S. not significant; **p<0.01, vehicle versus sulfasalazine or erastin in each media; $^{\dagger\dagger}p$<0.01, 200 μM versus 83 μM L-cystine-containing media under sulfasalazine or erastin treatment.

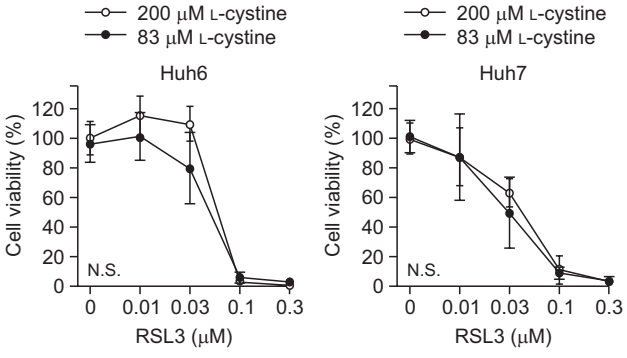

Fig. 3. Changes in cell viability by RSL3 in media containing 200 μM or 83 μM L-cystine in Huh6 and Huh7 cells. Huh6 and Huh7 cells were treated with RSL3 for 72 h in medium containing L-cystine at a concentration of 200 μM (white circles) (n=3) or 83 μM (black circles) (n=7 for Huh6; n=6 for Huh7). Viability was measured using CellTiter-Glo® assay. All data are expressed as the mean ± SD. N.S. not significant.

is transported by system x_c^- (Poltorack and Dixon, 2021). Extracellular L-cystine may be kept in equilibrium with the level of blood. The concentrations of L-cystine and L-cysteine in plasma from fasting healthy normal subjects are 79.4 ± 7.6 μM and 11.2 ± 0.9 μM, respectively (Chawla *et al*., 1984). So, the efficacy of system x_c^- inhibitor may be higher *in vivo* than *in vitro* condition. In addition to cancer, ferroptotic cell death is implicated in the pathology of neurodegenerative disease, inflammatory disease, and liver injury (Chawla *et al*., 1984; Bridges *et al*., 2012). The concentrations of L-cytine in serum are 49.1 μM in Parkinson's disease, 21.5 μM in sepsis, and 88.6 μM in cirrhosis, which were not different from corresponding control groups (Chawla *et al*., 1984; Su *et al*., 2015; Picca *et al*., 2019). As the concentrations of L-cystine in various pathophysiological conditions are micromolar ranges which is similar to or lower than the concentration of 83 μM used in our experiment, *in vitro* culture condition using commercial media could hide the vulnerability to ferroptotic cell death in pathophysiological conditions.

Cellular preference for L-cystine or L-cysteine is heterogeneous depending on the types of cyst(e)ine transporters on

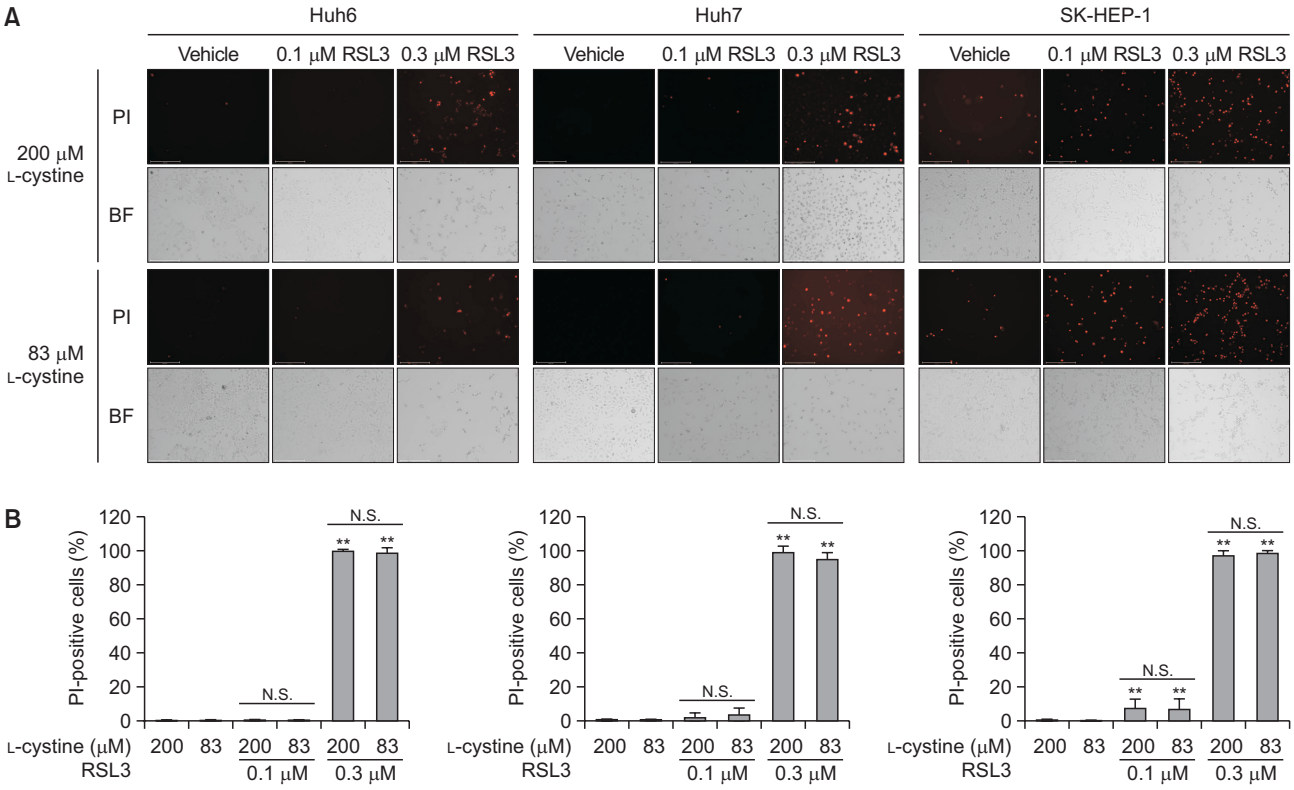

Fig. 4. RSL3-induced membrane permeabilization in medium containing a physiological level of L-cystine than normal medium. (A) RSL3 in media containing 200 μM or 83 μM L-cystine was exposed at the dose of 0.1 μM or 0.3 μM for 24 h in Huh6 and Huh7 cells or for 14 h in SK-HEP-1 cells (n=3). Cells were stained with 1 μg/mL propidium iodide (PI) for 20 min. (B) Three bright field (BF) and fluorescent field images of each group were randomly captured to quantify the percentage of PI-positive cells with the EVOS® cell imaging system. All data are expressed as the mean ± SD. ***p*<0.01, vehicle versus RSL3 in each media.

cell membrane. Extracellular L-cystine is transported via system x_c^-, whereas L-cysteine is transported into cells via sodium-dependent alanine-serine-cysteine transporter (ASCT) 1/2 and neutral amino acid transporter SNAT1/2/4 (Bridges *et al*., 2012). As L-cystine in culture medium is the only source of extracellular cyst(e)ine under cell culture conditions, murine B cells require β-mercaptoethanol or other sulfhydryl-containing compounds during *in vitro* culture to reduce L-cystine to L-cysteine, which eventually enter cells via neutral amino acid transporters (Broome and Jeng, 1973; Ishii *et al*., 1981). These cells can survive in co-culture with irradiated fibroblasts that take up and reduce L-cystine, and then provide L-cysteine to B cells (Falk *et al*., 1998). Mature neurons also exhibit limited L-cystine uptake capacity, but astrocytes can take up L-cystine via system x_c^- and subsequently release either L-cysteine or glutathione for neurons (Dringen *et al*., 1999; Wang and Cynader, 2000).

Huh6 and Huh7 cells are likely to have the capacity to take up both extracellular L-cystine and L-cysteine, and primarily dependent on L-cystine in culture condition, because inhibition of system x_c^- was efficacious to decrease cell viability and blunted by β-mercaptoethanol (Kim *et al*., 2019). This is supported by the observation that knockout of ASCT2 and L-type amino acid transporter 1 (LAT1) failed to substantially affect proliferation of Huh7 cells (Bothwell *et al*., 2018). To evaluate the effect of extracellular L-cysteine on ferroptosis, Huh6 and Huh7 cells are adequate as a ferroptosis-dependent model, because the growth retardation by sulfasalazine and erastin was significantly rescued by ferrostatin-1 (Kim *et al*., 2019). In contrast, the growth delay was not prevented by ferrostatin-1 in PLC/PRF/5 cells, indicating that this cell line may involve non-ferroptotic cell death by sulfasalazine and erastin.

In this study, both sulfasalazine and erastin were more efficacious for growth inhibition in low-L-cystine medium and the effects of sulfasalazine and erastin were reversed by L-cystine in media. Their mode of action for system x_c^- inhibition could be different each other. Sulfasalazine is extensively metabolized into sulfapyridine and 5-aminosalicylic acid in the intestine by azoreductase, but the inhibitory effects of system x_c^- are achieved by sulfasalazine, and not by these two metabolites (Gout *et al*., 2001; Bridges *et al*., 2012). Erastin inhibits system x_c^- via an indirect mechanism (Dixon *et al*., 2012). Analyses by affinity purification, metabolite profiling, and substrate antagonism showed that erastin binds to SLC7A5 (LAT1), and therefore interferes with system L (SLC7A5/SLC3A2) directly and system x_c^- (SLC7A11/SLC3A2) indirectly (Dixon *et al*., 2012). Erastin-induced ferroptosis is caused by the latter, and not by the former (Dixon *et al*., 2012). This study demonstrated extracellular L-cystine concentration is a determinant for the efficacy of system x_c^- inhibitor, but not for GPX4 inhibitor as the supply of cyst(e)ine for glutathione synthesis could not reverse the inhibitory effects of RSL3 on GPX4.

To overcome the weak binding potency and metabolic instability, there is increasing research interest in structure-ac-

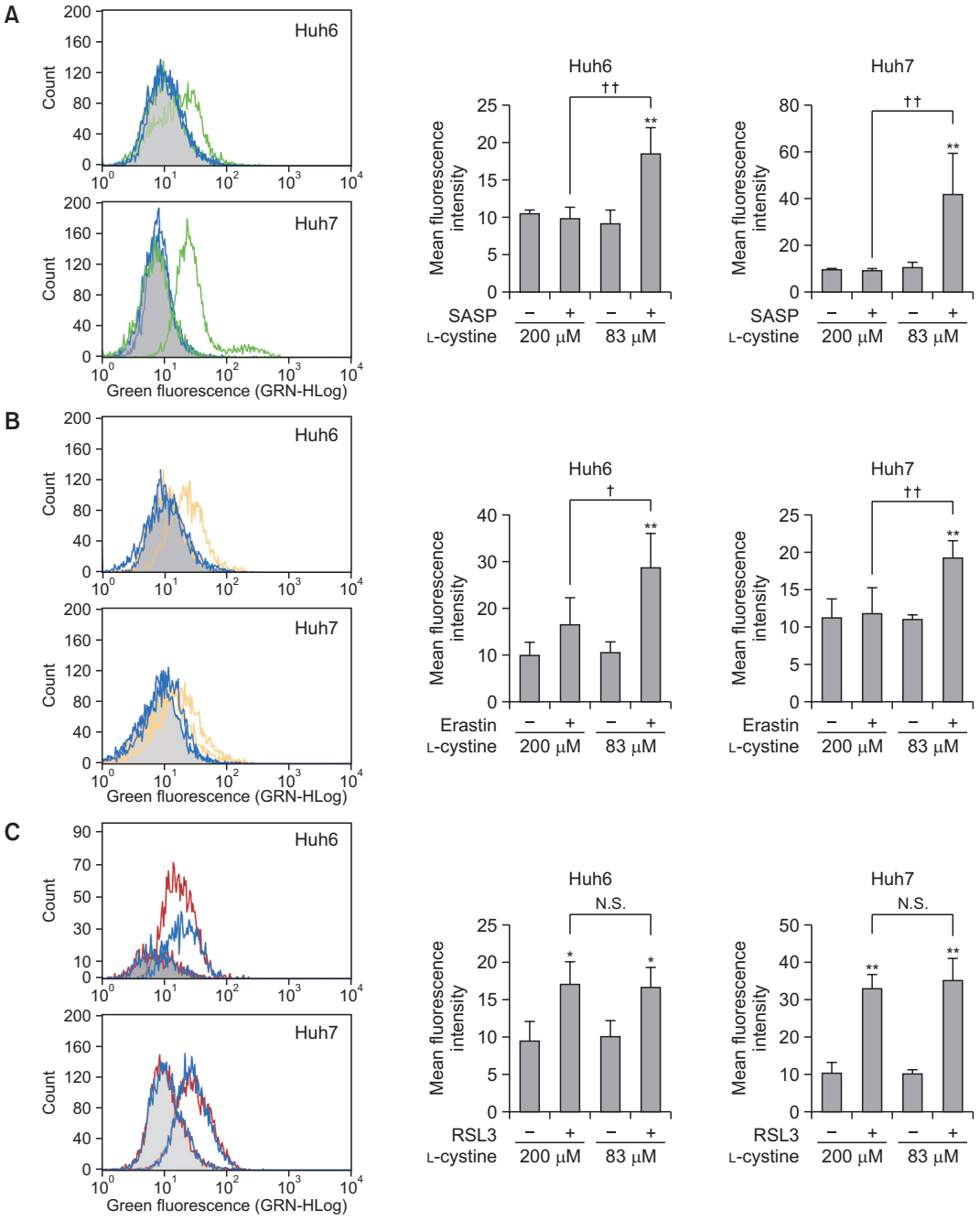

Fig. 5. Sulfasalazine- and erastin-induced lipid peroxidation were greater in medium containing a physiological level of L-cystine than normal medium. (A) Sulfasalazine (SASP) (green) or vehicle (blue) treatment was performed in cultures of Huh6 cells at a concentration of 400 μM for 48 h and Huh7 cells at a concentration of 300 μM for 24 h in media containing L-cystine at 200 μM (green fill) (n=3) or 83 μM (white fill) (n=6). (B) Erastin (yellow) or (C) RSL3 (red) was treated at a concentration of 10 μM and 0.3 μM, respectively, for 24 h in these cells (n=4 for vehicle, n=3 for erastin or RSL3). Cells were stained with C11-BODIPY® probe for 30 min and monitored with a Guava® easyCyte flow cytometer. Mean fluorescence intensities are expressed as the mean ± SD with representative images. N.S. not significant; *p<0.05, **p<0.01, vehicle versus sulfasalazine, erastin, or RSL3 in each media; $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, 200 μM versus 83 μM L-cystine-containing media under sulfasalazine, erastin, or RSL3 treatment.

tivity relationship analyses of sulfasalazine analogs and the development of metabolically stable analogues (Shukla *et al*., 2011; Larraufie *et al*., 2015; Zhang *et al*., 2019). High-throughput screening is currently underway to identify novel and potent system x_c^- inhibitors structurally distinct from sulfasalazine and erastin (Hassannia *et al*., 2019; Zhang *et al*., 2019). The traditional bioassays for system x_c^- inhibitors are cell-based [^{14}C]cystine uptake assay and growth inhibition assay. Routine culture media differ in their amino acid composition from body fluid and the tumor microenvironment, and the effects of com-

petitive inhibitors, such as sulfasalazine, could be obscured by the artificial concentrations of substrates or by large variation caused by inconsistent culture conditions. Our results suggest that the composition of culture medium in bioassays for system x_c^- inhibitor is important for the screening of large chemical libraries covering structurally diverse compounds.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

This study was financially supported by a research fund of Chungnam National University.

REFERENCES

Bridges, R. J., Natale, N. R. and Patel, S. A. (2012) System xc- cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. *Br. J. Pharmacol.* **165**, 20-34.

Broome, J. D. and Jeng, M. W. (1973) Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro*. Effects on mouse lymphoma cells in comparison with splenic lymphocytes. *J. Exp. Med.* **138**, 574-592.

Bothwell, P. J., Kron, C. D., Wittke, E. F., Czerniak, B. N. and Bode, B. P. (2018) Targeted suppression and knockout of ASCT2 or LAT1 in epithelial and mesenchymal human liver cancer cells fail to inhibit growth. *Int. J. Mol. Sci.* **19**, 2093.

Chawla, R. K., Lewis, F. W., Kutner, M. H., Bate, D. M., Roy, R. G. and Rudman, D. (1984) Plasma cysteine, cystine, and glutathione in cirrhosis. *Gastroenterology* **87**, 770-776.

Dixon, S. J., Lemberg, K. M., Lamprecht, M. R., Skouta, R., Zaitsev, E. M., Gleason, C. E., Patel, D. N., Bauer, A. J., Cantley, A. M., Yang, W. S., Morrison, B., 3rd and Stockwell, B. R. (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149**, 1060-1072.

Dixon, S. J., Patel, D. N., Welsch, M., Skouta, R., Lee, E. D., Hayano, M., Thomas, A. G., Gleason, C. E., Tatonetti, N. P., Slusher, B. S. and Stockwell, B. R. (2014) Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis. *Elife* **3**, e02523.

Dringen, R., Pfeiffer, B. and Hamprecht, B. (1999) Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. *J. Neurosci.* **19**, 562-569.

Falk, M. H., Meier, T., Issels, R. D., Brielmeier, M., Scheffer, B. and Bornkamm, G. W. (1998) Apoptosis in Burkitt lymphoma cells is prevented by promotion of cysteine uptake. *Int. J. Cancer* **75**, 620-625.

Guo, W., Zhao, Y., Zhang, Z., Tan, N., Zhao, F., Ge, C., Liang, L., Jia, D., Chen, T., Yao, M., Li, J. and He, X. (2011) Disruption of xCT inhibits cell growth via the ROS/autophagy pathway in hepatocellular carcinoma. *Cancer Lett.* **312**, 55-61.

Gout, P. W., Buckley, A. R., Simms, C. R. and Bruchovsky, N. (2001) Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the xc − cystine transporter: a new action for an old drug. *Leukemia* **15**, 1633-1640.

Hassannia, B., Vandenabeele, P. and Vanden, B. T. (2019) Targeting ferroptosis to iron out cancer. *Cancer Cell* **35**, 830-849.

Ishii, T., Bannai, S. and Sugita, Y. (1981) Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol *in vitro*. Role of the mixed disulfide of 2-mercaptoethanol and cysteine. *J. Biol. Chem.* **256**, 12387-12392.

Kim, J. Y., Kanai, Y., Chairoungdua, A., Cha, S. H., Matsuo, H., Kim, D. K., Inatomi, J., Sawa, H., Ida, Y. and Endou, H. (2001) Human cystine/glutamate transporter: cDNA cloning and upregulation by oxidative stress in glioma cells. *Biochim. Biophys. Acta* **1512**, 335-344.

Kim, D. H., Abdullah, M. and Lee, S. J. (2019) Mechanisms of growth inhibition by sulfasalazine and erastin in hepatocellular carcinoma cell lines. *Yakhak Hoeji* **63**, 152-159.

Kim, D. H., Kim, W. D., Kim, S. K., Moon, D. H. and Lee, S. J. (2020) TGF-β1-mediated repression of SLC7A11 drives vulnerability to GPX4 inhibition in hepatocellular carcinoma cells. *Cell Death Dis.* **11**, 406.

Larraufie, M. H., Yang, W. S., Jiang, E., Thomas, A. G., Slusher, B. S. and Stockwell, B. R. (2015) Incorporation of metabolically stable ketones into a small molecule probe to increase potency and water solubility. *Bioorg. Med. Chem. Lett.* **25**, 4787-4792.

Parkhitko, A. A., Jouandin, P., Mohr, S. E. and Perrimon, N. (2019) Methionine metabolism and methyltransferases in the regulation of aging and lifespan extension across species. *Aging Cell* **18**, e13034.

Picca, A., Calvani, R., Landi, G., Marini, F., Biancolillo, A., Gervasoni, J., Persichilli, S., Primiano, A., Urbani, A., Bossola, M., Bentivoglio, A. R., Cesari, M., Landi, F., Bernabei, R., Marzetti, E. and Lo Monaco, M. R. (2019) Circulating amino acid signature in older people with Parkinson's disease: a metabolic complement to the EXosomes in PArkiNson Disease (EXPAND) study. *Exp. Gerontol.* **128**, 110766.

Poltorack, C. D. and Dixon, S. J. (2021) Understanding the role of cysteine in ferroptosis: progress & paradoxes. *FEBS J.* doi: 10.1111/febs.15842 [Online ahead of print].

Seiler, A., Schneider, M., Förster, H., Roth, S., Wirth, K. E., Culmsee, C., Plesnila, N., Kremmer, E., Rådmark, O., Wurst, W., Bornkamm, G. W., Schweizer, U. and Conrad, M. (2008) Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab.* **8**, 237-248.

Shukla, K., Thomas, A. G., Ferraris, D. V., Hin, N., Sattler, R., Alt, J., Rojas, C., Slusher, B. S. and Tsukamoto, T. (2011) Inhibition of xc^- transporter-mediated cystine uptake by sulfasalazine analogs. *Bioorg. Med. Chem. Lett.* **21**, 6184-6187.

Su, L., Li, H., Xie, A., Liu, D., Rao, W., Lan, L., Li, X., Li, F., Xiao, K., Wang, H., Yan, P., Li, X. and Xie, L. (2015) Dynamic changes in amino acid concentration profiles in patients with sepsis. *PLoS ONE* **10**, e0121933.

Wang, X. F. and Cynader, M. S. (2000) Astrocytes provide cysteine to neurons by releasing glutathione. *J. Neurochem.* **74**, 1434-1442.

Yang, W. S., SriRamaratnam, R., Welsch, M. E., Shimada, K., Skouta, R., Viswanathan, V. S., Cheah, J. H., Clemons, P. A., Shamji, A. F., Clish, C. B., Brown, L. M., Girotti, A. W., Cornish, V. W., Schreiber, S. L. and Stockwell, B. R. (2014) Regulation of ferroptotic cancer cell death by GPX4. *Cell* **156**, 317-331.

Yagoda, N., Rechenberg, M. V., Zaganjor, E., Bauer, A. J., Yang, W. S., Fridman, D. J., Wolpaw, A. J., Smukste, I., Peltier, J. M., Boniface, J. J., Smith, R., Lessnick, S. L., Sahasrabudhe, S. and Stockwell, B. R. (2007) RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature* **447**, 864-868.

Yuk, H., Abdullah, M., Kim, D. H., Lee, H. and Lee, S. J. (2021) Necrostatin-1 prevents ferroptosis in a RIPK1- and IDO-independent manner in hepatocellular carcinoma. *Antioxidants* **10**, 1347.

Zhu, J., Berisa, M., Schwörer, S., Qin, W., Cross, J. R. and Thompson, C. B. (2019) Transsulfuration activity can support cell growth upon extracellular cysteine limitation. *Cell Metab.* **30**, 865-876.

Zhang, Y., Tan, H., Daniels, J. D., Zandkarimi, F., Liu, H., Brown, L. M., Uchida, K., O'Connor, O. A. and Stockwell, B. R. (2019) Imidazole ketone erastin induces ferroptosis and slows tumor growth in a mouse lymphoma model. *Cell Chem. Biol.* **26**, 623-633.e9.