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Ana Beatriz Celi

Universidad de Buenos Aires

Ana Mechali

Universidad de Buenos Aires

Natalia Beltramone

Universidad de Buenos Aires

Juan José Casal

Universidad de Buenos Aires

Claudia Capurro

Universidad de Buenos Aires

Gisela Di Giusto

Universidad de Buenos Aires

Paula Ford

Universidad de Buenos Aires

Valeria Rivarola

`mariv@fmed.uba.ar`

Universidad de Buenos Aires

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Targeting pH Regulation in Cancer: Combined Mild Alkalosis and NHE1 Inhibition as a Potential Therapy for Clear Cell Renal Cell Carcinoma

Ana Beatriz Celi^{1,2}, Ana Mechali^{1,2}, Natalia Beltramone^{1,2}, Juan José Casal^{1,2}, Claudia Capurro^{1,2}, Gisela Di Giusto^{1,2}, Paula Ford^{1,2} and Valeria Rivarola^{1,2*}

¹Universidad de Buenos Aires, Facultad de Ciencias Médicas, Departamento de Ciencias Fisiológicas. Laboratorio de Biomembranas. Buenos Aires, Argentina.

²CONICET - Universidad de Buenos Aires. Instituto de Fisiología y Biofísica Bernardo Houssay (IFIBIO Houssay). Buenos Aires, Argentina.

ORCID

Lic. Ana Beatriz Celi (ORCID number 0009-0001-4266-8477), acelicastillo@fmed.uba.ar. Argentina

Ana Mechali. animechali@gmail.com. Argentina

Natalia Beltramone (ORCID number 0009-0000-0701-7823). natibeltramone@gmail.com. Argentina

Dr. Juan José Casal (ORCID number 0009-0007-8200-1750). jcasal@fmed.uba.ar. Argentina

Dr. Claudia Capurro (ORCID number 0000-0003-3773-0202) claudiacapurro@fmed.uba.ar. Argentina

Dr. Gisela Di Giusto (ORCID number 0000-0002-7622-5172). gdigiusto@fmed.uba.ar. Argentina

Dr. Paula Ford (ORCID number 0000-0002-3656-0806). pford@fmed.uba.ar. Argentina

Dr. Valeria Rivarola (ORCID number 0000-0002-8879-1198). mariv@fmed.uba.ar. Argentina

Corresponding Author: Dr. Valeria Rivarola, Ph.D. Laboratorio de Biomembranas, IFIBIO Houssay, CONICET-UBA, Departamento de Ciencias Fisiológicas. Facultad de Medicina, Universidad de Buenos Aires. Paraguay 2155 piso 7 (C1121ABG) Buenos Aires, ARGENTINA. TEL: 54-11-52853315. e-mail: mariv@fmed.uba.ar

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Compliance with Ethical Standards

The authors of this manuscript certify that they have NO potential sources of conflict of interest (relationship, financial or otherwise) that might influence the author's objectivity in the subject matter or materials discussed in this manuscript.

Data linking

The KNIME workflow used in this study to perform total cell count is openly available on Figshare at [https://figshare.com/articles/software/Segment de acridina son naranjas knwfKnime workflow to count total cell number in photos of Acridine Orange Ethidium Bromide experiments/25913794](https://figshare.com/articles/software/Segment_de_acridina_son_naranjas_knwfKnime_workflow_to_count_total_cell_number_in_photos_of_Acridine_Orange_Ethidium_Bromide_experiments/25913794).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT and Grammarly to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Abstract

Acid-base homeostasis is critical for maintaining physiological functions. In cancer, an acidic tumor microenvironment, driven by altered cellular metabolism, plays a pivotal role in tumor progression by fostering aggressive phenotypes, immune evasion, and resistance to therapy, often at the detriment of surrounding normal tissues. The Na⁺/H⁺ exchanger isoform 1 (NHE1) is a key regulator of intracellular pH and a critical factor in cancer cell survival and proliferation. This study aimed to evaluate the effect of mild alkalosis, combined with NHE1 inhibition, on cell viability in normal renal cells and clear cell renal cell carcinoma (ccRCC) cells. Our findings reveal that this therapeutic combination selectively induces cell death in ccRCC cells while sparing normal renal cells. Mechanistically, we demonstrate that NHE1 activity is higher in ccRCC cells than in normal cells. Mild alkalosis enhances NHE1 activity in normal cells but inhibits its activity in cancer cells. Furthermore, prolonged alkaline exposure alters the subcellular localization of NHE1 in the plasma membrane, with distinct patterns observed between normal and cancer cells. These results suggest that targeting NHE1 activity in conjunction with alkalosis represents a promising strategy for ccRCC treatment, providing a potential therapeutic avenue to exploit the differential pH regulation between cancerous and normal cells.

Introduction

One of the hallmarks of cancer cells is their acidic microenvironment. Cancer cells develop adaptive mechanisms that allow them to evade acid-mediated toxicity, promoting their growth while compromising the viability of surrounding normal cells. Localized acidosis further enhances tumor progression by facilitating extracellular matrix degradation, which promotes invasion and suppresses immune responses to tumor antigens [1]. Among the key regulators of intracellular pH (pHi) in cancer cells is the isoform 1 of the Na⁺/H⁺ exchanger (NHE1) [2]. NHE1 plays a vital role in physiological processes such as epithelial salt transport, cell volume regulation, proliferation, and apoptosis [3]. However, its influence on apoptosis is context-dependent, as it can either promote or inhibit cell death depending on the specific cell type and conditions studied [4, 5].

In recent years, researchers have proposed neutralizing the acidic microenvironment of tumors to slow the progression of certain cancers. In silico simulations, which model changes in microenvironmental pH, suggest that increasing pH can reduce cancer progression rate [6]. Experimental studies in breast, prostate, and melanoma cancers further support this approach [7–10]. However, maintaining stable intracellular pH is critical for proper cell proliferation and death mechanisms under physiological conditions [11, 12]. Therefore, to establish optimal conditions for safely applying this strategy, the potential adverse effects of pH neutralization on healthy tissues must also be evaluated.

Renal cell carcinoma (RCC) includes a diverse group of cancers originating from renal epithelial cells and ranks among the ten most common cancers worldwide. Clear cell renal cell carcinoma (ccRCC) is the most prevalent subtype, accounting for 70–80% of RCC cases [13]. ccRCC has a high mortality rate in both men and women due to its resistance to chemotherapy and radiotherapy [14, 15]. Understanding the role of the cancer microenvironment and its interaction with transporters like NHE1 could offer new therapeutic strategies for ccRCC. For instance, Koltai et al. describe a case where a patient's ccRCC stabilized following treatment with repurposed drugs that neutralized the inverted pH gradient [16].

This study investigates the effects of mild alkalosis combined with NHE1 inhibition on cell death in normal and cancerous renal cells. We use three human renal cell models: HK-2, representing healthy proximal tubular epithelial cells; 786-O, derived from a primary clear cell renal cell carcinoma; and Caki-1, derived from a ccRCC metastasis. Our findings demonstrate that the combination of mild alkalosis and NHE1 inhibition selectively induces cell death in ccRCC cells while minimally affecting normal renal cells. Moreover, prolonged exposure to alkalosis downregulates NHE1 expression in ccRCC cells, suggesting a potential mechanism for this selective effect. These results provide insights into the interplay between extracellular pH modulation and NHE1 activity, offering a promising avenue for therapeutic strategies targeting ccRCC.

Materials and Methods

Cell culture

We cultured HK-2, 786-O, and Caki-1 cells using standard periodic subcultures on plastic supports. The culture media used were DMEM-F12 for HK-2 cells, RPMI for 786-O cells, and McCoy's 5A for Caki-1 cells, all supplemented with fetal bovine serum and antibiotics (Glube et al., 2007; Ryan et al., 1994; Willia et al., 1978).

To create alkaline conditions, we adjusted the culture media by adding NaOH. For NHE1 blockade experiments, we added 1 μ M HOE 694 (Aventis Pharma Deutschland GmbH, Germany) or vehicle (water, diluted 1:1000) to the medium.

Cell Death analysis using Acridine Orange and Ethidium Bromide staining

To quantify cell death, we washed the cells with 1X PBS and stained them with the fluorescent DNA-binding dyes acridine orange (AO, 15 μ g/ml) and ethidium bromide (EB, 500 μ g/ml) for 1 minute at room temperature. AO, which emits green fluorescence, penetrates both viable and dead cells, while EB, emitting red fluorescence, selectively marks dead cells. After staining, we washed the cells twice with PBS and immediately captured micrographs using an Olympus OPTIPHOT IMT-2 microscope equipped with an SPlan 10 PL 10X/0.30 NA objective. A Sony Alpha 3000 camera digitized the captures. We analyzed at least six fields from three independent coverslips for each condition.

To evaluate the percentage of cells displaying morphological features of cell death, we performed each experiment in triplicate and scored between 1,000 and 5,000 cells for each condition. We identified dead cells using established criteria such as nuclear morphological changes, chromatin condensation, and staining[20], ImageJ software facilitated image analysis and counting. We designed a KNIME workflow to perform total cell counts (see Data linking).

Cell Death Analysis using Annexin V and Propidium iodide staining

To assess cell death using an alternative method, we performed Annexin V/Propidium Iodide (PI) staining as described by Sánchez et al. [21]. We stained the cells with FITC-conjugated Annexin V (AV, BD Pharmingen) and Propidium Iodide, following the manufacturer's protocol. We captured micrographs with an Olympus OPTIPHOT IMT-2 microscope equipped with an SPlan 10 PL 10X/0.30 NA objective and digitized them using a Sony Alpha 3000 camera attached to the microscope. For each condition, we analyzed at least six fields from a minimum of three independent coverslips.

We classified cells based on staining patterns: AV-/PI- cells were considered viable, AV+/PI- cells were in early apoptosis, AV+/PI+ cells were in late apoptosis, and AV-/PI+ cells were necrotic.

Immunofluorescence studies

We conducted immunofluorescence studies on all cell lines. First, we seeded the cells onto coverslips and allowed them to grow in the presence or absence of 9.6 mM NaOH. In some experiments, we incubated the cells with 5 µg/ml wheat germ agglutinin Alexa 488 conjugate (WGA, Thermo Fisher Scientific, Cat# W11261). We then fixed the cells with 3% paraformaldehyde for 15 minutes and permeabilized them with 0.2% saponin at room temperature. Next, we incubated the samples overnight at 4°C with monoclonal anti-NHE1 (dilution 1:200, Santa Cruz Biotechnology, Cat# sc-518041, RRID: AB_3481247) and followed with a 2-hour incubation at room temperature with secondary antibodies (anti-mouse Alexa 594 conjugate, dilution 1:200, Thermo Fisher Scientific, Cat# A-11032, RRID: AB_2534091). To stain the nuclei, we used Hoechst (5 µg/ml) for 3 minutes and then mounted the coverslips with Vectashield mounting medium (Vector Laboratories Inc., CA, USA) at room temperature.

We captured and digitized the images using Fluoview FV1000 software on an Olympus FV1000 confocal microscope (objective UPLSAPO 40X/1.30 NA). We analyzed at least five fields from three independent coverslips per condition. Finally, we performed 3D reconstruction and colocalization analysis using Imaris software, calculating the amount of colocalization with the Mander's coefficient.

Intracellular pH Measurement and NHE1 Activity Assessment Using BCECF-AM and NH₄Cl Pre-Pulse Technique

For intracellular pH (pHi) measurements, we grew cells on glass coverslips and loaded them with 13 mM 2',7'-bis(2-carboxyethyl)-5-(and-6) carboxyfluorescein acetoxymethyl ester (BCECF-AM, Thermo Fisher Scientific, Cat# B1170) for 15 minutes at 37°C. Afterward, we washed away the excess dye. We then placed the chamber on a Nikon TE-200 epifluorescence inverted microscope (Nikon Plan Fluor 40X oil immersion objective lens) with a micro-incubator system and temperature controller (Harvard Apparatus Inc, MA, USA). Fluorescence data were collected every 10 seconds from a small circular region in the cells using a charge-coupled-device camera (Hamamatsu C4742-95) connected to a computer running the Metafluor acquisition program (Universal Imaging Corporation, PA, USA).

We determined intracellular pH by calculating the ratio of BCECF emission intensity at 535 nm. The dye was excited at ~490 nm, and its isosbestic point was set at ~440 nm. We calibrated the 490/440 BCECF fluorescence ratio by incubating the cells in a high K⁺ solution (140mM KCl, 4.6mM, NaCl, 1mM MgCl₂, 2mM CaCl₂, 10mM Hepes, 5mM glucose), followed by permeabilization with 5mM nigericin to equilibrate extracellular pH (pHo) with pHi. We then stepped the pH-bathing solution between 6.6 and 8.5. The 490/440 ratio remained linear within this pH range (r = 0.96, n = 6).

TABLE 1: Composition of solutions used for determination of pHi recovery.

Solution	Control	0 Na ⁺	NH ₄ Cl
----------	---------	-------------------	--------------------

NaCl	145	----	127
NaHCO₃	----	----	----
Hepes	30	30	30
KCl	3	3	3
CaCl₂	1.8	1.8	1.8
MgCl₂	----	----	----
MgSO₄	1	1	1
KH₂PO₄	1	1	1
K₂SO₄	1	1	1
NH₄Cl	----	----	20
TMA	----	147	----
glucose	5.6	5.6	5.6

All concentrations are given in mM; pH was adjusted to 7.4 at 37°C.

In some experiments, we monitored NHE1 activity by evaluating the recovery of pHi after an acid load, using the NH₄Cl pre-pulse technique as previously described [22–24]. We first exposed the cells to a control solution, followed by a 20 mM NH₄Cl pulse (see Table 1) and then Na⁺ removal (0 Na⁺ in Table 1). Afterward, we reintroduced Na⁺ to the bath and evaluated Na⁺-dependent pHi recovery mechanisms. We calculated acid fluxes using the following equation Eq. (A.1):

$$JH = \beta i \times \frac{dpH}{dt} \text{ Eq. (A.1)}$$

H⁺ represents the H⁺ flux, dpH/dt is the recovery rate from the acid load, and βi is the intracellular buffering power. We estimated intracellular H⁺ buffering power (βi) using the application of external permeant weak bases, as described by Roos, Boron, and Saleh [25, 26]. In this study, we used NH₄Cl, which permeates the cell membrane in its uncharged form but exists mainly in the ionized form once inside the cell. The ionization of this weak base absorbs H⁺ ions. Thus, NH₄Cl induces changes in pHi, which we used to estimate βi using the following equation Eq. (A.2):

$$\beta i(mM) = \frac{[NH_4]_0 \cdot 10^{(pH_o - pHi_p)}}{\Delta pHi} \text{ Eq. (A.2)}$$

Where ΔpHi is the change in pHi following the ammonium load, $[NH_4^+]_0$ is the concentration of external NH₄⁺, pH_o is the pH of the external solution, and pHi_p is the intracellular pH after the ammonium load.

Statistics

We presented the values as mean $\pm$ standard error of the mean (SEM), with "n" indicating the number of experiments conducted. To compare data between the two groups, we used an unpaired Student's t-test, considering differences significant at $p < 0.05$. For comparisons among multiple groups, we performed a one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test, defining significance as $p < 0.05$.

Results

Cancer cell death induced by alkali and NHE1 inhibition with minimal impact on healthy cells.

We used three human renal cell models: HK-2, representing healthy proximal tubular epithelial cells; 786-O, derived from primary clear cell renal cell carcinoma (ccRCC); and Caki-1, derived from a ccRCC metastasis. As expected, upon reaching confluence, the pH of the conditioned media (pHe) of normal HK-2 cells was less acidic than that of the cancer cell lines (pHe: HK-2 = 7.37 ± 0.02 , $n = 26$; 786-O = 7.04 ± 0.05 , $n = 24$; Caki-1 = 7.15 ± 0.05 , $n = 17$; one-way ANOVA followed by Tukey's multiple comparisons test: HK-2 vs. 786-O, $p < 0.0001$; HK-2 vs. Caki-1, $p < 0.01$).

We investigated the effects of neutralizing the acidic microenvironment by adding different concentrations of NaOH to the media for 72 hours. We then analyzed apoptosis percentages using the acridine orange/ethidium bromide staining method. As shown in Figure 1A, low NaOH concentrations (2.4–9.6 mM) caused minimal increases in cell death in HK-2 cells. In contrast, these concentrations led to a progressive increase in cell death in cancer cell lines. We selected 9.6 mM NaOH for 72-hour treatments in subsequent experiments.

Although the mild alkaline treatment did not significantly alter the pH of the HK-2-conditioned media, it was sufficient to neutralize the acidic microenvironment of the cancer cell lines, as shown in Figure 1B. Next, we explored the combined effects of NHE1 inhibition and mild alkalosis. To specifically inhibit NHE1, we used the HOE 694 inhibitor (1 μ M, Counillon & Pouyssegur, 2000). Without NaOH, NHE1 inhibition increased apoptosis in normal HK-2 cells. While NaOH alone slightly increased cell death in HK-2 cells, the combination of NHE1 inhibition and NaOH prevented NaOH-induced cell death in these cells. In contrast, NaOH-induced cell death was significantly higher in cancer cell lines compared to HK-2 cells, and NHE1 suppression did not alter this result (Figure 1C). Thus, the combination of mild alkali treatment and NHE1 inhibition selectively induced apoptosis in cancer cells with minimal damage to healthy cells.

Finally, annexin V/propidium iodide staining experiments confirmed that the cell death observed through acridine orange/ethidium bromide staining was primarily due to apoptosis (Figures 1D-E).

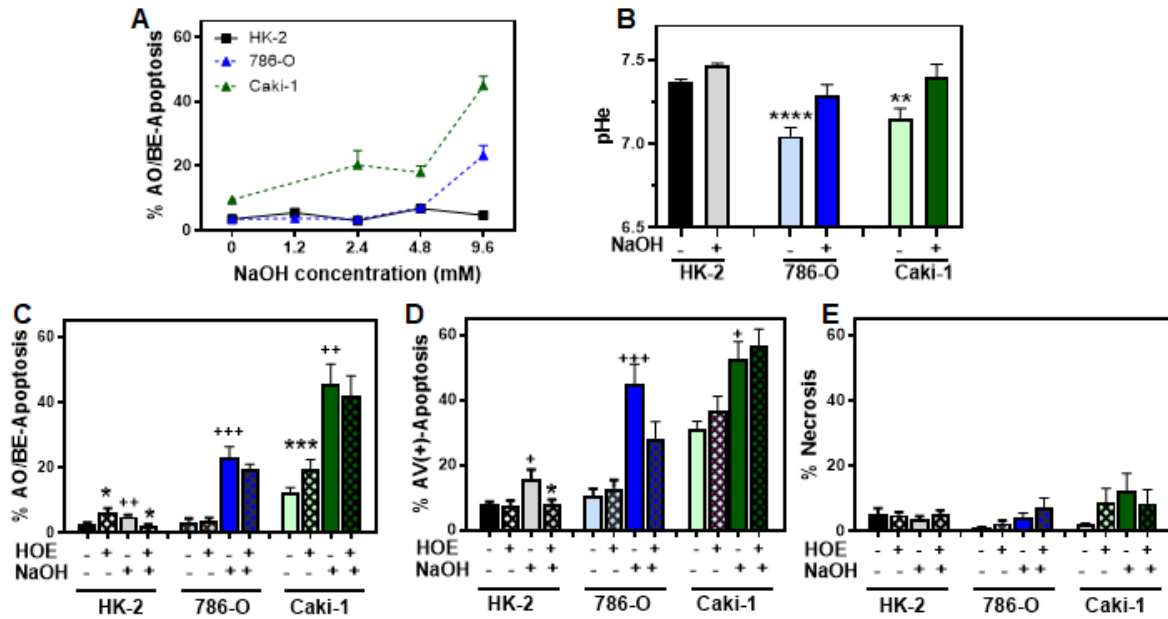


Figure 1 Effect of mild alkali and NHE1 activity on Cell Death in HK-2, 786-O, and Caki-1 cells. **A-** HK-2, 786-O, and Caki-1 cells were incubated for 72 hours with either vehicle or 1.2–9.6 mM NaOH. Apoptosis was quantified using the acridine orange/ethidium bromide staining method (AO/BE). **B-** Impact of NaOH on the pH of conditioned media after 72 hours with vehicle (-) or 9.6 mM NaOH (+). ** $p < 0.01$, *** $p < 0.0001$; $n = 103$. One-way ANOVA followed by Tukey's multiple comparisons test vs. HK-2 without NaOH. **C-E** Cells were treated with vehicle or 1 μ M HOE 694 (NHE1 inhibitor) in the presence or absence of 9.6 mM NaOH for 72 hours. **C-** Apoptosis quantified by acridine orange/ethidium bromide staining. **D-** Apoptosis was assessed using the annexin V method. **E-** Necrosis measured using the annexin V method. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, vehicle vs. NaOH treatment; * $p < 0.05$, *** $p < 0.001$, active vs. inhibited NHE1.

Enhanced NHE1 activity in cancer cells compared to healthy cells is suppressed by mild alkalosis.

We evaluated NHE1 activity in healthy and cancerous cell lines by monitoring intracellular pH (pHi) changes in BCECF-loaded cells. To quantify NHE1 activity, we employed the NH_4Cl pre-pulse technique. As shown in Figures 2A-C, 786-O cancer cells exhibited higher pHi, buffering capacity, and NHE1 activity than healthy HK-2 cells. In contrast, Caki-1 cells displayed elevated buffering capacity and NHE1 activity but did not exhibit an alkaline pHi.

Previous studies have reported that NHE1 in cancer cells undergoes an alkaline shift in its pH-sensing mechanism [28]. To determine whether this phenomenon also occurs in renal cancer cells, we plotted NHE1-dependent H^+ fluxes as a function of pHi. Figure 2D reveals that H^+ fluxes in cancer cells are shifted to the right, indicating an alkaline shift in the pH sensor of NHE1.

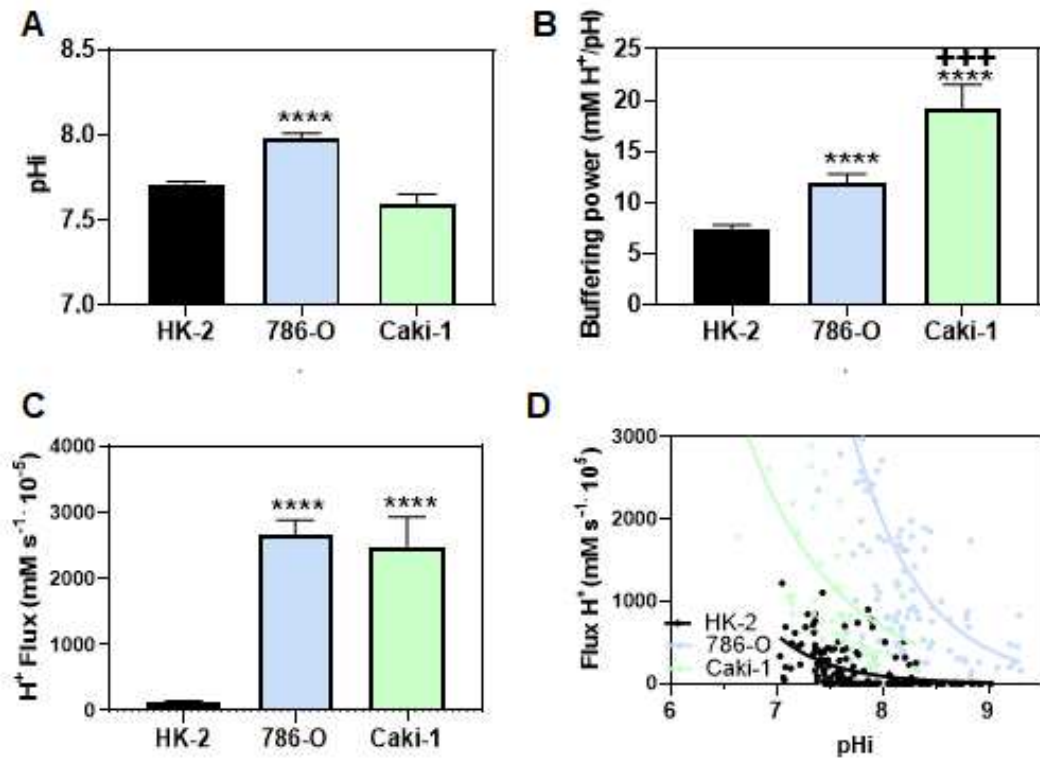


Figure 2 pH_i, buffering power and NHE1 activity in HK-2, 786-O, and Caki-1 cells.

A- Basal pH_i. **B-** Buffering power. **C-** NHE1-dependent H⁺ flux during the recovery of an ammonium pulse. One-way ANOVA followed by Tukey's multiple comparisons test for pH_i, Buffering Power, or H⁺ flux. **** p<0.0001, 786-O, or Caki-1 vs HK-2 cells. +++ p<0.001, Caki-1 vs 786-O cells. **D-** pH dependence in each cell line when grown in standard media.

We subsequently investigated whether NaOH affected NHE1 activity. NHE1 possesses two pH sensors (S. Reshkin et al., 2012). Alkali-induced modifications to NHE1 could result from allosteric changes, alterations in protein expression, or changes in the subcellular localization of NHE1. To explore these possibilities, we evaluated NHE1 activity in two conditions: cells grown in NaOH for three days to allow protein expression changes and cells exposed to NaOH for only five minutes before the NH₄Cl pre-pulse to assess rapid effects.

Figures 3D-F show that all cell types experienced allosteric inhibition of NHE1 activity upon brief exposure to NaOH despite no changes in pH_i (Figures 3A-C). However, after three days of growth in NaOH-containing media, NHE1 activity remained inhibited in cancer cells, while normal HK-2 cells exhibited a significant increase in NHE1 activity.

We also assessed whether NaOH influenced the sensitivity of the NHE1 pH sensor. In HK-2 cells, NaOH exposure did not alter the pH sensitivity of the NHE1 sensor. Conversely, even brief exposure to NaOH of cancer cells caused a leftward shift in NHE1 pH sensitivity, resulting in values comparable to those of normal cells (Figures 3G-I). This shift likely explains the rapid inhibition of NHE1 activity observed in cancer cells.

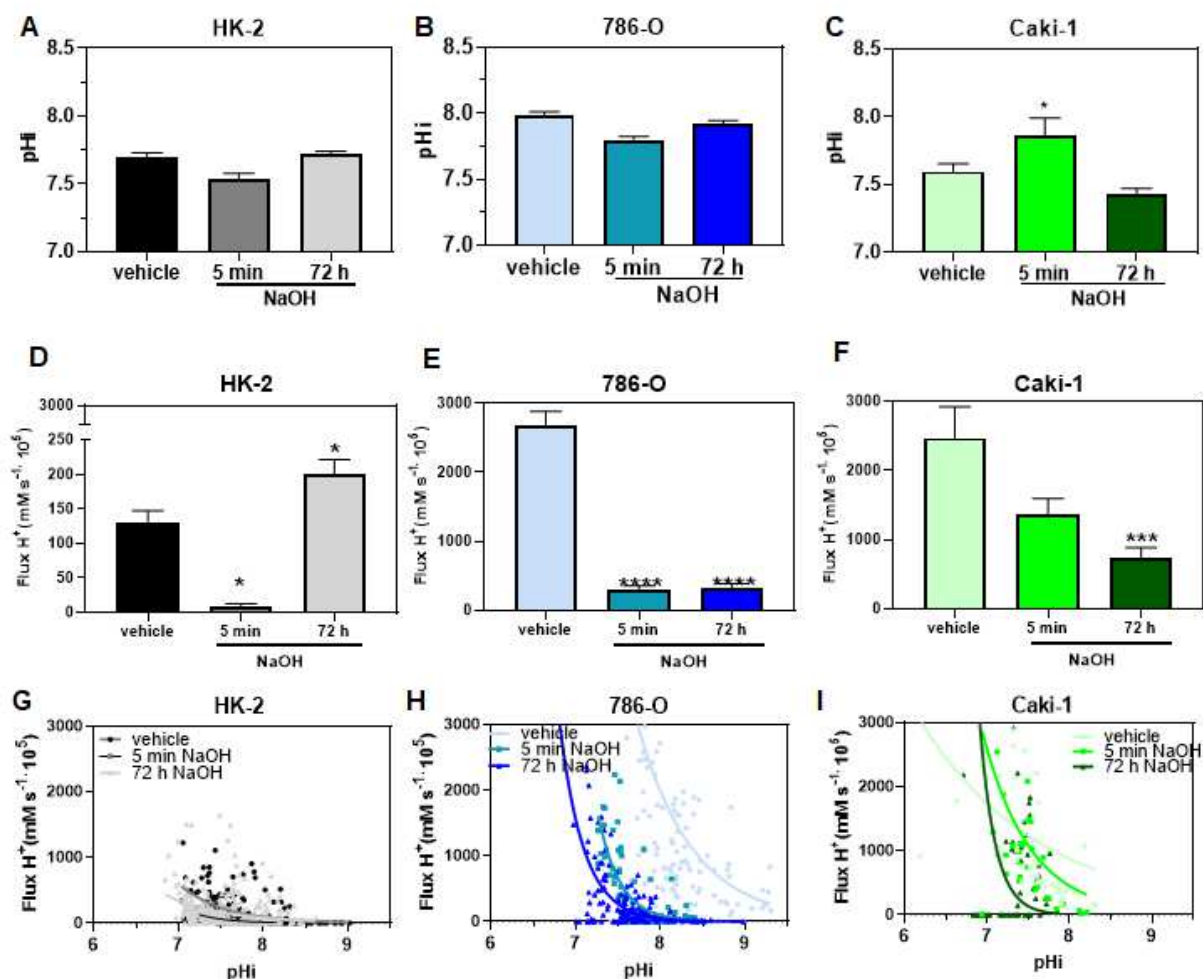


Figure 3 Effect of NaOH on pHi, NHE1-dependent H^+ flux and NHE1 pH sensitivity in HK-2, 786-O, and Caki-1 cells.

Cells were grown with Vehicle or 9.6 mM NaOH (for 72h, or 5 min), and pHi was measured with BCECF. **A-C pHi** **A-** HK-2 cells. **B-** 786-O cells. **C-** Caki-1 cells. **D-F-NHE1-dependent H^+ flux** was estimated by the ammonium pre-pulse technique. **D-** HK-2 cells. **E-** 786-O cells. **F-** Caki-1 cells. **G-I NHE1 pH sensitivity** **G-** HK-2 cells. **H-** 786-O cells. **I-** Caki-1 cells. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, vs vehicle. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line.

Differential NHE1 localization in cancer and healthy cells is altered by prolonged alkali exposure.

We investigated whether changes in NHE1 activity following NaOH exposure correlated with alterations in protein localization. Immunofluorescence studies were performed using cells stained with the plasma membrane marker WGA, and we analyzed the colocalization of WGA and NHE1. Figure 4 present representative images of stained cells and the Mander's coefficient for WGA-NHE1 colocalization, reflecting plasma membrane-associated NHE1 under each condition.

The Mander's coefficient was higher in cancer cells, confirming increased plasma membrane localization of NHE1. This finding correlates with the higher NHE1 activity observed in cancer cells compared to the lower activity in healthy cells. After a 5-minute exposure to NaOH, plasma membrane NHE1 localization remained comparable to control conditions across all cell lines, indicating that rapid changes in NHE1 activity in cancer cells were not due to altered protein localization.

However, prolonged exposure to NaOH resulted in significant changes in plasma membrane NHE1 localization. In healthy HK-2 cells, plasma membrane NHE1 levels increased after three days of NaOH treatment. In contrast, in 786-O cancer cells, plasma membrane NHE1 levels markedly decreased under the same conditions. Interestingly, metastatic Caki-1 cells did not exhibit changes in NHE1 localization following NaOH exposure.

These findings suggest that alterations in NHE1 localization may partially account for the observed changes in NHE1 activity after prolonged alkaline treatment.

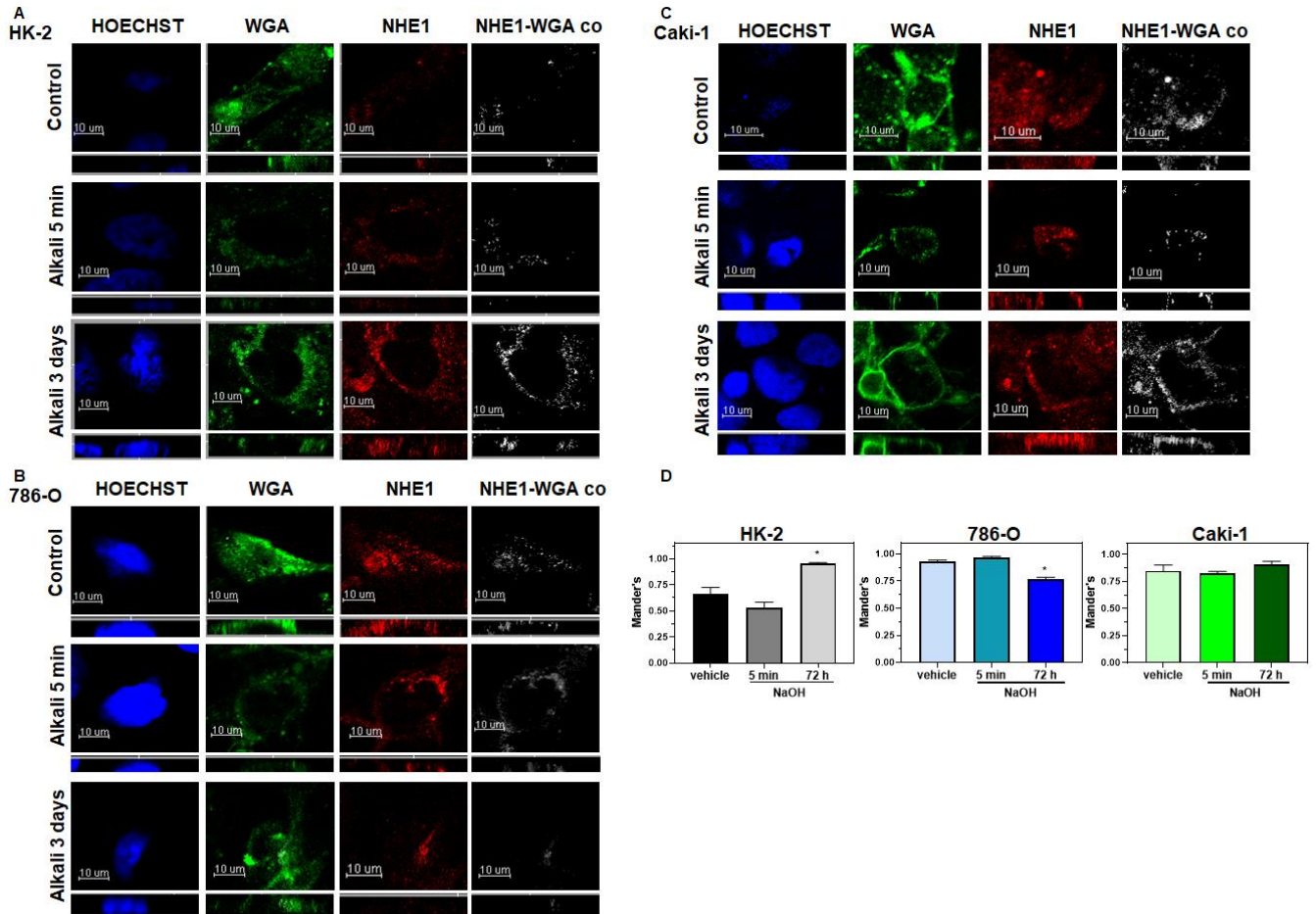


Figure 4: Localization of NHE1 in HK-2, 786-O, and Caki-1 cells

Cells were cultured under three conditions: Vehicle, 9.6 mM NaOH for 72 hours, or 5 minutes before NHE1 immunofluorescence analysis. The plasma membrane was stained using the membrane marker WGA. Representative images are shown for each condition. Nuclei are stained blue, WGA (plasma membrane) is green, NHE1 is red, and areas of WGA-NHE1 colocalization appear white. A- HK-2 cells. B- 786-O cells. C- Caki-1 cells. D- Colocalization analysis was performed and Mander's coefficient was calculated. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line. * $p < 0.05$, vs vehicle.

Discussion

Growing experimental evidence indicates that cancer-related pH alterations play a fundamental role in the etiopathogenesis of the disease [30]. Targeting these altered extracellular pH dynamics represents a promising strategy to destabilize cancer cells. Neutralizing extracellular pH may offer a novel therapeutic approach for clear cell renal cell carcinoma (ccRCC), a cancer type where conventional treatments often fail.

In this study, we demonstrate that neutralizing the extracellular microenvironment of ccRCC cells increases apoptosis in these cells. This finding aligns with previous reports showing that extracellular pH neutralization promotes cancer cell apoptosis across

various cancer types [7–10]. Specifically, in renal cell carcinoma, our results are consistent with earlier findings where a therapeutic strategy targeting cancer cell pH regulation achieved disease stabilization in a clinical case [16]. These observations support the potential of pH modulation as an effective adjunctive treatment for ccRCC and warrant further investigation.

Previous studies on pH neutralization have not thoroughly examined how normal cells respond to alkaline treatment. In this study, we show that healthy proximal epithelial cells increase apoptosis following mild alkalosis, a process dependent on the function of the NHE1 exchanger. This finding aligns with studies conducted on other cell types. For example, Cutaia et al. reported that human pulmonary arterial endothelial cells exhibit morphological changes and a marked increase in apoptosis under alkaline stress [31]. Similarly, Rivarola et al. demonstrated that apoptosis in collecting duct cells correlates with reduced cell growth and G2/M phase arrest after alkaline exposure. [32]. Our results reveal that healthy cells initially exhibit low levels of plasma membrane NHE1 and minimal NHE1-dependent H^+ flux. However, after three days of mild NaOH treatment, we observed increased plasma membrane localization of NHE1 and elevated NHE1-dependent proton flux. Given that alkalosis-induced apoptosis in healthy proximal cells depends on NHE1 activity, we hypothesize that the re-localization of NHE1 to the plasma membrane is a prerequisite for the apoptotic process.

ccRCC cancer cells exhibit high plasma membrane NHE1 levels, elevated NHE1-dependent proton flux, and characteristic extracellular acidosis. Previous studies have shown that an alkaline shift in the NHE1 proton sensor is an early and critical event in the malignant transformation of fibroblasts and keratinocytes [28]. Our findings confirm a similar pH sensor shift in ccRCC cells. This shift likely explains the sustained NHE1 activity at high intracellular pH, contributing to extracellular acidity. After a brief exposure to NaOH, we observed a sharp decrease in NHE1-dependent proton flux despite unchanged plasma membrane NHE1 levels. This decrease in NHE1 activity suggests that NHE1 undergoes allosteric inhibition as the pH sensitivity of the NHE1 proton sensor shifts to values comparable to those of healthy cells. Prolonged exposure to mild alkalosis further reduces plasma membrane NHE1 localization, particularly in 786-O cells. In summary, the alkaline-induced inhibition and altered localization of NHE1 in cancer cells may explain why NHE1 inhibition has a limited effect on cancer cell death when combined with alkalosis. We hypothesize that alkalosis-induced inhibition of NHE1 is a key factor in inducing cancer cell death.

What could explain the contrasting effects of NHE1 activity in healthy and cancer cells? NHE1 is involved in a wide range of cellular functions beyond ion transport, including volume regulation, actin remodeling, and cell migration. Its activity is also subject to multiple regulatory mechanisms, meaning that NHE1-dependent responses likely vary depending on the signals reaching the cell. In healthy cells, alkaline stress may activate NHE1, leading to cell death. In contrast, in cancer cells, the prolonged stress characteristic of this pathology, as described by Pedersen, may alter the response to alkaline stress [33].

NHE1 directly interacts with the cofactor proteins calcineurin B homologous protein 1 (CHP1) and CHP2 [34]. In vitro studies on lung carcinoma cells demonstrate that these cofactors regulate NHE1 activity. CHP1 exhibits ubiquitous localization, whereas CHP2 shows tissue-specific expression. Moreover, tumorigenic extracellular conditions, such as nutrient deprivation, modulate CHP2 expression [35]. These findings suggest that CHP2 interaction in cancer cells may contribute to the differential response of NHE1 to alkaline treatment observed between healthy and cancerous cells.

In conclusion, our study reveals that combining mild alkali treatment and NHE1 inhibition induces cancer cell death while minimizing damage to normal tissue. This therapeutic strategy offers a promising avenue for targeted therapy in clear cell renal cell carcinoma (ccRCC), potentially providing a novel approach for managing this challenging malignancy.

Author Contributions

Ana Celi conducted most of the validation, investigation, formal analysis and visualization.

Ana Mechali and Gisela Di Giusto contributed to some of the validation, investigation and formal analysis of pH experiments.

Natalia Beltramone was responsible for setting up the methodology of all cell models.

Juan José Casal developed the methodology, validation and software programming of KNIME workflow for automated cell counting from images and provided insights into all computer-based analyses and provided Data Curation.

Claudia Capurro assisted in Funding acquisition and Writing - Review & Editing.

Paula Ford and Gisela Di Giusto were involved in Funding acquisition, Conceptualization and Writing - Review & Editing.

Valeria Rivarola directed the project, so she is mainly involved in Conceptualization, Supervision, Formal analysis, Funding acquisition, Project administration and Writing - Original Draft and its Review & Editing.

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Figure Legends

Figure 1 Effect of mild alkali and NHE1 activity on Cell Death in HK-2, 786-O, and Caki-1 cells.

A- HK-2, 786-O, and Caki-1 cells were incubated for 72 hours with either vehicle or 1.2–9.6 mM NaOH. Apoptosis was quantified using the acridine orange/ethidium bromide staining method (AO/BE). **B-** Impact of NaOH on the pH of conditioned media after 72 hours with vehicle (-) or 9.6 mM NaOH (+). **p<0.01, ****p<0.0001; n=103. One-way ANOVA followed by Tukey's multiple comparisons test vs. HK-2 without NaOH. **C-E** Cells were treated with vehicle or 1 μM HOE 694 (NHE1 inhibitor) in the presence or absence of 9.6 mM NaOH for 72 hours. **C-** Apoptosis quantified by acridine orange/ethidium bromide staining. **D-** Apoptosis was assessed using the annexin V method. **E-** Necrosis measured using the annexin V method. One-way ANOVA followed

by Tukey's multiple comparisons test for each cell line. +p<0.05, ++p<0.01, +++p<0.001, vehicle vs. NaOH treatment; *p<0.05, ***p<0.001, active vs. inhibited NHE1.

Figure 2 pHi, buffering power and NHE1 activity in HK-2, 786-O, and Caki-1 cells. **A-** Basal pHi. **B-** Buffering power. **C-** NHE1-dependent H⁺ flux during the recovery of an ammonium pulse. One-way ANOVA followed by Tukey's multiple comparisons test for pHi, Buffering Power, or H⁺ flux. **** p<0.0001, 786-O, or Caki-1 vs HK-2 cells. +++ p<0.001, Caki-1 vs 786-O cells. **D-** pH dependence in each cell line when grown in standard media.

Figure 3 Effect of NaOH on pHi, NHE1-dependent H⁺ flux and NHE1 pH sensitivity in HK-2, 786-O, and Caki-1 cells.

Cells were grown with Vehicle or 9.6 mM NaOH (for 72h, or 5 min), and pHi was measured with BCECF. **A-C pHi** **A-** HK-2 cells. **B-** 786-O cells. **C-** Caki-1 cells. **D-F- NHE1-dependent H⁺ flux** was estimated by the ammonium pre-pulse technique. **D-** HK-2 cells. **E-** 786-O cells. **F-** Caki-1 cells. **G-I NHE1 pH sensitivity** **G-** HK-2 cells. **H-** 786-O cells. **I-** Caki-1 cells. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line. * p<0.05, *** p<0.001, **** p<0.0001, vs vehicle. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line.

Figure 4: Localization of NHE1 in HK-2, 786-O, and Caki-1 cells

Cells were cultured under three conditions: Vehicle, 9.6 mM NaOH for 72 hours, or 5 minutes before NHE1 immunofluorescence analysis. The plasma membrane was stained using the membrane marker WGA. Representative images are shown for each condition. Nuclei are stained blue, WGA (plasma membrane) is green, NHE1 is red, and areas of WGA-NHE1 colocalization appear white. **A-** HK-2 cells. **B-** 786-O cells. **C-** Caki-1 cells. **D-** Colocalization analysis was performed and Mander's coefficient was calculated. One-way ANOVA followed by Tukey's multiple comparisons test for each cell line. * p<0.05, vs vehicle.