Combination of lactate calcium salt with 5-indanesulfonamide and α-cyano-4-hydroxycinnamic acid to enhance the antitumor effect on HCT116 cells via intracellular acidification

KEUN-YEONG JEONG*, POONAM MANDER*, JAE JUN SIM and HWAN MOOK KIM

College of Pharmacy, Gachon Institute of Pharmaceutical Science, Gachon University, Incheon 406-840, Republic of Korea

Received January 13, 2015; Accepted December 3, 2015

DOI: 10.3892/ol.2016.4137

Abstract. Maintenance of a neutral intracellular pH (pH*i*) is favorable for the survival of tumors, and maintenance of highly acidic extracellular pH (pH*e*) facilitates tumor invasiveness. The aim of the present study was to investigate the antitumor effects of lactate calcium salt (CaLa), 5-indanesulfonamide (IS) and α-cyano-4-hydroxycinnamic acid (CA) via pH regulation in colon cancer cells. HCT116 cells were treated with CaLa, IS, CA and combinations of the three. Subsequently, the concentration of intracellular lactate was determined. pH*i* and pH*e* were measured using cell lysates and culture media. Colony formation assay, cell viability assay and western blot analysis were additionally performed to analyze the consequences of the pH changes. CaLa, IS, CA and combination treatments induced an increase in the concentration of intracellular lactate. Lactate influx into the tumor microenvironment produced an acidic pH*i* in colon cancer cells. Consequently, colony formation and cell viability were significantly decreased, as well as poly(adenosine diphosphate-ribose) polymerase degradation. The tumor microenvironment may be exploited therapeutically by disrupting the mechanism that regulates pH*i*, leading to cell apoptosis. The present study indicated that treatment with CaLa, IS and CA induced intracellular acidification via lactate influx, causing apoptosis of colon cancer cells. Additionally, the findings suggested that the combination of CaLa with IS and CA may enhance antitumor activity, and may provide a potential therapeutic approach for the treatment of colon cancer.

Correspondence to: Professor Hwan Mook Kim, College of Pharmacy, Gachon Institute of Pharmaceutical Science, Gachon University, 191 Hambangmoe-ro, Incheon 406-840, Republic of Korea
E-mail: hwanmook@gachon.ac.kr

*Contributed equally

Abbreviations: CRC, colorectal cancer, pH*i*, intracellular pH; pH*e*, extracellular pH; CA9, carbonic anhydrase 9; MCT, monocarboxylate transporter; CaLa, lactate calcium salt; CA, α-cyano-4-hydroxycinnamic acid; IS, 5-indanesulfonamide; PARP, poly(adenosine diphosphate ribose) polymerase; iLactate, intracellular lactate; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide

Key words: colon cancer, lactate calcium salt, carbonic anhydrase 9, monocarboxylate transporter 4, cellular pH, antitumor effect

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-associated mortality worldwide. According to mortality data from the National Center for Health Statistics (Hyattsville, MD, USA), 71,000 men and 65,000 women are estimated to be diagnosed with CRC in the USA each year, and approximately 26,000 men and 24,000 women succumb to disease annually (1). Treatment currently includes surgical resection if the tumor has not progressed to an advanced stage, cytotoxic chemotherapy and radiation. However, anticancer drugs prolong survival without eliminating the cancer, and they frequently have adverse effects (2).

A number of aspects of tumor function, growth and division are affected by the pH of the tumor microenvironment (3). The intracellular pH (pH*i*) of CRC tumors has been identified to be neutral to alkaline (7.0-7.4), whereas extracellular pH (pH*e*) is typically acidic (6.9-7.0) (4). Extracellular acidification in tumors is thought to be primarily due to lactate secretion following anaerobic glycolysis. Lactate is a significant regulator of cancer development and maintenance, and stimulates tumor angiogenesis (5). Metabolism of glucose leads to the production of high concentrations of lactate, and this results in an acidic microenvironment within a number of solid tumors (6).

The expression of carbonic anhydrase 9 (CA9) on the tumor cell membrane may contribute to an acidic pH*e*, as CA9 is able to form H^+ via catalyzing the reversible hydration of CO_2 to bicarbonate and a proton (7,8). The active site of CA9, which is located in the extracellular space, contributes to acidification of the extracellular environment during hypoxia and maintains a neutral pH*i* within the tumor (9). CA9 expression has been associated with tumor progression, aggressiveness and a poor prognosis; therefore, it has been proposed as a potential therapeutic target (10). Pharmacological inhibition of CA9 catalytic activity may be achieved via the use of specific CA9 inhibitors or monoclonal antibodies that interrupt pH regulation in cancer cells. This has been demonstrated to decrease tumor growth and metastasis (11). Several CA9-targeted agents are

in preclinical or clinical development, and this has established a precedent for a novel, pH-targeted therapy for the treatment of cancer (5,12).

In order to survive in the acidic microenvironment, the pH*i*-regulating system in tumor cells actively extrudes acids via the monocarboxylate transporter (MCT) (13). MCT4 is strongly expressed in tumors, and alterations in pH*i* have previously been reported to be produced by MCT4. The transport of H^+ across the plasma membrane and the uptake of lactate markedly increases with decreasing pH*e* (14). Studies have previously shown that lactate transport in MCT4-expressing cells is accompanied by changes in pH*i*. An increase in lactate concentration generates an increase in acidification of the pH*i*, indicating a concentration-dependent influx of H^+ (15,16). Therefore, MCT-targeted agents may additionally be good candidates for cancer therapy (17).

In the present study, HCT116 human colon cancer cells were treated with lactate calcium salt (CaLa) to investigate the antitumor effects of artificial lactate influx via pH regulation of the cancer cells. The combined effects of treatment with CaLa, 5-indanesulfonamide and α-cyano-4-hydroxycinnamic acid were additionally investigated in order to identify any enhanced antitumor effects due to MCT4 and CA9 inhibition.

Materials and methods

Cell lines and reagents. The HCT116 colon cancer cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a humidified atmosphere of 5% CO_2. CaLa, α-cyano-4-hydroxycinnamic acid (CA; a CA9 inhibitor) and 5-indanesulfonamide (IS; an MCT4 inhibitor) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-human antibodies against poly(adenosine diphosphate ribose) polymerase (PARP; cat no. 9542) and glyceraldehyde-3-phosphate dehydrogenase (cat no. 2118) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit polyclonal anti-human antibodies against CA9 (cat no. SC-25599) were acquired from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Lactate assay. Lactate concentration was measured using a Lactate Assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol (18). Briefly, HCT116 cells were seeded into 60-mm dishes at a concentration of $5x10^5$ cells/well and incubated at 37˚C for 24 h. Following incubation, cells were treated with RMPI 1640 media (control), CaLa, CA and IS for 24 h. Cells were washed using phosphate buffered-saline (PBS) and collected. The cells were subsequently centrifuged (694.4 x g, 5 min) and homogenized in assay buffer, and the total protein concentration was measured with a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Lactate concentration was measured by spectrophotometry and normalized to total protein. Sample solutions were incubated with the reaction agent for 30 min at room temperature. Subsequently, the absorbance of samples was measured with an Epoch microplate spectrophotometer (450 nm; cat no. 0211-3030, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Measurement of pHe. Changes in pH*e* were measured using a pH electrode (item no. EW-58823-36; Thermo Fisher Scientific, Inc.) (19). Briefly, $2.5x10^5$ HCT116 cells were seeded into 6-well plates with RPMI-1640 media and incubated for 24 h. Cells were cultured with IS, CA, CaLa and combinations of the three for 24 h. Media was collected, and pH*e* was measured.

Measurement of pHi. To measure pH*i*, cells were seeded at $2.5x10^3$ cells/well into 96-well plates with RPMI-1640 media and incubated for 24 h. Cells were cultured with IS, CA, CaLa and combinations of the three for 24 h. Subsequently, pH-sensitive pHrodo™ Red AM (Molecular Probes; Thermo Fisher Scientific, Inc.) was incubated with the HCT116 cells. Briefly, fluorescence was measured at an excitation wavelength of 555 nm and emission wavelength of 580 nm, analyzed and converted to pH*i* using a nigericin calibration curve.

Colony formation assay. HCT116 cells were seeded into 6-well plates with RPMI-1640 media at a density of $5x10^2$ cells/well and incubated at 37˚C. Following incubation and treatment with CaLa, CA and IS, cells were allowed to grow for 7 days to form colonies. The cells were washed twice using PBS, and stained with hematoxylin. Colony morphology was observed with an optical microscope (Eclipse TS100; Nikon, Tokyo, Japan).

Cell viability assay. HCT-116 cells were seeded at a density of 5,000 cells/well in 96-well plates and treated with RMPI 1640 media (control), CaLa (5 mM), IS (1 mM) and CA (5 mM) for 7 days. The cell viability assay was performed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; 5 mg/ml; Biosesang Seongnam, Korea). MTT reduction was assessed using an Epoch Micro-Volume Spectrophotometer System (Bio-Tek Instruments, Inc.) at 460 nM.

Western blot. Cultured cells were washed twice using cold PBS and lysed on ice in lysis buffer (Thermo Fisher Scientific, Inc.) containing 1 M Tris-HCl (pH 7.4), 5 M NaCl, 0.5 mM ethylenediaminetetraacetic acid, NP-40, NaF, deoxycholate, 0.1% Triton X-100 and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN, USA). Protein samples of 20 μg were loaded on 8% polyacrylamide gels and subjected to electrophoresis. Protein bands were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked with 0.1% Tween 20 and 5% skimmed milk protein in PBS for 1 h at room temperature. Membranes were subsequently probed using primary polyclonal antibodies (Dilutions: PARP, 1:1,000; CA9, 1:5,000; GAPDH, 1:5,000). Following incubation with goat anti-rabbit IgG antibody (cat no. bs-0295G-HRP; 1:5000 dilution; Bioss, Inc., Woburn, MA, USA), immunoblots were developed with western blot detection reagents (GE Healthcare Life Sciences, Chalfont, UK) and exposed to X-ray film (Agfa-Gevaert N.V., Leverkusen, Germany), according to the

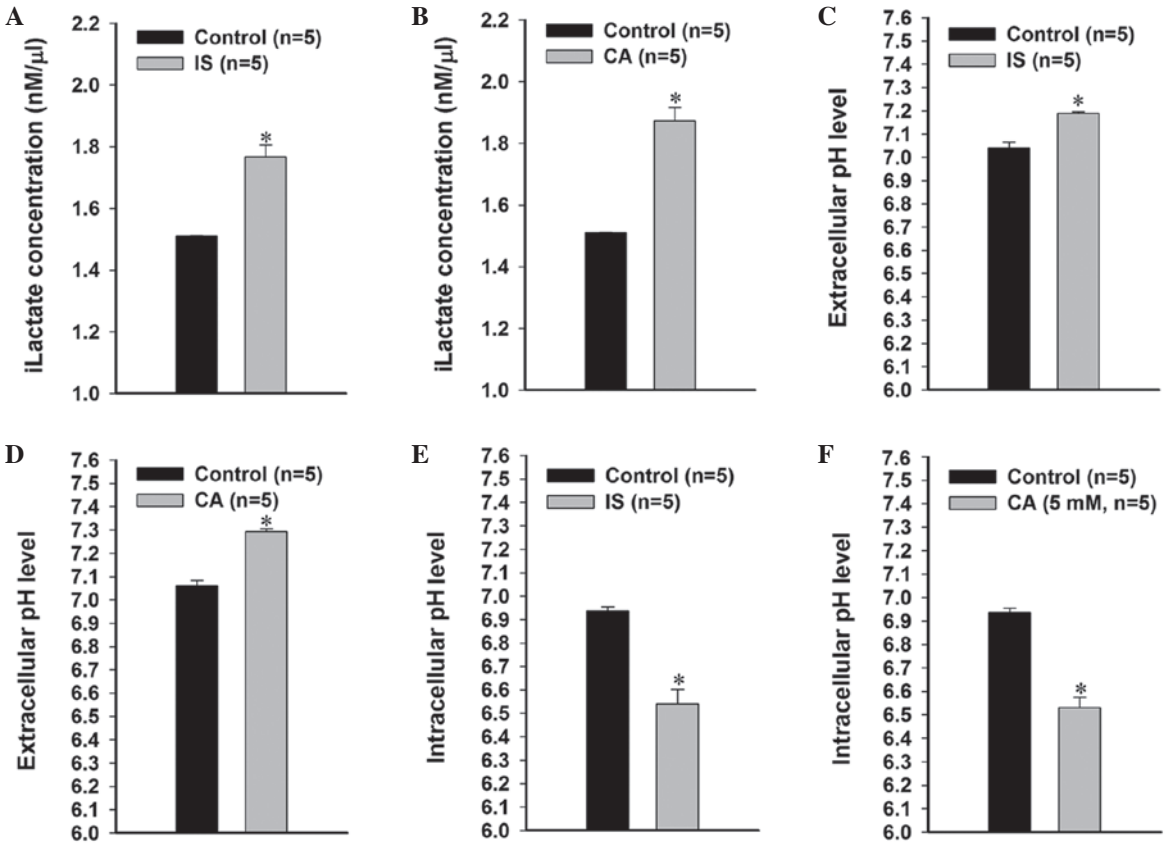

Figure 1. Measurement of iLactate accumulation and cellular pH following inhibition of CA9 and MCT4 in colon cancer cells. IS was used to inhibit CA9, and CA was used to inhibit MCT4. The graphs show the effects of (A) IS treatment and (B) CA treatment on iLactate concentration; the effects of (C) IS treatment and (D) CA treatment on extracellular pH; and the effects of (E) IS treatment and (F) CA treatment on intracellular pH. Control cells were treated with RPMI 1640 media. Results are presented as the mean ± standard deviation. *P<0.05 vs. control. iLactate, intracellular lactate; CA9, carbonic anhydrase 9; MCT, monocarboxylate transporter; IS, 5-indanesulfonamide; CA, α-cyano-4-hydroxycinnamic acid.

manufacturer's protocol. Densitometry measurements were performed using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical significance was analyzed using the Student's t-test depending on the normality of the data. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SigmaStat (version 3.5; Systat Software Inc., San Jose, CA, USA).

Results

Lactate accumulation occurs via CA9 and MCT4 inhibition. In order to investigate the role of lactate transport via MCT4 and CA9 activity, HCT116 cells were cultured in 1 mM CA- or 5 mM IS-containing media. IS treatment led to a significant increase (P=0.044) in the concentration of intracellular lactate (iLactate) compared with the control group (Fig. 1A). CA treatment also resulted in a significant increase to 1.77 nm/μl (P=0.009) in the concentration of iLactate compared with the control group (1.51 nm/μl; Fig. 1B).

IS and CA affect the regulation of pH in colon cancer cells. To determine the role of CA9 and MCT4 in pH regulation, HCT116 colon cancer cells were cultured in 1 mM CA- or 5 mM IS-containing media. pH*e* was observed to be significantly increased (IS, 7.19±0.006; CA, 7.29±0.012; P=0.016) following treatment with IS and CA (Fig. 1C andD). By contrast, IS or CA treatment induced a significant decrease (IS, 6.94 ± 0.017; CA, 6.21±0.045; P=0.007) in the pH*i* (Fig. 1E and F).

CaLa treatment affects the pH-associated tumor microenvironment. iLactate concentration, pH*e* and pHi were measured in colon cancer cells following 5 Mm CaLa treatment. iLactate was significantly increased to 0.285 nm/μl (P=0.001) following CaLa treatment compared with the control group (0.216 nm/μl; Fig. 2A). The pH*e* was similar in the control and CaLa-treated groups (Fig. 2B). However, pH*i* was significantly decreased to 6.50±0.020 (P<0.001) in the CaLa-treated group compared with the control group (6.94±0.017; Fig. 2C). This indicated that lactate-induced acidification was facilitated by the addition of CaLa to colon cancer cells.

Combined treatment with CaLa, IS and CA affects the pH-associated tumor microenvironment of colon cancer cells. A treatment strategy for combination treatment of CaLa (5 mM) with IS (1 Mm) and CA (5 mM) was established. Initially, cells were exposed to CaLa, IS and CA separately and incubated for 12 h. Subsequently, the cells were washed using PBS and treated to form combinations of CaLa+IS, CaLa+CA or CaLa+IS+CA. On the final day of the experiment, the iLactate concentration, pH and cell viability were measured (Fig. 3A). iLactate concentration was significantly increased

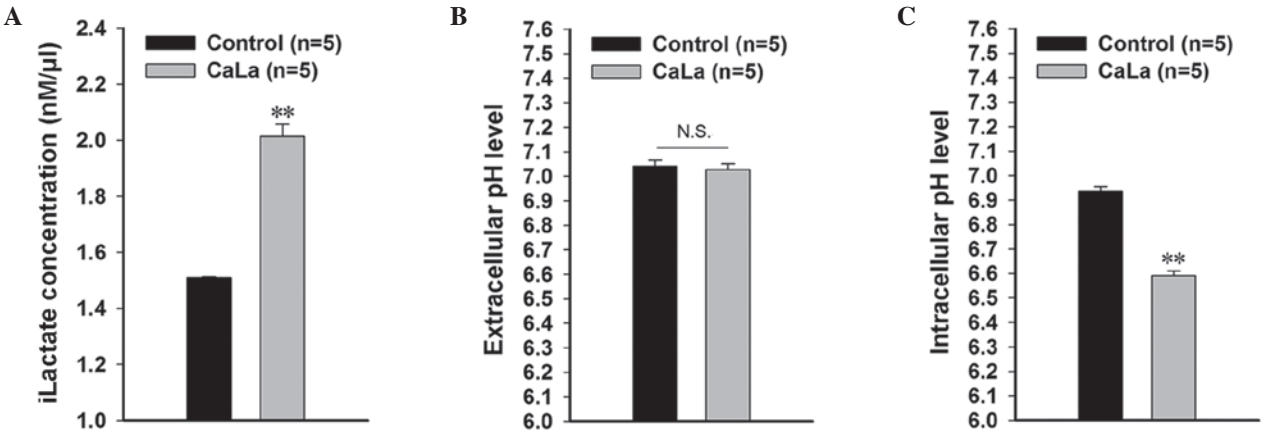

Figure 2. Measurement of iLactate accumulation and cellular pH following treatment with CaLa. (A) Effect of CaLa treatment on iLactate accumulation. (B and C) Effect of CaLa treatment on the cellular pH. Control cells were treated with RPMI 1640 media. Results are presented as the mean ± standard deviation. **P<0.001 vs. control. iLactate, intracellular lactate; CaLa, lactate calcium salt; N.S., not significant.

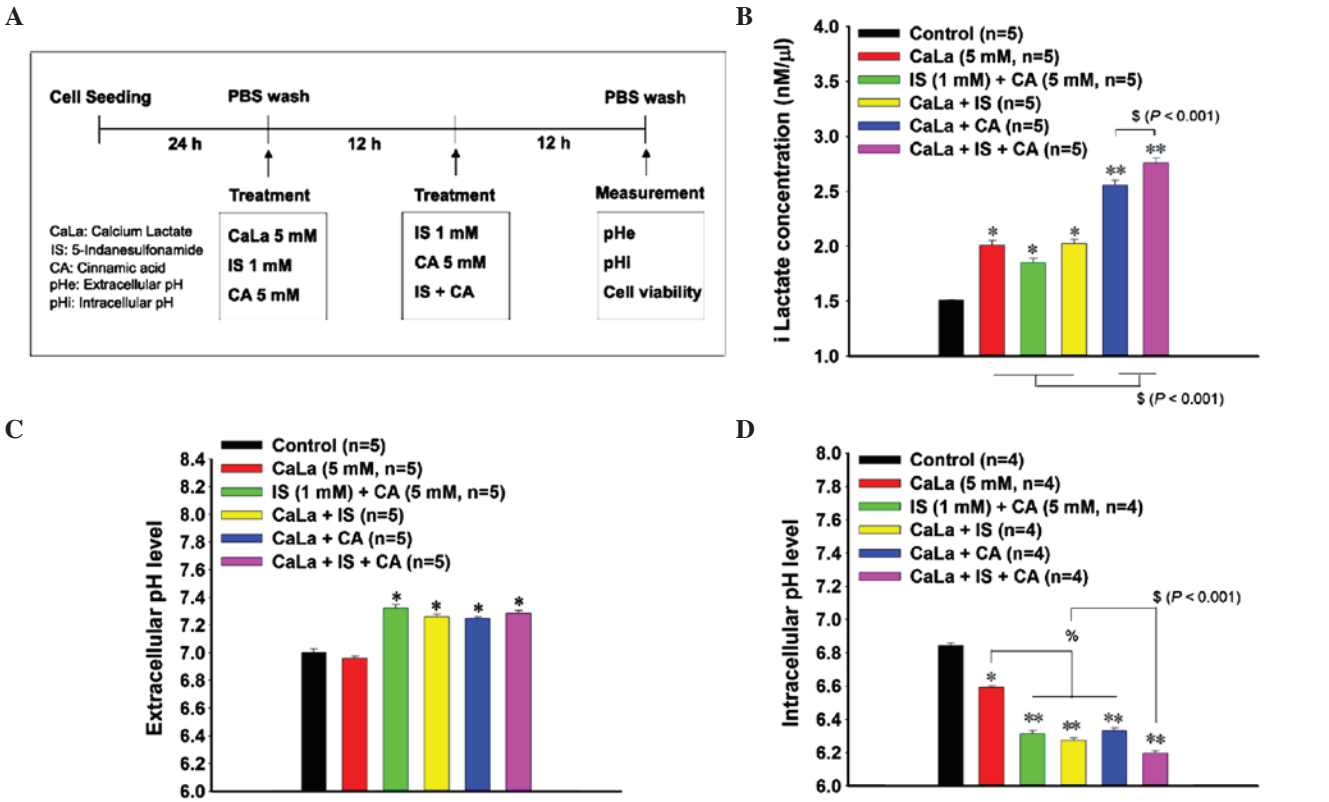

Figure 3. Combined effects of treatment with CaLa, IS and CA on lactate accumulation and cellular pH. (A) Schematic representation of a strategy for combined treatment with CaLa, IS and CA. Effect of combined treatment on (B) iLactate accumulation, (C) extracellular pH and (D) intracellular pH. IS was used as a carbonic anhydrase 9 inhibitor, and CA was used as a monocarboxylate transporter 4 inhibitor. Control cells were treated with RPMI 1640 media. Results are presented as the mean ± standard deviation. *P<0.05 and **P<0.001 vs. control; %P<0.05; $P<0.001. CaLa, lactate calcium salt; IS, 5-indanesulfonamide; CA, α-cyano-4-hydroxycinnamic acid; iLactate, intracellular lactate; pHe, extracellular pH; pHi, intracellular pH; PBS, phosphate-buffered saline.

in the CaLa-treated (2.01 nm/μl; P=0.017), IS+CA-treated (1.85 nm/μl; P=0.016) and CaLa+IS-treated (2.02 nm/μl; P=0.017) groups compared with the control group (1.51 nm/μl, Fig. 3B). The CaLa+CA-treated (2.55 nm/μl) and Ca+IS+CA-treated (2.75 nm/μl) groups demonstrated a greater increase in the iLactate concentration, which indicated an increased effect on lactate content (P<0.001; Fig. 3B). According to the pH*e* results, the IS+CA-treated (7.32±0.02), CaLa+IS-treated (7.26±0.02), CaLa+CA-treated (7.25±0.018) and CaLa+IS+CA-treated (7.29±0.021) groups demonstrated an increased pH*e* compared with the control group (7.00±0.026) and CaLa-treated group (6.96±0.015; Fig. 3C). The pH*i* was significantly lower (6.84±0.015; P=0.018) following CaLa treatment (6.59±0.019) compared with the control group (6.84±0.015, Fig. 3D). Similarly, treatment with IS+CA (6.31±0.018), CaLa+IS (6.27±0.015) and CaLa+CA (6.33±0.018) induced a decrease in the pH*i* compared with the CaLa-treated group (6.59±0.019). The greatest decrease in

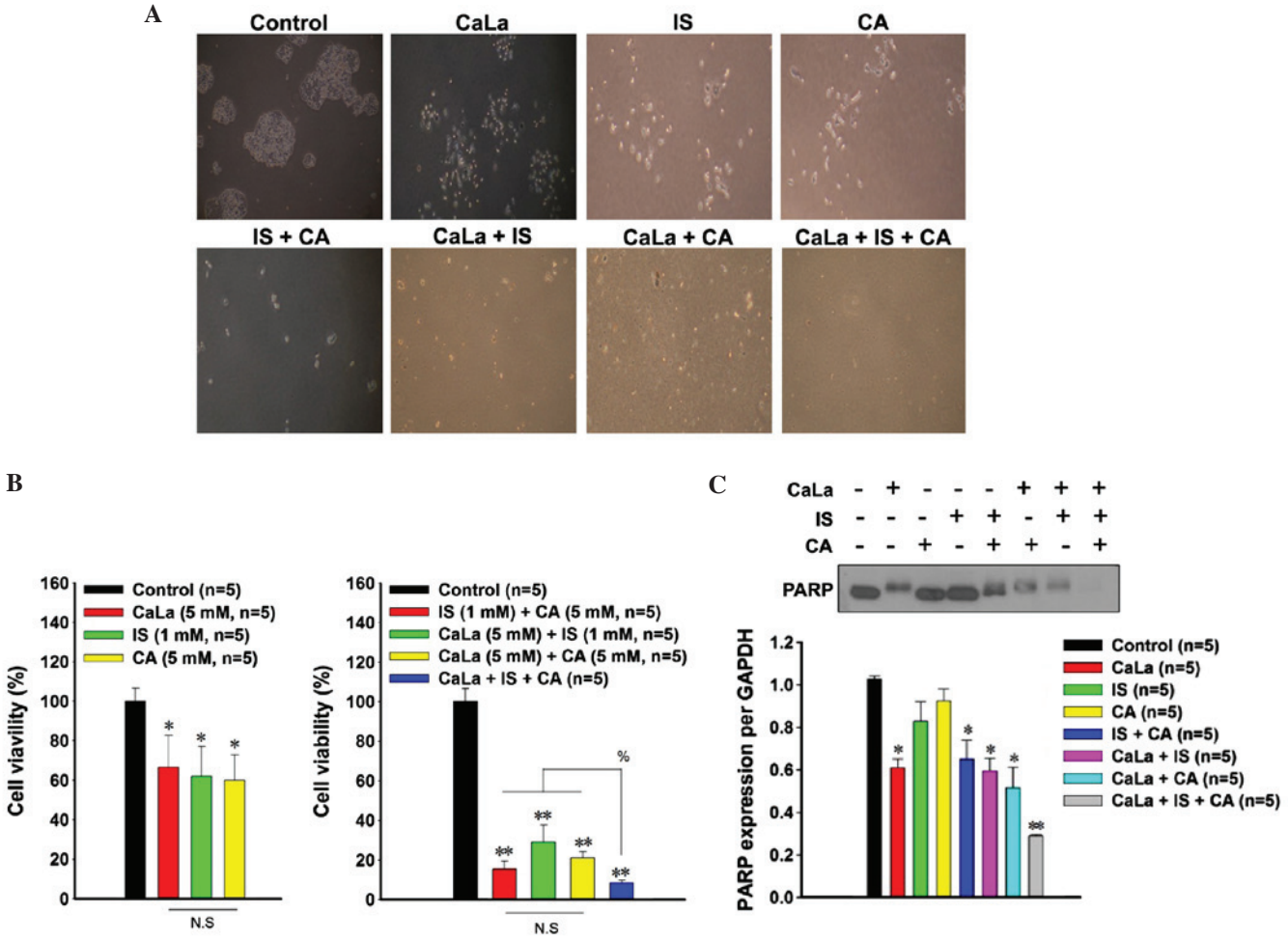

Figure 4. Combined effects of treatment with CaLa, IS and CA on the viability of colon cancer cells. (A) Microscopic observation of the morphological structure of colon cancer cells during the colony formation assay. (B) Inhibitory effects of single or combination treatment with CaLa, IS and/or CA on the viability of colon cancer cells. (C) Western blot analysis for PARP following single or combination treatment with CaLa, IS and CA (measured relative to GAPDH). IS was used to inhibit carbonic anhydrase 9 and CA was used to inhibit monocarboxylate transporter 4. Control cells were treated with RPMI 1640 media. Results are presented as the mean ± standard deviation. *P<0.05 and **P<0.001 vs. control; $^{\#,\$}$P<0.001. CaLa, lactate calcium salt; IS, 5-indanesulfonamide; CA, α-cyano-4-hydroxycinnamic acid; PARP, poly(adenosine diphosphate ribose) polymerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

pH*i* was observed with the three-agent combination treatment of CaLa+IS+CA (6.19±0.015; Fig. 3D). The decrease in pH*i* signified lactate-induced intracellular acidification.

Treatment with CaLa, IS and CA has a combined effect on the viability of colon cancer cells. Colony formation was markedly decreased following single treatment with CaLa, IS or CA compared with controls. Additionally, treatment with combinations of the agents (IS+CA, CaLa+IS, CaLa+CA or CaLa+IS+CA) completely inhibited colony formation compared with the control group (Fig. 4A). The cytotoxic effects on colon cancer cells were compared using an MTT assay. Relative to the control group (defined as 100% cell viability), decreased cell viability was observed following administration of a single-agent treatment with CaLa (66.61±16.17; P=0.029), IS (62.01±15.01; P=0.028) or CA (59.9±13.13; P=0.023) (Fig. 4B). Administration of two different agents (IS+CA, 15.42±4.18; CaLa+IS, 29.01±8.52; or CaLa+CA, 21.01±3.26) significantly reduced cell viability (P<0.001), whilst the three-agent combination of CaLa+IS+CA (8.42±1.31) led to a greater reduction in cell viability (P<0.001) compared with the two-agent combinations.

PARP is involved in acid-induced cell death. In order to investigate the mechanism underlying the decrease in viability of colon cancer cells, the expression of PARP was determined using western blot analysis (Fig. 4C). Among the single treatments, only the CaLa-treated group (0.70±0.04) demonstrated a decrease in PARP expression compared with the control group (1.03±0.01). Similarly, PARP was significantly decreased (P=0.017) following treatment with the two-agent combinations (IS+CA, 0.65±0.08; CaLa+IS, 0.79±0.06; and CaLa+CA, 0.72±0.09). The three-agent combination of CaLa+IS+CA resulted in a greater reduction (0.38±0.008) in the expression of PARP compared with CaLa-only treatment and two-agent combination treatments.

Discussion

In the present study, the effects of CaLa on the pH microenvironment of colon cancer cells were investigated. The findings revealed that lactate was able to induce intracellular acidification via MCT4 and CA9 inhibition. CaLa increased the intracellular lactate concentration, which led to a more acidic pH*i*. These conditions may contribute to the inhibition

of cancer cell survival. Combination treatment of CaLa with CA and IS led to a marked decrease in the viability of colon cancer cells. The current findings demonstrate that CaLa may potentiate the activity of CA9 and MCT4 inhibitors, providing *in vitro* evidence for the utility of CaLa as a potential pharmacological agent for the treatment of colon cancer.

The majority of cancer cells produce lactate under aerobic conditions, which indicates a state of active glycolysis (20). The intracellular lactate shuttle is important in maintaining the redox balance within cells (21). Lactate overproduction by tumors may be due to exaggerated glycolysis and a decreased clearance capacity caused by an impaired capacity for oxidative phosphorylation (22). Lactate accumulation has been proposed as a marker of malignancy in certain types of human cancer (23).

CA is known to inhibit MCT4, preventing monocarboxylate uptake and therefore inhibiting extracellular lactate transport (24). In addition, IS may block lactate transport via inhibition of CA9 activity (4). Therefore, intracellular lactate is increased following treatment with CA and IS. Lactate accumulation in tumor cells is accompanied by changes in the cytosolic pH, leading to acidification (25).

The primary function of CA9 in cancer is maintenance of pH homeostasis, which is associated with acidification of the tumor microenvironment and promotion of cancer cell migration and invasiveness. An additional function of CA9 is to maintain the intracellular pH under hypoxic conditions (9). Expression of CA9 has been proposed to be a marker of hypoxia and an indicator of poor prognosis (25). MCT offers a mechanism for achieving low pH*e*, while maintaining an alkaline pH*i* (4). This mechanism has been well-demonstrated using an MCT4 inhibitor, which led to an alkaline pH*e* and an acidic pH*i* (5,26). Previous reports have demonstrated that lactate transport in MCT4-expressing cells was accompanied by changes in the cytosolic pH (16). The high levels of lactate produced by cancer cells are usually removed by MCTs. MCT transport depends on pH, intracellular vs. extracellular lactate concentration and the levels of additional MCT substrates. The cotransport of protons with lactate prevents the toxic buildup of lactate and the acidification of the intracellular environment. Accordingly, lactate transport by MCTs is a therapeutic vulnerability of cancer cells, as intracellular acidosis poses a threat to cell survival (5).

In the present study, CaLa was used to induce iLactate accumulation in colon cancer cells. CaLa was observed to pass easily through tumor cell membranes. As a number of cancer cells are able to utilize calcium, a calcium-bound form of lactate was used. MCT4 channels were able to facilitate the transport of CaLa out of tumor cells. Therefore, inhibition of MCT4 led to increased intracellular acidification following CaLa treatment.

PARP is important in the maintenance of genetic integrity in response to DNA damage, and has been implicated in cell death induced by DNA damage (27). PARP is the substrate for the majority of caspases *in vitro,* and it has been reported that intracellular acidification may lead to apoptotic cell death (28). Apoptotic stimuli, including cancer chemotherapies, are capable of inducing intracellular acidification (29). Direct induction of an acidic pH*i* reportedly triggers the classic hallmarks of apoptosis, including nuclear condensation, cytoplasmic vacuolization and endonuclease-mediated DNA degradation (30). These observations suggest a direct association between acidification and a decrease in the viability of colon cancer cells. In the present study, downregulation of PARP activity was significant in the CaLa-treated group. Furthermore, combination treatment with CaLa+IS+CA led to enhanced inhibition of PARP activity.

In conclusion, the present study demonstrated that combination treatment with CaLa+IS+CA is able to induce intracellular acidification in colon cancer cells via maintenance of lactate accumulation, and to inhibit the viability of colon cancer cells. Therefore, it is proposed that combined treatment with CaLa+IS+CA may enhance antitumor activity compared with single-agent treatment, and this may provide a potential approach for the treatment of colon cancer via regulation of pH*i*.

Acknowledgements

The present study was supported by the Gachon Institute of Pharmaceutical Sciences Research Fund 2013 (Gachon University, Incheon, Republic of Korea).

References

1. Siegel R, Desantis C and Jemal A: Colorectal cancer statistics, 2014. CA Cancer J Clin 64: 104-117, 2014.
2. Benito M and Díaz-Rubio E: Molecular biology in colorectal cancer. Clin Transl Oncol 8: 391-398, 2006.
3. Parks SK, Chiche J and Pouyssegur J: pH control mechanisms of tumor survival and growth. J Cell Physiol 226: 299-308, 2011.
4. Swietach P, Patiar S, Supuran CT, Harris AL and Vaughan-Jones RD: The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths. J Biol Chem 284: 20299-20310, 2009.
5. Doherty JR and Cleveland JL: Targeting lactate metabolism for cancer therapeutics. J Clin Invest 123: 3685-3692, 2013.
6. Semenza GL: Tumor metabolism: Cancer cells give and take lactate. J Clin Invest 118: 3835-3837, 2008.
7. Kato Y, Ozawa S, Miyamoto C, Maehata Y, Suzuki A, Maeda T and Baba Y: Acidic extracellular microenvironment and cancer. Cancer Cell Int 13: 89, 2013.
8. Swietach P, Vaughan-Jones RD and Harris AL: Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev 26: 299-310, 2007.
9. McDonald PC, Winum JY, Supuran CT and Dedhar S: Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. Oncotarget 3: 84-97, 2012.
10. Gu MJ and Kwon KW Carbonic anhydrase IX expression is associated with favorable prognostic factors in small intestinal carcinoma. J Histochem Cytochem 62: 205-210, 2014.
11. McIntyre A, Patiar S, Wigfield S, Li JL, Ledaki I, Turley H, Leek R, Snell C, Gatter K, Sly WS, *et al*: Carbonic anhydrase IX promotes tumor growth and necrosis *in vivo* and inhibition enhances anti-VEGF therapy. Clin Cancer Res 18: 3100-3111, 2012.
12. Dubois L, Peeters S, Lieuwes NG, Geusens N, Thiry A, Wigfield S, Carta F, McIntyre A, Scozzafava A, Dogné JM, *et al*: Specific inhibition of carbonic anhydrase IX activity enhances the *in vivo* therapeutic effect of tumor irradiation. Radiother Oncol 99: 424-431, 2011.
13. Chiche J, Ilc K, Laferrière J, Trottier E, Dayan F, Mazure NM, Brahimi-Horn MC and Pouysségur J: Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res 69: 358-368, 2009.
14. Nguyen TT and Bonanno JA: Bicarbonate, NBCe1, NHE, and carbonic anhydrase activity enhance lactate-H+ transport in bovine corneal endothelium. Invest Ophthalmol Vis Sci 52: 8086-8093, 2011.
15. Nakayama Y, Torigoe T, Inoue Y, Minagawa N, Izumi H, Kohno K and Yamaguchi K: Prognostic significance of monocarboxylate transporter 4 expression in patients with colorectal cancer. Exp Ther Med 3: 25-30, 2012.
16. Dimmer KS, Friedrich B, Lang F, Deitmer JW and Bröer S: The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 350: 219-227, 2000.

17. Gotanda Y, Akagi Y, Kawahara A, Kinugasa T, Yoshida T, Ryu Y, Shiratsuchi I, Kage M and Shirouzu K: Expression of monocarboxylate transporter (MCT)-4 in colorectal cancer and its role: MCT4 contributes to the growth of colorectal cancer with vascular endothelial growth factor. Anticancer Res 33: 2941-2947, 2013.
18. Hussien R and Brooks GA: Mitochondrial and plasma membrane lactate transporter and lactate dehydrogenase isoform expression in breast cancer cell lines. Physiol Genomics 43: 255-264, 2011.
19. Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf dem Keller U, Leung S, Huntsman D, *et al*: Targeting tumor hypoxia: Suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. Cancer Res 71: 3364-3376, 2011.
20. Pacchiano F, Carta F, McDonald PC, Lou Y, Vullo D, Scozzafava A, Dedhar S and Supuran CT: Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. J Med Chem 54: 1896-1902, 2011.
21. Draoui N and Feron O: Lactate shuttles at a glance: From physiological paradigms to anti-cancer treatments. Dis Model Mech 4: 727-732, 2011.
22. Zheng J: Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review). Oncol Lett 4: 1151-1157, 2012.
23. Touisni N, Maresca A, McDonald PC, Lou Y, Scozzafava A, Dedhar S, Winum JY and Supuran CT: Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. J Med Chem 54: 8271-8277, 2011.
24. Klier M, Andes FT, Deitmer JW and Becker HM: Intracellular and extracellular carbonic anhydrases cooperate non-enzymatically to enhance activity of monocarboxylate transporters. J Biol Chem 289: 2765-2775, 2014.
25. Ditte Z, Ditte P, Labudova M, Simko V, Iuliano F, Zatovicova M, Csaderova L, Pastorekova S and Pastorek J: Carnosine inhibits carbonic anhydrase IX-mediated extracellular acidosis and suppresses growth of HeLa tumor xenografts. BMC Cancer 14: 358, 2014.
26. Lim KS, Lim KJ, Price AC, Orr BA, Eberhart CG and Bar EE: Inhibition of monocarboxylate transporter-4 depletes stem-like glioblastoma cells and inhibits HIF transcriptional response in a lactate-independent manner. Oncogene 33: 4433-4441, 2014.
27. Kim MY, Zhang T and Kraus WL: Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev 19: 1951-1967, 2005.
28. Zanke BW, Lee C, Arab S and Tannock IF: Death of tumor cells after intracellular acidification is dependent on stress-activated protein kinases (SAPK/JNK) pathway activation and cannot be inhibited by Bcl-2 expression or interleukin 1beta-converting enzyme inhibition. Cancer Res 58: 2801-2808, 1998.
29. Lagadic-Gossmann D, Huc L and Lecureur V: Alterations of intracellular pH homeostasis in apoptosis: Origins and roles. Cell Death Diff 11: 953-961, 2004.
30. Thammasit P, Sangboonruang S, Suwanpairoj S, Khamaikawin W, Intasai N, Kasinrerk W, Tayapiwatana C and Tragoolpua K: Intracellular Acidosis promotes mitochondrial apoptosis pathway: Role of EMMPRIN down-regulation via specific single-chain Fv intrabody. J Cancer 6: 276-286, 2015.