

Acidic pH at physiological salinity enhances the antitumor efficacy of lenvatinib, a drug targeting vascular endothelial growth factor receptors

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Abstract. Anti-angiogenic therapies have several clinical benefits for patients with tumors. The acidic environment of tumor cells degrades the extracellular matrix, releases several growth factors, such as vascular endothelial growth factor (VEGF), and induces angiogenesis. By contrast, a high-salt diet induces osmotic stress and salt-sensitive hypertension in patients treated with anti-angiogenic drugs. However, the consequences of various salinity conditions in combination with the pH of the tumor microenvironment have not yet been characterized, at least to the best of our knowledge, and are thus the focus of the present study. Herein, molecular dynamics simulations were performed between lenvatinib and VEGF receptor 2 (VEGFR2) at two different pH levels (acidic pH, 6.5; basic pH, 7.5) and three different salinity conditions (0.15, 0.30 and 0.45 M). The results suggested that, compared with the basic pH, the acidic pH reduced the binding affinity of lenvatinib to VEGFR2 and induced tumor cell viability by enhancing the expression of cyclin-dependent kinase 2 (CDK2), fatty acid synthase (*FASN*), DnaJ heat shock protein family (Hsp40) member C9 (*DNAJC9*), *c-JUN*, *Bcl-xL* and dual specificity phosphatase 6 (*DUSP6*). It was also observed that physiological salinity (0.15 M) reversed the tumorigenic effect of the acidic pH by enhancing the binding affinity of lenvatinib, decreasing cell viability and migration, inhibiting the expression of *CDK2*, *FASN*, *DNAJC9*, *c-JUN*, *Bcl-XL*, *BAX* and *DUSP6*, and increasing the expression of *VEGFR2* in MDA-MB-231 breast cancer cells. However, opposite results were obtained under the same salinity conditions at a basic pH. These results suggest that physiological salinity

conditions at an acidic pH enhance the antitumor efficacy of antiangiogenic drugs targeting VEGFR2. Overall, the present study suggests that the salinity and pH of the tumor environment play a crucial role in the antitumor efficacy of therapies targeting VEGFR2-mediated angiogenesis.

Introduction

Cancer is a life-threatening disease with a marked incidence (20 million) and mortality (10 million) as per Globocan 2022 (1). Angiogenesis plays an indispensable role in tumorigenesis due to the formation of new blood vessels from pre-existing blood vessels. Therefore, anti-angiogenic therapies are considered effective in the treatment of cancer. These therapies target various growth factors and their receptors of tumor cells (2,3). Vascular endothelial growth factor (VEGF) has been identified as a crucial regulator of both physiological and pathological angiogenesis, and the increased expression of VEGF is associated with the poor prognosis of patients with a number of types of cancer, such as liver, breast, lung cancer, etc. (4-7). To date, eleven anti-VEGF/VEGFR drugs have been approved for use in the treatment of cancer (axitinib, cabozantinib, pazopanib, sunitinib, sorafenib, ramucirumab, lenvatinib, bevacizumab, regorafenib, vandetanib, ziv-aflibercept) (3,8). For instance, lenvatinib has been used in combination therapies including immunotherapy and has exhibited unprecedented results (9-12). Lenvatinib inhibits the kinase activities of VEGF receptors (VEGFRs), such as VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4). However, strategies targeting VEGF and VEGFR have partially failed for two major reasons: i) The precise mechanisms of neo-angiogenesis are not yet clear; and ii) the abrogation of blood supply restricts drug delivery. Therefore, improvements in their efficacy are required (13).

The tumor microenvironment (TME) is classically acidic due to the Warburg effect (high rate of glucose uptake) and poor perfusion. Therefore, the production of organic acids increases due to the dependency of tumor cells on anaerobic glycolysis, even in the presence of oxygen, and the increased H⁺ ions are expelled by the upregulation of the sodium/hydrogen exchanger on the cell membrane, which creates an acidic environment (14). A pH of 6.2-6.9 has been reported for the

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extracellular TME of tumor cells (15). The pH of normal tissues varies between 7.3-7.4 (15). Acidity has been shown to enhance angiogenesis in tumor cells (16). It has been shown that the acidic pH of the TME degrades the extracellular matrix, releases several growth factors, including VEGF, and thus promotes angiogenesis (16). Similarly, an acidic pH has been shown to promote angiogenesis via the upregulation of matrix metalloproteinases and pro-angiogenic factors in human melanoma cells and in BALB/c nude mice (17). Previously, the role of an acidic pH in reducing the efficacy of anti-VEGFR2 therapy (sunitinib) has also been described (18). By contrast, research has suggested a role for hypertonic saline in the abrogation of tumors (19). For example, hypertonic saline attenuates tumor cell metastasis (20). However, the effects of salinity on anti-VEGFR therapies with changes in the pH of the TME have not yet been described, at least to the best of our knowledge.

The present study demonstrates that the interaction of lenvatinib with VEGFR2 induces the protonation of the D1046 and E885 residues of the lenvatinib-VEGFR2 complex. These residues have been previously reported to play a critical role in the binding of sunitinib to VEGFR2 via the formation of hydrogen bonds (21). The present study also demonstrates that an acidic pH reduces the binding affinity of lenvatinib to VEGFR2. In addition, the findings presented herein demonstrate that physiological salinity conditions can reverse the tumorigenic effects of acidic pH by reducing tumor cell viability, thus enhancing the antitumor efficacy of anti-angiogenic drugs targeting VEGFR2.

Materials and methods

Molecular dynamics (MD) simulations. The CASTp 3.0 tool was used to identify the binding pockets of lenvatinib with VEGFR2 (available online at <http://cast.engr.uic.edu>). The NAMD tool with CHARMM force fields (<https://www.ks.uiuc.edu/Research/namd/>) was used for MD simulations at pH 6.5 and 7.5, and three different salinity conditions (0.15, 0.30 and 0.45 M) (22). The structure of VEGFR2-lenvatinib (3WZD) was obtained from the Protein Data Bank. The system was solvated using TIP3P water molecules in an orthorhombic box. The system was then minimized and neutralized by the addition of various concentrations (0.15, 0.30 and 0.45 M) of sodium (Na⁺) and chloride (Cl⁻) ions using CHARM-GUI (23,24). The H⁺ server was used to examine the protonation states of both complexes at pH 6.5 and 7.5 (25). The simulation was initiated for 50 nsec at a constant temperature of 300 K. The simulation results are saved at a frequency of 2 fs. The VMD energy tool was used to examine the binding affinity of the lenvatinib-VEGFR2 complex. RMSD, RMSF, and Rg were calculated and plotted using the VMD, Bio3D v2.3-0 package (<http://thegranlab.org/bio3d/>) and R Studio. DCCM plots were plotted using the DCCM argument of Bio3D v2.3-0 package in R studio.

Cells and cell culture. The MDA-MB-231 cells were (National Centre for Cell Science, Pune, India) obtained from the National Center for Cell Science (NCCS). The MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; MilliporeSigma) supplemented with 10% v/v fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and

1% penicillin-streptomycin (PePn-Strep) (MilliporeSigma). The MDA-MB-231 cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. When the cells reached 90% confluency, they were subcultured in fresh growth medium for cytotoxicity and gene expression assays.

In-vitro cytotoxicity assay. A phase I/II trial of lenvatinib plus pembrolizumab plus fulvestrant for ER-positive/HER2-negative metastatic breast cancer is ongoing (NCT06110793). Moreover, a recent study demonstrated the therapeutic potential of lenvatinib in breast cancer (26). Therefore, the present study opted to use the MDA-MB-231 cells. The MDA-MB-231 cells were seeded on a 96-well cell culture plate at a concentration of 0.5x10⁵ cells/ml for 24 h. The following day, the cells were treated with 40 µmol/l lenvatinib (MerckMillipore) under three different salinity conditions (0.15, 0.30 and 0.45 M) in combination with two different pH levels (acidic pH, 6.5; basic pH, 7.5) for 24 h at 37°C with 5% CO₂. To obtain media at specific pH levels (pH 6.5 and pH 7.5), drops of 0.1 M HCl (HiMedia) or 0.1 M NaOH (HiMedia) were added gradually to the media until the desired pH level was reached (27). The salinity conditions (0.15, 0.30 and 0.45 M) were achieved by diluting 1 M stock solution of NaCl (HiMedia) in culture media (19). The concentration of lenvatinib was selected based on previous studies (28,29). Subsequently, 20 µl of 5 mg/ml MTT (MilliporeSigma) was added to each well, mixed and incubated for 4 h at 37°C. The supernatants were removed and 100 µl DMSO were added to each well to dissolve the purple crystal formazan. The absorbance was measured on a microplate reader (BioTek Instruments, Inc.) at a wavelength of 570 nm to estimate cell viability. The percentage of cell viability is expressed as the mean ± SEM of four independent experiments.

In-vitro wound healing assay. The MDA-MB-231 cells (1.0x10⁶ cells/well) were seeded in 24-well plates and grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum (FBS) and 1% penicillin-streptomycin for 24 h at 100% confluency. Serum starvation was not used since it has been shown that the presence of 10% FBS has no influence on the scratch area (30). The assay was performed as previously described (31,32). A scratch was then made using a sterile 20-200 µl pipette tip in each well, and the detached cells were removed using 500 µl phosphate-buffered saline (PBS) (HiMedia Laboratories, LLC) and gently shaken for 1-2 min. The cells were treated with 40 µmol/l lenvatinib under physiological salinity conditions (0.15 M) in combination with two different pH (acidic pH, 6.5; basic pH, 7.5) for 24 h at 37°C with 5% CO₂. Prior to image acquisition, the plates were washed with PBS. A pre-warmed medium or sample was then added again, and images were obtained. Scratch closure was monitored and imaged at 24 h using a Nikon Eclipse Ti-2E microscope (Nikon Corporation) at x10 magnification and 1/3,700 sec exposure time. ImageJ software (version 1.54h) was used to measure the scratch area (National Institutes of Health).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the MDA-MB-231 cells using TRIzol reagent (cat. no. 79306; Qiagen, Inc.) as previously described (33). Complementary DNA (cDNA)

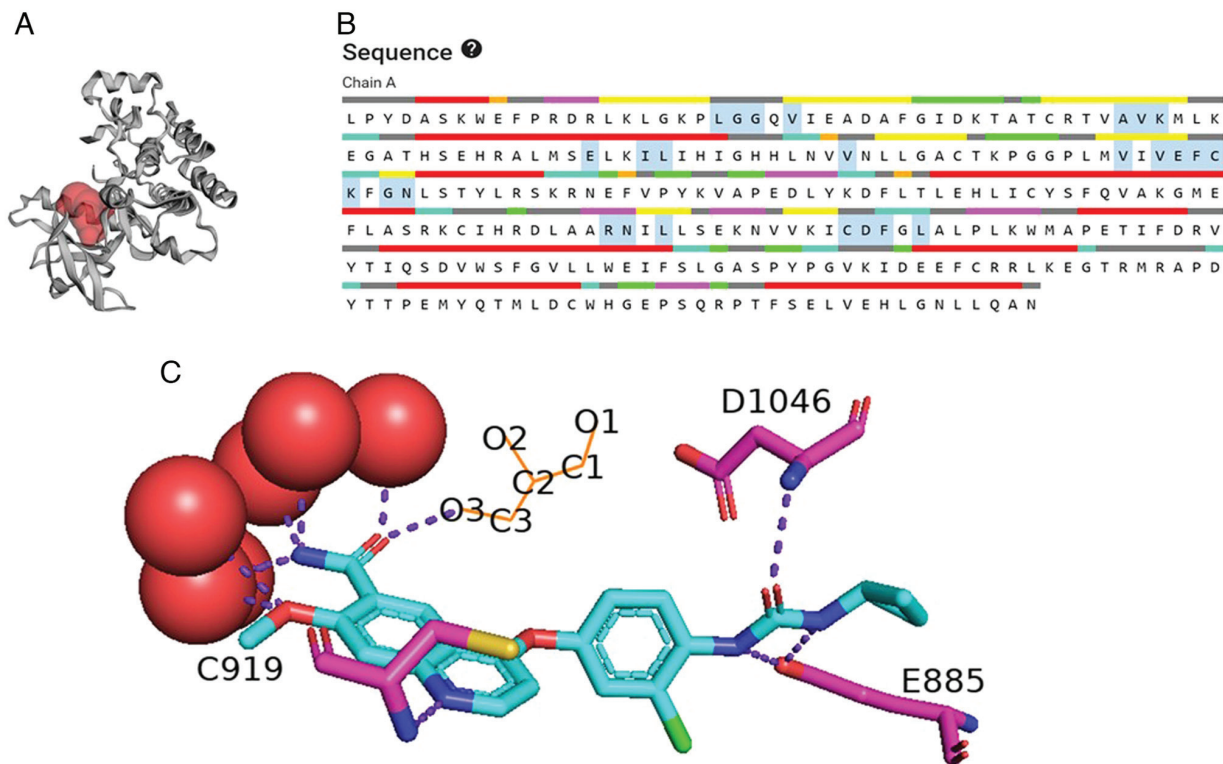


Figure 1. Binding pocket identification by CASTp server: (A) The binding sites of lenvatinib (magenta) to VEGFR2 protein (grey). (B) Crystal color shaded boxes highlight the amino acid residues present in the binding site. (C) Molecular docking of lenvatinib to VEGFR2. Cyan color represents lenvatinib; magenta color stick represents different amino acid residues of VEGFR2, purple color showing hydrogen bonds between lenvatinib and VEGFR2, red spheres represent water molecules and orange line indicates glycerol. VEGFR2, vascular endothelial growth factor receptor 2.

was synthesized from 100 ng RNA using a G-Biosciences cDNA synthesis kit (cat. no. 786-5020). qPCR was performed in triplicate with 2X SYBR-Green qPCR Master Mix from G-Biosciences (cat. no. 786-5062) under the following conditions, apart for *Bcl-xL* and *BAX*: 95°C for 3 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The conditions for *Bcl-xL* were as follows: 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec and 62°C for 60 sec. The conditions for *BAX* were the following: Holding at 50°C for 2 min, 95°C for 4 min, followed by 45 cycles at 95°C for 10 sec, 54°C for 5 sec, 72°C for 5 sec, and 83°C for 15 sec. The relative expression levels of the target genes were calculated using the comparative Cq method (relative expression = $2^{-\Delta\Delta C_q}$) using β -actin as an internal control (34). The following primer sequences were used to amplify cyclin-dependent kinase 2 (*CDK2*), fatty acid synthase (*FASN*), DnaJ heat shock protein family (Hsp40) member C9 (*DNAJC9*), *c-JUN*, dual specificity phosphatase 6 (*DUSP6*) and β -actin: Human *CDK2* forward, 5'-CTGCCATTCTCATCGGGTC-3' and reverse, 5'-ATTTGCAGCCCAGGAGGATT-3'; human *FASN* forward, 5'-CTTCCGAGATTCCATCCTACGC-3' and reverse, 5' TGGCAGTCAGGCTCACAAACG 3'; human *DNAJC9* forward, 5'-CTCTCCTGTGCTCACCAAG-3' and reverse, 5'-AGCCAGCTCTTCTTCCGAAC-3'; human *c-JUN* forward, 5'-GTGCCGAAAAGGAAGCTGG-3' and reverse, 5'-CTGCGTTAGCATGAGTTGGC-3'; human *DUSP6* forward, 5'-TCCCTGAGGCCATTCTTTTCATAGATG-3' and reverse, 5'-GCA GCTGACCCATGAAGTTGAAGT-3'; human *Bcl-xL* forward, 5'-TCAGGCTGCTTGGGATAAAG-3' and reverse, 5'-AGG

CTTCTGGAGGACATTTG-3'; human *BAX* forward, 5'-GGA CGAACTGGACAGTAACATGG-3' and reverse, 5'-GCA AAGTAGAAAAGGGCGACAAC-3'; human *VEGFR2* forward, 5'-GGACTCTCTCTGCCTACCTCAC-3' and reverse, 5'-GGCTCTTTCGCTTACTGTTCTG-3'; and human β -actin forward, 5'-GGACTTCGAGCAAGAGATGG-3' and reverse, 5' AGCACTGTGTTGGCGTACAG 3'.

Statistical analysis. Each experiment was repeated four times, and the results are presented as the mean $\pm$ SEM. Differences in cell viability and migration were assessed using one-way analysis of variance followed by the Bonferroni-post hoc test. OriginPro (Version:2019b) was used for all statistical analyses. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

High salt concentrations induce the protonation of aspartic acid 1046 and glutamic acid 885 residues in the VEGFR2-lenvatinib complex. The lenvatinib binding pockets were predicted using CASTp 3.0 (available online at <http://cast.engr.uic.edu>). The binding pocket of lenvatinib (3WZD) is lined by L840, G841, G846, V848, A866, V867, K868, E885, I888, L889, V899, V914, V916, E917, F918, C919, K920, G922, N923, L1032, N1033, L1035, C1045, D1046, F1047 and L1049 (Fig. 1A and B). The area and volume of the binding pocket were 331.787Å² and 245.871 Å³, respectively. The amino acids occupying the binding pocket are illustrated in Fig. 1A and B.

Table I. Acid dissociation constant (pKa) of D1046 and E885 amino acid residues of VEGFR2-lenvatinib complex (3WZD) and VEGFR2 alone.

A, pKa of D1046				
Salinity	VEGFR2-lenvatinib	VEGFR2	VEGFR2-lenvatinib	VEGFR2
	pH 6.4	pH 6.4	pH 7.5	pH 7.5
Control	0	0	0	0
0.15 M	4	0	3.9	0
0.30 M	4.38	0	4.36	0
0.45 M	4.54	0.13	4.56	0.19

B, pKa of E885				
Control	0	0	0	0
0.15 M	1.47	1.2	1.46	1.19
0.30 M	2.04	0.32	2.05	0.35
0.45 M	2.38	0.66	2.38	0.7

Table II. Analysis of root mean square deviation and radius of gyration of VEGFR2-lenvatinib complex (3WZD) at two different pH and three different salinities (0.15 M, 0.30 M, 0.45 M).

VEGFR2-lenvatinib		
A, RMSD		
Salinity	pH 6.4	pH 7.4
Control	1.54	1.53
0.15 M	1.55	1.50
0.30 M	1.51	1.61
0.45 M	1.53	1.49

B, Radius of gyration		
Control		
0.15 M	40.34	40.33
0.30 M	40.26	40.24
0.45 M	40.01	40.25

The H⁺⁺ server indicated that compared to VEGFR2 alone, the pKa value of the D1046 residue of VEGFR2 in the VEGFR2-lenvatinib complex (PDB: 3WZD) was high at every salinity condition at both acidic and basic pH. Similar results were obtained for the E885 residue of VEGFR2 in the VEGFR2-lenvatinib complex (Table I). These results demonstrate that the binding of lenvatinib to VEGFR2 induces the protonation of the D1046 and E885 residues of VEGFR2 under different saline conditions at both acidic and basic pH. The results of molecular docking suggested that lenvatinib forms a polar interaction with both the D1046 and E885 residues of

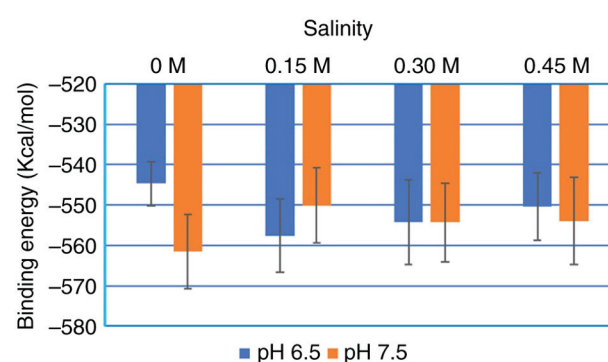


Figure 2. Effect of pH either alone or in combination with the indicated salinities on the binding affinity of lenvatinib to VEGFR2. VEGFR2, vascular endothelial growth factor receptor 2.

VEGFR2 (Fig. 1C). Therefore, the high protonation of these residues may affect the binding affinity of lenvatinib for VEGFR2 by affecting hydrogen bond formation.

Effect of D1046 and E885 protonation on the binding affinity of lenvatinib with VEGFR2 at various salt concentrations and pH. To observe the effects of various salinities on the binding affinity of lenvatinib to VEGFR2, MD simulations were performed using the protonated state of the VEGFR2-lenvatinib complex. The results demonstrated that, compared with the acidic pH, the basic pH enhanced the binding affinity of lenvatinib to VEGFR2 under no salinity conditions (pH 6.5: -544±11.56; pH 7.5: -561±9.18). However, the difference was not significant (P=0.28). The same pattern was not observed for physiological salinity conditions (0.15 M). At 0.15 M salinity, the binding affinity was higher with the acidic pH compared with the basic pH (pH 6.5 and 0.15 M salinity: -557±9.14; pH 7.5 and 0.15 M salinity: -547±9.25; Fig. 2). No major changes in binding affinity were observed for other salinity conditions (pH 6.5 and 0.30 M salinity: -554±7.32; pH 6.5 and 0.45 M

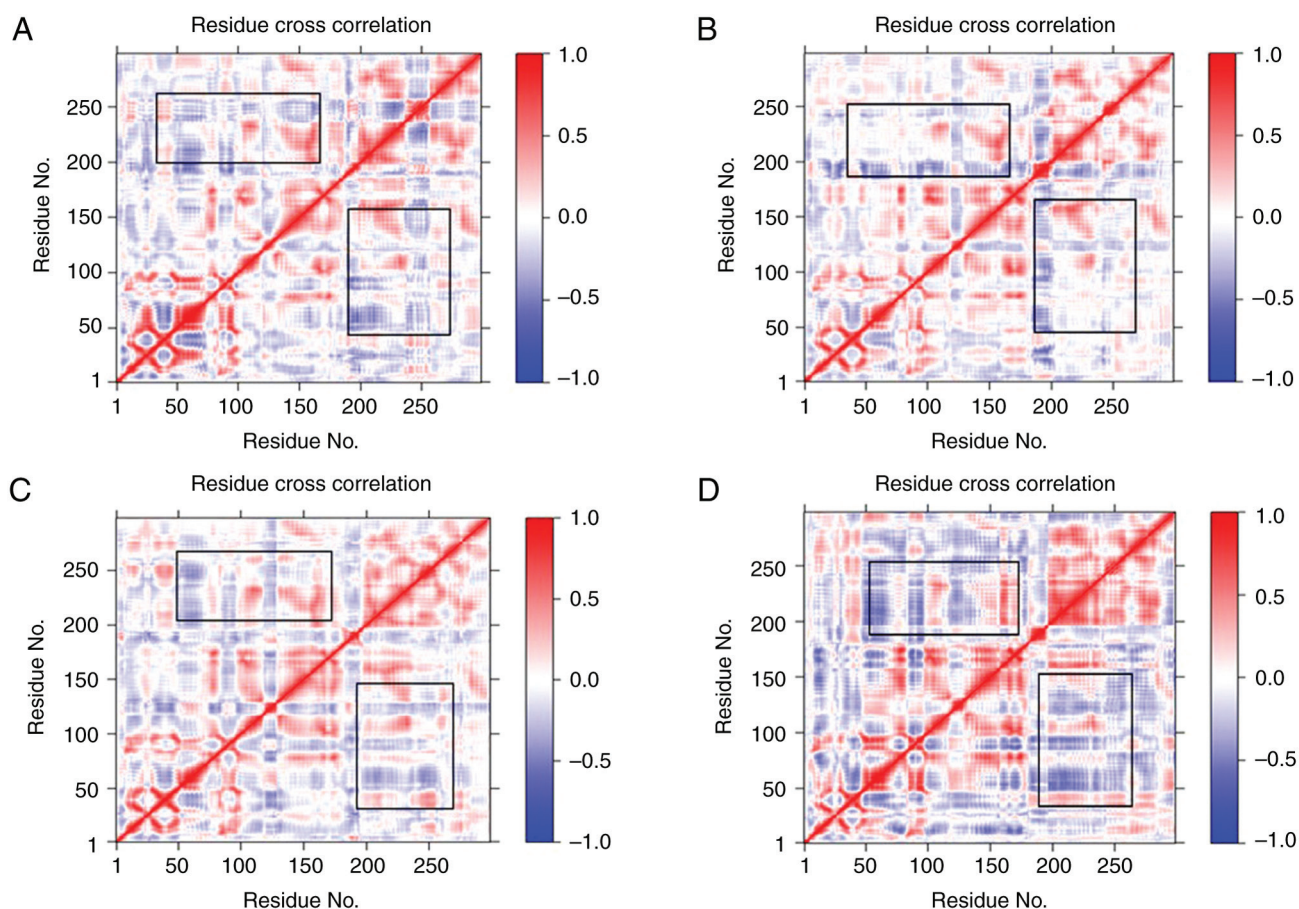


Figure 3. Dynamical cross-correlation map of KDR-lenvatinib (3WZD) at (A) pH 6.4 (B) pH 7.5 (C) pH 6.4 with 0.15 M salinity (D) pH 7.5 with 0.15 M salinity. The squared box highlights the variance in the correlated motions. Relatively higher anti-correlated movement (blue color) of amino acid residues is observed at pH 7.5 simulation in the presence of (D) 0.15 M salinity when compared to (C) pH 6.5 at 0.15 salinity.

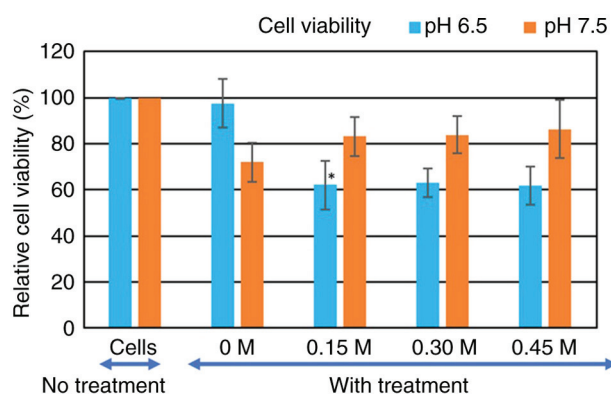


Figure 4. Effect of pH either alone or in combination with the indicated salinities on the cell viability of lenvatinib treated MDA-MB-231 breast cancer cells. * $P < 0.05$ vs. pH 6.5 and no salinity.

salinity: -550 ± 8.43 ; pH 7.5 and 0.30 M salinity: -554 ± 6.64 ; pH 7.5 and 0.15 M salinity: -554 ± 10.12 ; Fig. 2; $P = 0.75$). It was also observed that, compared with the non-salinity conditions, various salinities and pH did not significantly affect the RMSD and radius of gyration of the VEGFR2-lenvatinib complex (Table II).

Overall, these results suggest that an acidic pH in combination with 0.15 M salinity or basic pH alone induce the binding affinity of lenvatinib to VEGFR2 when compared with basic

pH at 0.15 M salinity and acidic pH, respectively. However, the difference was not found to be significant.

Dynamical cross-correlation map (DCCM) analysis of lenvatinib at physiological salinity. Since differences in binding energy and RMSD were more prominent in the case of the lenvatinib-VEGFR2 complex under physiological (0.15 M) and no salinity conditions, DCCM was conducted for this complex under these conditions only. Dynamic cross-correlation measures the atomic fluctuations or displacements of amino-acid residues with respect to other residues in the same protein. The values of correlation were between -1 and 1. Positive 1 (+1) stands for complete correlation (red), negative 1 (-1) indicates complete anti-correlation (blue), and 0 indicates no correlation (white). Plots were generated automatically using the Bio3D package in the R Studio software (<http://thegrantlab.org/bio3d/>).

When comparing the DCCM of simulations of the protonated complex of 3WZD at acidic and basic pH at 0.15 M salinity, it was observed that the residues in range 1013-1063 (in figure residues no. 200-250) and residues in range 865-865 (in figure residues no. 50-150) in chain A of the complex exhibited an anti-correlated direction of dynamical movement at 0.15 M salinity and pH 7.5 in comparison to same salinity conditions at pH 6.5 (Fig. 3). These were the regions flanking the binding pocket (DI046: in figure residue number

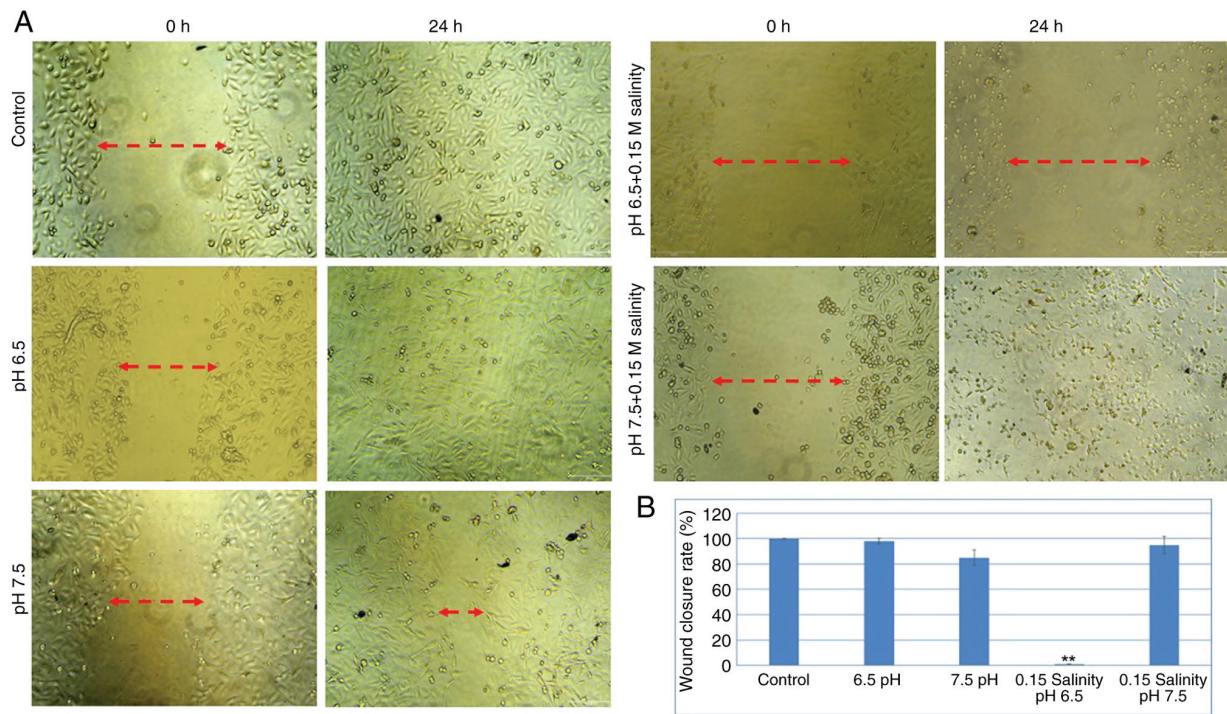


Figure 5. Effect of pH either alone or in combination with the indicated salinities on the cell migration of MDA-MB-231 breast cancer cells treated with lenvatinib (40 μ mol/l) for 0 and 24 h. (A) Representative images of migrating cells under microscope at x10 magnification, before and after injury. Red lines with arrows indicate wound area being measured. (B) Bar graph representation of wound closure rate (%). The experiments were repeated four times. ** $P < 0.01$ vs. pH 6.5 and no salinity.

D233) of the complex (Fig. 3B and D). This anti-correlation resulted in wayward movement of the binding pocket, leading to the opening of the lenvatinib-binding cleft, and thus, may be responsible for the low binding affinity at basic pH and 0.15 M salinity. In addition, this anti-correlation was observed at pH 6.5 compared to pH 7.5 under no salinity conditions (Fig. 3A and C).

Effect of salinity and pH on the anti-proliferative efficacy of lenvatinib in triple-negative breast cancer. It was hypothesized that as the binding affinity of lenvatinib for VEGFR2 is lower at pH 6.4, it may enhance tumor cell viability. To examine this hypothesis, cancer cells were treated with lenvatinib at pH 6.5 and 7.5. The MTT proliferation assay demonstrated that lenvatinib reduced tumor cell proliferation at pH 7.5 (72 vs. 100%) compared with that at pH 6.5 (97 vs. 100%) (Fig. 4). However, cell proliferation was reduced at 0.15 M saline conditions and pH 6.4 when compared to same salinity conditions at basic pH (pH 6.5 and 0.15 M salinity: 62%; pH 7.5 and 0.15 M salinity: 83%). By contrast, an 11% increase in cell viability was observed at basic pH and 0.15 M salinity (pH 7.5 and 0.15 M salinity: 83%; pH 7.5: 72%) (Fig. 4).

Effect of salinity and pH on cell migration in triple-negative breast cancer. In the wound healing assay, the migration of the MDA-MB-231 cells was examined in response to the mechanical scratch wound at physiological salinity conditions at two different pH levels. Images of the scratch areas from the time points 0 and 24 h are illustrated in Fig. 5. The results of the wound healing assay demonstrated that the migration of the cells was completely inhibited at pH 6.5 with 0.15 M salinity at

24 h compared with pH 6.5 and no salinity (pH 6.5: $98 \pm 2.34\%$; pH 6.5 + 0.15 M salinity: $1 \pm 0.24\%$; $P < 0.01$). Migration was also inhibited at pH 7.5 compared with pH 6.5 (pH 6.5: $98 \pm 2.34\%$; pH 7.5: $85 \pm 6.23\%$). It was also observed that salinity conditions at pH 7.5 induced cell migration compared with pH 7.5 and no salinity (pH 7.5: $85 \pm 6.23\%$; pH 7.5 + 0.15 M salinity: $95 \pm 6.84\%$) (Fig. 5A and B). Overall, these findings indicate that salinity conditions at acidic pH inhibit the migration of MDA-MB-231 cells.

Effect of physiological salinity on the expression of anti-cancer genes at two different pH levels. To investigate the mechanisms underlying the reduced cell viability under acidic conditions at 0.15 M salinity, the expression levels of seven cancer-associated genes (*CDK2*, *FASN*, *DNAJC9*, *c-JUN*, *DUSP6*, *Bcl-xL*, *BAX*) were measured in TNBC cells along with *VEGFR2*. Using RT-qPCR, it was found that compared with the acidic pH alone, 0.15 M salinity at an acidic pH enhanced the expression of *VEGFR2* (pH 6.5: 0.70 ± 0.21 fold; pH 6.5 and 0.15 M salinity: 1.20 ± 0.12 fold) and *DNAJC9* (pH 6.5: 0.54 ± 0.03 fold; pH 6.5 and 0.15 M salinity: 1.65 ± 0.78 fold) and reduced the expression of *CDK2* (pH 6.5: 1.33 ± 0.34 fold; pH 6.5 and 0.15 M salinity: 0.73 ± 0.23 fold), *FASN* (pH 6.5: 3.97 ± 1.03 fold; pH 6.5 and 0.15 M salinity: 1.45 ± 0.45 fold), *c-JUN* (pH 6.5: 3.58 ± 0.98 ; pH 6.5 and 0.15 M salinity: 1.72 ± 0.13 fold), *DUSP6* (pH 6.5: 1.81 ± 0.45 fold; pH 6.5 and 0.15 M salinity: 1.21 ± 0.15 fold), *Bcl-xL* (pH 6.5: 0.46 ± 0.11 fold; pH 6.5 and 0.15 M salinity: 0.02 ± 0.02 fold). The expression of apoptotic protein, *BAX*, increased at acidic pH and physiological salinity, when compared to acidic pH alone (pH 6.5: 0.017 ± 0.003 fold; pH 6.5 and 0.15 M salinity: 0.15 ± 0.04 fold)

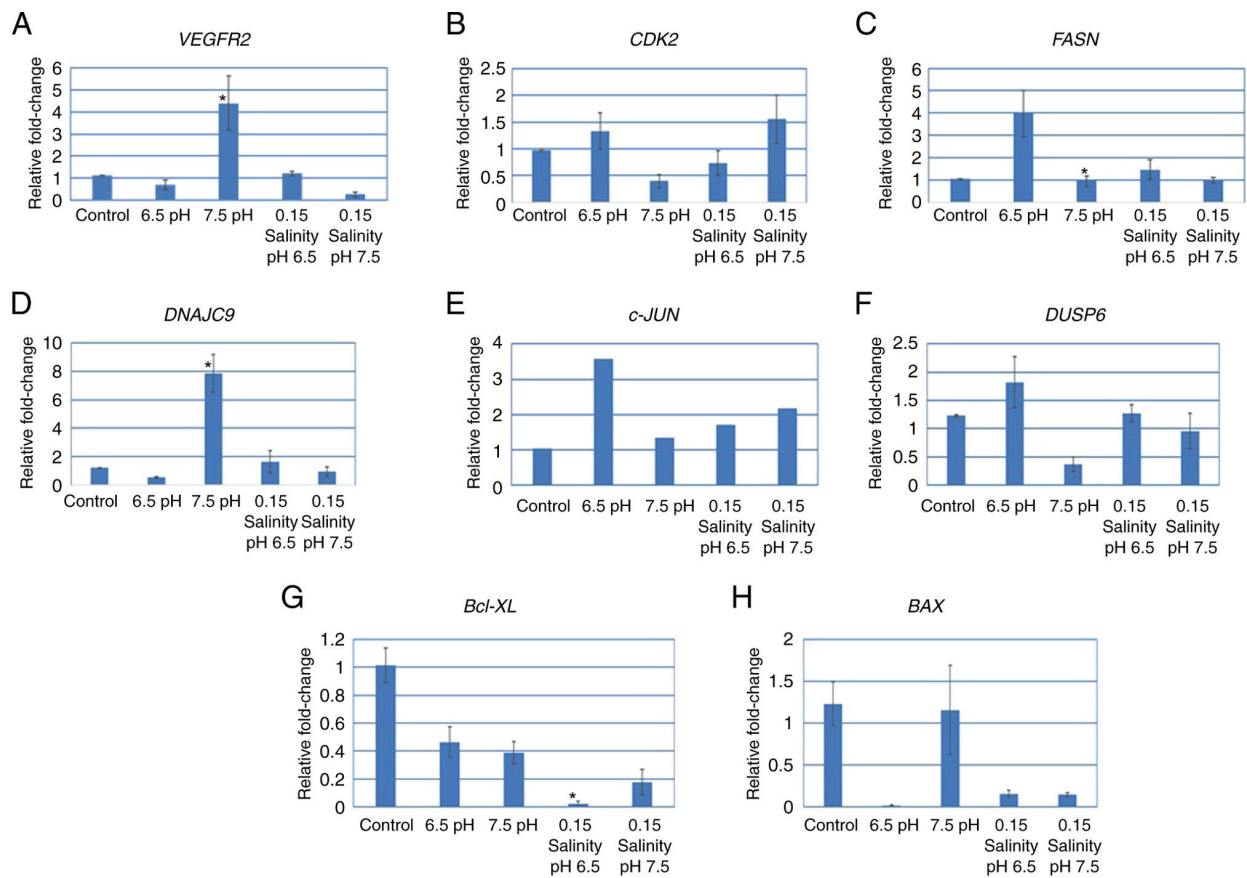


Figure 6. Effect of pH either alone or in combination with the indicated salinities on the expression of (A) *VEGFR2*, (B) *CDK2*, (C) *FASN*, (D) *DNAJC9*, (E) *c-JUN*, (F) *DUSP6*, (G) *Bcl-xL*, and (H) *BAX* genes. * $P < 0.05$ vs. pH 6.5 and no salinity. *CDK2*, cyclin-dependent kinase 2; *FASN*, fatty acid synthase; *DNAJC9*, DnaJ heat shock protein family (Hsp40) member C9; *DUSP6*, dual specificity phosphatase 6.

(Fig. 6). Similar results were obtained at a basic pH in the absence of salinity conditions when compared with an acidic pH (*VEGFR2*: pH 6.5: 0.70 ± 0.21 fold; pH 7.5: 4.39 ± 1.23 fold; $P < 0.04$), (*CDK2*: pH 6.5: 1.33 ± 0.34 fold; pH 7.5: 0.39 ± 0.12 fold), (*FASN*: pH 6.5: 3.97 ± 1.03 fold; pH 7.5: 0.95 ± 0.23 fold; $P < 0.04$), (*DNAJC9*: pH 6.5: 0.54 ± 0.03 fold; pH 7.5: 7.86 ± 1.32 fold; $P < 0.01$), (*c-JUN*: pH 6.5: 3.58 ± 0.98 fold; pH 7.5: 1.34 ± 0.32 fold), (*DUSP6*: pH 6.5: 1.81 ± 0.45 -fold; pH 7.5: 0.37 ± 0.13 fold), *Bcl-xL* (pH 7.5: 0.38 ± 0.08 fold; pH 7.5 and 0.15 M salinity: 0.17 ± 0.09 fold) and *BAX* (pH 7.5: 1.15 ± 0.53 fold; pH 7.5 and 0.15 M salinity: 0.14 ± 0.02 fold) (Fig. 6). These results suggest that either a basic pH in the absence of salinity or an acidic pH in the presence of physiological salinity will enhance the treatment efficacy of lenvatinib by reducing the expression of tumorigenic genes.

Discussion

The efficacy of anti-angiogenic therapies is not high and efforts are required to enhance the efficacy of these drugs. For example, lenvatinib has been used in combination with immune checkpoint therapies and has shown enhanced efficacy in early phase clinical trials (9). Excess salt intake has recently been shown to significantly inhibit tumor growth in two independent murine tumor transplantation models by completely blocking murine-derived immunosuppressive

cells (MDSCs) (35). An increase in the number of MDSCs in peripheral blood, lymphoid tissue and tumor sites has been found to be significantly associated with unfavorable outcomes and the shorter survival of patients with multiple cancer types such as breast cancer, lung cancer, multiple myeloma (36). There is increasing evidence to indicate that MDSCs may be an effective target for combatting immune resistance and for harnessing immune checkpoint blockade (36,37). These studies suggest a possible role of a high-salt diet (HSD) in enhancing the efficacy of anti-angiogenic drugs when used in combination with immune checkpoint blockade therapy. It has been shown that the acidic pH of the tumor microenvironment decreases the efficacy of sunitinib by reducing the expression of *VEGFR2* (16,18). However, only a limited number of studies have suggested a role for high salt intake in inhibiting tumor growth by enhancing antitumor immunity (35,38). The dual role of a HSD has been shown to regulate angiogenesis. For instance, a HSD inhibits angiogenesis during chronic muscle stimulation (39), but can also enhance skin lymphangiogenesis (40). It has also been shown that anti-angiogenic drugs such as sunitinib block the VEGF pathway via Na^+ drainage, leading to Na^+ accumulation in the skin and salt-sensitive hypertension (40). However, the role of salinity at different pH levels in the treatment efficacy of anti-angiogenic therapies has not yet been reported, at least to the best of our

knowledge. In the present study, it was found that, compared with the slightly basic conditions (pH 7.5), the acidic pH (pH 6.5) reduced the efficacy of lenvatinib by reducing its affinity to VEGFR2. These results support the findings of previous research, which demonstrated that a prominent *in vitro* angiogenic response is observed at a low pH when stimulated by exogenous growth factors in the rat aortic ring model (41). The effect of acidic pH on binding affinity was reversed in the presence of physiological salinity conditions. Moreover, the same conditions of acidic pH at 0.15 M salinity reduced the viability of triple-negative breast cancer cells. Contrasting results were obtained for basic pH and 0.15 M salinity. These results suggested that acidic conditions enhanced the efficacy of lenvatinib in the presence of 0.15 M salinity. These observations suggest that a slight increase in intratumoral pH can enhance the efficacy of drugs targeting VEGFR2-mediated angiogenesis. However, basic conditions in the presence of salt are not advantageous due to the anti-correlated motion of the amino acid residues around the binding pocket of VEGFR2. This anti-correlated motion results in the opening of the lenvatinib-binding cleft. It appears that dynamics at pH 7.5 and physiological salinity conditions reduced the binding of lenvatinib with VEGFR2.

Lenvatinib binds to VEGFR2, and any changes in the expression of VEGFR2 affect its binding to VEGFR2. Therefore, the present study first compared the expression values of VEGFR2 at two different pH levels and 0.15 M salinity conditions. The high expression of VEGFR2, either at a basic pH alone or acidic pH at 0.15 M salinity, suggests a possible reason for the high binding affinity of lenvatinib to VEGFR2 under both conditions. These results support the findings of previous research, which demonstrated that the basic pH of the TME enhanced the expression of VEGFR-2 by endothelial cells and therefore potentiated the anti-tumor effects of sunitinib (18). Mechanisms other than the increased expression of VEGFR-2 by tumor cells also play a crucial role in tumor resistance to anti-VEGF therapies. For instance, anti-angiogenic drugs lead to a tumor-resistant phenotype via non-VEGF-mediated vascularization and the secretion of various pro-angiogenic factors (42). Moreover, *CDK2*, *c-JUN*, and *FASN* are expressed at high levels in proliferating endothelial cells, thereby regulating angiogenesis (43-46). These genes regulate tumorigenesis; the present study also observed that 0.15 M salinity at an acidic pH decreased the expression of *CDK2*, *DUSP6*, *c-JUN*, and *BAX* when compared to acidic conditions only. Contrasting results were obtained under basic pH conditions at physiological salinity in comparison with the basic pH. Surprisingly, we also observed increased expression of DNAJC9 at acidic conditions and physiological salinity, suggesting that lenvatinib enhance HSP70 promoted TNF-mediated apoptosis in breast cancer cells. Previous studies have shown that DNAJC9 induce HSP70 expression which may lead to TNF-mediated apoptosis (47,48). These results highlight the mechanisms that may be involved in the efficacy of anti-VEGF therapies at an acidic and basic pH. It has been well-established that acidity promotes angiogenesis, the cancer stem cell phenotype, drug resistance and mutation rates, and hinders immune cell functions (49). For example, an acidic TME abrogates the activation of cytotoxic T- and natural killer cells. An acidic pH inhibits the maturation of dendritic

cells from myeloid precursors, thus hindering antigen presentation (49). The acidic environment of tumor cells can be created using carbonic anhydrase IX (CAIX) (50). However, molecules targeting CAIX have been shown to enhance the efficacy of anti-VEGF therapies. These results suggest that tumor acidity may play a role in decreasing the efficacy of antiangiogenic drugs (51). A number of other proteins also play critical roles in regulating the pH of the TME (52,53). Previous reports have demonstrated that sodium bicarbonate can be used to increase the extracellular pH in tumor xenografts, and it ultimately favors antitumor responses of immune cells by enhancing vascular density and recruitment of immune cells to the tumor site (18,54). However, to the best of our knowledge, the role of salinity with changes in the pH of tumor cells has not yet been investigated. The present study demonstrated that 0.15 M salinity at an acidic pH reduced cell viability and migration by inhibiting the expression of *CDK2*, *c-JUN*, *DUSP6*, *Bcl-xL*, and by enhancing the expression of *VEGFR2*. These results further highlight that 0.15 M salinity conditions can enhance the antitumor efficacy of anti-VEGF drugs at an acidic pH, and may thus be advantageous due to the acidic environment of tumor cells.

In conclusion, the present study demonstrates that 0.15 M salinity enhances the antitumor efficacy of anti-VEGF drugs at an acidic pH. Since cancer cells are in an acidic condition, the present study suggests that the use of physiological salinity conditions can enhance the antitumor efficacy of anti-angiogenic drugs. The findings further demonstrated that salinity may play a key role in anti-angiogenic treatments targeting VEGFR2. It is suggested that the efficacy of anti-angiogenic drugs used either alone or in combination with ICB therapies should also be explored in the presence of salinity conditions. However, the present study has the following main limitations: i) The efficacy of only one drug on single cancer cells was evaluated in the presence of various pH levels and salinity conditions; and ii) the data were not validated in small animals. Therefore, the authors aim to focus on these limitations in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SP performed all the analyses. BP performed the cell culture experiment. MP performed RT-qPCR. RG was responsible for the conceptualization of the study and manuscript drafting. All the authors have read and approved the final manuscript. SKP and RG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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