**Neutralization of Acidic Tumor Microenvironment (TME) with Daily Oral Dosing of Sodium Potassium Citrate (K/Na Citrate) Increases Therapeutic Effect of Anti-cancer Agent in Pancreatic Cancer Xenograft Mice Model**

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**Extracellular pH (pHe) of tumor cells is characteristic of tumor microenvironment (TME).** Acidic TME impairs the responses of tumors to some anti-cancer chemotherapies. In this study, we showed that daily oral dosing of sodium potassium citrate (K/Na citrate) increased blood HCO$_3^-$ concentrations, corresponding to increase of HCO$_3^-$ concentrations and pHs in urine, and neutralized the tumor pHe. Neutralization of acidic TME by alkaline substance like HCO$_3^-$, an active metabolite of K/Na citrate, well potentiated the therapeutic effect of anticancer agent TS-1®, an orally active 5-fluoro-uracil derivative, in Panc-1 pancreatic cancer-xenograft murine model. Neutralization of acidic TME by using an alkaline K/Na citrate is a smart approach for enhancement of the therapeutic effects of anticancer agents for pancreatic cancer in the end stage.

**Key words**  acidic extracellular pH; tumor microenvironment (TME); sodium potassium citrate; cancer chemotherapy; pancreatic cancer

**INTRODUCTION**

Several reports showed that the interstitial extracellular pH (pHe) in tumor is acidic (pH 6.2–6.9) rather than normal tissues (pH 7.3–7.4). This is caused in part of the overstimulation of several ion transporters such as Na$^+$/H$^+$ exchanger (NHE-1), Na$^+$-dependent and independent HCO$_3^-$/Cl$^-$ exchangers and the mono-carboxylate transporter (MCT), which increase H$^+$ ions in extracellular space and acidify the pHe in tumors. In addition, the Warburg effect, a tumor-associating anabolic glycolysis, is one of the principal factors inducing acidic tumor microenvironment (TME) in the tumor extracellular region. It was reported that the acidic TME is relating to tumor progression and metastasis.

The acidic TME is relating to tumor responses to anticancer chemotherapeutic agent, which results in aggressive phenotype of tumors characterized by chemoresistance and suppress apoptosis caused by activating nuclear factor-kappaB (NF-κB) and Bcl-2-associated X protein (BAX). The acidic TME also causes p53-dependent apoptosis and harbors p53-mutated cancer cells that lose the apoptotic potency and are not responsive to the apoptosis-inducing anticancer drug.

Alkaline product of sodium potassium citrate (K/Na citrate) has been used for clinical treatment of the metabolic acidosis in patients. In human body, K/Na citrate is metabolized to HCO$_3^-$ in the intestine after oral dosing of K/Na citrate. The HCO$_3^-$ buffer system plays an important role of maintaining the homeostatic pH in the blood through balancing of the composition of carbonic acid (H$_2$CO$_3$), HCO$_3^-$ and carbon dioxide (CO$_2$), as consequence, contributes to modulation of tumor pHe. It is assumed to be increased serum HCO$_3^-$ concentrations and deliver excess HCO$_3^-$ into the tumors after oral administration of K/Na citrate. The HCO$_3^-$ would trap an H$^+$ ion and forms H$_2$CO$_3$ in the tumor interstitial space, resulting in the neutralization of tumor pHe. Renal filtration regulates blood levels of HCO$_3^-$ through glomerular filtration and acid secretion.

TS-1® is an oral anticancer drug consisting of combinations of tegafur, 5-chloro-2,4-dihydroxypridine (CDHP) and potassium oxonate, having some clinical success in the treatment of patients with malignant cancers including pancreatic cancer. TS-1® is known to maintain therapeutic concentrations of 5-fluouracil (5-FU) in blood and the tumor via metabolism of a prodrug tegafur in the TS-1®. We investigated the effect of oral administration of K/Na citrate on the serum HCO$_3^-$ concentrations along with uric HCO$_3^-$ concentrations and uric pHs, and on the tumor pHe. And the therapeutic effect of the combination of daily oral dosing of TS-1® with daily oral dosing of K/Na citrate in a Panc-1 human pancreatic cancer xenograft murine model.

**MATERIALS AND METHODS**

**Materials** K/Na citrate, a mixture composed of 2 mol potassium citrate, 2 mol sodium citrate and 1 mol citric acid hydrate was purchased from Nippon Chemiphar (Tokyo, Japan). TS-1® was obtained from Taiho Pharmaceutical (Tokyo, Japan). All other reagents were of analytical grade.

**Cells and Animals** Colon-26 murine colorectal carcinoma cell line (RCB2657) and Panc-1 human pancreatic carcinoma cell line (RCB2095) were purchased from the RIKEN Bioresource Center (Ibaraki, Japan). The cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Corning, NY, U.S.A.), 100 units/mL penicillin and 100 µg/mL streptomycin (ICN Biomedicals, CA, U.S.A.) in a 5% CO$_2$/air incubator at 37°C.

BALB/c mice (female, 5 weeks old) and BALB/c nu/nu...
mice (female, 5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were housed under controlled environmental conditions (constant temperature and humidity, and a 12-h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University.

**Tumor-Bearing Mice** Colon-26 tumor-bearing model was established by subcutaneous inoculation of Colon-26 cells (2×10^6 cells/mouse) at a flank region of BALB/c mice. Panc-1 tumor-bearing mouse model was established by subcutaneous inoculation of Panc-1 cells (5×10^6 cells/mouse) at a flank region of BALB/c nude mice. All animal experiments were initiated when the tumors reached 50–100 mm³ in size.

**Measurement of Serum HCO₃⁻, Urine HCO₃⁻ and Urine pH** BALB/c mice were orally administered with a dose of K/Na citrate (2500 mg/kg). At selected time points post administration (0, 0.5, 1, 2, 4, 6, 8, and 24 h), blood was collected from the postcaval vein of the mice. Serum samples were obtained by centrifugation of the blood (3000 rpm, 4 °C, 15 min) following by incubation for 30 min at room temperature. The serum HCO₃⁻ concentrations were measured using the HCO₃⁻ measurement kit (Diacolor® CO₂ clinical diagnostic reagent, TOYOBO, Osaka, Japan). At the same time points, urine was collected by pushing on the lower abdominal region of the mice. The urine HCO₃⁻ concentrations were measured using Diacolor® CO₂. Urine pH values were measured with the pH test paper (pH 5.5–9.0, AS ONE, Osaka, Japan).

**Measurement of Intratumor pH Using a Microelectrode** Colon-26 tumor-bearing mice were orally administered with a dose of K/Na citrate (500 mg/kg) for 14 d. The interstitial pH in the tumor was measured using microelectrode with pH meter as referred the previous publications. The interstitial pH in the tumor was measured using microelectrode (MI-408B TIP, Microelectrodes) into the center of the tumor. Electrodes were calibrated before the measurements using standard pH 4.01 and 7.00 buffers. One measurement was taken at each mouse and plotted (n = 7–9).

**Therapeutic Effects of Oral Administration of K/Na Citrate Combined with Oral Administration of TS-1® Treatment in a Panc-1-Xenograft Murine Model** Panc-1 tumor-bearing mice were orally administered with 14 daily doses of K/Na citrate (500 mg/kg/d) combined with TS-1® (18 mg/kg/d). Volume of tumors and body weight of the treated mice were recorded twice weekly. Tumor growth inhibition [TGI (%)] was calculated using the following formula (RTV: relative tumor volume).²⁹

\[
TGI = \left(1 - \frac{RTV}{RTV_{control}}\right) \times 100
\]

**RESULTS**

**HCO₃⁻ Urine HCO₃⁻ in Serum and pHs in Urine after Oral Administration of K/Na Citrate** It is known that oral K/Na citrate produces HCO₃⁻ in the body and helps to correct the acid buildup in the blood.¹¹ The dosage of K/Na citrate was decided by reference to the previous publication.²⁰ A single administration of K/Na citrate, given to naive mice, clearly increased serum HCO₃⁻ concentrations until 2 h post administration, and then gradually decreased down to base over 48 h (Fig. 1A). The administration of K/Na citrate rapidly increased HCO₃⁻ concentrations in urine by 1 h post administration and then decreased down to the base level until 6 h (Fig. 1B). In addition, the administration of K/Na citrate increased urine pH to approx. 8.5 within 2 h post administration and then fell back to pH 5.9 within 6 h (Fig. 1C), which is highly consistent with the change of urine HCO₃⁻ concentrations. These results indicate that an oral administration of K/Na citrate may increase the pH of extracellular fluid in vivo by increasing blood HCO₃⁻ concentrations.

**Determination of Tumor pH after Administration of K/Na Citrate** As shown in Fig. 2A, pHs in subcutaneous region or intratumor region of Colon-26 tumors were directly measured with pH electrode probes as described in several literatures.¹⁷,¹⁸ Subcutaneous pH in normal mice, as a reference, was neutral (pH 7.15 ± 0.07) (Fig. 2B). The tumor pH in non-treated mice was acidic (pH 6.67 ± 0.08), which is consistent with the other observations.¹⁷,²³ Meanwhile, after treatment with K/Na citrate, the tumor pH was clearly increased up to pH 6.90 ± 0.11, compared to that in non-treatment mice. These results confirm that daily administrations of K/Na citrate in-

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Fig. 1. Serum and Urine HCO₃⁻ Concentrations and Urine pH after Oral Administration of K/Na Citrate

BALB/c mice were orally administered with a dose of K/Na citrate (2500 mg/kg). At selected time points post administration (0, 0.5, 1, 2, 4, 6, 8, and 24 h), blood and urine were collected from the treated mice. (A) Serum HCO₃⁻ concentrations, (B) urine HCO₃⁻ concentrations, and (C) Urine pHs were determined. The data are means ± standard deviation (S.D.) (n = 5–6).
crease the pH of extracellular fluid of solid tumors.

**Combined Treatment with Oral K/Na Citrate and Oral TS-1® in Panc-1 Xenograft Mouse Model** Effect of neutralization of tumor pH by oral K/Na citrate on *in vivo* tumor growth was investigated when combined with TS-1®. Neither K/Na citrate alone nor TS-1® inhibited the growth of tumor under our experimental condition. Meanwhile, the combined treatment showed suppression of the growth of Panc-1 tumor (Fig. 3A). These indicate that neutralization of tumor pH by K/Na citrate administration increased the therapeutic effect of TS-1®, which did not show any therapeutic effect by mono-treatment. In the K/Na citrate treatment group (mono-treatment or combined treatment), any significant body weight changes were observed (Fig. 3B), indicating that the 14d the K/Na citrate treatment was entirely tolerable.

**DISCUSSION**

In the current study, we showed an oral administration of K/Na citrate increases serum HCO₃⁻ concentrations, corresponding to urine HCO₃⁻ concentrations and urine pHs (Fig. 1). In addition, we showed that chronic oral administrations of K/Na citrate (14d) successfully neutralize the tumor pH (Fig. 2). It has been reported that acidic TME can impair the responses to anticancer chemotherapy.²² One can hypothesize that therapeutic interventions designed for increasing tumor pH may be able to improve therapeutic outcomes of some anticancer agents. We tested this hypothesis using Panc-1 xenograft murine model and TS-1® that is used for advanced pancreatic cancer in Japan.²³ It was reported that the tumor acidity facilitates the epithelial mesenchymal transition (EMT) of pancreas cancer,²⁰ which plays a role in the development of drug resistance against a cytotoxic chemotherapy including 5-FU, an active pharmaceutical ingredient in TS-1®, via over expression of Zeb-1, a transcriptional factor, and poor expres-

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**Fig. 2. Tumor pH after Oral Administration of K/Na Citrate**

Colon-26 tumor-bearing mice were orally administered with a dose of K/Na citrate (500mg/kg) for 14d. Then, the interstitial pH in the tumor was measured using microelectrode with pH meter. (A) A reference electrode and a pH electrode were stabbed into the subcutaneous region and the center of tumor, respectively. (B) One measurement was taken at each mouse and plotted as a violin plot (n = 7–9). ***p < 0.001 vs. subcutaneous, ###p < 0.001 vs. intratumor (control). (Color figure can be accessed in the online version.)

**Fig. 3. Effect of Increasing pH on Therapeutic Effects of Low-Dose TS-1® in Pancreatic Cancer Xenografts**

Panc-1 tumor-bearing mice were orally administered with TS-1® (18mg/kg/d, daily) for 2 weeks, combined with K/Na citrate (500mg/kg/d, daily). (A) Tumor volumes and (B) body weights of mice were recorded twice weekly. The data are means ± S.D. (n = 8, *p < 0.05 vs. control).

| Treatment               | TGI (%) | RTV     |
|-------------------------|---------|---------|
| Control                 | —       | 9.5 ± 0.9|
| K/Na citrate            | 13.4    | 8.3 ± 0.6|
| TS-1®                   | 14.9    | 8.1 ± 0.7|
| Combination             | 32.4    | 6.4 ± 0.3*|

*p < 0.05 vs. control.
sion of E-cadherin, a cell adhesion protein.\textsuperscript{25} We confirmed that increasing tumor pH by oral K/Na citrate potentiates the therapeutic efficacy of TS-1\textsuperscript{18} without any systemic adverse effects (Fig. 3). Recently, the Japanese National Cancer Center reported that the 3-year survival rate of the pancreatic cancer patients with end stages at stage III and stage IV was 11.9 and 2.5\%, respectively.\textsuperscript{19,22,23}\textsuperscript{20} In clinical cases, it must be difficult to treat with a full dose of anti-cancer agent, such as TS-1\textsuperscript{18}, for pancreatic cancer patients with the end stage. In the present study, we newly investigated that the therapeutic effect of a decreased dose of TS-1\textsuperscript{18}, that showed no effects by monotherapy, was potentiated under the condition of TME neutralized by alkaline K/Na citrate on Panc-1 pancreatic cancer xenograft model.

Orally administered K/Na citrate is absorbed from the intestinal tract into blood and dissociated into its constituent ions. The citrate anion is excreted through the urine, causing a shift in the electrical equilibrium.\textsuperscript{27} In order to recover this homeostasis, blood HCO\(_3\)\(^{-}\) concentrations increase accompanied with a decrease of serum H\(^{+}\) ions,\textsuperscript{11} corresponding to decreasing H\(^{+}\) ions in tumor tissue. Accordingly, oral K/Na citrate can produce HCO\(_3\)\(^{-}\) in appearing in, or disappearing from, the blood (Fig. 1A). It is well known that excess HCO\(_3\)\(^{-}\) in the blood is eliminated through glomerular filtration into urine; renal filtration regulates blood levels of HCO\(_3\)\(^{-}\) and acid secretion.\textsuperscript{15} Subsequently, urine HCO\(_3\)\(^{-}\) concentrations and urine pHs were increased in conjunction with increases in serum HCO\(_3\)\(^{-}\) concentrations. Interestingly, urine HCO\(_3\)\(^{-}\) concentrations and pHs rapidly dropped to base levels (Figs. 1B, C). This might be due to the presence of excess citrate ions secreted in urine that would tend to acidify the urine.

In recent days, there have been several clinical reports suggesting that internal acid-base balances are important for the health status of patients and may be involved in the therapeutic outcomes. In colorectal cancer patients undergoing resection of their primary tumors, the group with low serum HCO\(_3\)\(^{-}\) concentrations showed a lower 30-d overall survival than the group with normal HCO\(_3\)\(^{-}\) levels.\textsuperscript{28} In addition, in advanced pancreatic cancer patients treated with an alkaline diet with supplementary HCO\(_3\)\(^{-}\), the median overall survival was significantly longer in the patients with alkaline urinary pH (\(p<7.0\)) than those with acidic urinary pH (\(\leq 7.0\)) (16.1 and 4.7 months, respectively; \(p<0.05\)).\textsuperscript{29} The neutralization of acidic TME via increasing serum HCO\(_3\)\(^{-}\) concentrations by oral chronic K/Na citrate might make patients better in physical condition and subsequently prevent the malignant tumor growth.

In conclusion, we demonstrated that the treatment with K/Na citrate neutralizes the acidic tumor’s interstitial pH through elevating serum HCO\(_3\)\(^{-}\) concentrations, corresponding to increased urine HCO\(_3\)\(^{-}\) concentrations and urine pHs. Increasing tumor pH by oral K/Na citrate potentiates the therapeutic efficacy of TS-1\textsuperscript{18} without any severe systemic adverse effects. Our results imply that K/Na citrate, when orally and chronically administered, improve therapeutic effect of some types of chemotherapeutic agents by modulating tumor pH.

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Conflict of Interest Kiyoshi Eshima is President at Delta-Fly Pharma, Inc. No potential conflicts of interest were disclosed by the other authors.

REFERENCES

1) Griffiths JR. Are cancer cells acidic? Br. J. Cancer, 64, 425–427 (1991).
2) Wike-Hooley JL, Haveman J, Reinhold HS. The relevance of tumor pH to the treatment of malignant disease. Radiother. Oncol., 2, 342–366 (1984).
3) Maddox H. Regulation of intracellular pH in eukaryotic cells. Biochem. J., 250, 1–8 (1988).
4) Warburg O. On the origin of cancer cells. Science, 123, 309–314 (1956).
5) Cardone RA, Casavola V, Reshkin SJ. The role of disturbed pH dynamics and the Na\(^{+}/\)H\(^{+}\) exchanger in metastasis. Nat. Rev. Cancer, 5, 786–795 (2005).
6) Xie R, Wang H, Jin H, Wen G, Tuo B, Xu J. NHE1 is upregulated in gastric cancer and regulates gastric cancer cell proliferation, migration and invasion. Oncol. Rep., 37, 1451–1460 (2017).
7) Tavares-Valente D, Baltazar F, Moreira R, Queiros O. Cancer cell bioenergetics and pH regulation influence breast cancer cell resistance to paclitaxel and doxorubicin. J. Bioenerg. Biomembr., 45, 467–475 (2013).
8) Park H, Lyons JC, Griffin RJ, Lim BU, Song CW. Apoptosis and cell cycle progression in an acidic environment after irradiation. Radiat. Res., 153, 295–304 (2000).
9) Williams AC, Collard TJ, Paraskeva C. An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. Oncogene, 18, 3199–3204 (1999).
10) Starke A, Corsena A, Köhler T, Knubben J, Kraenzlin M, Uebelhart D, Wuthrich RP, von Rechenberg B, Muller R, Ambuhl PM. Correction of metabolic acidosis with potassium citrate in renal transplant patients and its effect on bone quality. Clin. J. Am. Soc. Nephrol., 7, 1461–1472 (2012).
11) McNaughton L, Cedaro R. Sodium citrate ingestion and its effects on maximal anaerobic exercise of different durations. Eur. J. Appl. Physiol. Occup. Physiol., 64, 36–41 (1992).
12) Robey IF, Baggett BK, Kirkpatrick ND, Roe DJ, Dosescu J, Sloane BF, Hashim AI, Morse DL, Raghunand N, Gatenby RA, Gillies RJ. Bicarbonate increases tumor pH and inhibits spontaneous metastases. Cancer Res., 69, 2260–2268 (2009).
13) Wesson LG Jr, Anslow WP Jr. Relationship of changes in glomerular filtration, plasma chloride and bicarbonate concentrations and urinary osmotic load to renal excretion of chloride. Am. J. Physiol., 180, 237–248 (1955).
14) Kanbe T, Kishimoto Y, Tsunoda H, Imamoto R, Mukouyama T, Kaneti G, Dahan N, Lupu-Haber Y, Suss-Toby E, Weiss-Messer E, Fujisawa T, Egashira H, Koizumi S, Iwasaki S, Chiba K, Kawauchi T, Egashira H, Koizumi K, Fujisawa J, Arakawa T, Momma K, Rokutan H, Horiguchi S, Hishima T. Malignant acinar-endocrine carcinoma of the pancreas treated with S-1. Clin. J. Gastroenterol., 6, 459–464 (2015).
15) Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. Phase II study of S-1 in patients with advanced biliary tract cancer. Br. J. Cancer, 91, 1039–1044 (2004).
16) Estrella V, Chen T, Lloyd M, Wijkowiak J, Cormell HH, Ibrahim-Hassan A, Bailey K, Balagurunathan Y, Rothberg JM, Sloane Br, Johnson J, Gatenby RA, Gillies RJ. Acidity generated by the tumor microenvironment drives local invasion. Cancer Res., 73, 1524–1535 (2013).
17) Asmanal-Masarweh H, Koren L, Zinger A, Yaari Z, Krisnky N, Kaneti G, Dahan N, Lupu-Haber Y, Suss-Toby E, Weiss-Messer E, Young Scientists (19K16415).
18) Japan Society for the Promotion of Science, Grant-in-Aid for Young Scientists (19K16415).
27) N. Schlesinger-Laufer, I. Shainsky-Roitman, A. Schroeder. Sodium bicarbonate nanoparticles modulate the tumor pH and enhance the cellular uptake of doxorubicin. *J. Control. Release*, **296**, 1–13 (2019).

19) M. Walls, S. M. Baxi, P. P. Mehta, K. K. Liu, J. Zhu, E. Estrella, H. Li, C. Zientek, M. Zong, S. Smeal, M. J. Yin. Targeting small cell lung cancer harboring PIK3CA mutation with a selective oral PI3K inhibitor PF-4989216. *Clin. Cancer Res.*, **20**, 631–643 (2014).

20) I. Sasagawa, T. Nakada, M. Ishigooka, Y. Kubota, T. Sawamura. Effect of standardized mixture of potassium and sodium citrate and citric acid (Uralyt-U) on the correction of postoperative acidosis in patients who underwent ureterosigmoidostomy. *Nephron*, **66**, 477–478 (1994).

21) D. C. Hinshaw, L. A. Shevde. The tumor microenvironment innately modulates cancer progression. *Cancer Res.*, **79**, 4557–4566 (2019).

22) J. Barar, J. Omidi. Dysregulated pH in tumor microenvironment checkmates cancer therapy. *Biosimilars*, **3**, 149–162 (2013).

23) K. Sudo, K. Nakamura, K. Yamaguchi. S-1 in the treatment of pancreatic cancer. *World J. Gastroenterol.*, **20**, 15110–15118 (2014).

24) S. Deng, X. Li, Y. Niu, Y. Zhu, S. Jin, Y. Deng, J. Chen, J. Liu, Y. He, C. Yin, T. Yang, Z. Tao, J. Xiong, J. Wu, H. Wang, C. Zhao. MiR-652 inhibits acidic microenvironment-induced epithelial–mesenchymal transition of pancreatic cancer cells by targeting ZEB1. *Oncotarget*, **6**, 39661–39675 (2015).

25) T. Arumugam, N. Ramachandran, V. Fournier, K. F. Wang, L. Marquis, L. Abbruzzese, J. E. Gallick, G. E. Logsdon, C. D. McConkey, D. J. Choi, W. R. Abbruzzese, J. L. Gallick, G. E. Logsdon, C. D. McConkey, D. J. Choi. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. *Cancer Res.*, **69**, 5820–5828 (2009).

26) J. M. Kowalchuk, S. A. Maltais, H. R. Hughson. The effect of citrate loading on exercise performance, acid-base balance and metabolism. *Eur. J. Appl. Physiol. Occup. Physiol.*, **58**, 858–864 (1989).

27) K. C. Chan, C. L. Diakos, A. Engel, H. C. Chan, D. L. H. Pavlakis, N. Gill, A. Clarke. Serum bicarbonate is a marker of peri-operative mortality but is not associated with long term survival in colorectal cancer. *PLos One*, **15**, e0238466 (2020).

28) Y. Hamaguchi, T. Narui, H. Wada. Effects of alkalinization therapy on chemotherapy outcomes in metastatic or recurrent pancreatic cancer. *Anticancer Res.*, **40**, 873–880 (2020).