Systemic alkalinisation delays prostate cancer cell progression in TRAMP mice

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**ABSTRACT**

The microenvironment of solid tumours is extremely acidic and this condition arises since the precancerous stage. This acidic milieu could therefore provide a useful target for both prophylactic and therapeutic approaches. In TRAMP transgenic mice, an in vivo model of prostate adenocarcinoma (AC), oral administration of alkaline water was devoid of unwanted side effects, and when started from an early age was as effective as NaHCO$_3$ in significantly delaying tumour progression, while when started when prostate tumours were already present, a nonstatistically significant trend in the same direction was detected. These findings indicate that the use of alkalinizing drugs should be considered for chemoprevention and, in association with standard chemotherapy, for treatment of human prostate AC.

**Introduction**

During tumour development, the microenvironment becomes progressively acidic due to different and often concomitant mechanisms: local hypoxia resulting from poor blood perfusion, increased flux of carbons through fermentative glycolysis, and the release by cancer cells of lysosome content into the extracellular matrix (ECM)\textsuperscript{1-3}. Indeed, extracellular acidosis in human solid tumours can reach pH values as low as 6.5. Likely, adaptation of cancer cells to an acid microenvironment occurs early during cancer progression, as pre-cancer cells undergo a metabolic switch in ATP generation, from oxidative phosphorylation to glycolysis. Since the latter pathway provides a much lower energy gain, transformed cells greatly increase their glucose uptake to meet their amplified metabolic requirements, resulting in intracellular lactate accumulation and the excretion of H$^+$ by proton transporters\textsuperscript{4}, causing progressive acidification of the extracellular milieu. This acidified habitat supports cancer cells with a stabilized glycolytic phenotype, which in turn leads to sustained generation of metabolic acids, even in well-oxygenated conditions, and to selection of cancer cells resistant to acid-mediated apoptosis\textsuperscript{5}. It has been hypothesised that these mechanisms lead to a competitive advantage of cancer cells toward normal bystander cells that cannot survive in an increasingly acidic microenvironment\textsuperscript{1-2}. Besides being toxic to normal cells, acidosis can stimulate invasion and metastatization by degrading and remodelling the ECM, increasing angiogenesis through the release of vascular endothelial growth factor, and inhibiting the immune response\textsuperscript{5,7-10}.

It is likely that targeting the driver functions that confer selective advantages to tumour cells can be a suitable alternative approach for cancer therapy. The reversal of pH gradient in cancer cells is increasingly considered as a hallmark of virtually all cancers, and a potential target for new anti-tumours therapies\textsuperscript{11}. In particular, alkalinizing treatment with existing molecules such as proton pump inhibitors (PPIs) and buffers, such as NaHCO$_3$, citrate or TRIS has been a proposed for human therapy\textsuperscript{2}. This approach has been supported by a clinical study on companion animals with spontaneous tumours in which the PPI lansoprazole, administered at high dose and combined with a water alkali, has proven effective in enhancing tumour response to metronomic chemotherapy\textsuperscript{12}, and by two clinical trials, in either osteosarcoma\textsuperscript{13} or metastatic breast cancer patients\textsuperscript{14}, where the administration of the PPI esomeprazole either improved the local effect of neoadjuvant chemotherapy or prolonged the time to progression and the overall survival rate in treated patients. Coming to the effect of alkali agent as monotherapy, it has been demonstrated that treatments with NaHCO$_3$ or lysine inhibit human mammary and prostate metastases, respectively, in mouse xenograft models\textsuperscript{15,16}, while the oral administration of a commercially available water alkali significantly reduced tumour growth in a syngeneic melanoma mouse model\textsuperscript{17}. However these results should be confirmed in a more physiological model, i.e. in transgenic animals spontaneously developing tumours.

TRAMP mice are the best model available so far for pharmaceutical studies on prostate carcinoma, since 100% of these animals display spontaneous multistage prostate carcinogenesis, with histological and molecular features similar to those present in human prostate cancer\textsuperscript{18,19}. Not surprisingly, the TRAMP model has been used to successfully test the chemopreventive efficacy of several natural anticancer agents such as green tea, grape, garlic, cabbage, tomato, hop\textsuperscript{20-26}. The efficacy of microenvironment alkalinization in such a model was assessed by Ibrahim-Hashim and co-authors. They found that NaHCO$_3$ in drinking water prevented the onset of prostate cancer in transgenic TRAMP mice, albeit it was ineffective in treating established tumours\textsuperscript{27}. Despite this excellent proof-of-principle demonstration, the authors themselves correctly stated that the administration of this regime to humans would be unadvisable, due to the unwanted side effects resulting from sustained intake of high doses of NaHCO$_3$. Moreover, sodium bicarbonate cannot be considered the ideal buffer for tumour
treatment and prevention for many reasons. First, NaHCO₃ is not a potent buffer molecule inasmuch as it can reach no more than pH 8.5 in a water solution. Moreover, it is unbalanced in term of electrolyte equilibrium, containing exclusively Na⁺, and therefore exposing to potential side effects in prolonged treatment regimens, including cardiovascular and renal dysfunctions. Lastly, at the concentration proposed in the Ibrham-Hashim’s paper it would result disgusting when used for oral administration, independently from the disease condition. We have therefore tested in the same TRAMP model, the anti-tumour effect of a water alkalinizer (AlkaWater®) through which the best pH condition can be reached in water solution (from pH 9.0 to 10.0) depending on the tumour and systemic pH; moreover, the taste of alkalinized water is comparable to that of either tap and mineral water and the solution is balanced in terms of electrolytes, containing Na⁺, K⁺, Ca²⁺ and Mg²⁺. The results have shown that the administration of alkaline water was devoid of any unwanted side effects, and when started from an early age was as effective as NaHCO₃, significantly delaying tumour progression, while when started when prostate tumours were already present showed a trend in the same direction.

Materials and methods

Cell culture and reagent

Tramp C1 prostate carcinoma cell lines (ATCC, Rockville, MD) were cultured in DMEM containing 10% FCS, glutamine and penicillin/streptavidin.

The alkaline stock solution contained NaCl and KOH (AlkaWater®) and was diluted 1:1000 in tap water to obtain a drinking solution at pH 9.5, and 1:300 for a solution at pH 10.5.

NaHCO₃ was dissolved in tap water at the concentration of 200 mM, as previously described²⁷.

Animals and anti-acid treatment

Animal studies and research protocols were reviewed and approved by the Ethics Committee of the IRCCS San Martino-IST and were conducted in accordance with the current Italian regulations and guidelines for the care and use of laboratory animals (D.L. 26/2014).

For in vivo studies, we used two different models, the spontaneous prostate tumour model developing in the transgenic TRAMP mice and xenograft model with TRAMP C1 cells.

TRAMP mice were maintained in heterozygosity by crossing C57Bl/6 TRAMP females with C57Bl/6 wild type males (Charles River Laboratories, Calco, Italy), and transgene verification was carried out when newborn mice reached 3 weeks of age using DNA extraction from tail clipping as previously described¹⁹. Transgene-verified mice and xenograft model with TRAMP C1 cells (ATCC, Rockville, MD) were cultured in DMEM containing 10% FCS, glutamine and penicillin/streptavidin.

Histology and immunohistochemical analysis

For histology and immunohistochemistry analysis, samples were fixed in 4% neutral buffered formalin, embedded in paraffin and cut to obtain 3–4 μm thick sections. Slides were then either stained with haematoxylin and eosin (H&E) for pathological analysis or processed for immunohistochemistry. All the evaluations were done in blind and scored independently by two investigators. Samples were examined for the presence of low or high grade PIN, well-differentiated adenocarcinoma (AC), phyllodes-like tumour (PHY) and neuroendocrine tumour (NE)¹⁹,²⁰.

Immunohistochemistry was performed using rabbit monoclonal anti-Ki67 or rabbit polyclonal anti-CD3 (Abcam, Cambridge, UK). Primary antibodies were diluted 1:150 and 1:100 respectively in PBS containing 1%BSA, and incubated for 1 h at RT. After washing, the slides were incubated with a biotinylated anti-rabbit secondary antibody (Pierce, Thermo Fisher Scientific, Waltham, MA) followed by peroxidase-conjugated streptavidin (BioSPA Biochemical, Milano, Italy). Samples were then stained using the Vectastain DAB Kit (Vector Laboratories, Burlingame, CA). To quantify proliferation, five randomly selected fields on each H&E-stained sections, from four different animals, were blindly photographed, with an oil-immersion 100× objective. Positive nuclei were counted and expressed as percentage of the total nuclei present in each field.

Statistical analysis

All statistical analyses were done with the IBM SPSS Statistic Software version 20 or 2×2 contingency table calculator (http://vassarstats.net/tab2x2.html).
Results

Alkaline water administration delays prostate AC progression in TRAMP mice

To evaluate toxicity of the treatment, we initially administered alkaline water at pH 10.5 to 5 C57/B6 male mice for two months, and looked for signs of toxicity in terms of pain, suffering or distress. In particular, we monitored behavioural signs and found no changes in posture, gait, activity and interactions with the environment. Moreover, we found no alteration in clinical signs, such as coat condition, nasal or ocular discharge, swollen eyes, increased respiratory rate, dyspnœa, tremors, excessive urination and consistency of faeces. Similarly, we did not register any significant difference in bw when treated mice were compared with control mice receiving tap water. At the time of necropsy, we macroscopically checked internal organs, with particular attention to liver and kidneys. No sign of toxicity was found (data not shown).

Afterward, we started to assess the therapeutic effect of alkaline water administration. Water consumption varied among the groups, with the NaHCO3-treated animals that doubled their intake (13.2 ± 1.47 ml/day/animal), compared to control group (7.25 ± 0.43 ml/day/animal). Conversely, the water intake for both groups, with the NaHCO3-treated animals that doubled their intake (13.2 ± 1.47 ml/day/animal), compared to control group (7.25 ± 0.43 ml/day/animal).

Afterward, we started to assess the therapeutic effect of alkaline water administration. Water consumption varied among the groups, with the NaHCO3-treated animals that doubled their intake (13.2 ± 1.47 ml/day/animal), compared to control group (7.25 ± 0.43 ml/day/animal). Conversely, the water intake for both “prevention” and “therapy” groups was slightly lower (5.8 ± 1.12 ml/day/animal and 5.2 ± 0.66, respectively).

At the established experimental end point (32 weeks of age), we sacrificed and thoroughly necropsied all animals. We found no animals with evidence of oedema or abnormal organ size, in target and non-target organs, however we observed hydronephroses in 11 out of the 29 (38%) animals fed with NaHCO3.

The animals in the “prevention” group displayed a significant (p = .017) decrease in UG/bw, with respect to the animals in the “control” group, while no difference with “control” group was found in the animals belonging to the “therapy” group (p = .26); the mice in the “bicarbonate” group showed a sharp decrease in the UG/bw ratio with respect to controls (p = .001) (Figure 1).

All collected tumours were then examined for both histological and immunohistochemical analyses. Tissue sections, stained with H&E, were evaluated in a blind assay by two different researchers. In order to evaluate the progression of the disease, we recorded the different type of lesions found on each slide (Figure 2).

Overall tumour incidence was 100% in all groups but comparison between “control” and “prevention” groups demonstrated that our treatment produced a decrease in the incidence of high grade PIN from 83 to 29.5% (p = .001), and a corresponding increase in the incidence of low grade PIN from 4.17 to 36.4% (p = .001) (Figure 3(a)). Also the administration of alkaline water with the “therapy” scheme affected PIN progression, and albeit statistical significance was not achieved, there was a clear reduction in the high to low PIN ratio, as compared to the “control” group (Figure 3(b)). The “bicarbonate” group, showed a significant decrease, with respect to “control” group, in the incidence of high grade PIN (from 83 to 52% p = .020), and a corresponding increase in the incidence of low grade PIN (from 4.17 to 44% p = .01).

As it regards AC incidence, we found with respect to “control” group, a reduction of 12.8% in the “prevention” group, of 46.7% in the “therapy” group and of 61.6% in the “bicarbonate” group.

We detected no statistically significant difference in PHY or NE incidence among the groups.

Immunohistochemical analyses evidenced no changes in tumour cell proliferation, measured by Ki67 staining, as well as in the distribution and amount of tumour infiltrating T cells between treated group and controls (data not shown).

Alkaline water administration delays tumour growth in xenotransplants of an androgen independent prostate cancer cell line

C57/B6 wild type male mice injected s.c. with TRAMP C1 syngeneic cells, were randomly divided into two groups that received either tap water or alkaline water at pH 9.5, and tumour growth was monitored (Figure 4). The growth of tumour nodules in treated mice was delayed respect to control mice. The delay was statistically significant till day 14 from cell injection (p = .018, p = .001 and p = .017 for day 10, 12 and 14 respectively). From day 17, the difference became not statistically significant, due to increase in standard error. Histology on tumours collected from the mice at the end of experiment, showed no differences in the histological types between the two groups (data not shown). Also, immunohistochemistry did not show any difference in tumour cell proliferation and tumour infiltration by T cells between the two groups (data not shown).

Discussion

The effect of alkaline water treatment confirms that the alkalinisation of the microenvironment has a prophylactic effect on prostate cancer progression, as already observed by Ibrahim-Hashim et al.27 with NaHCO3 using a small number of animals. We have followed this therapeutic strategy in a much larger cohort of mice and also using a different alkalinizing agent. We have found that oral administration of alkaline water or NaHCO3 to TRAMP mice, beginning at four weeks of age, are equal in inducing a delay in the progression of prostate AC, with reduction in the incidence over time of both high grade PIN and AC. Confirming previous results with NaHCO3, both the alkalinizing agents we tested the induced growing tumours to retain a more differentiated low grade PIN for longer.

However, the preventive treatment with alkaline water has the advantage to be devoid of the long-term unwanted side effects of a high dose NaHCO3. As a matter of fact, we have found nephropathy in a significant percentage of NaHCO3-treated mice. In these animals, an excess of water intake was also detected, suggesting possible long-term impact on blood pressure, and on cardiac and renal function. Instead, no tissue or organ impairment was detected in the two groups of mice treated with alkaline water. Indeed, the administration of high dose of NaHCO3 regimens to humans would be unadvisable27, making alkaline water a safer alternative.
The effect of the “therapy” scheme was less significant, however, there was a clear trend toward a delay in PIN progression and a reduction in AC incidence. This conclusion is supported by the results obtained with xenografts, a model of early tumour, which showed that alkaline water treatment delays tumour growth. These results are comparable to those obtained with NaHCO₃ treatments in a mouse model of mammary tumour 3. These findings are encouraging, since no therapeutic effect was detected when NaHCO₃ was administered to TRAMP mice with the same scheme 27.

It is of note that PHY tumours and NE tumours did not respond to the alkalinizing treatments. It remains to be elucidated whether these tumours do not rely on an acidic microenvironment for their growth, or if they have escape mechanisms that allow them to counter the effect of alkalinizing agents. However, the prostate...
tumours in humans are almost all ACs, the NE tumour accounting for about 4% of the total29 and the PHY tumours of the prostate being very rare32, therefore alkaline water may be considered a promising low-cost therapeutic approach that should be taken into account for prophylaxis and, combined with standard chemotherapy, for treatment of human prostate cancer. The few clinical trials available to date suggest that the anti-acidic/alkalinizing approach may well represent an efficient way to implement the existing anticancer therapies13,34.

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Disclosure statement
The authors report no conflicts of interest.

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