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The effect of prothrombin, the precursor of thrombin, on the proliferation and migration of colorectal cancer cells

Marija Cumbo*, Sofija Dunjić Manevski, Maja Gvozdenov, Martina Mia Mitić, Valentina Đorđević and Branko Tomić

Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11000 Belgrade, Serbia

***Corresponding author**: marijacumbo@imgge.bg.ac.rs

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Abstract: Thrombotic disorders are some of the main comorbidities in cancer patients. So far, research has indicated that thrombin, a key regulator of hemostasis, contributes to cancer progression. However, data on its origin in tumor microenvironments remain elusive. Based on previous research, we analyzed the RNA and protein expression of prothrombin, a precursor of thrombin, in selected colorectal cancer (CRC) cell lines. Since the effect of prothrombin in cancer development has not been previously reported, we treated the cells for 24 h and 48 h with different prothrombin concentrations and assessed the effect on cell proliferation and migration. Our results show that the tested CRC cell lines expressed prothrombin and that prothrombin inhibited proliferation and migration. The presented results suggest that prothrombin may contribute to CRC etiopathology and could serve as a potential diagnostic biomarker and therapeutic target. The mechanisms underlying prothrombin expression in cancer cells, potential prothrombin activation, and the underlying processes driving the described effects warrant further investigation.

Keywords: prothrombin, cancer, protein expression, cell proliferation, cell migration

Abbreviations: venous thromboembolism (VTE); colorectal cancer (CRC); protease-activated receptors (PARs); quantitative real-time PCR (qPCR); glyceraldehyde 3-phosphate dehydrogenase (GAPDH); enzyme-linked immunosorbent assay (ELISA); confidence interval (CI); receptor for advanced glycation end products (RAGE)

INTRODUCTION

Despite significant advancements in the understanding of cancer biology and therapeutic procedures, cancer remains a major global health concern and one of the most challenging medical issues, accounting for nearly 10 million deaths in 2020 [1]. The intertwined nature of cancer and coagulation has been recognized for more than 150 years, with venous thromboembolism (VTE) being the second leading cause of death in cancer patients. Cancer patients are at a 5-fold higher risk of developing VTE compared to the general population, a factor that is also associated with a poorer prognosis [2,3]. Besides numerous studies, the exact mechanisms of this interconnection have yet to be fully elucidated.

Prothrombin is a precursor of thrombin, a multifunctional serine protease with a central role in regulating hemostasis [4]. Apart from its main role in coagulation, data suggest that thrombin significantly influences cancer pathophysiology, acting through both coagulation-dependent and independent mechanisms [5]. Namely, through the activation of protease-activated receptors (PARs), thrombin can impact the growth and progression of cancer. PAR receptors are a widely expressed receptor family whose activation leads to an expression of different factors regulating processes such as cell proliferation and cell death. The mentioned processes together lead to the promotion of cancer cell invasiveness and dissemination [6,7]. Furthermore, thrombin exerts a proangiogenic effect by stimulating metalloproteinases, which contributes to vascular remodeling, and by upregulating the expression of growth factors and receptors that promote angiogenesis in both cancer and different stromal cells [8]. In addition, through platelet activation and the formation of fibrin clots, thrombin provides a protective

microenvironment, shielding cancer from the immune system and allowing its further progression [9].

Although prothrombin is predominantly expressed in the liver and secreted into the bloodstream, there is evidence of (pro)thrombin in cancer tissue [10,11]. Wojtukiewicz et al. showed that Fragment 1+2, a by-product of prothrombin activation to thrombin, was found in cancer tissue of the stomach, pancreas, and larynx carcinoma, thus indicating local activation of prothrombin [12]. Considering that tumors, including CRC, are comprised of different types of cells, such as epithelial, mesenchymal, etc. [13], it is challenging to elucidate the specific origin of prothrombin. In our previous study, prothrombin expression was examined *in vitro* in cell lines originating from different types of cancer. The results indicated that the Caco-2 cell line originating from colorectal cancer, expresses prothrombin, whereas other cell lines do not [14].

CRC is a prevalent type of malignancy and the third leading cause of cancer-related death worldwide, but the underlying mechanism of its progression and metastasis is still not fully understood [1,15]. This study aimed to determine further whether different colon cancer cell lines express prothrombin and to assess the impact of exogenous prothrombin on these cells.

MATERIALS AND METHODS

Cell culture and experimental materials

Permanent CRC cell lines used were: Caco-2 (ATCC® HTB-37™), SW480 (ATCC® CCL-228™), SW620 (ATCC® CCL-227™), HCT116 (ATCC® CCL-247™) and HT29 (ATCC® HTB-38™). Also, the HepG2 (ATCC® HB-8065™) cell line, originating from hepatocellular carcinoma, was used. The cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM, high glucose for CRC lines and low glucose for HepG2; Sigma, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and an antibiotic/antimycotic mix consisting of penicillin, streptomycin, and amphotericin B at a final concentration of 100 µg/µL (Gibco, USA). All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2.

During the expression analysis assays, the cell medium was enriched with 5 µg/mL vitamin K (Konakion M, Roche, Switzerland). In all performed assays, the growth medium was replaced with DMEM/Ham's Nutrient Mixture F12 medium (DMEM/F12; Sigma, USA) at corresponding times. Human prothrombin (Invitrogen, USA) was used for cell treatments, with appropriate vehicle control of 50% glycerol (Merck, USA).

RNA expression analysis

Prothrombin gene expression was assessed with quantitative real-time PCR (qPCR) analysis. Cells were seeded and left to reach a 70% confluence level when the cell medium was replaced with serum-free DMEM/F12. Total RNA was isolated after 24 h using TRI Reagent™ solution (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA template in a 20 µL reaction mix using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Relative quantification of prothrombin mRNA expression was performed using the TaqMan Gene Expression Assay (Applied Biosystems, USA). A 10 µL reaction consisted of 1×Universal PCR MasterMix, 1×TaqMan Gene Expression Assay, and 1.5 µL of cDNA. Expression levels of prothrombin mRNA were standardized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Protein expression analysis

Prothrombin levels in cell lysate and medium were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Novus Biologicals, USA). Upon reaching 70% confluence, the cell medium was replaced with serum-free DMEM/F12 and incubated for 24h. Collected cell lysates and mediums were prepared and analyzed according to the manufacturer's protocol. The developed signal in the ELISA assay was measured using a spectrophotometer Infinite 200 PRO multiplate reader (TECAN, Switzerland). The observed concentrations on the standard curve used to determine the prothrombin concentration were in the range of 0.344-22 ng/mL.

MTT assay

The effects of prothrombin on cell viability were measured using the MTT assay. Briefly, cells were plated in 96-well plates using standard culture medium. After 24 h, the culture medium was replaced with a serum-free DMEM/F12 medium containing increasing

concentrations of prothrombin (0-0.5 mg/mL), and cells were treated for 24 h and 48 h. After treatment, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) (the final concentration was 500 µg/mL) for 2 h at 37°C. The formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO; Serva, Germany), and cell viability was measured using a spectrophotometer Infinite 200 PRO multiplate reader (TECAN, Switzerland) at an optical density (OD) of 570 nm, with a background reduction of 630 nm. All assays were conducted a minimum of three times.

Wound healing assay

A wound-healing assay was used to assess the effect of prothrombin on cell migration. Cells were seeded in 35 mm Petri dishes (Sarstedt, Germany) and grown to full confluence. A scratch in the cell monolayer was made using a 200 µL pipette tip. After wounding, the cells were washed to remove debris produced and treated with serum-free DMEM/F12 medium containing prothrombin. Images of the wounded area were taken at 0 h, 24 h, and 48 h using a DM IL LED Inverted Microscope (Leica Microsystems, Germany), and the wound area was measured with Image J software (version 1.53k; National Institute of Health, USA) [16]. All assays were conducted a minimum of three times.

Statistical analysis

The results obtained were analyzed using Microsoft Office Excel 2016 and GraphPad software (version 9.0). For data that showed a normal distribution, one-way ANOVA was followed by Tukey's multiple comparisons test; otherwise, the Kruskal-Wallis test, followed by Dunnett's test, was used. Data were presented as the mean or median±95% confidence interval (CI).

RESULTS

Prothrombin expression analysis

Relative expression of the prothrombin gene in different cell lines was assessed by qPCR. Relative quantification values (RQ) of prothrombin mRNA are shown in Table 1 and Fig. 1A. To determine the presence of prothrombin protein in cell lysates and cell medium, the ELISA assay was used. The concentration of prothrombin in the lysates and medium was calculated according to the standard curve, and the obtained results are shown in Fig. 1B, C. All samples were in the detection range of the assay. As a positive control for qPCR and ELISA assays, the HepG2 cell line was used (results in the Supplementary Materials), as its prothrombin expression has already been confirmed [17]. Prothrombin expression was confirmed in all analyzed CRC cell lines.

Table 1. Values for relative mRNA expression levels of the prothrombin gene in analyzed CRC cell lines.

	CRC Cell lines				
	Caco2	**HCT116**	**HT29**	**SW620**	**SW480***
Avg Ct	23.53	33.94	28.03	27.88	31.79
Avg dCt	4.78	6.47	9.08	10.28	13.77
RQ	508.16	156.77	25.69	11.21	1.00
RQ min	435.95	101.42	22.47	9.59	0.84
RQ max	592.32	242.34	29.36	13.10	1.20

*Calibrator; Avg – Average; Ct – threshold; RQ – relative quantification values

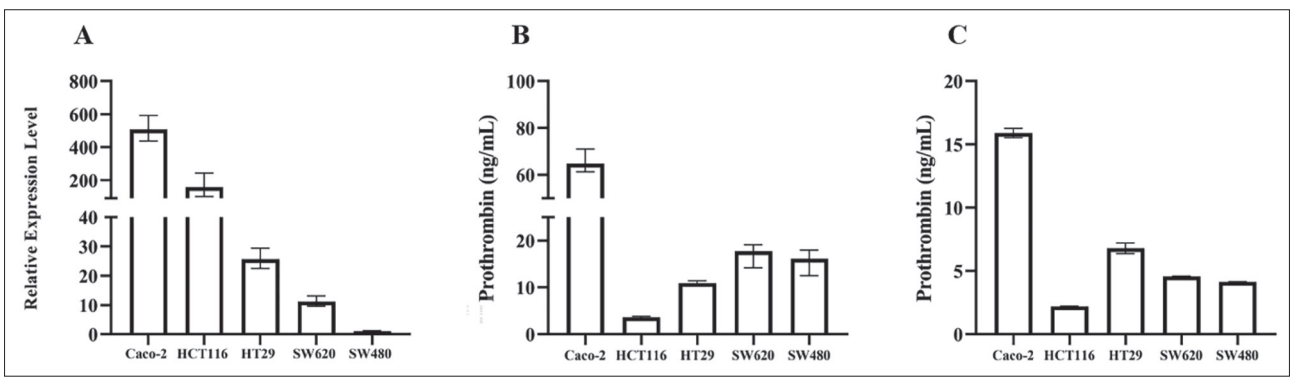

Fig. 1. Analysis of prothrombin expression in different CRC cell lines. **A** – Relative mRNA expression levels of the prothrombin gene in different cell lines. SW480 cell line was used as a calibrator, with the lowest expression of the gene. Results are presented as RQ with 95% CI. **B** – Prothrombin protein levels in cell lysate. **C** – Prothrombin protein levels in cell medium. ELISA results are presented as median±95% CI.

Effects of prothrombin on cell viability and proliferation

An MTT test was employed to investigate the effects of prothrombin on cell viability and proliferation. Prothrombin was used in concentrations of 0.02 mg/mL, 0.1 mg/mL, and 0.5 mg/mL, where 0.1 mg/mL corresponds to prothrombin concentration in normal plasma. A statistically significant inhibitory effect of the highest concentration was detected across all cell lines. However, use of the relevant vehicle control (50% glycerol) at corresponding concentrations indicated that the vehicle control itself exhibited an inhibitory effect in SW480 and SW620 cell lines. Except for SW480, the lowest concentration of prothrombin, as well as the one corresponding to the physiological level, did not yield significant results compared to untreated cells in all lines. The results are presented in Fig. 2.

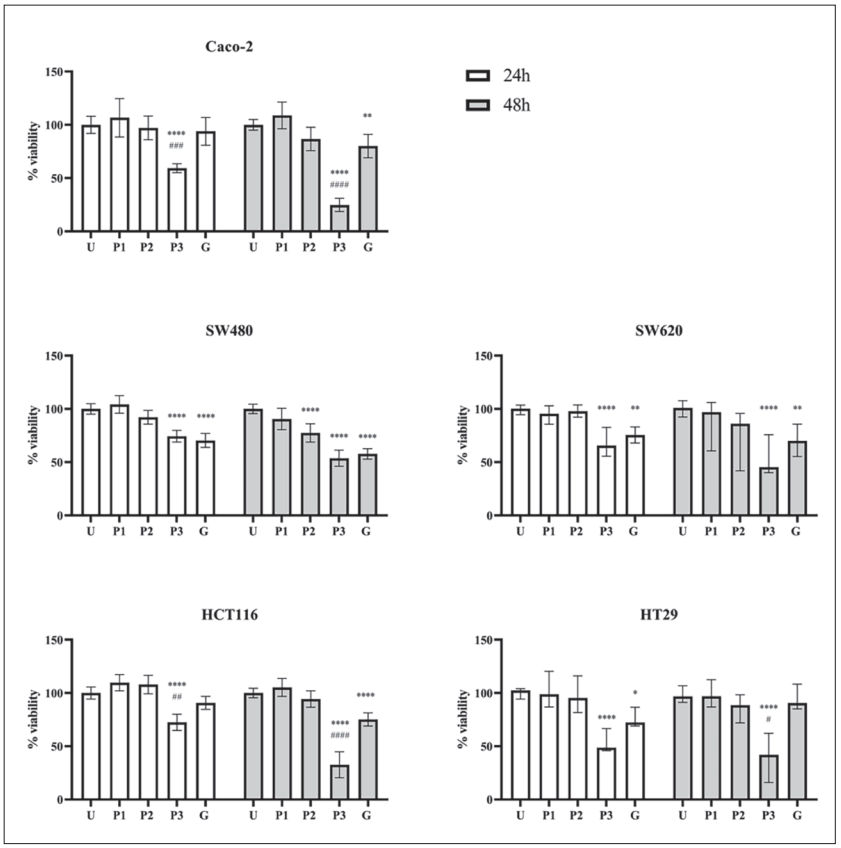

Fig. 2. MTT results of prothrombin effects on cell viability and proliferation. All treatments are expressed as % of untreated cells for corresponding time point (100%). Data are shown as mean ± 95% CI (for Caco-2, SW480, and HCT116) or as median ±95% (for SW620 and HT29). U – untreated cells, P1 – cells treated with prothrombin 0.02 mg/mL, P2 – cells treated with prothrombin 0.1 mg/mL, P3 – cells treated with prothrombin 0.5 mg/mL, G – cells treated with 50% glycerol corresponding to P3. * – comparison to untreated cells (U), # – comparison to cells treated with glycerol (G). */# P<0.05, **/## P<0.01, ***/### P<0.001, ****/#### P<0.0001.

Effects of prothrombin on cell migration

The migratory potential of cells following prothrombin treatment was evaluated by the wound healing assay. A concentration of prothrombin corresponding to the physiological concentration from plasma (0.1 mg/mL) was used. The Caco-2 cell line showed statistically significant migration inhibition upon treatment at both time points (Fig. 3). At both 24 h and 48 h, prothrombin demonstrated an inhibitory effect on cell migration, as evidenced by the wider wound area after treatment. However, this effect was not detected in other cell lines (Supplementary Materials).

DISCUSSION

The well-established connection between coagulation and malignancy implicates thrombin, a key regulator of hemostasis, that may contribute to cancer progression by influencing various tumor characteristics, such as growth and dissemination [5,18]. Although thrombin's role in these processes has been investigated in detail, its origin in the tumor microenvironment remains to be elucidated. Our previous research showed that only Caco-2 originating from CRC exhibited prothrombin expression among the examined cell lines at both the RNA and protein levels [14]. In a study by Sierco et al., Fragment 1+2 detection was conducted in human colon cancer samples, indirectly suggesting the potential presence and local activation of prothrombin colorectal cancer *in vivo* [19]. Although prothrombin half-life is longer than other coagulation factors [20], there is no evidence of prothrombin accumulation and activation in tumor tissues.

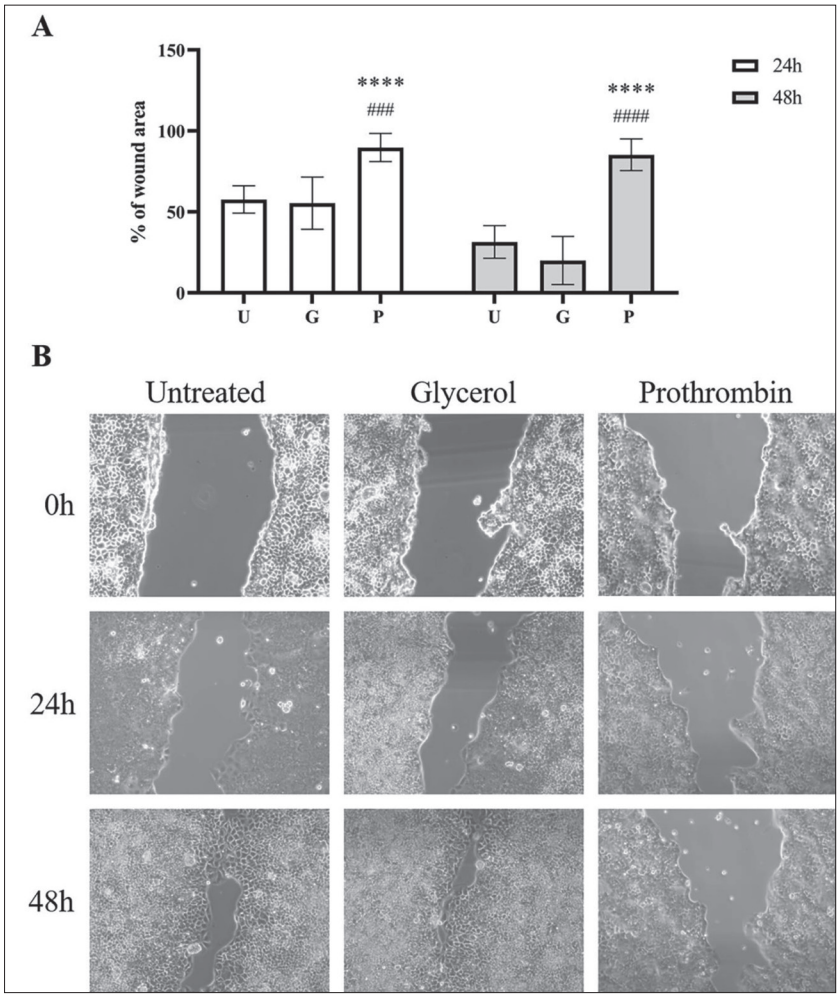

Fig. 3. Effect of prothrombin on migration potential of Caco-2 cell line. **A** – All wound areas at 24h and 48h are expressed as % of the same area at 0h (100%). Values are expressed as mean ± 95% CI. **B** – Representative results of the experiment. U – untreated cells, G – cells treated with glycerol, P – cells treated with prothrombin 0.1 mg/mL. * – comparison to untreated cells (U), # – comparison to cells treated with glycerol (G). ***/### P<0.001, ****/#### P<0.0001.

Considering previous literature data and our previous findings, we further investigated prothrombin's expression in colorectal cancer *in vitro*. Since our expression analysis was performed using cell lines grown in a serum-free medium, we eliminated the possibility of prothrombin originating from serum. This study used different epithelial colorectal cancer cells, all originating from different development stages of colorectal cancer [21]. As established, SW480 corresponds to Duke's type B-stage, and SW620 to Duke's type C-stage. Further, these two cell lines originated from the same patient, with SW480 isolated from primary cancer and SW620 isolated from lymphatic metastasis one year later [22]. The HT29 cell line corresponds to Duke's type C-stage, and HCT116 to Duke's type D-stage [23,24]. However, the developmental stage from which Caco-2 originates remains unknown [25]. According to the results, all examined cell lines express prothrombin at both the RNA and protein levels. In addition, the ELISA assay in the cell medium confirmed that the cell lines also secrete the protein.

Colorectal cancer is recognized for its significant diversity, characterized by considerable heterogeneity. Disease heterogeneity is reflected at various levels, such as primary tumor location, genetic or epigenetic signatures, and tumor microenvironment, all of which can be used to predict disease progression, therapy response, and overall survival prognosis [26-28]. Another source of heterogeneity is the transcriptional profile of cancer cells. Dynamic spatiotemporal variation in the tumor microenvironment can lead to varying expression profiles between the same type of tumor in different patients (inter-tumoral heterogeneity), as well as differing expression profiles among tumor cells in the same patient (intra-tumoral heterogeneity) [29]. Considering that the used CRC cell lines expressed prothrombin, further research into the mechanisms regulating prothrombin expression is needed, which could lead to the establishment of prothrombin as a potential biomarker for disease development. In favor of this is the finding that the peptide fragment of prothrombin was found to be significantly higher in the serum of CRC patients compared to healthy controls, indicating it can be used as a serum biomarker [30].

We treated cancer cells with different concentrations of prothrombin to determine whether prothrombin could be exerting an effect on them. Besides the evidence of thrombin's presence in the tumor microenvironment, the concentration of thrombin and

prothrombin from which this thrombin is generated is uncertain. Our results show that all cell lines have significantly inhibited proliferation and viability when exposed to higher concentrations of prothrombin. In certain cell lines like SW480 and SW620, the response induced by the concentration of the vehicle control is comparable, making it difficult to attribute the observed effect solely to prothrombin in those cells. This response in these lines may also be linked to their shared origin from the same patient. The lowest and physiological concentrations of prothrombin had no effect, except in the SW480 line at the 48-h time point. Evidence suggests that glycerol, although a non-toxic substance found in organisms, has a dose-dependent inhibitory effect on proliferation in some cell lines by mechanisms including inhibition of transition from G1 to S phase and apoptosis induction [31,32]. However, the mechanism behind the different responses to glycerol in different CRC cell lines remains unclear. Although all cell lines exhibited a consistent response to prothrombin regarding their proliferation and viability levels, only the Caco-2 cell line responded to treatment in the wound-healing assay. Prothrombin exerted a significant inhibitory effect on cell monolayer migration compared to both untreated cells and the corresponding vehicle control at both time points.

Whether the obtained results are due to prothrombin activation to thrombin in the cell medium remains to be investigated. *In vivo*, prothrombin to thrombin activation in the cancer microenvironment could be connected to the presence of tissue factor (TF) expressed at the cell membrane of various types of tumors, as well as the cells examined in this study [33-35]. Additionally, research has shown that prothrombin could be autocatalytically activated, potentially bypassing the need for other components of the coagulation cascade in the activation process [37].

Although literature data supports thrombin's stimulatory effect on cell proliferation and migration *in vitro*, there are data indicating a concentration-dependent biphasic effect of thrombin. Zain et al. showed that concentrations up to 0.5 U/mL stimulated the mentioned stimulative processes *in vitro*, whereas higher concentrations resulted in an inhibitory effect [38]. Considering previous studies on thrombin, our results suggest that its effects may indeed be dependent on the concentration to which the cancer cells are exposed, but further investigation is needed.

In the study conducted by Degani et al., prothrombin was identified as one of the most abundant human plasma ligands for the receptor for advanced glycation end products (RAGE) [39]. RAGE is a multiligand receptor present in different types of cells and is associated with the occurrence and progression of various inflammatory diseases [40]. The presence of this receptor in CRC cells has been linked with the growth and migration of these cells [41]. Interestingly, prothrombin binds to RAGE through the Gla domain, which is essential for its activation, meaning that through this interaction, prothrombin activation is impaired [39]. The results of these studies suggest that the observed effects of prothrombin treatment could stem from prothrombin itself rather than from its activation to thrombin, which opens a new avenue for future investigations.

The presented results lay the groundwork for exploring more intricate questions regarding the mechanisms behind prothrombin expression in cancer, its modes of action and activation within the tumor microenvironment, and the consequences of these phenomena. To our knowledge, the concentrations of either prothrombin or thrombin in the microenvironment of different types of tumors are unknown. Hence, the significance of prothrombin production by cancer cells and the effects of prothrombin noted *in vitro* have yet to be examined *in vivo*. Establishing a correlation between prothrombin and disease progression could potentially elevate prothrombin status as a robust prognostic tool and lead to the development of new therapeutics or repurposing existing drugs, particularly direct oral anticoagulants. This notion is reinforced by data demonstrating that the administration of various anticoagulant therapies successfully impeded the impact of thrombin on the progression of diverse tumor types. Using dabigatran and argatroban, direct thrombin inhibitors, cell proliferation and migration of breast cancer and glioblastoma cells stimulated by thrombin were reversed [42,43]. Similar results were obtained *in vivo* using the xenograft model in mice [42,44].

CONCLUSIONS

In this study, we evaluated prothrombin expression in different cell lines originating from colorectal cancer in a continuation of our previous work. Confirmation of prothrombin gene and protein expression at varying

levels in the analyzed cell lines underscores its potential role in the development and progression of this type of cancer. We also examined the effects of prothrombin on cancer cells *in vitro*. The results show that the applied concentrations induced an inhibitory response in cell proliferation and migration. To our knowledge, our study is the first to investigate the effect of prothrombin on cancer cells specifically, rather than focusing on its active form, thrombin. The noted effects of prothrombin, in contrast to extensive data from previous studies on its active form, thrombin, underscore the need for further studies to elucidate the mechanisms governing both its expression and levels of activation in the tumor microenvironment.

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Author contributions: MC, BT, and VDJ conceptualized the research and the manuscript. MC wrote the manuscript. MC, SDM, MG, and MMM cultured the cell lines, and performed qPCR, and ELISA. MC performed the MTT and wound-healing assays. MC and BT analyzed the results, and VDJ contributed to the evaluation and interpretation of the results. BT and VDJ oversaw the study and manuscript supervision. All authors have revised and approved the final manuscript.

Conflict of interest disclosure: None of the authors have a conflict of interest to disclose.

Data availability: Data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Cumbo%20et%20al_Raw%20dataset.pdf

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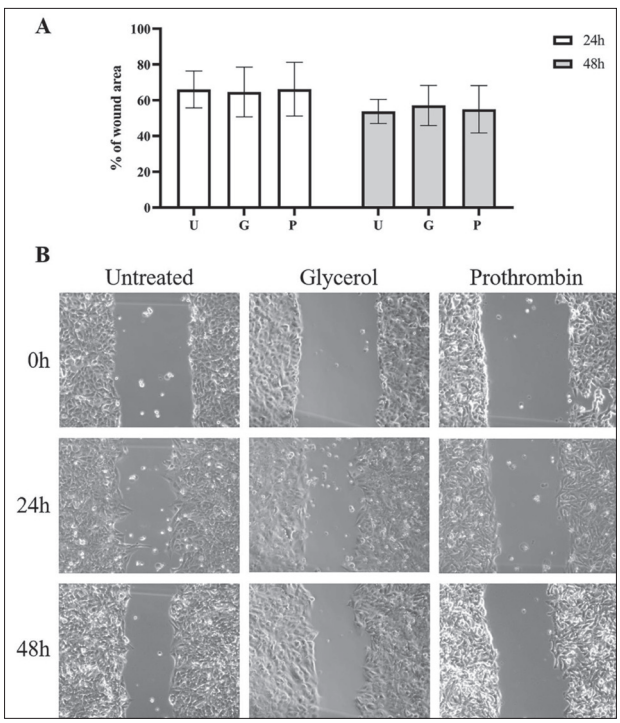
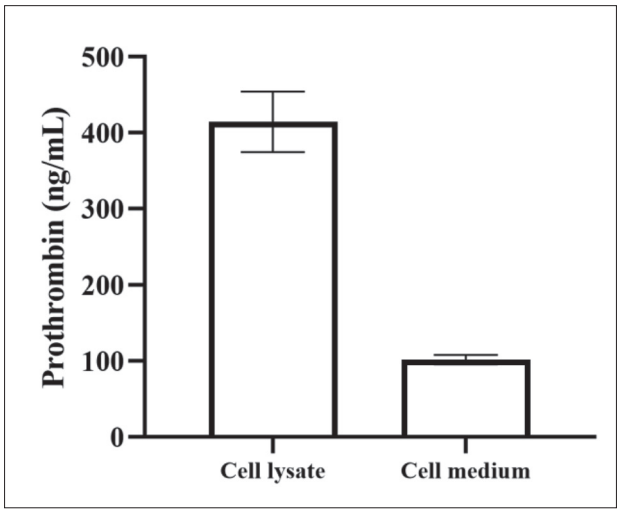
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Values for relative mRNA expression levels of the prothrombin gene HepG2 cell line.

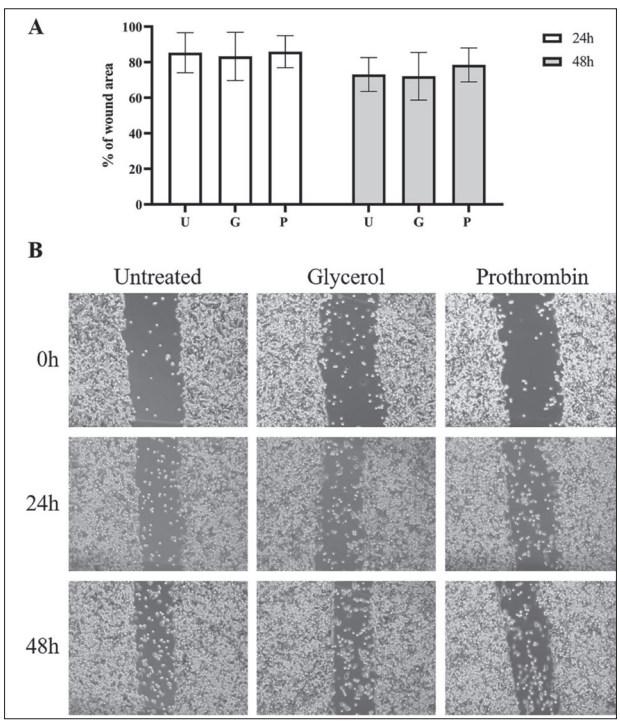
	HepG2
Avg Ct	19.49
Avg dCt	1.07
RQ	6614.73
RQ min	4807.66
RQ max	9101.02



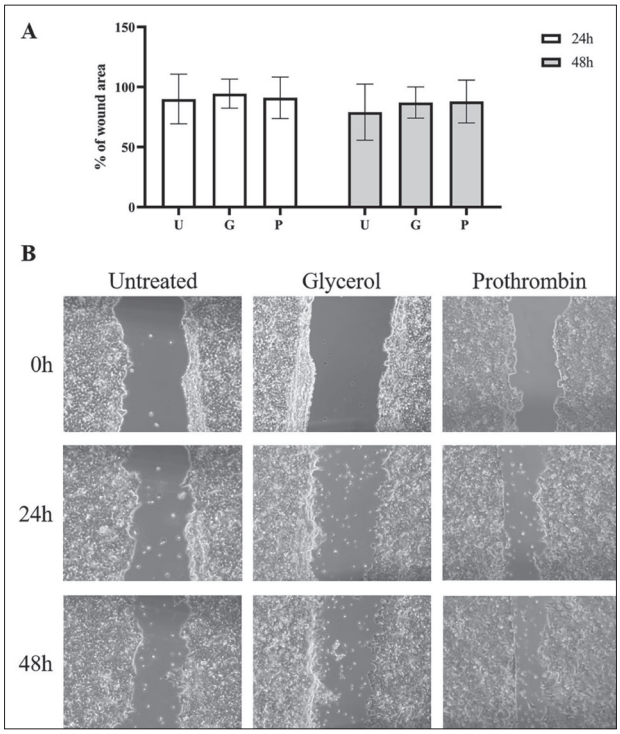
Supplementary Fig. S2. Effect of prothrombin on migration potential of the SW480 cell line. **A** – All wound areas at 24 h and 48 h are expressed as the % of the same area at 0 h (100%). Values are expressed as the mean±95% CI. **B** – Representative results of the experiment. U – untreated cells, G – cells treated with glycerol, P – cells treated with prothrombin 0.1 mg/mL.



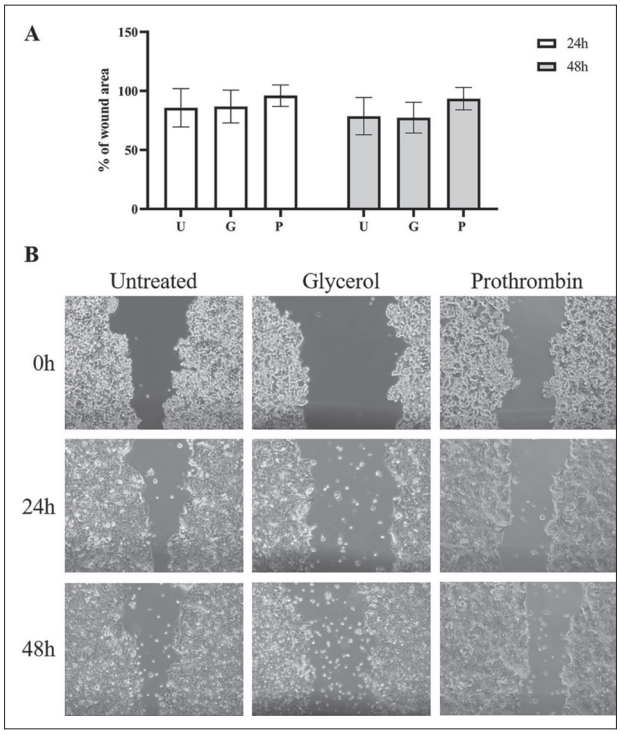
Supplementary Fig. S1. Prothrombin levels in cell lysate and cell medium of the HepG2 cell line. Results are presented as the median±95% CI.



Supplementary Fig. S3. Effect of prothrombin on migration potential of the SW620 cell line. **A** – All wound areas at 24 h and 48 h are expressed as the % of the same area at 0 h (100%). Values are expressed as the mean±95% CI. **B** – Representative results of the experiment. U – untreated cells, G – cells treated with glycerol, P – cells treated with prothrombin 0.1 mg/mL.



Supplementary Fig. S4. Effect of prothrombin on migration potential of the HCT116 cell line. **A** – All wound areas at 24 h and 48 h are expressed as the % of the same area at 0 h (100%). Values are expressed as the mean±95% CI. **B** – Representative results of the experiment. U – untreated cells, G – cells treated with glycerol, P – cells treated with prothrombin 0.1 mg/mL.



Supplementary Fig. S5. Effect of prothrombin on migration potential of the HT29 cell line. **A** – All wound areas at 24 h and 48 h are expressed as the % of the same area at 0 h (100%). Values are expressed as the mean±95% CI. **B** – Representative results of the experiment. U – untreated cells, G – cells treated with glycerol, P – cells treated with prothrombin 0.1 mg/mL.