

# Effects of boric acid on invasion, migration, proliferation, apoptosis and miRNAs in medullary thyroid cancer cells

#### Onurcan Yıldırım ( dronurcanyildirim@gmail.com )

Ege University Faculty of Medicine: Ege Universitesi Tip Fakultesi https://orcid.org/0009-0006-2998-4877

#### Mücahit Seçme

Ordu Üniversitesi Tıp Fakültesi: Ordu Universitesi Tip Fakultesi

#### Yavuz Dodurga

Pamukkale Üniversitesi Tıp Fakültesi: Pamukkale Universitesi Tip Fakultesi

#### Gülçin Abban Mete

Pamukkale Üniversitesi Tıp Fakültesi: Pamukkale Universitesi Tip Fakultesi

#### Semin Melahat Fenkci

Pamukkale Üniversitesi Tıp Fakültesi: Pamukkale Universitesi Tip Fakultesi

#### Research Article

Keywords: Medullary thyroid cancer, boric acid, TT cells, Apoptosis, miRNA

Posted Date: July 21st, 2023

#### DOI: https://doi.org/10.21203/rs.3.rs-3164400/v1

License: (a) This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

## Abstract Background

Medullary thyroid cancer (MTC) is an aggressive, chemoresistant form originating from the thyroid parafollicular C cells, has spurred interest in alternative treatments like boric acid, a boron-based compound has demonstrated anti-carcinogenic effects.

## **Materials and Methods**

Cell viability were determined using 2,3-bis(2-methoxy-4 nitro-5- sulfophenyl- 2H-tetrazolium- 5carboxanilide (XTT) assay.. Total RNA was isolated with Trizol reagent for gene and miRNA analysis via reverse transcription polymerase chain reaction (RT-PCR). Terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) and comet assays evaluated boric acid's impact on apoptosis and genotoxicity, respectively. We also examined its influence on cell invasion, colony formation, and migration using matrigel- chamber, colony formation, and wound healing assays.

### Results

50% lethal dose (IC50) of boric acid was 35 µM at 48 hours. Real-time PCR showed changes at apoptosis-related genes, and miRNAs post-treatment. Significant increases in the expression of *NOXA, apoptotic protease activating factor 1 (APAF-1), Bcl-2-associated X protein (Bax), caspase-3,* and *caspase-9,* which are associated with apoptosis, were observed. Additionally, the expression of *B-cell lymphoma 2 (bcl2), B- cell lymphoma- extra-large (bcl-xl),* and *microRNA-21 (miR-21),* which are linked to the aggressiveness of MTC, was significantly reduced. The TUNEL assay revealed a 14% apoptosis rate, while assays showed a 30.8% decrease in cell invasion, a 67.9% decrease in colony formation, reduced cell migration, and increased DNA breaks post-treatment.

## Conclusions

In conclusion, our findings suggest that boric acid may have potential as an anticancer agent in medullary thyroid cancer and other cancers with similar mechanisms.

#### Introduction

Medullary thyroid cancer is a rare type of thyroid cancer that produces calcitonin hormone and originates from parafollicular C cells of neural crest origin. It accounts for 5–10% of all thyroid cancer types. The mechanism of medullary thyroid cancer is defined within the activation of RET. Sporadic medullary

carcinoma (sMTC) consist of 75% cases of medullary carcinoma. The remaining 25% cases are familial medullary carcinoma (hMTC) which is specified with RET oncogene [1].

Genetic diagnosis in hMTC provides an opportunity to provide a possible cure with total prophylactic thyroidectomy. However, this is different when most sMTC are diagnosed, and metastases are present to lymph nodes and distant organs, particularly bone, liver and lung. Today, a surgical protocol consisting of total thyroidectomy and lymph node dissection is applied in both types. The effectiveness of systemic chemotherapy is low, and the side effect profile is high, and tyrosine kinase inhibitors had limited effects in advanced metastatic MTC [2].

RET oncogene mutations reveal some level of explanatory mechanisms in explaining MTC tumorigenesis, but these are not sufficient. The role of RET in MTC progression is still poorly illuminated and RET has been shown to cooperate with different signal transduction pathways, especially in the metastatic context [3].

In this context, in recent years, studies on MicroRNAs and genes related to cell cycle and apoptosis have been very much to reveal progression, tumor development and similar in MTC and other types of cancer [2, 4].

Boron as a non-metal element does not exist in the form of a pure element in nature. Borax, boric acid, colemanite, kernite, ulexite and borate are different forms of boron structures. Absorbed boron is found mostly as Boric acid in the human's systems [5].

Previous in vivo and in vitro studies showed that boron has anti-cancer effects. These effects are also existing in the prostate cancer [6, 7], colon cancer [8], lung cancer [9], breast cancer [10], and in malign melanoma [11].

The aim of this study is to evaluate the anticancer effect of boric acid on TT medullary thyroid cancer cells in vitro and try to understand molecular mechanism of boric acid activity. For this purpose, the effects of boric acid on cell cycle control and apoptosis genes, migration, invasion, proliferation and some of miRNA's connected with medullary thyroid cancer were evaluated.

#### Material and methods

## Cell culture

Thyroid medullary carcinoma TT cell line (ATCC, CRL 1803<sup>™</sup>) and human thyroid fibroblast (HTF; ScienCell Cat No: 3730) cell lines were used in this study. Both cell lines were cultured under suitable conditions at 37°C in 5% CO2 and cells were grown in Dulbecco Modified Eagle Medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Capricorn Scientific), 20 units/ml penicillin and 20 µg/mL streptomycin, 0,1 mM amino acid solution (Biological Industries) and 1mM sodium pyruvate (Biological Industries). Different concentrations (10µM, 20µM, 35µM, 50µM, 75µM,

## $100\mu$ M, $200\mu$ M, $500\mu$ M) of boric acid (Etimaden) were applied to the cells in a time and dose-dependent manner.

## Cell proliferation XTT assay

Effects of boric acid on cell proliferation in TT medullary thyroid carcinoma and Human thyroid fibroblast were detected by XTT (2,3-bis (2-methoxy-4 nitro-5- sulfophenyl)- 2H-tetrazolium-5-carboxanilide) assay according to manufacturer procedure (Cell Proliferation Assay with XTT Reagent-Cell Proliferation Kit;

Biotium cat no: 30007). TT and HTF cells were seeded into 96-well plates at a concentration of  $1 \times 10^4$  cells per well. After 24 hours of incubation, the cells were treated with 10 µM, 20 µM, 35 µM, 50 µM, 75 µM, 100 µM, 200 µM, 500 µM concentrations of boric acid during 24, 48 and 72h. Dose concentration range was selected by using references in the literature as Boric acid exert anti-cancer effect in poorly et al 2019 and Barranco WT and Eckert CD 2004 [6, 12]. Untreated cells were used as control cells. After the incubation period, XTT mixture added and then formazan formation was determined spectrophotometrically at 450 nm (reference wavelength 630 nm) by a microplate reader (Biotek). Viability (%) was calculated using the background- corrected absorbance as follows:

Viability (%) = Absorbance of experiment well / Absorbance of control well × 100

IC50 doses of boric acid on TT cells were evaluated by GraphPad Prism 8 computer programme. IC50 dose was used as dose group in other studies such as invasion, migration, TUNEL, Real-Time PCR and comet assay.

## RNA isolation, cDNA synthesis, and real-time PCR (RT-PCR)

Total RNA from control and dose group of TT medullary thyroid carcinoma cells were isolated by Trizol Reagent (Invirogen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA), according to the manufacturer's protocol.

*Caspase-3, caspase-9, B- cell lymphoma 2 (Bcl- 2), B- cell lymphoma- extra-large (Bcl-xl), apoptotic protease activating factor 1 (APAF-1), Bcl-2-associated X protein (Bax), NOXA genes were used for cell cycle, and cell apoptosis pathway expression analysis in this study and expression profiles were calculated using the beta- actin (house-keeping gene) as the reference. Real-time PCR tests were performed by according to the SYBR Green qPCR Master Mix (Applied Biosystem, USA) protocol. RT-PCR assay was performed using gene- specific primers. The sequences of primers were given in Supplementary Materials (Supplementary Table 1).* 

miRNA expression change was also determined by using RT-PCR. miRNA cDNA synthesis kit (abm) was used for cDNA synthesis and subsequently relative quantification of *hsa-miR-21-5p*, *hsa-miR-224-5p* was analyzed by RT-PCR according to evagreen (abm) master mix (abm) protocol. miRNAs expressions were normalized to U6 as the human endogenous control.

## TUNEL assay

The apoptotic effects of boric acid in TT medullary thyroid cancer cells were assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. The control and dose group of TT cells were fixed with 4% (w/v) paraformaldehyde, and the cell apoptosis was analyzed using a commercial kit (Tunel In Situ Cell Detection Kit, AAT bioquest) according to the manufacturer's protocol. TT cells were stained with Hoechst dye and then observed under a fluorescence microscope (Olympus Inc., Tokyo, Japan). The total cells and TUNEL-positive cells were counted in 10 randomly selected fields in the fluorescence microscope. The results were expressed as a percentage of TUNEL-positive cells defining the ratio of apoptotic cells to the total cells.

## Matrigel-Invasion assay

Analyzing the number of invasive chambers were performed using Matrigel invasion chambers (Thermo scientific LOT: 159467) following the manufacturer's instructions. Briefly,  $2x10^5$  cells with serum-free DMEM were plated onto the upper chambers of Matrigel-coated filter inserts, and serum-containing DMEM medium (500 µL) was added to the lower chambers as a chemoattractant. After incubation at 37°C for 24h, filter inserts were removed from the wells. A cotton-tipped swab removed the non-invasive cells on the upper

surface of the filter. The cells that invaded the lower surface of the filter were fixed with methanol for 10 minutes (min) and stained with crystal violet. Using an inverted microscope, the cells that invaded in the lower surface of the filter were counted. The assays were performed in triplicate. The percentage of invasion was computed using the following equation:

 $Invasion (\%) = \frac{\text{The number of cells in matrixel matrix basement membrane}}{\text{The number of cells in control membrane}} x100$ 

## **Colony formation assay**

Colony formation assay was performed to examine the colony formation of TT cells treated with boric acid. Cells at the exponential growth phase were digested with trypsin and counted using the trypan blue dye exclusion test. Then, the cells were suspended in a DMEM medium containing 10% fetal bovine serum. The seeding of the cells into a six-well plate was done at a density of 10<sup>3</sup> cells/well. The medium was changed every three days for 2 to 3 weeks. When macroscopically visible colonies appeared in the culture dish, colonies were fixed in methanol for 10 minutes and stained with crystal violet. The morphology of colonies was observed under a microscope, and the numbers of colonies were counted.

#### Wound-healing migration assay

Anti-migratory effects of boric acid on TT medullary thyroid cancer cells were determined by woundhealing assay. The control and dose group TT cells were seeded in 60×15 mm style cell culture dishes at 10<sup>6</sup> cells per well and allowed to adhere overnight at 37°C with 5% CO2. After the growing cell layers had reached about 90% confluence, a scratch was made via sterile 200-µL plastic pipette tip. The cells were washed with 2 ml serum-free DMEM to remove all detached cells. Cells were further incubated for 24 hours with an IC50 dose of boric acid. Then evaluated the closure at 24 hours using a light microscope. Images of the TT cell proliferation were taken. The assays were performed in triplicate.

## Comet assay

Effects of boric acid on DNA damage and genotoxic situations in TT medullary thyroid cancer cells were evaluated. Slides were prepared by covering with high-melting agarose (HMA). Control and dose group cells were dissolved in PBS after tripsinization and cell suspension was mix with low-melting agarose in the microcentrifugation tube. All slides were immersed in freshly prepared cold lysing solution [2.5M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris (pH 10) and 10% DMSO] and incubated at 75 minutes 4°C). Following incubation, the slides were placed in an electrophoresis buffer [0.3 M NaOH, 1mMNa2EDTA; pH 13] for 20 min at 4°C. Electrophoresis process were performed at (25 V (300 mA, approx. 0.74 V/cm)) for 35 min at 4°C. Then, the slides were washed three times with dH20, and incubated in neutralization buffer [0.4 M Tris; pH 7.5] for 5 minutes each. The slides were incubated in methanol for 5 minutes at – 20°C to fix the cells to the slides. After the staining of slides with ethidium bromide, cells were evaluated by fluorescence a microscope (Nikon) with the comet assay IV Version 4.3.2 for the Basler FireWire programme. Head length (µm), tail length (µm), head density (%), tail density (%) and tail moment parameters were measured.

## Statistical analysis

Real-time PCR data were evaluated by the  $\Delta\Delta$ Ct method for the statistical analysis of the findings and quantified with a computer program. The comparison of the groups was performed with the "Volcano Plot" analysis, from "RT<sup>2</sup> Profiles PCR Array Data Analysis", which is assessed statistically using the "student *t*- test." IBM SPSS V21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) analysis program was used to perform the parametric and nonparametric analysis of dose and control groups. *P*< 0.05 was considered to indicate statistical significance.

#### Results

## Anti-Proliferative effects of boric acid in TT medullary thyroid cancer

The anti-proliferative activity of boric acid in TT medullary thyroid cancer cells was determined by XTT assay. Live cells reduced tetrazolium to orange colored formazan. In this way, the effect of boric acid on the TT cell line was investigated depending on time and dose and the IC50 dose of boric acid was found to be  $35\mu$ M in the 48th hour (Fig. 1).

## mRNA expression of genes and miRNA by real-time PCR

cDNAs, which are synthesis from total RNA from control and dose group cells were performed, and Real Time PCR was used to detect the expression of genes and proteins which are missioned in apoptosis pathways and cell cycles and some of miRNAs, which are related with medullary thyroid cancer. It was observed that boric acid in TT cell line caused a significant increase in the apoptosis-related gene expression of *caspase-3, caspase-9, Bax, NOXA, APAF-1* which are inducing apoptosis and a significant decrease in the apoptosis related protein expression of *bcl-2* and *bcl-xI* which are negative regulator of apoptosis (Table 1).

#### Table 1 The mRNA expression changes of apoptosis genes, and miRNAs mRNA expression in TT cancer cells treated with boric acid compared with the control group cells. Data were obtained by RT-PCR assay via ΔΔCt method in RT2 Profiles PCR Array Data Analysis online program.

Apoptosis Related Genes			
Gene Names	Fold Regulation	p-value	
Caspase-3	2.3613	0.015	
Caspase-9	1.3332	0.046	
BAX	4.2143	0.024	
BCL2L	-2.1713	0.038	
BCLXL	-2.1624	0.002	
NOXA	3.0652	0.022	
APAF1	7.8504	0.030	
Apoptosis Related miRNAs			
miRNAs Names	Fold Regulation	p-value	
hsa-miR-21-5p	-4.3648	0.020	
hsa-miR-224-5p	-8.9801	0.004	

\*BAX: BCL2 Associated X, Bcl-2: B-cell lymphoma 2, BCL2L: B-cell lymphoma-extra-large, NOXA: Phorbol- 12-myristate-13-acetate-induced protein 1, APAF1: Apoptotic Peptidase Activating Factor 1, p < 0.05 statistically significant.

## Apoptotic effects of boric acid in the TT cell line

TUNEL test was used to check the data about apoptosis. In the light of the data obtained, the percentage of apoptotic cells was compared between the dose and the control group. In the control group, the rate of

apoptotic cells was determined as 4%, and in the dose group in which IC50 (35 μM) dose of boric acid was added, the rate of apoptotic cells was determined as 14% (Fig. 2).

## Anti-invasive effect of boric acid in the TT cell line

Matrigel invasion chamber assay was used to detect of effect boric acid on invasion in the TT cell line. It observed that boric acid inhibited the invasion of dose groups, compared with the control groups. According to the results, the invasion capacities of the control cells were  $63.22 \pm 3.5\%$ , while the percentage of invasion was  $32.44 \pm 2.5\%$  in the dose group administered boric acid (Fig. 3) (p = 0.046).

## Effects of boric acid on colony formation of the TT cell line

In order to understand the effect of boric acid on colony formation, colonies larger than 0.1 cm were evaluated. While the average colony in the control group cells was 412, the number of colonies was determined as 134 in the dose group cells treated with boric acid ( $35 \mu$ M). This significant decrease was expressed graphically after the counting process, and it was found that the number of colonies was suppressed by 67.9% in TT cells with boric acid exposure. The colony images of the cells were evaluated, it was remarkable that the number of colonies between the control group colony number and the boric acid administered dose group was significantly different, and that there was significant decrease in the number of colonies of the TT cell line after boric acid (Fig. 4).

### Effects of boric acid on migration in the TT cell line

Wound healing migration assay was used for detecting of the effect of boric acid on migration in the TT cell

line. Cells in the control and dose groups were photographed at 0 and 24 hours. As a result of the experiment, it has been shown that boric acid inhibits cell migration more than the control group (Fig. 5).

## Detection of DNA damage caused by boric acid in the TT cell line

Comet assay was used for observed to DNA damage of the TT cell line caused by boric acid. Damage in DNA was assessed by measuring the head and tail length, density and tail moment, which occurred after the migration of the DNA, whose supercoil structure was impaired, towards the anode in gel electrophoresis. Significant changes were observed in head and tail length, density, and tail moment (Table 2, Fig. 6).

Table 2 Comet Assay - Genotoxicity Test Results				
	Control Group	Dose Group	p-value	
Head length (µm)	48.72 ± 10.29	44.31 ± 9.22	0.024	
Tail length (µm)	38.15 ± 12.5	44.68 ± 11.48	0.002	
Head density (%)	84.04 ± 11	66.92±16.86	< 0.001	
Tail density (%)	15.96 ± 11	33.07 ± 16.86	< 0.001	
Tail moment	3.2 ± 2.78	7.4 ± 4.38	< 0.001	
*p < 0.05 statistically significant				

#### Discussion

This study investigates the potential anti-cancer properties of boric acid, focusing specifically on its effects on medullary thyroid cancer cells. We examined the influence of boric acid on cell migration, invasion, proliferation, and the expression of cell cycle and apoptosis-related genes and miRNAs. Since there are no prior studies on boric acid's direct impact on medullary thyroid cancer cells, comparisons were made with similar experiments involving other cancer cell lines from the literature [13].

We evaluated the cytotoxic effects of boric acid on TT cells, determining an IC50 dose of 35 µm at 48 hours. This dose was used for subsequent tests to assess changes in variables compared to control groups. We studied the expression of apoptosis and cell cycle regulating genes and proteins in both control and treated TT cell groups to understand boric acid's anti-cancer effects. The expression of certain miRNAs related to MTC was also examined.

We studied the activation of caspase-3, a cysteine-aspartic acid protease family member, in conjunction with caspase-8 and caspase-9, across both extrinsic and intrinsic apoptotic pathways [14, 15].

In an in-vitro study by Barranco and Eckhert in 2004, it was shown that activity and expression of *caspase-3* did not change after treatment with boric acid in DU-145 cells [16]. However, Hacioglu et al. showed a significant dose-depended increase in expression of *caspase-3* in DU-145 cells [6].

Scorei et al. determined that calcium fructoborate and boric acid inhibits proliferation of breast cancer cells and they showed that calcium fructoborate acid increases expression and activity of *caspase-3*, but they didn't detect any increase of *caspase-3* in cells which treated with boric acid [16]. According to our results, we observed a significant increase in expression of *caspase-3* and *caspase-9*.

The Bcl-2 gene family includes Bcl-xl and Bcl-2, anti-apoptotic proteins that block cytochrome c release, and Bax, a protein promoting release of pro-apoptotic factors from mitochondria [17]. Hinze et al. showed that *Bcl-2* was strongly expressed, and *Bcl-xl* was moderately expressed in medullar thyroid cancer [18].

Hacioglu et.al detected an increase in expression of *Bax* in DU-145 cells which treated with boric acid [6]. In a study examining the effects of borax, a salt of boric acid, on hepatocellular cancer cells, apoptosis was found to be induced in cells after borax, and it was demonstrated that *Bax* increased and *Bcl-2* decreased [19]. In our study, a significant increase was determined in the mRNA expression of *Bax* and a decrease in the mRNA expression of *Bcl-2* and *Bcl-xl* in the boric acid treatment group cells compared with the control group cells.

NOXA gene is a pro-apoptotic member of the Bcl-2 protein family The expression of NOXA is regulated by

*p53* which is a tumor suppressor gene [20].

Previous research showed that the RET proto-oncogene negatively regulates NOXA, a pro-apoptotic gene, via transcription factor 4 (ATF4) [21]. APAF-1, another apoptosis-related factor, combines with cytochrome c to form a complex stimulating caspase 9, leading to cell apoptosis [22]. Our results showed a significant increase in NOXA and APAF-1 in cells treated with boric acid. This, along with similar studies, suggests that boric acid triggers apoptosis in TT cells by increasing pro-apoptotic genes and reducing anti-apoptotic gene expression.

Furthermore, boric acid and its apoptotic effects on TT cells were confirmed by TUNEL assay. According to the results, apoptotic cell percentage were compared between the dose group and the control group. In the control group, the rate of apoptotic cells was determined as 4%, and in the dose group in which IC50 (35  $\mu$ M) dose of boric acid was added, the rate of apoptotic cells was determined as 14%.

Pennelli et al.'s study on the PDCD4/miR-21 pathway in medullary thyroid cancer found a consistent link between increased miR-21 expression and MTC development. Higher miR-21 levels were associated with elevated calcitonin levels, lymph node metastasis, advanced, and resistant disease [23]. In a study by Y.H.Chu et al. involving 42 MTC cases, there was significant over-expression of miR-21 in MTC cells compared to normal thyroid tissue. Moreover, after removing miR-21 and MALAT-1 in MTC cell culture, a significant decrease in cell proliferation and invasion was observed [24]. In a study by Mian et al., which included 34 sporadic MTC, 6 hereditary MTC, and 2 C cell hyperplasia cases, and examined miRNA profiles in familial and sporadic MTC, miR-21 levels were found to be 4.2 times higher in diseased tissue compared to normal thyroid tissue [25]. Recent research has revealed that *miR-21* supports cell proliferation by suppressing tumor suppressor genes such as *PTEN*, *RECK*, *PDCD4*, and *TPM1* [26, 27]. In our study, in the TT cell line treated with boric acid (35  $\mu$ M), *miR-21* was found to be reduced 4.3 times compared to the control group. With the decrease in *miR-21*, the elimination of pressure on tumor suppressor genes can be thought to induce apoptosis. In the same study, we found *miR-224* 8.9 times reduced compared to the control group.

In the same study, we found *miR-224* 8.9 times reduced compared to the control group. In studies on *miR-224* in MTC, the increase of *miR-224* has been associated with the absence of lymph node metastasis, low-stage disease and good prognosis [28, 29]. However, in studies conducted on different types of cancer such as breast cancer, cervical cancer and lung cancer, the increase of *miR-224* expression has

been associated with poor prognosis, cancer aggressiveness and advanced disease [30]. Although studies on MTC show that *miR-224* is associated with good prognosis and low-stage disease, in our study, it was found that *miR-224* expression decreased in TT cells with boric acid added. As a result of tests performed during the study, if the boric acid is thought to decrease proliferation and induce apoptosis in TT cells, studies involving MTC and *miR-224* related mostly patient and healthy control groups are needed.

The comet assay test was used to detect the damage done by boric acid on DNA. According to the test results, it was determined that the head length and density decreased, the tail length and density increased, and the tail moment increased in the dose group where boric acid was applied compared to the control group. This result shows that boric acid induces cell death by increasing DNA damage in TT cells.

#### Conclusion

Carcinogenesis is a very complex process considering the functioning of genes and proteins involved in apoptosis, enzymatic reactions. This study has a power in order to demonstrate of ant anti-carcinogenic effect of boric acid in multiple aspects in cell culture of MTC. In our study, it was determined that boric acid reduces proliferation, induces apoptosis, reduces invasion and colony formation in TT medullary thyroid cancer cells. When all the results are evaluated, the idea arises, that boric acid can be used as a possible therapeutic agent in both medullary thyroid cancer and maybe other types of cancer. It is important in this respect to accelerate and expand in vitro and in vivo studies.

#### Declarations

#### Acknowledgements

The authors would like to acknowledge the financial support of the Pamukkale University Scientific Research Projects Coordination Unit (PAUBAP) (Project No.: 2018TIPF040). Furthermore, the manuscript was presented as preprint in Research square platform with before.

#### Author contribution

All authors whose names appear on the submission made substantial contributions to the conception or design of the work. Project administration: SMF. Literature research: All authors. Data analysis: OY. MS. YD. GAM. Experimental studies: OY. MS. YD. GAM. Manuscript writing and editing: All authors.

#### Funding

This work was supported by Pamukkale University Scientific Research Projects Coordination Unit (PAUBAP) (Project No: 2018TIPF040).

Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Conflict of Interest: The authors declare that they have no conflicts of interest.

Ethical approval: Thyroid medullary carcinoma TT cell line (ATCC, CRL 1803<sup>TM</sup>) and human thyroid fibroblast (HTF; ScienCell Cat No: 3730) cell line, which are provides de-identified samples, were used in this study. This study was reviewed and deemed exempt by our Pamukkale University Institutional Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

#### References

- 1. Wells SA, Jr, et al. Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma. Thyroid. 2015;25(6):567–610. https://doi.org/10.1089/thy.2014.0335
- Bartz-Kurycki MA, et al. Medullary thyroid carcinoma: recent advances in identification, treatment, and prognosis. Ther Adv Endocrinol Metab. 2021;12:20420188211049611. https://doi.org/10.1177/20420188211049611
- Salvatore D, Santoro M, Schlumberger M. The importance of the RET gene in thyroid cancer and therapeutic implications. Nat Rev Endocrinol. 2021;17(5):296–306. https://doi.org/10.1038/s41574-021-00470-9
- Galuppini F, et al. MicroRNAs in Medullary Thyroid Carcinoma: A State of the Art Review of the Regulatory Mechanisms and Future Perspectives. Cells. 2021;10(4):955. https://doi.org/10.3390/cells10040955
- 5. Nielsen FH, Eckhert CD. Boron. Adv Nutr. 2020;11(2):461–462. https://doi.org/10.1093/advances/nmz110
- Hacioglu C, et al. High Concentrations of Boric Acid Trigger Concentration-Dependent Oxidative Stress, Apoptotic Pathways and Morphological Alterations in DU-145 Human Prostate Cancer Cell Line. Biol Trace Elem Res. 2020;193(2):400–409. https://doi.org/10.1007/s12011-019-01739-x
- Henderson KA, et al. Boric acid induces cytoplasmic stress granule formation, elF2α phosphorylation, and ATF4 in prostate DU-145 cells. Biometals. 2015;28(1):133–141. https://doi.org/10.1007/s10534-014-9809-5
- Sevimli M, et al. Boric acid suppresses cell proliferation by TNF signaling pathway mediated apoptosis in SW-480 human colon cancer line. J Trace Elem Med Biol. 2022;71:126958. https://doi.org/10.1016/j.jtemb.2022.126958
- 9. Cebeci E, Yüksel B, Şahin F. Anti-cancer effect of boron derivatives on small-cell lung cancer. J Trace Elem Med Biol. 2022;70:126923. https://doi.org/10.1016/j.jtemb.2022.126923
- 10. Scorei R, et al. Comparative effects of boric acid and calcium fructoborate on breast cancer cells. Biol Trace Elem Res. 2008;122(3):197–205. https://doi.org/10.1007/s12011-007-8081-8

- 11. Acerbo AS, Miller LM. Assessment of the chemical changes induced in human melanoma cells by boric acid treatment using infrared imaging. Analyst. 2009;134(8):1669–1674. https://doi.org/10.1039/b823234b
- 12. Barranco WT, Eckhert CD. Boric acid inhibits human prostate cancer cell proliferation. Cancer Lett. 2004; 216:21–26. https://doi.org/10.1016/j.canlet.2004.06.001.
- Kahraman E, Göker E. Boric acid exert anti-cancer effect in poorly differentiated hepatocellular carcinoma cells via inhibition of AKT signaling pathway. J Trace Elem Med Biol. 2022; 73:127043. https://doi.org/10.1016/j.jtemb.2022.127043.
- Asadi M, Taghizadeh S, Kaviani E, Vakili O, Taheri-Anganeh M, Tahamtan M, Savardashtaki A. Caspase-3: Structure, function, and biotechnological aspects. Biotechnol Appl Biochem. 2022; 69(4):1633–1645. https://doi.org/10.1002/bab.2233.
- Li P, Zhou L, Zhao T, Liu X, Zhang P, Liu Y, Zheng X, Li Q. Caspase-9: structure, mechanisms and clinical application. Oncotarget. 2017; 8(14):23996–24008. https://doi.org/10.18632/oncotarget.15098.
- Scorei R, Ciubar R, Ciofrangeanu CM, Mitran V, Cimpean A, Iordachescu D. Comparative effects of boric acid and calcium fructoborate on breast cancer cells. Biol Trace Elem Res. 2008; 122:197-205. https://doi.org/10.1007/s12011-007-8081-8.
- 17. Kale J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the dance towards death. Cell Death Differ. 2018; 25(1):65–80. https://doi.org/10.1038/cdd.2017.186.
- Hinze R, Gimm O, Taubert H, Bauer G, Dralle H, Holzhausen HJ, Rath FW. Regulation of proliferation and apoptosis in sporadic and hereditary medullary thyroid carcinomas and their putative precursor lesions. Virchows Arch. 2000; 437:256-263. https://doi.org/10.1007/s004280000233.
- 19. Wei Y, Yuan FJ, Zhou WB, et al. Borax-induced apoptosis in HepG2 cells involves p53, Bcl- 2, and Bax. Genet Mol Res. 2016; 15. https://doi.org/10.4238/gmr.15028300.
- 20. Morsi RZ, Hage-Sleiman R, Kobeissy H, Dbaibo G. Noxa: Role in Cancer Pathogenesis and Treatment. Curr Cancer Drug Targets. 2018; 18(10):914–928. https://doi.org/10.2174/1568009618666180308105048.
- 21. Bagheri-Yarmand R, Krishna M, Sinha KM, Gururaj AE, et al. Novel Dual Kinase Function of the RET Proto-oncogene Negatively Regulates Activating Transcription Factor 4-mediated Apoptosis. J Biol Chem. 2015; 290:11749-11761. https://doi.org/10.1074/jbc.M114.619833.
- 22. Shakeri R, Kheirollahi A, Davoodi J. Apaf-1: Regulation and function in cell death. Biochimie. 2017; 135:111–125. https://doi.org/10.1016/j.biochi.2017.02.001.
- 23. Pennelli G, Galuppini F, Barollo S, Cavedon E, Bertazza L, Fassan M, et al. The PDCD4/miR- 21 pathway in medullary thyroid carcinoma. Hum Pathol. 2014; 46:50-57. https://doi.org/10.1016/j.humpath.2014.09.006.
- Chu YH, Hardina H, Schneiderb DF, Chenc H, Lloyda RV. MicroRNA-21 and long non- coding RNA MALAT1 are overexpressed markers in medullary thyroid carcinoma. Exp Mol Pathol. 2017; 103:229-236. https://doi.org/10.1016/j.yexmp.2017.10.002.

- 25. Mian C, Pennelli G, Fassan M, Balistreri M, Barollo S, Cavedon E, et al. MicroRNA profiles in familial and sporadic medullary thyroid carcinoma: preliminary relationships with RET status and outcome. Thyroid. 2012; 22:890–896. https://doi.org/10.1089/thy.2012.0045.
- 26. Sun LH, Tian D, Yang ZC, Li JL. Exosomal miR-21 promotes proliferation, invasion and therapy resistance of colon adenocarcinoma cells through its target PDCD4. Sci Rep. 2020; 10(1):8271. https://doi.org/10.1038/s41598-020-65207-6.
- 27. Feng YH, Tsao CJ. Emerging role of microRNA-21 in cancer. Biomed Rep. 2016; 5:395-402. https://doi.org/10.3892/br.2016.747.
- Cavedon E, Barollo S, Bertazza L, Pennelli G, Galuppini F, Watutantrige-Fernando S, Censi S, Iacobone M, Benna C, Vianello F, Zovato S, Nacamulli D, Mian C. Prognostic Impact of miR-224 and RAS Mutations in Medullary Thyroid Carcinoma. Int J Endocrinol. 2017; 9:4915736. https://doi.org/10.1155/2017/4915736.
- 29. Yu L, Zhang J, Guo X, Li Z, Zhang P. MicroRNA-224 upregulation and AKT activation synergistically predict poor prognosis in patients with hepatocellular carcinoma. Cancer Epidemiol. 2014; 38:408-413. https://doi.org/10.1016/j.canep.2014.05.001.
- 30. Wang H, Zhu LJ, Yang YC, Wang ZX, Wang R. MiR-224 promotes the chemoresistance of human lung adenocarcinoma cells to cisplatin via regulating G (1)/S transition and apoptosis by targeting p21(WAF1/CIP1). Br J Cancer. 2014; 111:339-354. https://doi.org/10.1038/bjc.2014.157.



#### Figures

#### Figure 1

Effect of Boric acid on the viability of TT cell line at different time and doses. The cells were treated with boric acid at different concentrations and time intervals and their proliferation was assessed by XTT assay. Data are the average results of three independent experiments. \*IC50 dose of boric acid in TT thyroid cancer cells was detected  $35\mu$ M at  $48^{th}$  hour



Control



Dose group

#### Figure 2

Fluorescence microscopy images at 20x magnification of control and dose group cells after hoechst staining. The red arrows show the apoptotic cells



Control

Dose group

#### Figure 3

Crystal viole stained images of invaded cells in control and boric acid treated cells (Magnification: 40x)



#### Figure 4

TT cells Control and Dose group colony image



#### Figure 5

Wound healing assay results showed that boric acid reduced cell migration. Control and dose (35  $\mu$ M BA) images at 0, 16, and 24 h were presented



Control

Dose group

#### Figure 6

Comet images of the control and dose group were given. Comet assay: DNA damage in TT cells after 48 h exposure to boric acid at 35  $\mu$ M dose: Head length, head intensity, tail length, tail intensity, tail moment, tail migration demonstrative images of genotoxicity

#### **Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

• SUPLEMENTARYTABLE.docx