Original article

# Targeting thymidine phosphorylase inhibition in human colorectal cancer xenografts

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A B S T R A C T

Human thymidine phosphorylase (hTP) is overexpressed in several solid tumors and is commonly associated with aggressiveness and unfavorable prognosis. 6-(((1,3-Dihydroxypropan-2-yl)amino)methyl)-5-iodopyrimidine-2,4 (1$H$,3$H$)-dione (CPBMF-223) is a noncompetitive hTP inhibitor, which has been described as a tumor angiogenesis inhibitor. The present study investigated the effects of CPBMF-223 in a xenograft tumor induced by human colorectal carcinoma cells (HCT-116). Additionally, CPBMF-223 capacity to reduce cell migration, its toxicological profile, and pharmacokinetic characteristics, were also evaluated. The intraperitoneal treatment with CPBMF-223 markedly prevented the relative tumor growth with an efficacy similar to that observed for 5-fluorouracil. Interestingly, number of vessels were significantly decreased in the treated groups. Moreover, CPBMF-223 significantly reduced the migration of cell line HCT-116. In the Ames assay and in an acute oral toxicity test, the molecule did not alter any evaluated parameter. Using the zebrafish toxicity model, cardiac and locomotor parameters were slightly changed. Regarding the pharmacokinetics profile, CPBMF-223 showed clearance of 9.42 L/h/kg after intravenous administration, oral bioavailability of 13.5%, and a half-life of 0.75 h. Our findings shed new light on the role of hTP in colorectal cancer induced by HCT-116 cell in mice, pointing out CPBMF-223 as, hopefully, a promising drug candidate.

## 1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, resulting in 1.8 million new cases in 2018. According to The Global Cancer Observatory (GLOBOCAN), it is the second most prevalent cancer in women and the third in men claiming almost 900,000 lives annually [1]. Although the recent advances in oncology therapy, this pathology is responsible for approximately 10% of all cancer incidence and mortality [1,2]. Although the overall survival of patients diagnosed with CRC has improved in recent years, less than 15% of patients with metastatic CRC have an average 5-year overall survival [3,4]. Neo-vascularization, including tumor angiogenesis, is a key process involved in the progression of cancer and metastasis. This concept was first described in the 1970s and is still considered a hallmark of cancer, being

an important pharmacological target for new cancer drugs [5,6].

The human thymidine phosphorylase (hTP), also known as platelet-derived endothelial cell growth factor (PD-ECGF), belongs to the pyrimidine nucleoside phosphorylase family and its main function is to ensure the functionality of the pyrimidine nucleoside pathway rescue required for DNA replication and repair [7,8]. In addition, the enzyme is responsible for the catalysis of reversible phosphorylation of thymidine to 2-deoxy-α-D-ribose-1-phosphate (2DDR1P) and thymine in the presence of inorganic phosphate [9]. It can be expressed in different cells, including endothelial cells, fibroblasts, lymphocytes, and macrophages [10]. In particular, several types of solid tumors such as gastric, esophageal, breast, bladder, cervical, lung, glioblastoma, ovarian, and colorectal have been shown to overexpress hTP enzyme which is associated with unfavorable prognosis, tumor aggressiveness, invasiveness and metastasis [10–12]. The biological effects are directly linked to proangiogenic and anti-apoptotic activity, triggered by hTP and 2-deoxyribose-D-ribose (2DDR), a downstream mediator, formed from nonenzymatic dephosphorylation of the product 2DDR1P [13–15].

The proangiogenic and anti-apoptotic activity in cancer progression makes hTP inhibition an attractive target for anticancer drug discovery. In the early 2000s, Fukushima and collaborators described a potent competitive hTP inhibitor, 5-chloro-6-(1-[2-iminopyrrolodinyl]methyl) uracil hydrochloride (TPI), with concentration of inhibitor that reduces enzyme velocity by half ($IC_{50}$) of 0.035 μM [16]. TPI associated with cytotoxic agent trifluorothymidine (TFT), a thymidine-based nucleoside analog, is the unique hTP inhibitor approved for clinical use by the Food and Drug Administration (FDA) (2015).

Recently we reported novel hTP inhibitors designed to mimic the transition state of the activated complex produced in the enzymatic catalysis. The novel pyrimidine-2,4-diones inhibited hTP activity with $IC_{50}$ value ranging from 0.12 to 87 μM. The 6-(((1,3-Dihydroxypropan-2-yl)amino)methyl)-5-iodopyrimidine-2,4(1*H*,3*H*)-dione (CPBMF-223; Fig. 1F) was the most potent compound presenting an $IC_{50}$ of 0.12 ± 0.01 μM with a noncompetitive inhibition mode regarding both substrates [17]. So far, to the best of our knowledge, CPBMF-223 is the most potent inhibitor of hTP described in the literature with a noncompetitive inhibition mechanism [17]. Furthermore, the CPBMF-223 exhibited no apparent toxicity in cytotoxicity and genotoxicity assays (alkaline comet), moderate in vitro metabolism rate and low probability of drug-drug interaction. Interestingly, in a human glioblastoma xenographic model, the compound was able to significantly reduce the relative tumor volume (RTV) when intraperitoneally administered to nude mice. The reduction of tumor volume was mediated by the reduction of vascularization accessed through vascular endothelial growth factor (VEGF) immunolabeling and vessel number evaluations [17].

Based on these findings, the present study describes the antitumor potential of CPMBF-223 using human colorectal carcinoma cells (HCT-116) in a female and male nude mice model. Additionally, the compound ability to inhibit HCT-116 cell migration was also studied. Furthermore, the evaluation of toxicological properties of the compound was expanded by using Ames assay, zebrafish (*Danio rerio*) toxicity models, and an acute oral toxicity model in mice. Finally, the oral pharmacokinetic profile of CPBMF-223, as a preliminary step to test different routes of further drug administration, was also presented.

## 2. Materials and methods

### 2.1. Drugs and cell culture

CPBMF-223 was obtained as previously described [17]. 5-Fluorouracil (5-FU) was obtained from Sigma–Aldrich (St. Louis, MO, USA). TPI was obtained from Med Chem Express (Princeton, NJ, USA). Immortalized human colorectal carcinoma (HCT-116) cell line was obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM -

Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), gentamicin (0,08 mg/mL, INLAB, Diadema, SP, Brazil), and 0,1% fungizone (Gibco, Grand Island, NY, USA). Posteriorly, the HTC-116 cells were incubated at 37 °C, relative humidity of 95%, and 5% $CO_2$.

### 2.2. Mice

Female and male BALB/c nude mice (CbyJ.Cg-Foxn1[nu]/J; 4–8 weeks old; $n = 44$) were used for in vivo antitumor assay; matrices of these animals were a donation from Sociedade Beneficente Israelita Brasileira Albert Einstein (São Paulo, SP, Brazil). Female CF1 mice (8–12 weeks old) were used for acute oral evaluation test ($n = 19$) and pharmacokinetics study ($n = 36$). All mice were obtained from the Center of Experimental Biological Models from Pontifícia Universidade Católica do Rio Grande do Sul (CeMBE/PUCRS). The animals were maintained at controlled humidity (40–60%) and temperature room ($24 \pm 2$ °C), under a 12/12 h light/dark cycle. Food and water were provided ad libitum. All experimental protocols were approved by the Animal Ethics Committee from Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS) (protocol number: 8731). In addition, we followed the Brazilian guideline for production, maintenance or use of animals in teaching or scientific research, from the National Council for the Control of Animal Experimentation (CONCEA, Brasília, DF, Brazil) and the last published ARRIVE Guideline to report in vivo experiments [18].

### 2.3. Tumor growth in mice

#### 2.3.1. Tumor xenograft model

HCT-116 cells ($5 \times 10^6$) in 200 μL of Geltrex (Gibco, Grand Island, NY, USA) and culture media (1:1) were subcutaneously (s.c.) inoculated into a right hind flank of male and female nude mice [19]. After 7–8 days of cell implantation, the animals were randomly divided into experimental groups and the treatments were started. Animals were randomized into three groups: vehicle control (0,9% NaCl), CPBMF-223 (50 mg/kg, 5 days/week) and 5-FU (30 mg/kg, every other day (q.o.d)) [17,20]. Treatments were intraperitoneally (i.p.) administrated for 3 weeks.

Tumors were measured every other day using a digital caliper. Tumor volume was calculated as V = 0.5 × length (mm) × width$^2$, while RTV was calculated using the following formula: RTV = (tumor volume on the measured day) / (tumor volume on day 0). The solid tumor growth inhibition (TGI) on specific day as TGI = [1 − (RTV of treated group) / (RTV of the control group)] × 100 (%) was also calculated to evaluate toxicity, the body weight change (BWC) was estimated as BWC = [(body weight on the last day) – (body weight on day 0)] / (body weight on day 0) × 100 (%) [17]. After 21 days of treatment, mice were euthanized, the tumor tissues were excised, and the blood collected through abdominal aorta in EDTA tube (BD Vacutainer, Franklin Lakes, NJ, USA). A separate control group was treated with vehicle (sodium chloride (NaCl) 0,9%) by i.p. route also for 21 days.

##### 2.3.1.1. Criteria of inclusion and exclusion.
In order to reduce the variation presented in tumor response to antiangiogenic drugs, inclusion and exclusion criteria were inserted in this study. Animals with tumor volume below 20 mm$^3$ after 7–8 days of cell implantation, were not included in the experiment ($n = 2$). Mice with a variation above of three standard errors of the mean, or animals with the tumor without growing at the appropriate location were excluded of the study ($n = 3$) [18,21].

### 2.4. Cell migration assay

HCT-116 ($1,8 \times 10^6$) cells were seeded in 6-wells plate until 90–100% confluences were achieved. Using a 200 μL micropipette tip was scratched a gap line across the center of the cell's monolayers. The
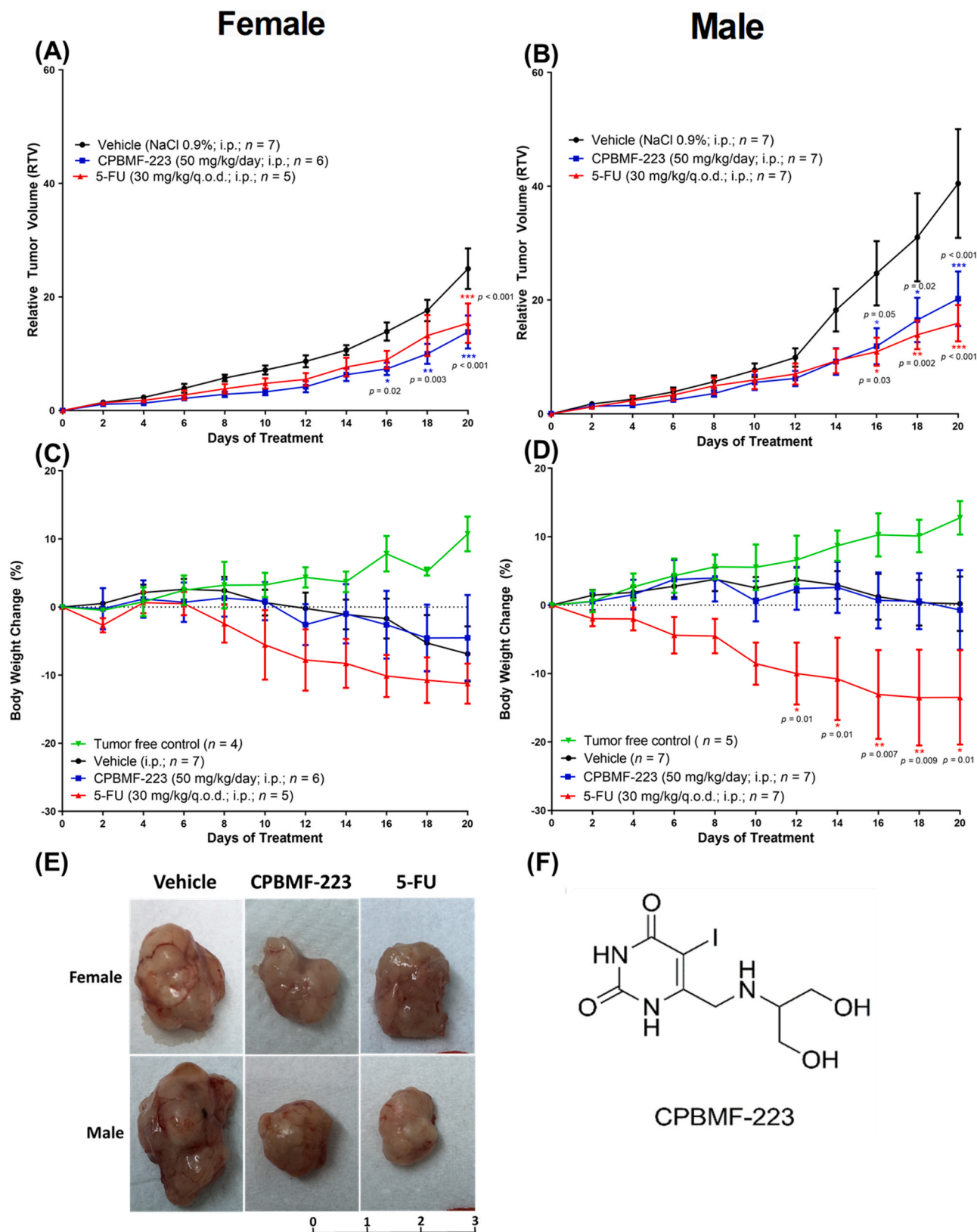
**Fig. 1.** Effects of treatments with CPBMF-223 (50 mg/kg/day, 5 days/week, 3 weeks) and 5-fluorouracil (5-FU; 30 mg/kg/day, every other day), by intraperitoneal (i.p.) route, on tumor relative volume (RTV) and body weight changes (%) in nude mice injected with human colorectal carcinoma (HCT-116). RTV curve during treatment for female (A) and male (B) mice. Body weight changes curve for female (C) and male (D) animals. Representative pictures to show the sizes of the tumors excised on day 21 (E). Chemical structure of 6-(((1,3-dihydroxypropan-2-yl)amino)methyl)-5-iodopyrimidine-2,4(1*H*,3*H*)-dione (CPBMF-223) (F). 5-FU treatment was used as a positive control drug for colorectal cancer. Data were expressed with the mean ± standard error of the mean. Statistical analysis was performed by two-way analysis of variance, followed by Bonferroni post-test. Data significant in relation to vehicle (negative control group) at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. q.o.d.: every other day.

cells were washed with PBS to remove detached and dead cells, followed by exposure to three concentrations of CPBMF-223 (50, 100 or 250 μM) or TPI (100 μM) in DMEM with 10% of FBS. DMEM with 10% of FBS was used as a positive control of migration. The images of the gap area were captured immediately (0 h), and after 4, 8, 12, 24 and 48 h with ×4 magnification using an inverted microscope (Olympus IX71). The analysis of the images was performed using the software ImageJ 1.8.0 (NIH, Bethesda, MD, USA). The gap distance of five sites per image was measured [22] ($n = 3$–4).

## 2.5. Immunohistochemistry and histological analysis

Tumor samples were fixed in 4% formaldehyde solution for over 24 h and then embedded in paraffin. The slides were incubated with primary polyclonal vascular endothelial growth factor (VEGF) antibody (1:300, Cat. PA5-16754, Cat. SK2482305B; Thermo Fisher Scientific, Waltham, MA, USA) and monoclonal Ki-67 antibody (SP6) (1:300, Cat. MA5-14520, Lot. SK2478526A; Thermo Fisher Scientific, Waltham, MA, USA). The immunohistochemistry (IHC) and hematoxylin-eosin (HE) analysis were performed as previously described [17].

## 2.6. Liver injury enzymes

Blood samples were collected via aortic puncture and centrifuged at 4000 rpm for 15 min at 4 °C. Plasma samples were divided into aliquots and stored at −80 °C. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (GAMMA-GT) levels were measured using commercial kits (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil). AST and ALT were measured with a UV kinetic methodology in a 340 nm and GAMMA-GT using a colorimetric method in 405 nm (SpectraMax M2e, Molecular Devices, San Jose, CA, USA).

## 2.7. Toxicity assays

### 2.7.1. Salmonella/microsome mutagenicity assay (Ames assay)

For the *Salmonella* mutagenicity test, the pre-incubation method was performed with or without metabolic activation using S9 mix (4% S9 fraction) (MOLTOX, Boone, NC, USA) [23,24]. *Salmonella typhimurium* strains TA98, TA97a, TA100, TA102, and TA1535 were obtained from MOLTOX (Boone, NC, USA). CBPFM-223 was dissolved in dimethylsulfoxide (DMSO), and concentrations used were determined according to a previous experiment in TA100 strain, with and without metabolization, where cytotoxicity was observed at concentrations higher than 2000 μg/plate (data not shown). Therefore, the defined concentrations were 100, 250, 500, 1000, and 2000 μg/plate. Firstly, 100 μL of bacterial culture ($1$–$2 \times 10^9$ cells/mL) were incubated with different CPBMF-223 concentrations in the absence or presence of S9 mix for 20 min at 37 °C, without shaking. After the incubation time, they were added to a test tube 2 mL of soft agar (0.6% agar, 0.5% NaCl, histidine 50 μM, biotin 50 μM, pH 7.4, at 42 °C) and poured immediately onto a plate of minimal agar (1.5% agar, Vogel-Bonner E medium, containing glucose 2%) and incubated at 37 °C for 48 h before counting the revertant colonies. In the absence of metabolic activation, as a positive control for TA98, TA97a, and TA102 strains 4-nitroquinoline--oxide (4-NQO, 0.5 μg/plate) was used, while for TA100 and TA1535, sodium azide (NaN3, 1 μg/plate) was employed. For all strains in the presence of metabolic activation was used 2-aminoanthracene (2-AA, 2 μg/plate) as a positive control. Assays were repeated once, and the plating for each dose was conducted in triplicate.

### 2.7.2. Zebrafish: animal maintenance and treatment

Embryos and larvae, 0–5 days post-fertilization (dpf), wild-type *Danio rerio*, from the AB background were obtained from the breeding colony. The animals were maintained in recirculating systems (Zebtec, Tecniplast, Pontremoli, MS, Italy) with reverse osmosis filtered water equilibrated to reach the species standard temperature (28 ± 2 °C), pH

(7.0–7.5), and ammonia, nitrite, nitrate, and chloride levels. Animals were subjected to a light/dark cycle of 14/10 h, respectively [25]. For breeding, females and males (1:2) were placed in breeding tanks overnight separated by a transparent barrier that was removed after the lights went on the following morning. The fertilized eggs retained in the fitted tank bottom were collected, sanitized, and immediately subjected to the treatment. Water used in the experiments was obtained from a reverse osmosis apparatus (18 MΩ/cm) and was reconstituted with marine salt (Crystal Sea, Marinemix, Baltimore, MD, USA) at 0.4 ppt. The total organic carbon concentration was 0.33 mg/L. The total alkalinity (as $CO_3^{2-}$) was 0.030 mEq/L. During fish maintenance, water parameters were monitored daily and maintained in the following ranges: pH: 6.5–7.5, conductivity: 400–600 μS, ammonium concentration: < 0.004 ppm, and temperature: 25–28 °C. All protocols were approved by the Animal Ethics Committee from Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS, Porto Alegre, RS, Brazil; protocol number 7249).

Embryos were placed in Petri dishes (40 embryos per dish), for neurotoxicity experiments, or in 96 well plates, for cardiac analyzes; and subjected to acute treatment at doses of 0 (vehicle, fish water), 50, 250, and 500 μM for five days, from 1 h post-fertilization (hpf) to 5 dpf. Animals were monitored daily for survival rate using an inverted stereomicroscope.

#### 2.7.2.1. Cardiotoxicity and cardiac evaluation. Each animal was monitored daily for cardiac alterations, and the heartbeat rate was analyzed at 2 and 5 dpf under a stereomicroscope ($n = 18$). Treated larvae and control were placed in 96 well plates with their heart rate was monitored for 60 s. For all procedures, temperature was kept stable at 28 °C [26].

#### 2.7.2.2. Neurotoxicity and exploratory behavior evaluation. Five dpf larvae were individually placed in a 24-well plate filled with 3 mL of water or respective solution treatment for exploratory performance analysis during a 5 min session following 1 min acclimation ($n = 30$). The performance was recorded for automated analysis using EthoVision XT software (version 11.5, Noldus), which is able to track the swimming activity of the animals at a rate of 15 positions per second. Total distance traveled (cm), velocity (cm/s), time outside area (s), and absolute turn angle were considered the main parameters of exploration of a new environment [27].

### 2.7.3. Acute oral toxicity

The study was performed according to the Organization for Economic Cooperation and Development (OECD) Guideline for testing of chemicals, no. 423 for the assessment of acute oral toxicity-acute toxic class method [28]. The method can be performed in a single oral dose of substance, or multiple doses given within 24 h. The acute toxicity range of the test substance depends on the mortality status of the animals, which supports finding the toxicity category of the drug tested as well as to define the exposure ranges where the median lethal oral dose (LD50) is expected.

After 3 h of fasting, three female CF1 mice were exposed to 300 mg/kg of CPBMF-223 by the single oral route, and food was withheld for further 2 h. It is recommended to use this starting dose when there is no information on the substance to be tested. Animals were observed for clinical signals, including changes in eyes and mucous membranes, skin, and fur, and also circulatory, respiratory, autonomic and central nervous systems, somatomotor activity, and behavior patterns had special attention the first 4 h after treatment and daily for 14 days. After 14 days, the mice were euthanized and subjected to a gross pathological examination for signals of toxicity. The kidneys, liver, and spleen were harvested and weighed. In case 2–3 animals treated with 300 mg/kg did not shown toxicity characteristics or mortality, three additional mice received the same dose, followed by a higher dose of 2000 mg/kg ($n = 6$) that was performed in two doses of 1000 mg/kg administered

with an interval of 4 h. An additional group of animals were evaluated as control, receiving only the vehicle (0.9% NaCl) as treatment ($n = 8$).

### 2.8. Data analysis

The results obtained in the mouse model were expressed as the mean ± standard error of the mean, and the statistical analysis of the data were performed by using one- or two-way analysis of variance, depending on the experimental protocol, followed by Bonferroni's or Dunnet's post-test. *Salmonella* mutagenicity tests are expressed as mean ± standard deviation and were determined by one-way analysis of variance, followed by Dunnet's post-test. The zebrafish protocols are evaluated by mean ± standard error of the mean and analyzed by one-way followed by Tukey post-test. *p* values < 0.05 were considered significant. All tests and the construction of graphs were performed using the GraphPad Prism (San Diego, CA, USA).

## 3. Results

### 3.1. CPBMF-223 reduces the tumor growth in HCT-116 colorectal cancer xenograft mice

The activity of CPBMF-223 in colorectal cancer was investigated by subcutaneous (s.c.) implantation of HCT-116 cells into the right hind flank of mice. CPBMF-223 (50 mg/kg) was administrated for 21 days in 3 cycles of 5 days a week, and the reference drug 5-fluorouracil (5-FU; 30 mg/kg) was administrated every other day, both by intraperitoneal (i.p.) route. Interestingly, for both female and male mice, RTV curves grew slower, when compared to the positive control group (vehicle) (Fig. 1A, B, E). The same result was observed when combined both sexes (Fig. SA1). For females, treatment with CPBMF-223 was able to significantly inhibit tumor growth rate in 47 ± 8%, 43 ± 9% and 45 ± 12% on days 16, 18, and 20, respectively; in turn, 5-FU was responsible for inhibiting 38 ± 14% on day 20 (Fig. 1A and Table 1). Similarly, the inhibition percentages for CPBMF-223-treated males were 52 ± 13%, 47 ± 13%, and 50 ± 12% and for 5-FU-treated males were 56 ± 10%, 55 ± 8% and 61 ± 8% (Fig. 1B and Table 1). Considering all data (two sexes), the inhibition was approximately 50% of tumor growth, for both compounds in the last three measurements (Fig. S1A and Table S1).

In addition, the pharmacological treatment with CPBMF-223 did not cause any body weight changes compared to the tumor or tumor-free untreated control groups. On the other hand, male mice treated with 5-FU had their body weight reduced compared to the tumor vehicle control and to the no tumor vehicle control (Figs. 1C, D and S1B).

### 3.2. CPBMF-223 reduce HCT-116 cell migration in vitro

The cell migration assay was performed after the in vitro cytotoxicity

assay to ensure the CPBMF-223 non-toxic concentrations (data not shown). Concentrations of 100 and 250 µM of CPBMF-223 and 100 µM of TPI (hTP inhibitor) significantly reduced the migration of HCT-116 cell after 48 h of treatment (Fig. 2). The CPBMF-223 inhibited the cell migration in 8.2 ± 2.2%, 10.4 ± 6.1% and 24.2 ± 3.5%, for 50, 100 and 250 µM, respectively. Under the same experimental conditions, 100 µM concentration of TPI inhibited 17.6 ± 4.4% of cell migration when compared with positive control (PC; DMEM with 10% FBS).

### 3.3. Effect of CBPMF-223 on VEGF, Ki-67, and vessel density

VEGF and Ki-67 immunohistochemistry (IHC) was performed to evaluate the expression levels of these promoters of angiogenesis and cell proliferation factors in HCT-116 tumors [17]. In the VEGF immunolabeling evaluation, no changes were observed in male or female groups, however when the both sexes were jointly assessed, 5-FU group showed some reduction compared with vehicle group (Figs. 3A, D and S2A). Regarding Ki-67 immunolabeling, it was reduced in nude male mice tumors by CPBMF-223 and 5-FU treatment (Figs. 3E and S2B) with 12.1 ± 2% and 9.9 ± 2.2% of inhibition, respectively. This effect was not observed for females or when the female and male groups data were associated (Figs. 3B and S2B). Next, the slice of mice tumor was stained with hematoxylin-eosin (HE) for visualization and counting of vessel numbers. As illustrated in Fig. 3C, F, G, the number of vessels in the colorectal tumor induced by HCT-116 were significantly decreased in both treatment groups, CPBMF-223 and 5-FU for both sexes. The inhibition percentages for CPBMF-223 were 26.9 ± 8.3%, 31.5 ± 6.5% and 29.5 ± 5.2%, and for 5-FU were 53.3 ± 5.1%, 36.5 ± 4.1 and 44.2 ± 3.5% for female, male and both sexes mixed, respectively (Tables 2 and S2). Representative images from HE labeling were depicted in Fig. 3G. Additionally, IHC images for VEGF and Ki-67 antibodies were also described in the Supporting information (S3A, B).

### 3.4. CPBMF-223 did not increase the liver enzyme levels

Regarding the toxicological safety profile, CPBMF-223 did not cause any changes in liver enzymes from mice plasma samples (Table 3). In contrast, 5-FU showed a reduction in alanine aminotransferase (ALT) values when compared to the vehicle group (Table 3). In addition, the tumor negative control group had a small increase in aspartate aminotransferase (AST) values when compared to tumor-free control (Table S3).

### 3.5. CPBMF-223 shows acceptable safety profile from in vitro and in vivo evaluations

In the Ames assay, CPBMF-223 was tested in five different concentrations (100–2000 µg/plate). The test was performed in five *S. typhimurium* strains: TA1535 and the corresponding isogenic strain TA100 which detect base-pair substitutions (DNA target leucine (GAG) by proline (GGG)), TA102 which detect crosslinking, oxidative and alkylating mutagens by an ocher mutation TAA in this *hisG* gene, and TA98 (DNA target –C–G–C–G–C–G–C–G-; –1) and TA97a (DNA target -C-C-C-C-C-C-; +1 cytosine) which detect frameshift mutations. In addition, aiming to mimic the human metabolic process was included S9 mix incubation with the compound. As shown in Table 4, CPBMF-223 did not induce mutagenicity effects with any concentration or any strain in the presence or absence of metabolic activation. Moreover, the positive control group for DNA damage increased the corresponding revertant colonies over the vehicle control group. Important to mention that 5-FU has been shown to be mutagenic to some strains of *Salmonella typhimurium* and *Saccharomyces cerevisiae*. Additionally, this drug has also been described as mutagenic in the micronucleus test on mouse bone marrow cells [29].

Using zebrafish as a toxicity model, we aimed to observe some parameters such as cardiotoxicity, neurotoxicity, and behavioral variation.

**Table 1**
Inhibitory effects of intraperitoneal administration of CPBMF-223 and 5-fluorouracil (5-FU) on tumor growth inhibition (TGI).

| Experimental group | | TGI (%) | | |
|---|---|---|---|---|
| | | Day 16 | Day 18 | Day 20 |
| **Female** | CPBMF-223 ($n = 6$) | 47 ± 8* | 43 ± 9** | 45 ± 12** |
| | 5-FU ($n = 5$) | 38 ± 11 | 25 ± 20 | 38 ± 14*** |
| **Male** | CPBMF-223 ($n = 7$) | 52 ± 13* | 47 ± 13** | 50 ± 12*** |
| | 5-FU ($n = 7$) | 56 ± 10* | 55 ± 8*** | 61 ± 8*** |

CPBMF-223: 50 mg/kg/day, 5 days/week, 3 weeks; 5-fluorouracil (5-FU): 30 mg/kg/day, every other day, for 3 weeks. Data were expressed as the mean ± standard error of the mean. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Bonferroni post-test.
  * *p* < 0.05 significantly different from vehicle group (negative control).
  ** *p* < 0.01 significantly different from vehicle group (negative control).
  *** *p* < 0.001 significantly different from vehicle group (negative control).
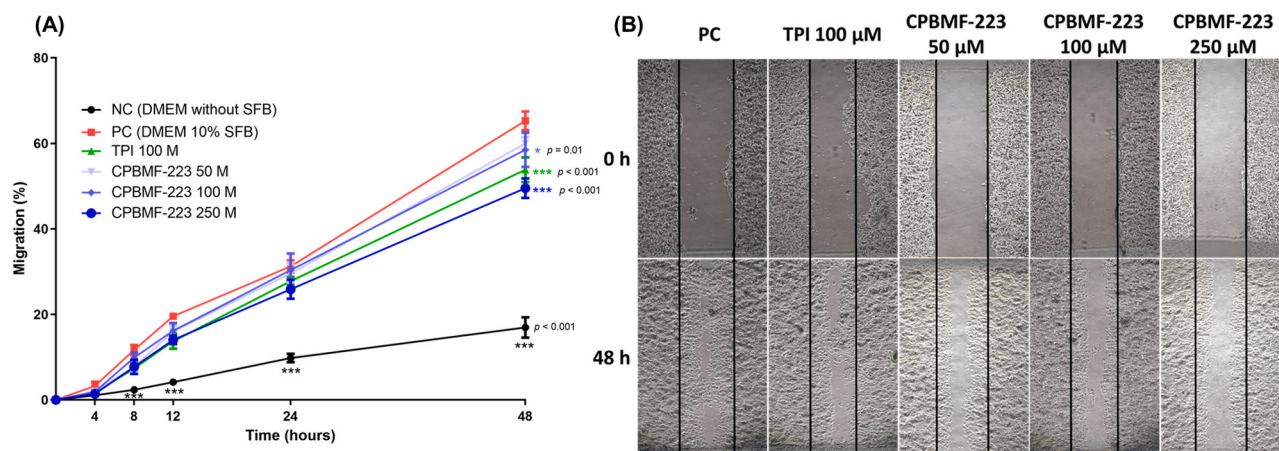
**Fig. 2.** Migration assay in HCT-116 cell over 48 h of exposure to CPBMF-223 and TPI (competitive inhibitor of hTP). Measurement of HCT-116 cell migration (%) over time (0, 4, 8, 12, 24 and 48 h) (A) and representative images of migration after 48 h of treatment (B). DMEM with 10% FBS was used as positive control of migration. Five independent measurements per picture were performed in order to calculate the percentage of migration, considering time 0 of each treatment with 0%. Data were expressed with the mean ± standard error of the mean. Statistical analysis was performed by two-way analysis of variance, followed by Bonferroni post-test. Data significant in relation to positive control (DMEM 10% FBS) at *$p < 0.05$ and ***$p < 0.001$.

The cardiac effects of CPBMF-223 were assessed and this compound decreased heart rate in the animals with 2 days postfertilization (dpf), 6.2 ± 0.9% and 6.6 ± 1%, at concentrations of 250 and 500 μM, respectively (Fig. 4A). Of note, this result was partially reversed at 5 dpf, where only the concentration of 500 μM was still responsible for changing this parameter (9.5 ± 0.8%) (Fig. 4B). Furthermore, the locomotor activity was used as a parameter to evaluate possible neurological impairment at 5 dpf. All three dosages tested resulted in a significant decrease in the distance and velocity of the free-swimming of the zebrafish when compared to the negative control, with no substantial differences between the different concentrations tested. The reductions of the total distance traveled parameter were of 36.3 ± 5.8%, 30 ± 4.7%, and 26.6 ± 7.3%, considering velocity parameter, the reductions were of 27.5 ± 3.8%, 27.6 ± 3.9%, and 19.6 ± 5.9% in the animals exposed to 50, 250, and 500 μM of CPBMF-223, respectively (Fig. 4C, D). An additional locomotor parameter measured was the absolute turn angle, a parameter that is sensitive to measure of locomotor coordination. The highest concentration (500 μM) was able to statistically increase the absolute turn angle by 42.4 ± 8.5% compared to the control (Fig. 4E). Otherwise, we analyzed as a behavioral parameter related to anxiety, the time that the animal remains on the periphery of the well. In acutely treated animals, the evaluation showed no significant differences between groups evaluated (Fig. 4F).

Next, the mice's acute oral toxicity of CPBMF-223 was tested using an Organization for Economic Cooperation and Development (OECD) protocol. The starting dose was 300 mg/kg in a single oral administration on female mice. In this primary test, no clinical signs of toxicity or mortality were observed within 14 days after administration. The protocol was repeated with the same dosage, and one animal was found dead on the first day, whereas the other two animals which received the same CPBMF-223 dosage showed no visual toxicity. Following recommendations from the protocol, the test was carried out with the administration of 2000 mg/kg, and no clinical sign of toxicity was observed. The surviving animals in the treated groups with an oral dose of CPBMF-223 (300 or 2.000 mg/kg) did not display any bodyweight loss, or kidney, liver, or spleen weights loss when compared to the control values (Table 5). Additionally, a necropsy examination revealed no treatment-related gross pathological changes in the carcasses of the mice. It is noteworthy that 5-FU shows LD$_{50}$ of 230 mg/kg and 115 mg/kg when orally administrated to rats and mice, respectively [30].

### 3.6. CPBMF-223 pharmacokinetic profile

A previously published HPLC methodology was used to quantify CPBMF-223 concentrations in mouse plasma [17]. Pharmacokinetic parameters of CPBMF-223 were obtained after i.v. and oral administration of 25 mg/kg and 250 mg/kg by using non-compartmental analysis (Table S4 and Fig. S4). Moreover, once the data was obtained after administration by the i.v. route, along with data previously published [17], it was possible to calculate the i.p. route data of clearance and bioavailability (Table S4).

The clearance was similar for the three administration routes, and bioavailability *F* value were found to be 13.52% and > 100% to oral and i.p. routes, respectively (Table S4). Following an oral administration of CPBMF-223 to CF-1 male mice, the peak concentration ($C_{max}$) (3.12 μg/mL) was achieved after 0.25 h (time to peak plasma concentration; $t_{max}$). The elimination rate constant (Ke) and the elimination half-life ($t_{1/2}$) values were 0.93 h$^{-1}$ and 0.75 h. The area under the compound plasma concentration from time 0 versus time curve and extrapolated to infinity (AUC$_{0 \to t}$ and AUC$_{0 \to \infty}$) were 2.6 μg h/L and 3.59 μg h/L. The area under the first moment of the plasma concentration versus time curve and extrapolated to infinity (AUMC$_{0 \to t}$ and AUMC$_{0 \to \infty}$) were 1.74 and 4.27 μg h$^2$/L, while the mean residence time (MRT) was 1.19 h in mice (Table S4). The intravenous (i.v.) route showed a short $t_{1/2}$ (0.45 h), a moderate volume of distribution (Vd) (3.24 L/kg) and a high clearance (CL) (9.42 L/h/kg). The remaining pharmacokinetic parameters were: Ke = 1.53 h$^{-1}$, AUC$_{0 \to t}$ = 2.36 μg h/L, AUC$_{0 \to \infty}$ = 2.65 μg h/L, AUMC$_{0 \to t}$ = 0.43 μg h$^2$/L, AUMC$_{0 \to \infty}$ = 0.91 μg h$^2$ h/L and MRT = 0.34 h (Table S4). The CPBMF-223 binding to plasma proteins was not influenced by the drug concentration (5 and 40 μg/mL) and was approximately 34.4 ± 1.07%.

## 4. Discussion

Herein, we evaluated the cytotoxicity of CPBMF-223 on HCT-116 cells by neutral red uptake assay. The in vitro incubation with different concentrations (25–500 μM) of the compound did not affect the viability of the cells (data not shown), which is in agreement with previously reported data [17]. By contrast, CPBMF-223 attenuated the tumor growth rate in a xenograft colorectal cancer mouse model induced by HCT-116 cells. It is important to mention that the hTP enzyme is overexpressed in the HCT-116-induced cancer model [31]. Treatment with CPBMF-223 showed a significant delay in growth tumor rate after sixteen days of treatment and remained reduced until the end
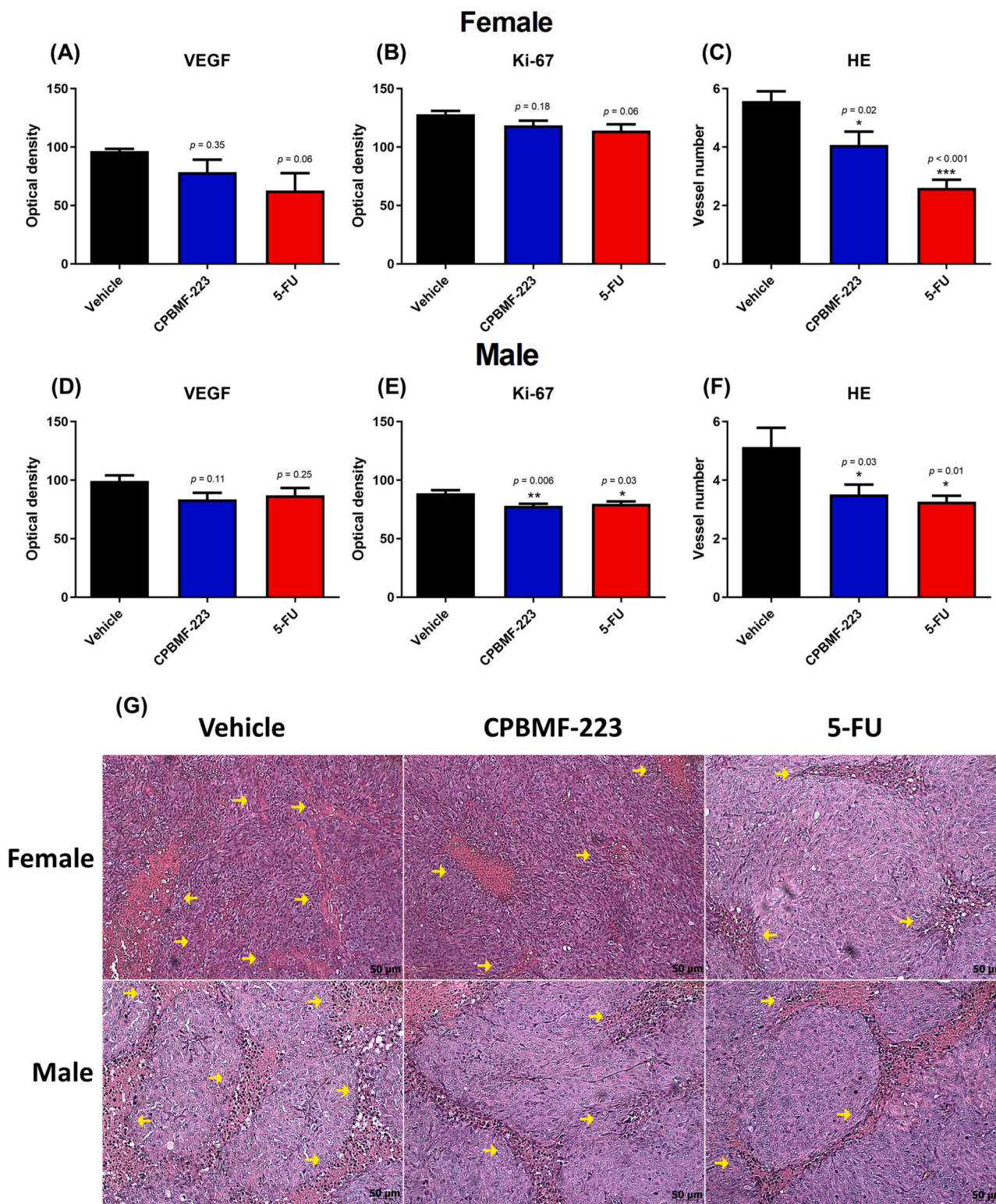
**Fig. 3.** Effects of treatment with CPBMF-223 (50 mg/kg/day, 5 days/week, 3 weeks) and 5-fluorouracil (5-FU; 30 mg/kg, every other day) by intraperitoneal (i.p.) route. Immunolabeling for vascular endothelial growth factor (VEGF; angiogenesis marker) (A and D) and Ki-67 (cell proliferation marker) (B and E); and hematoxylin-eosin (HE) for number of blood vessels (C and F) (angiogenesis marker) present in the tumor tissue for female and male nude mice. Representative images of HE in different treatment groups and sexes (G). 5-FU treatment was used as a positive control drug for colorectal cancer. Results are expressed as optical density or number of vessels and represent the mean ± standard error of the mean for five images captured per tumor (x200 for HE and VEGF; x400 for Ki-67). Statistical analysis was determined by one-way analysis of variance, followed by Dunnet's post-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ significantly from vehicle group (negative control). $n = 5$–7 animals per group.

**Table 2**
Inhibitory effects of CPBMF-223 and 5-fluorouracil in immunohistochemistry and histological analysis.

| Experimental group | | Percentage of inhibition (%) | | |
|---|---|---|---|---|
| | | VEGF | Ki-67 | HE |
| **Female** | CPBMF-223 ($n = 6$) | 18.5 ± 11 | 7.2 ± 3 | 26.9 ± 8⁻ |
| | 5-FU ($n = 5$) | 34.9 ± 11 | 10.6 ± 4 | 53.3 ± 5*** |
| **Male** | CPBMF-223 ($n = 7$) | 15.9 ± 6 | 12.1 ± 2** | 31.5 ± 7* |
| | 5-FU ($n = 7$) | 12.2 ± 6 | 9.9 ± 2* | 36.5 ± 4* |

CPBMF-223: 50 mg/kg/day, 5 days/week, 3 weeks; 5-fluorouracil (5-FU): 30 mg/kg/day, every other day, 3 weeks; by intraperitoneal route. Vascular endothelial growth factor (VEGF): angiogenesis marker, Ki-67: cell proliferation marker, hematoxylin-eosin (HE): histological analysis of blood vessel number. Data were expressed as the mean ± standard error of the mean. Statistical analysis was performed by one-way analysis of variance followed by Dunnet's post-test.

\* $p < 0.05$ significantly different from vehicle group (negative control).

\*\* $p < 0.01$ significantly different from vehicle group (negative control).

\*\*\* $p < 0.001$ significantly different from vehicle group (negative control).

**Table 3**
Changes in circulating alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GAMMA-GT) in female and male nude mice.

| Experimental group | | AST activity (U/L) | ALT activity (U/L) | GAMMA-GT activity (U/L) |
|---|---|---|---|---|
| **Female** | Vehicle ($n = 4$) | 27.9 ± 9.6 | 7 ± 1.4 | 15.7 ± 0.8 |
| | CPBMF-223 ($n = 5$) | 16 ± 2.4 | 5.8 ± 1.2 | 17.8 ± 1.3 |
| | 5-FU ($n = 5$) | 22.2 ± 5.1 | 6.4 ± 1.2 | 17.1 ± 0.6 |
| | Tumor free control ($n = 4$) | 8.8 ± 1 | 4.6 ± 0.5 | 17 ± 1.4 |
| **Male** | Vehicle ($n = 7$) | 13.2 ± 1.1 | 4.6 ± 0.3 | 18.6 ± 2 |
| | CPBMF-223 ($n = 7$) | 14.1 ± 2.8 | 4.1 ± 0.3 | 17.7 ± 1 |
| | 5-FU ($n = 6$) | 10.5 ± 1.6 | 3.3 ± 0.1** | 17.8 ± 1.4 |
| | Tumor free control ($n = 5$) | 7.8 ± 0.6 | 3.9 ± 0.3 | 16.2 ± 1.6 |

The animals received CPBMF-223 (50 mg/kg/day, 5 days/week) and 5-fluorouracil (5-FU; 30 mg/kg, every other day) by intraperitoneal (i.p.) route for 3 weeks. Serum levels of ALT, AST and GAMMA-GT were expressed as the mean ± standard error of the mean. Statistical analysis was determined by a one-way analysis of variance, followed by Dunnet's post-test. n=4–7 animals per group.

\*\* $p < 0.01$ significantly from the vehicle group (negative control).

of the experiment for both male and female mice. Moreover, no apparent toxicological variations were observed in body weight and in hepatic enzyme AST, ALT, and GAMMA-GT levels. It is noteworthy that our results indicated that CPBMF-223 was effective in both sexes, following US National Institute of Health policies for excluding possible sex bias [32].

Afterward, the CPBMF-223 ability to reduce cell migration in vitro was also evaluated. The migration assay has been used to test compounds in endothelial cells for pro-migratory or anti-migratory activity [33]. Migration capacity of cancer cells is an essential process to tumor invasion, angiogenesis and metastasis [34]. The results indicated that CPBMF-223 (100 e 250 μM) and TPI (100 μM) were able to reduce the migration process in human colorectal cancer cells after 48 h. In view of this interesting result, further studies are needed to better investigate the possible effects of CPBMF-223 on invasion and metastasis process.

Seeking to determine whether the compound was able to alter the expression levels of promoters of angiogenesis and cell proliferation factors, VEGF and Ki-67 immunohistochemistry was performed. In addition, the density of vessels in the tumor tissue was also assessed. Our results demonstrated that CPBMF-223 was not able to alter the immunolabeling of VEGF; however, it was effective to reduce the vessels and

cell proliferation (male Ki-67) in the colorectal tumor model. These results are in agreement with those pointed in the glioblastoma xenograft model induced by U-87 MG cells, indicating a reduction of blood vessel density mediated by this hTP inhibitor [17].

Several hTP inhibitors have already shown to be effective in preclinical models [35–39]. In an important study, TPI treatment was able to inhibit 30.8% of the tumor growth in the mice xenograft model using human KB epidermoid carcinoma cells. Interestingly, in this model, TPI was not able to inhibit the angiogenesis through the microvessels density evaluation. Similarly, results presented in the xenograft model induced by colorectal adenocarcinoma (HT-29) showed that TPI reduced the tumor volume and weight, increasing the apoptotic cells, but with no reduction of VEGF expression (immunohistochemistry and mRNA (RT-PCR)) and microvessel density [40].

Considering the vessel density reduction exhibited by 5-FU, has been described that this chemotherapy drug or others 5-FU-based drugs [41], could reduce angiogenesis in colorectal and breast cancer by induction of the thrombospondin-1 protein, which is known as an endogenous angiogenesis inhibitor [41,42]. It is important to mention that the differences found in CPBMF-223 treatments (glioblastoma and colorectal cancer), were that the glioblastoma model showed not only a reduction in the number of vessels, but also reduction in VEGF immunostaining, possibly due to different types of tumors. Different cell types may express different levels of hTP, possibly linked to the diverse genetic profile [10,17,43]. These findings suggest that another angiogenic factor (beyond VEGF) might be involved in the angiogenesis process in CPBMF-223 inhibition. In addition, as suggested for TPI [37], CPBMF-223 may play an important role in initial stages of tumor angiogenesis through the remodeling of the pre-existing vasculature and the other angiogenic factors might be involved in angiogenesis after reaching a certain tumor volume. It is important to mention that protocols were different in the time of treatment (14 and 21 days) and cancer cells used (U-87 MG and HCT-116).

To determine the toxicity profile of the new hTP inhibitor, toxicological studies were accomplished with CPBMF-223 in a panel of safety evaluations. Interestingly, the Ames assay showed that the compound did not induce base-pair substitution mutation, as observed by no increasing his+ revertant colonies in all *S. typhimurium* strains. Moreover, CPBMF-223 did not induce gene mutation in prokaryotic cells in the Ames test, until the concentration of 2000 μg/plate. These findings are in agreement with previous reports showing a lack of genotoxic effects for CPBMF-223 in the comet assay [17]. Similarly, TPI was found no mutagenic effects in the *S. typhimurium* strains TA100, TA98, TA1535, and TA 1537 and *E. coli* WP2uvrA in concentrations up to 5000 μg/mL, with or without metabolic activation [44].

Cardiotoxicity is one of the major concerns for oncological treatments. Many chemotherapeutic agents are potentially directly cardiotoxic, as a 5-FU (CCR drug treatment), which has been reported to cause drug-induced cardiac ischemia in patients [45]. Our results suggested the possible cardiac alterations at high concentrations of CPBMF-223, which reduced the heartbeat rate of zebrafish (*Danio rerio*) at 2 dpf (250 and 500 μM). The cardiac effects were partially reverted when observed at 5 dpf as only the highest dose was able to reduce the above rate. This reversal may be correlated to the adaptive capacity of the zebrafish heart induced by improved development of animals at 5 dpf [46]. Furthermore, Zebrafish larvae are capable of free swimming from 3 dpf, together with brain development and the central nervous system [47]. Therefore, locomotor patterns and behaviors were evaluated at 5 dpf after acute exposition to CPBMF-223. Our results demonstrated that exposure to CPBMF-223 altered the locomotor activity in the early stages of zebrafish development, with a decrease in the distance traveled and the mean speed at all concentrations (50, 250, and 500 μM), but there is no difference between the groups. In addition, the absolute body turn angle during movement was increased in animals treated with the highest concentration. In contrast, nothing was observed in the analyzed behavioral pattern. It is to highlight that the

**Table 4**

Induction of $his^+$ revertants in *S. typhimurium* strains by CPBMF-233 with and without metabolic activation (S9 mix).

| S. typhimurium strains | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Substance | Concentration (μg/plate) | TA98 | | TA97a | | TA100 | | TA1535 | | TA102 | |
| | | Rev/plate[a] | MI[b] | Rev/plate[a] | MI[b] | Rev/plate[a] | MI[b] | Rev/plate[a] | MI[b] | Rev/plate[a] | MI[b] |
| **Without metabolic activation (−S9)** | | | | | | | | | | | |
| NC[c] | – | $35.7 \pm 10.0$ | – | $113.0 \pm 16.5$ | – | $79.0 \pm 5.6$ | – | $5.7 \pm 2.1$ | – | $345.0 \pm 20.2$ | – |
| **CPBMF-223** | 100 | $39.7 \pm 5.8$ | 1.11 | $132.3 \pm 26.7$ | 1.17 | $77.0 \pm 13.3$ | 0.97 | $7.0 \pm 1.0$ | 1.23 | $453.0 \pm 50.3$ | 1.31 |
| | 250 | $38.3 \pm 9.5$ | 1.07 | $138.3 \pm 35.6$ | 1.22 | $82.3 \pm 14.4$ | 1.04 | $4.7 \pm 0.6$ | 0.82 | $342.0 \pm 59.2$ | 0.99 |
| | 500 | $38.0 \pm 7.1$ | 1.06 | $125.5 \pm 46.0$ | 1.11 | $83.5 \pm 14.7$ | 1.06 | $9.7 \pm 1.5$ | 1.70 | $383.0 \pm 7.9$ | 1.11 |
| | 1000 | $24.0 \pm 1.4$ | 0.67 | $114.3 \pm 38.5$ | 1.01 | $89.3 \pm 23.7$ | 1.13 | $7.8 \pm 0.6$ | 1.37 | $327.7 \pm 37.8$ | 0.95 |
| | 2000 | $22.3 \pm 5.9$ | 0.62 | $89.0 \pm 13.9$ | 0.79 | $88.3 \pm 9.8$ | 1.12 | $5.7 \pm 0.6$ | 1.00 | $360.0 \pm 16.6$ | 1.04 |
| PC[d] | 0.5 (4-NQO) 1 (NaN₃) | **87.5 ± 13.4** \*\*\* | **2.45** | **335.5 ± 54.5** \*\*\* | **2.97** | **720.5 ± 31.8** \*\*\* | **9.12** | **876.5 ± 61.5** \*\*\* | **153.8** | **2407.0 ± 213.5** \*\*\* | **6.98** |
| **With metabolic activation (+S9)** | | | | | | | | | | | |
| NC[c] | – | $31.0 \pm 7.2$ | – | $77.5 \pm 6.4$ | – | $109.3 \pm 19.0$ | – | $9.3 \pm 3.5$ | – | $445.3 \pm 28.5$ | – |
| **CPBMF-223** | 100 | $35.3 \pm 8.3$ | 1.14 | $63.0 \pm 2.8$ | 0.81 | $102.7 \pm 14.4$ | 0.94 | $9.3 \pm 2.5$ | 1.00 | $444.3 \pm 21.0$ | 1.00 |
| | 250 | $33.0 \pm 2.6$ | 1.06 | $65.3 \pm 18.5$ | 0.84 | $84.3 \pm 25.6$ | 0.77 | $7.0 \pm 1,7$ | 0.75 | $368.0 \pm 21.7$ | 0.83 |
| | 500 | $33.3 \pm 8.5$ | 1.07 | $91.7 \pm 8.5$ | 1.18 | $70.3 \pm 11.9$ | 0.64 | $11.7 \pm 6.0$ | 1.26 | $381.0 \pm 62.1$ | 0.86 |
| | 1000 | $26.0 \pm 5.3$ | 0.84 | $81.7 \pm 35.8$ | 1.05 | $75.7 \pm 4.7$ | 0.69 | $8.3 \pm 3.1$ | 0.89 | $402.3 \pm 56.9$ | 0.90 |
| | 2000 | $34.3 \pm 3.5$ | 1.11 | $65.7 \pm 13.5$ | 0.85 | $66.0 \pm 18.5$ | 0.60 | $9.5 \pm 0.7$ | 1.02 | $328.0 \pm 15.6$ | 0.74 |
| PC[d] | 2 (2-AA) | **170.5 ± 26.2** \*\*\* | **5.50** | **299.5 ± 41.7** \*\*\* | **3.86** | **465.5 ± 7.8** \*\*\* | **4.26** | **69.0 ± 7.1**\*\*\* | **7.42** | **1428.0 ± 55.7** \*\*\* | **3.21** |

Statistical analysis was performed in relation to the negative control using a one-way analysis of variance followed by Dunnet's post-test.

[a] Number of revertants/plate: mean of two independent experiments ± standard deviation.

[b] MI: mutagenic index (no. of $his^+$ induced in the sample/no. of spontaneous $his^+$ in the negative control).

[c] NC: negative control (10 μL dimethylsulfoxide used as a solvent for the CPBMF-223).

[d] PC: positive control (−S9) sodium azide (NaN₃) to TA100 and TA1535; 4-nitroquinoline-oxide (4-NQO) to TA97a, TA98 and TA102; (+S9) 2-aminoanthracene (2-AA).

\*\*\* $p < 0.001$.

concentrations used in zebrafish assays were based on cytotoxicity and genotoxicity tests previously reported by our group [17]. As shown below, these concentrations are far above the maximum concentrations of CPBMF-223 already found in pharmacokinetic assays and very above concentrations needed to inhibit hTP activity.

Regarding the results of acute oral toxicity, the dose of 300 mg/kg was responsible for the death of one animal, which is acceptable by the OECD protocol. For the other animals tested at doses of 300 and 2000 mg/kg, no changes in the general activity, awareness, and sensitivity of the mice were observed. The mice in the treated groups did not show weight changes when compared to the vehicle control group after 14 days, which confirms the low toxicity profile of CPBMF-223. In addition, no significant changes were observed in the liver, kidney, or spleen weights. The acute oral toxicity uses pre-defined doses and allows to classify the test compound according to the Globally Harmonized System (GSH) for the classification of the chemical. Based on the obtained results, CPBMF-223 was classified as category 5, where median lethal oral dose (LD$_{50}$) is above 2000 mg/kg [28]. A similar result was found for the TPI; no mortality was observed after a single dose in female and male rats, and showed LD$_{50}$ greater than 2000 mg/kg [48].

The oral and i.p. bioavailability determined for CPBMF-223 were low and high, respectively. The *F* value for i.p. is consistent with that presented by the TPI, both seem to have bioavailability greater than 100% through this route of administration in mice [49]. The i.p bioavailability *F* determined for CPBMF-223 may be related to loss of substance during the i.v. route of administration. Accordingly, the loss can occur before the substance reaches the bloodstream because drugs administered by i.v. route can be extensively metabolized by the lungs, and consequentially, reduce the drug availability (plasma drug concentration) [50]. Theoretically, a drug administered by the i.v. route has 100% bioavailability, however studies have been shown that the bioavailability by the i.v. route is not always complete [50,51], corroborating with our results. Future investigations need to be conducted to observe potential factors underlying the i.p bioavailability of CPBMF-223 observed in mice.

Post-i.v. clearance (CL) was found to be rapid, indicating the probability of low oral bioavailability *F* and compatibly short half-life (t$_{1/2}$), which was confirmed by our results. The i.v. and oral results for Ke and t$_{1/2}$ here presented, together with previously reported by pharmacokinetic results about i.p. route and metabolic stability, confirm the rapid/moderate elimination rate of this compound [17].

Thus, we show that treatment with CPBMF-223, the most effective noncompetitive inhibitor of hTP described in the literature, was able to reduce the growth of the colorectal tumor in nude mice. Interestingly, the compound showed a reduction in HCT-116 migration cell, reduction in cell proliferation markers and in the number of blood vessels in the tumor. In addition, the treatment does not show any alteration in body weight or in liver injury enzymes. Moreover, the CPBMF-223 exhibited no mutagenesis in *Salmonella*/microsome assay and the absence of acute oral toxicity. Using zebrafish for toxicity assays, some cardiac and locomotor changes were observed in high concentrations of compound, which were above the maximum concentrations already found in mouse plasma. Further studies to evaluate the mechanism of action and using the oral route for CPBMF-223 alone or in combination will be needed. Finally, our results shed new light on the role of hTP enzyme in the colorectal tumor model, pointing out CPBMF-223 as a promising drug candidate for cancer management.
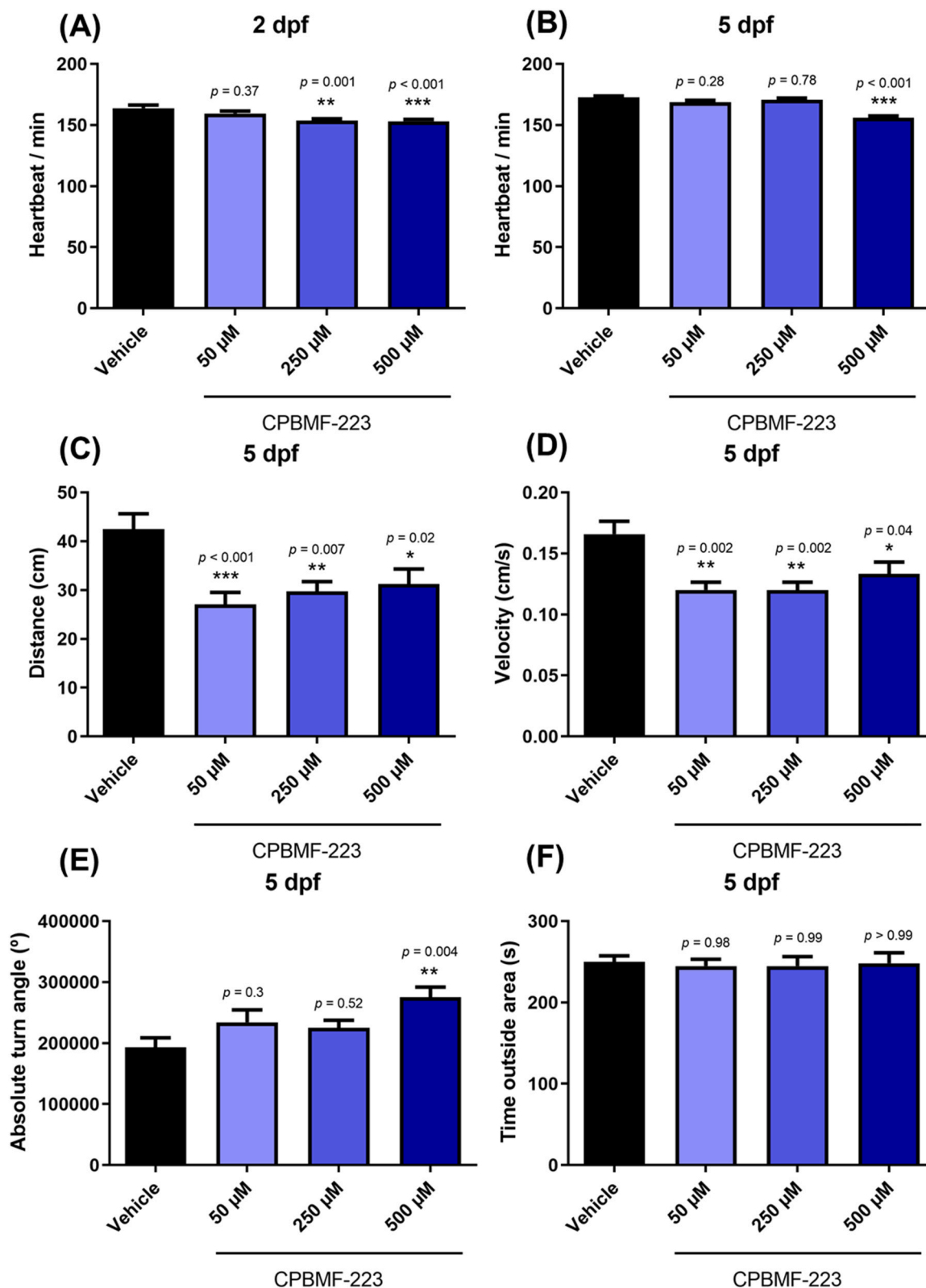
## Funding

**Fig. 4.** Effect of CPBMF-223 (50–500 μM) acute treatment in early stages of heartbeat, exploratory behavior, and neurotoxicity in zebrafish larvae. The heartbeat was evaluated in 2 and 5 days postfertilization (dpf) (A and B), while distance (C), velocity (D), angular velocity (E) and frequency outside area (F) were evaluated in 5 dpf. Data are expressed mean ± standard error of the mean from 18 (Heartbeat) or 30 animals tested individually for each group and were analyzed by one-way analysis of variance, followed by Tukey post-test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significantly from vehicle group (negative control).

**Table 5**
Acute toxicity evaluation of CPBMF-223 in mice.

| Experimental group | Body weight (g) | | Organ weight/body weight (mg/g) | | |
|---|---|---|---|---|---|
| | Day 0 | Day 14 | Kidney | Liver | Spleen |
| **Vehicle (n = 8)** | 25 ± 0.9 | 27.4 ± 1.4 | 13 ± 0.6 | 45.7 ± 1.7 | 3.7 ± 0.3 |
| **CPBMF-223 300 mg/kg (n = 5)** | 24.5 ± 0.9 | 26.8 ± 0.7 | 13.6 ± 0.5 | 47.4 ± 2 | 4.4 ± 0.4 |
| **CPBMF-223 2.000 mg/kg (n = 6)** | 25.7 ± 0.5 | 28.8 ± 1 | 13.5 ± 0.5 | 47.6 ± 0.9 | 4.7 ± 0.3 |

Effects of CPBMF-223 on body weight and kidney, liver and spleen weights of CF1 mice, after a single oral administration of 300 mg/kg or oral administration of 2.000 mg/kg in two doses (4 h interval between doses). Data representing the mean ± standard error of the mean.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111672.

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