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PRECLINICAL STUDIES

CPBMF65, a synthetic human uridine phosphorylase-1 inhibitor, reduces HepG2 cell proliferation through cell cycle arrest and senescence

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Summary

Hepatocellular carcinoma (HCC) is the most prevalent type of tumor among primary liver tumors and is the second highest cause of cancer-related deaths worldwide. Current therapies are controversial, and more research is needed to identify effective treatments. A new synthetic compound, potassium 5-cyano-4-methyl-6-oxo-1,6-dihydropyridine-2-olate (CPBMF65), is a potent inhibitor of the human uridine phosphorylase-1 (hUP1) enzyme, which controls the cell concentration of uridine (Urd). Urd is a natural pyrimidine nucleoside involved in cellular processes, such as RNA synthesis. In addition, it is considered a promising biochemical modulator, as it may reduce the toxicity caused by chemotherapeutics without impairing its anti-tumor activity. Thus, the objective of this study is to evaluate the effects of CPBMF65 on the proliferation of the human hepatocellular carcinoma cell line (HepG2). Cell proliferation, cytotoxicity, apoptosis, senescence, autophagy, intracellular Urd levels, cell cycle arrest, and drug resistance were analyzed. Results demonstrate that, after incubation with CPBMF65, HepG2 cell proliferation decreased, mainly through cell cycle arrest and senescence, increasing the levels of intracellular Urd and maintaining cell proliferation reduced during chronic treatment. In conclusion, results show, for the first time, the ability of a hUP1 inhibitor (CPBMF65) to reduce HepG2 cell proliferation through cell cycle arrest and senescence.

Keywords Human uridine phosphorylase-1 · HepG2 cells · Hepatocellular carcinoma · Cell cycle arrest · Senescence

Introduction

Hepatocellular carcinoma (HCC) is the fourth most common cancer and the second highest cause of cancer-related deaths in the world, with an incidence rate that has tripled in the United States in the last three decades [1]. It is a silent disease, usually detected at an advanced stage, reducing the survival rate to approximately 14% within a five-year period [2]. Currently, surgical resection has been considered a key treatment for HCC, but there is recurrence in approximately 80% of patients within a five-year period, mainly due to latent multicentric carcinogenesis or intrahepatic metastasis [3]. In addition, the use of this treatment in multinodular HCC is controversial, with literature suggesting transplantation as the best alternative [3–5].

Considering that new, effective treatments are needed, novel drugs are being developed using specific defined molecular targets to reach metabolic pathways of the tumor. Potassium 5-

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cyano-4-methyl-6-oxo-1,6-dihydropyridine-2-olate (CPBMF65) is one of these drugs, synthesized and produced at the Pontifical Catholic University of Rio Grande do Sul (PUCRS). The drug was previously tested by Renck et al.[6], in order to verify the 5-fluorouracil (5-FU) toxicity decrease in SW-620 cells. This new molecule is an inhibitor of human uridine phosphorylase-1 (hUP1), which controls uridine (Urd) cell concentration through the reversible phosphorolysis of uridine (Urd) to uracil and ribose-1-phosphate in the presence of inorganic phosphate, using the pyrimidine salvage pathway [7].

Urd is a natural pyrimidine nucleoside involved in cellular processes, such as RNA synthesis, and is also a promising biochemical modulator, as it may reduce the toxicity caused by chemotherapeutics, such as 5-FU, without impairing its anti-tumoral activity [8, 9]. High doses of Urd are necessary to achieve a protective effect for the adverse events of chemotherapy, although the exogenous administration of Urd is not well tolerated, considering that very high doses would have to be administered in order to obtain the desirable effect, due to the rapid degradation caused by hUP1 [8, 10]. Taking that into account, selective inhibitors of hUP1 have been suggested to increase Urd levels [6]. Thus, the objective of this study was to evaluate the effects of the hUP1 inhibitor, CPBMF65, on HepG2 cell proliferation, as well as the mechanisms involved in its effects. A better understanding of its actions and mechanisms may contribute to the development of new therapeutic targets for the treatment of HCC.

Materials and methods

HepG2 cell culture

Human HCC HepG2 cells (Banco de Células do Rio de Janeiro – BCRJ, code: 0103) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life-technologies, USA), supplemented with fetal bovine serum (FBS, 10%; Gibco, Life-technologies, USA) and 1% penicillin/streptomycin antibiotics (ATB; Gibco, Life-technologies, USA) and maintained in a humidified incubator at 37 °C and 5% CO_2. Cells were seeded in 96-, 24-, or 6-well plates, depending on the assay, with a cell density of 2.5×10^4, 5×10^4, or 1.2×10^6 cells/well, respectively. All experiments were performed in triplicate.

Peripheral blood mononuclear cell (PBMC) preparation

The PBMCs were used to verify whether treatment with CPBMF65 would influence viability or induce toxicity in normal human cells. The PBMCs were isolated from the blood of healthy humans by gradient centrifugation on Ficoll-Paque (GE Halthcare, USA). A total of 12 mL of heparinized blood was diluted 1:2 with saline solution. Then, 2 mL of Ficoll-Paque was added to 6 mL of the previous dilution and centrifuged at 720 x g and 22ºC for 20 min. PBMCs were collected (cloudy layer) from the diluted blood/Ficoll-Paque interface. Afterward, the cell pellet was washed twice in 10 mL of phosphate buffered saline (PBS). Cells were re-suspended in Roswell Park Memorial Institute (RPMI 1640; Gibco, Life-technologies, USA), supplemented with 0.15% garamycin (Schering-Plough, USA) and 20% homologous serum at a final density of 1.6×10^6 cells/mL and maintained in humidified incubator at 37 °C and 5% CO_2 [11]. All experiments were performed in triplicate.

Treatment with CPBMF65 and Urd

HepG2 cells were seeded in 96-well plates and incubated with medium alone or with crescent concentrations of CPBMF65 (7.5, 15, 45, 90, 135, and 180 μM diluted in medium) and Urd (1.5 and 10 mM diluted in medium). After 48 h of treatment, the dose-response curve and cytotoxicity were evaluated. A previous time-curve and the concentrations chosen were based on a previous study performed on SW-620 cells [6].

Evaluation of cellular proliferation

HepG2 cells were seeded in 96-well plates (2.5×10^4 cells/well) for 48 h and PBMCs were seeded in 96-well plates (1.6×10^6 cells/well) for 96 h. After treatment, the number of viable cells was determined by mixing 25 μL of cell suspension and 25 μL of 0.4% trypan blue (Sigma-Aldrich, USA) using a Neubauer hemocytometer and optical microscope (Nikon Optiphot, Japan). The trypan blue dye exclusion assay was used to determine the number of viable cells present in the cell suspension, considering that living cells have intact cell membranes that exclude certain dyes, such as trypan blue [12]. Non-viable cells were not counted, considering that a number inferior to 1% was demonstrated. The results were expressed as the total number of living cells per well.

Measurement of lactate dehydrogenase (LDH)

The cytotoxicity of the treatments was evaluated by the presence of the enzyme LDH, since the release of LDH (located in the cytoplasm) in the culture medium is considered evidence of cell membrane rupture. HepG2 cells were seeded in 96-well plates (2.5×10^4 cells/well) and treated with CPBMF65 for 48 h using concentrations of 90, 135, and 180 μM. HepG2 cells were also seeded in 96-well plates (2.5×10^4 cells/well) and treated with Urd for 48 h, using concentrations of 5 and 10 mM. Enzyme activity was measured in both supernatants and lysates using the colorimetric LDH kit (Labtest, Brazil). As a control of cell lysis, we used Tween® 20% (Sigma-Aldrich,

USA) in the culture medium. LDH release was calculated by measuring the absorbance at 492 nm using an Elisa microplate reader (Biochrom EZ Read 400 Microplate Reader).

Evaluation of apoptosis

The PE-Annexin V/7AAD (BD Biosciences, USA) dual label assay was performed to quantify cells in apoptosis. HepG2 cells were seeded in 96-well plates (2.5×10^4 cells/well) and treated with CPBMF65 (90 μM) and 40 μM of cisplatin (CDDP; a positive control for apoptosis; Fauldcispla®, Cisplatina, Libbs Farmacêutica Ltda, Brazil). After 48 h of treatment, cells were trypsinized, washed with ice-cold PBS, and resuspended in binding buffer at a cell density of 1×10^6 cells/mL. PE-Annexin V (5μL) and 7-AAD (5μL) were added, and cells were homogenized and incubated, while protected from light, for 15 min at room temperature. After incubation, the samples were analyzed using flow cytometry in the FACSCanto II Flow Cytometer (BD Bioscience, USA). Data were analyzed in the FlowJo 7.2.5 software program (Tree Star Inc., USA). The analysis allowed for discrimination between necrotic cells (Annexin V- / 7-AAD+), late apoptosis (Annexin V+ / 7-AAD+), and early apoptosis (Annexin V+ / 7-AAD).

Evaluation of autophagy

The formation of acidic vesicle organelles (AVOs) was analyzed using acridine orange (AO) dye (Sigma-Aldrich, USA). The dye emits green fluorescence in the nucleus and cytoplasm and red fluorescence when AVO formation occurs [13], indicating that the cells are in an autophagic process. HepG2 cells were seeded in 96-well plates (2.5×10^4 cells/well) and treated with CPBMF65 (90 μM). Cisplatin (20 μM) was used as a positive control (Fauldcispla®, Cisplatina, Libbs Farmacêutica Ltda, Brazil). After 48 h of treatment, cells were desalted from the culture dish with trypsin and incubated with AO (1 μg/mL) in culture medium, protected from light, for 15 min at room temperature. Briefly, cells were then visualized with a fluorescence microscope (IX71 Inverted Microscope, Olympus, Japan), and the percentage of AVOs, as well as the intensity of orange fluorescence, were measured with a FACSCanto II flow cytometer (BD Bioscience, USA) and evaluated using FlowJo 10.0.8 software (Tree Star Inc., USA).

Evaluation of senescence

The nuclear morphometric analysis (NMA) method is based on the measurement of the size and shape of the nucleus of eukaryotic cells *in vitro*. This technique enables the evaluation of the number of cells in senescence. HepG2 cells were seeded in 24-well plates (5×10^4 cells/well) and treated with CPBMF65 (90 μM) or Urd (5 and 10 mM). The culture medium was discarded, and four steps were performed: (1) labeling of the nuclei with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent dye that binds strongly to the adenine rich regions and thymine in DNA sequences [14]; (2) acquisition of images using an inverted fluorescence microscope (20x microscope objective; Eclipse TE2000-S, Nikon, Japan). At least 200 nuclei in three independent experiments were analyzed at each data point. Each experiment contained three internal replicates and five fields were captured per well; (3) obtainment of the morphometric data (Image Pro Plus, Media Cybernetics, USA); (4) data analysis (Excel 2013, Microsoft, USA), according to the protocol described by Filippi-Chiela et al. [15]. As a positive control, 150 μM of hydrogen peroxide (H_2O_2) was used [16].

Quantification of intracellular uridine

HepG2 cells were seeded in 24-well plates (5×10^4 cells/well) and treated with selected doses of CPBMF65 (90 μM) and Urd (5 and 10 mM). After 48 h of treatment, counts were made using trypan blue (Sigma-Aldrich, USA), and each group was adjusted to the amount of 7×10^5 cells. Cells were washed three times with PBS and disrupted by sonication. The intracellular uridine was then quantified through high performance liquid chromatography with a diode array detector (HPLC-DAD; Thermo Scientific, USA). A calibration curve was made from 0.625 to 20 μM with an uridine standard (Sigma-Aldrich, USA) and centrifuged at 13,000 rpm for 30 min in Amicon microtubes (3 kDa molecular weight cutoff). The samples (100 μL) were injected into a Sephasil Peptide C18 ST 250 × 4.6 mm, 5 μm, 100 Å column (GE HealthCare, USA) maintained at 20 °C, and the flow rate was set at 0.5 mL/min with a mobile phase of 0.1% acetic acid. Under these conditions, the retention time of uridine was 31.7 min with a linear relationship ($r > 0.99$) between the peak area and uridine concentration, normalized by the saline group concentration.

Evaluation of the cell cycle

The FITC BrdU Flow Kit (BD Biosciences, USA) was used to evaluate if treatments would interfere with the cell cycle. Briefly, HepG2 cells were seeded in 96-well plates (2.5×10^4 cells/well) and incubated for 24 h in culture medium only, without FBS. Afterward, treatment with selected doses was performed. After 48 h of treatment with CPBMF65 (90 μM) or Urd (5 and 10 mM), cells were incubated with bromodeoxyuridine (BrdU) and fixed with BD Cytofix/Cytoperm buffer (BD Biosciences, USA). After fixation, cells were treated with DNase to expose the incorporated BrdU. Subsequently, the total DNA was labeled with 7-AAD. The labeled cells were analyzed on the FACSCanto II flow cytometer (BD Biosciences, USA), and the results were

analyzed using the program FlowJo 7.6.5 (Tree Star Inc., USA). As a positive control, H_2O_2 (150 μM) was used.

Drug resistance analysis

The cumulative population doubling (CPD) assay was used to evaluate the proliferation rate and regrowth of HepG2 cells after treatment. HepG2 cells were seeded in 6-well plates (1.2×10^6 cells/well) and treated with CPBMF65 (180 and 90 μM) or Uridine (5 and 10 mM). Cisplatin (20 μM; Fauldcispla®, Cisplatina, Libbs Farmacêutica Ltda, Brazil) was used as a positive control. After 48 h of treatment, cells were harvested and seeded with cell densities of 12×10^4, 9×10^4, 6×10^4, and 3×10^4 cells/well in 24-well plates and incubated for 48 h at 37 °C in a 5% CO_2 incubator. Cells were retreated at days 1, 3, 5, and 8 for 48 h, followed by cell counting (trypan blue dye exclusion assay). Control cells and wells that were not retreated received fresh medium with 10% FBS in the same time interval. Cells were counted on the third, fifth, seventh, and tenth days after seeding [17]. CPD of each interval was determined according to the formula PD = [log N (t) - log N (to)]/log 2, where N (t) is the number of cells at the time count, and N (to) is the number of cells seeded. The sum of PDs was then plotted against the time of the culture.

Statistical analysis

Results were presented using descriptive statistics (mean ± standard deviation). For the comparison of means between groups, a one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test for multiple comparisons, was used. In the presence of asymmetry, the nonparametric correspondent was used. The significance level was set at $p < 0.05$ with a 95% confidence interval, and all data were analyzed using SPSS (Statistical Package for Social Sciences) version 15.0. (SPSS Inc., IBM, USA).

Results

Treatment with CPBMF65 decreases HepG2 cell proliferation without causing cytotoxicity

Firstly, we assessed the antiproliferative effect of CPBMF65 using concentrations of 7.5, 15, 45, 90, 135, and 180 μM in HepG2 cells. Figure 1a shows decreased proliferation when concentrations of 90, 135, and 180 μM were tested. The membrane integrity of CPBMF65-treated HepG2 cells was also analyzed by measuring LDH in both the cell supernatant and lysate. Cells were treated with CPBMF65 (90, 135, and 180 μM), considering the significant results from our previous experiment, in order to determine its toxicity. There were no significant differences in LDH release between groups, indicating that there was no loss of plasma membrane integrity in the cells when compared to the control (Fig. 1b). Next, we decided to investigate whether treatment with CPBMF65 would change cell viability and, also, if there was toxicity to normal human PBMCs. Therefore, PBMCs were also treated with 7.5, 15, 45, 90, 135, and 180 μM of CPBMF65 and toxicity, using trypan blue, was analyzed. No significant results were obtained when comparing treatment groups, demonstrating that there was no viability decrease in the primary culture of PBMCs (Fig. 1c).

CPBMF65 treatment promotes senescence in HepG2 cells

Trypan blue and LDH assay results have shown no cytotoxic response to CPBMF65 treatment. Thus, a third assay using PE-Annexin V/7AAD double-labeling for apoptosis evaluation was performed. Once again, the concentration of 90 μM was chosen, considering it is the lowest dose with a significant antiproliferative effect. CDDP (40 μM) was used as a positive control. Quantification by flow cytometry showed no significant differences between the group treated with CPBMF65 and the control groups. The positive control (CDDP, 40 μM) presented a 45% apoptosis increase (Fig. 2a and b). Autophagy was also investigated in HepG2 cells using the AO dye, as this cellular mechanism involves degradation of unnecessary or non-functional components. CDDP (20 μM) was chosen as a positive control based on previous studies from our laboratory [13, 18]. Cells were quantified using the flow cytometer and visualized under a fluorescent microscope (Fig. 2c and d). There was no significant difference between the control and CPBMF65 groups. However, the positive control, CDDP (20 μM), presented a significant increase of 43% in autophagic cells. We also investigated whether treatment with CPBMF65 would induce senescence, another mechanism that may be involved in decreasing cell proliferation. A significant senescence increase of 9% in HepG2 cells treated with CPBMF65 was observed, while the positive control group (treated with H_2O_2, 150 μM) presented a 16% senescence increase (Fig. 2e and f).

Evaluation of the uridine intracellular levels in HepG2 cells treated with CPBMF65 and uridine

Considering that CPBMF65 is an inhibitor of uridine phosphorylase, we investigated if treatment with Urd would present the same effect in HepG2 cells. Results showed that HepG2 cells treated with 90 μM of CPBMF65 and both 5 and 10 μM of Urd presented a significant increase in the intracellular uridine when compared to the non-treated control. CPBMF65 also presented a significant difference when

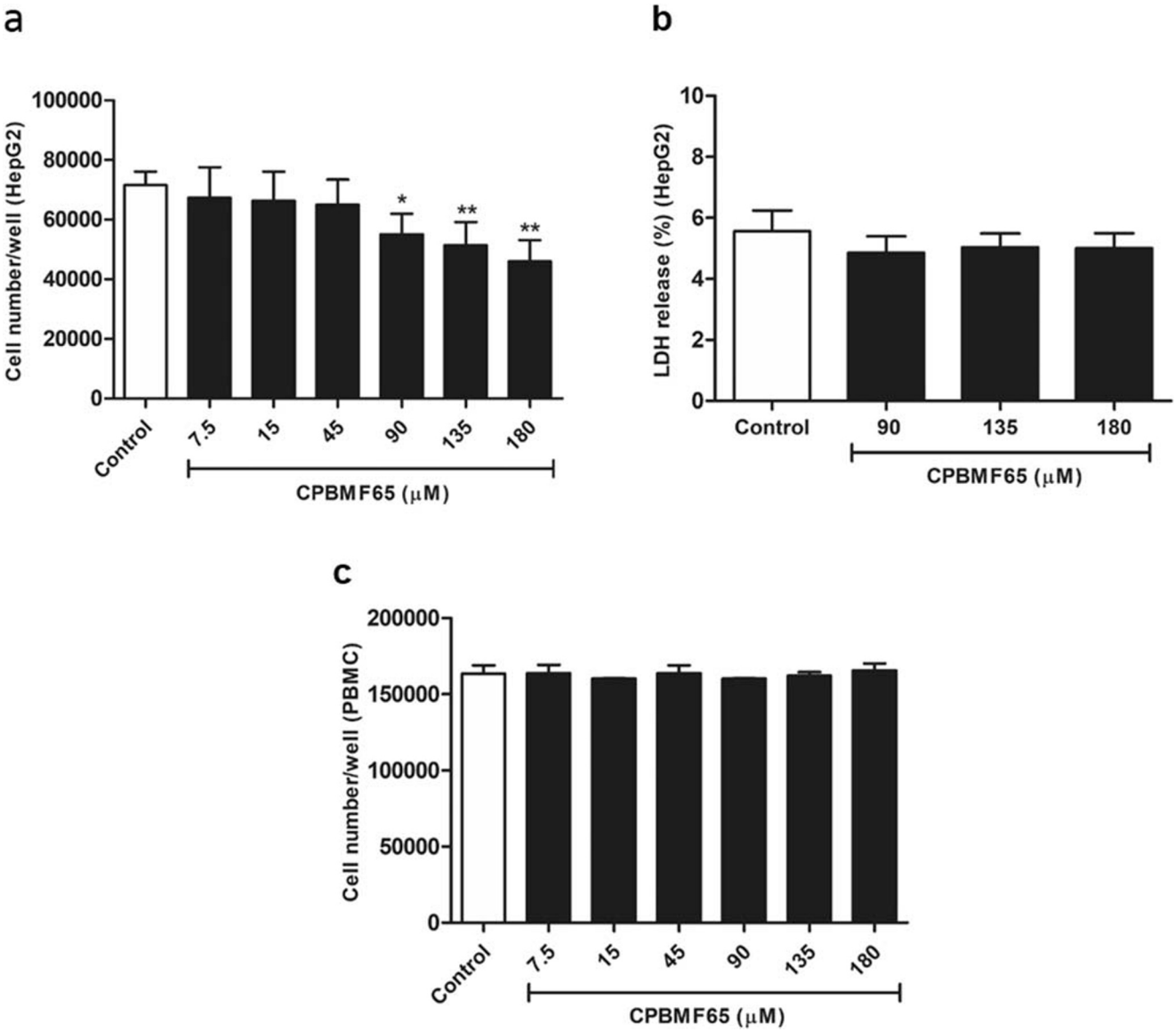

Fig. 1 HepG2 cells treated with CPBMF65 showed reduced cell proliferation without cytotoxicity induction. **a** HepG2 cells treated with CPBMF65 (7.5–180 μM). After 48 h of treatment, cell viability was assessed by direct cell counting. **b** Percentage of LDH release by HepG2 cells after 48 h of treatment with CPBMF65 (90, 135, and 180 μM). **c** PBMCs treated with CPBMF65 (7.5–180 μM) for 48 h. Data represent the mean ± standard deviation (SD), n = 03. *p < 0.05; **p < 0.01

compared to 10 mM Urd, as well as 5 mM Urd when compared to 10 mM Urd (Fig. 3).

Uridine treatment also promotes reduction of HepG2 cell proliferation through senescence induction, without causing cytotoxicity

Considering that CPBMF65 induced a reduction in the number of cells through senescence, we also evaluated if treatment with Urd would decrease cell proliferation and induce senescence. A significant decrease of cell proliferation using 5 and 10 mM of Urd was demonstrated (Fig. 4a). Moreover, data demonstrated that these concentrations did not show cytotoxicity in HepG2 cells (Fig. 4b). In order to explore if the Urd treatment was also capable of inducing senescence, the NMA assay was performed. Results demonstrated that 5 and 10 mM of Urd, as well as 150 μM of H_2O_2 (positive control), induced an increase of senescence in HepG2 cells (Fig. 4c and d).

Treatment with CPBMF65 and uridine promotes cell cycle arrest in HepG2 cells

After demonstrating that CPBMF65 and Urd were capable of inducing senescence, we decided to evaluate its

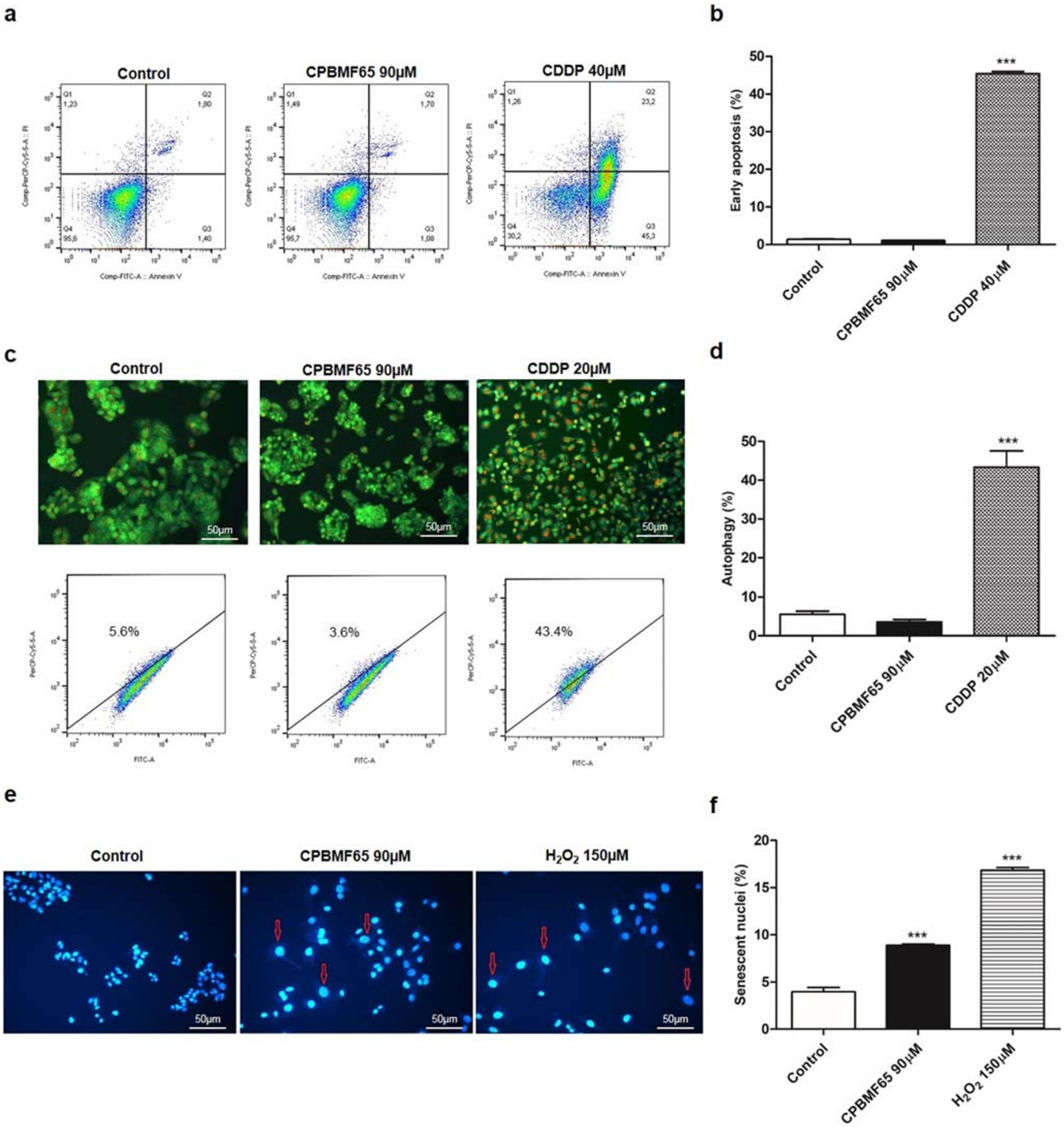

Fig. 2 HepG2 cells treated with CPBMF65 showed an increase of senescent cells without causing apoptosis and autophagy. **a** Representative flow cytometry plots showing apoptosis and **b** graph percentage of early apoptosis cells exposed to CPBMF65 and cisplatin (positive control). **c** Representative flow cytometry plots and images of nuclei from control cells and cells exposed to CPBMF65 and cisplatin (positive control). (AO stain on the lysosome is shown in orange and the nuclei in green; 200x magnification.) **d** Graph percentage of autophagic cells. **e** Representative images of nuclei from control cells and cells exposed to CPBMF65 and cisplatin (positive control). Red arrows indicate senescent nuclei. (f) DAPI-stained nuclei were analyzed for size and irregularity, and the percentage of senescent cells is show. Data represent the mean ± SD, n = 03. ***$p < 0.001$

treatment effects on cell cycle arrest. A significant increase in the G0/G1-phase and a decrease in the S and G2/M-phases, compared to the control, were seen after treatment, indicating that there is a cell cycle arrest when HepG2 cells are treated with CPBMF65 and Urd (Fig. 5).

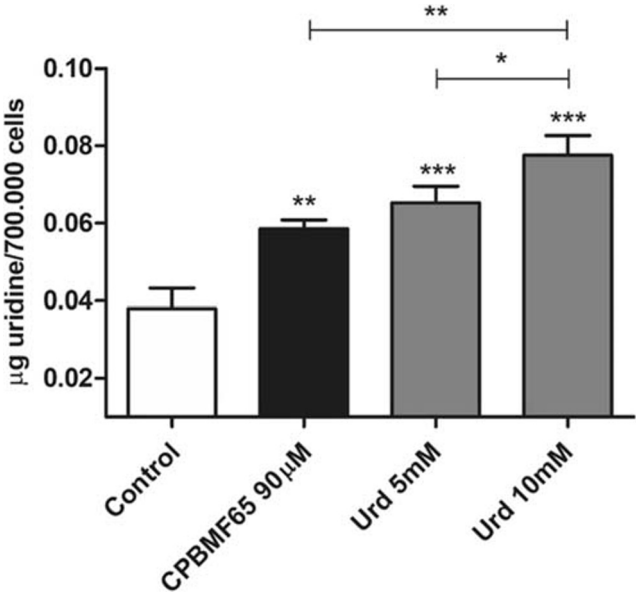

Fig. 3 HepG2 cells treated with CPBMF65 and uridine showed a significant increase in intracellular levels of uridine. Data represent the mean ± SD, n = 03. **p < 0.01; ***p < 0.001

Response of HepG2 cells exposed to long-term treatment with CPBMF65 and uridine

Finally, we evaluated the long-term response of HepG2 cells exposed to treatment with CPBMF65 and Urd at the highest doses. A decrease of cell proliferation was found when HepG2 cells were treated with CPBMF65 (90 and 180 µM) and Urd (10 mM; Fig. 6). CDDP (20 µM) was used as a positive control. Treatment with a single dose of CPBMF65 (180 µM) and retreatment with 90 µM suppressed the regrowth of HepG2 cells during the period of analysis, as well as the positive control CDDP (20 µM). In contrast, treatment with CPBMF65 (90 µM) and Urd (10 mM) did not induce this suppression. Additionally, cells that were retreated with multiple doses of CPBMF65 demonstrated a stable arrest of cell growth and a continuous reduction in the number of cells, suggesting a decrease of cell proliferation, which is in agreement with the NMA and cell cycle analyses (Fig. 6).

Discussion

The development of HCC involves the association of distinct mechanisms of the hepatocarcinogenesis process, leading to the progressive transformation of normal hepatocytes into highly malignant derivatives [19]. Early-stage HCC is responsive to curative treatment, including surgical resection and liver transplantation [4, 5]. Nevertheless, HCC is a major global health problem, being the fourth most common cause of cancer-related death worldwide [1], highlighting the need to research new treatments. In the present study, we have demonstrated, for the first time, the ability of CPBMF65 to modulate mechanisms directly involved in the progression of this tumor *in vitro*.

CPBMF65 is a new molecule that was used, isolated, and tested, for the first time, to explore its antiproliferative effects. Treatment with CPBMF65 yielded a reduction in cell proliferation from the concentration of 90 µm in HepG2 cells when compared to the untreated control group. Therefore, aiming to demonstrate that this effect was not due to cytotoxicity, the LDH levels were measured using concentrations that were able to reduce the number of cells. Our results did not demonstrate correlation between the reduction of cell proliferation and cell death resulting from plasma membrane damage in HepG2 cells. HepG2 cells are commonly used in drug metabolism and hepatotoxicity studies [20]. Considering that the treatment with CPBMF65 did not demonstrate toxicity in HepG2 cells, its safety in non-tumor cells was also investigated. PBMCs were used as a model, and results demonstrated no changes in viability in response to the same CBPMF65 treatment.

Reduction of proliferation in HepG2 cells may be caused by necrosis (cytotoxicity), autophagy, apoptosis, cell cycle arrest, or senescence. Autophagy plays a dual role in tumor promotion and suppression [21]. Once the tumor is established, autophagy helps the tumor to survive and grow, since the basal level of autophagy is increased in cancer cells. This is caused by stress-induced DNA and cellular damage induced by unregulated proliferation [22]. However, at the beginning of this tumor process, autophagy can phagocytize the mutated cells and suppress the tumor by isolating damaged organelles, allowing cell differentiation, as well as increasing and promoting cancerous cell death [23]. In addition, reduction of proliferation may be related to senescence or apoptosis, considering that while autophagy decreases, apoptosis or senescence increases [23]. Our results have shown that the reduction in the HepG2 cell number in response to CPBMF65 treatment was not via apoptotic programmed death activation, nor was there any activation of the autophagy mechanism, which could be associated with some cell defense mechanism in response to treatment.

Another mechanism involved in the tumor response is cell cycle arrest. When cells are exposed to mild stress, the cell cycle can be disrupted, causing growth inhibition and recruitment of DNA repair synthesis machinery. This response allows cells to restore damage induced by cell stress. However, when cells receive a greater stress stimulus that is uncontrollable, it may result in apoptosis or senescence, which are the main routes that limit the growth of tumors, preventing the proliferation of defective cells [24]. Our results have shown an increase in senescent cells, as well as cell cycle arrest in the

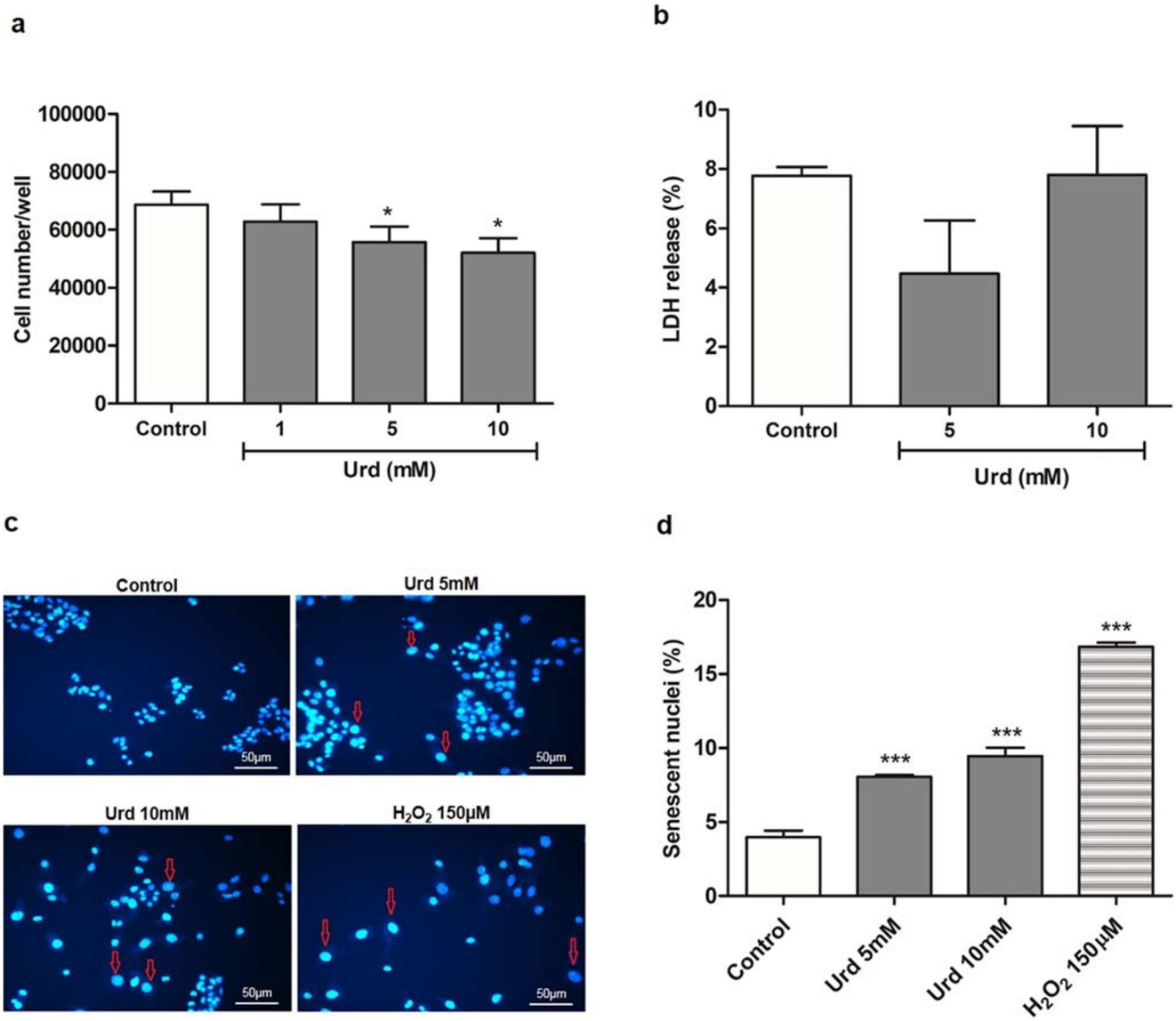

Fig. 4 HepG2 cells treated with uridine showed reduced cell proliferation without affecting viability and an increase in senescent cells. **a** HepG2 cells treated with uridine (1, 5, and 10 mM). After 48 h of treatment, cell viability was assessed by direct cell counting. **b** Percentage of LDH release by HepG2 cells after 48 h of treatment with uridine (5 and 10 mM). **c** Representative images of nuclei from control cells and cells exposed to uridine and H_2O_2 (positive control). Red arrows indicate senescent nuclei. **d** DAPI-stained nuclei were analyzed for size and irregularity, and the percentage of senescent cells is show. Data represent the mean ± SD, n = 03. *p < 0.05; ***p < 0.001

G0/G1-phase, demonstrating the arrest of cell proliferation via induction of cell senescence.

Considering that cell cycle dysregulation is one common characteristics of human cancer, targeting the mitotic phase of the cell cycle may be an effective approach to cancer therapy [25–29]. Many drugs currently used to treat cancer induce senescence, including cisplatin and bleomycin. The mechanism of action of these senescence-inducing drugs is usually linked to DNA damage [30–32]. The cell cycle is controlled by a family of proteins, known as cyclin-dependent kinases and cyclins, and their inhibitors have been found to induce senescence [33, 34]. These data support the fact that inhibitors of the cell cycle can induce senescence and be used as tumor suppressors.

CPBMF65 is an inhibitor of uridine phosphorylase, which creates the possibility of increased intracellular uridine during treatment. This means that when intracellular Urd is increased, it is possible to reduce cancer cell proliferation, considering that this increase reflects a partial halt of the salvage pathway. Cancer cells up-regulate energy metabolic pathways to produce high energy levels and to ensure the availability of essential cellular components for rapid cell proliferation, such as amino acids, nucleotides, and lipids [35]. The salvage pathway operates to recover bases and nucleosides generated from

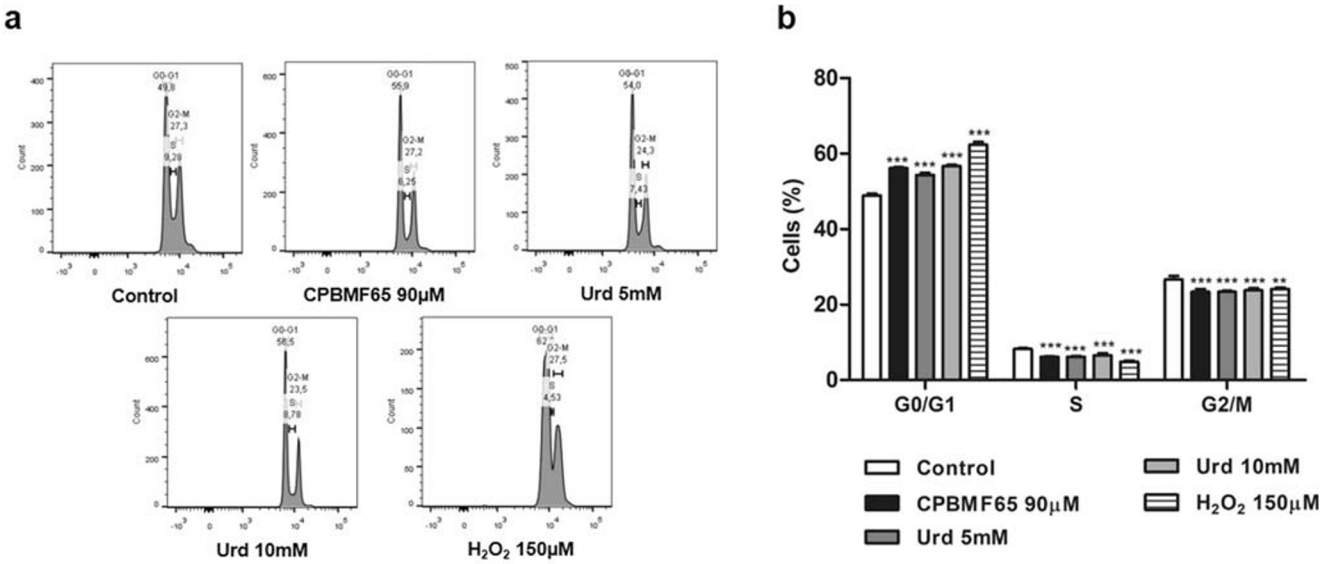

Fig. 5 HepG2 cells treated with CPBMF65 and uridine induced a G0/G1-phase cell cycle arrest. (a) Representative flow cytometry plots showing the cell cycle distribution. (b) Graph distribution of cells (%) between G0/G1, S, and G2 phases in response to treatments. Data represent the mean ± SD, n = 03. ***p < 0.001

the damage of DNA and RNA. The salvaged bases can be then transformed into nucleotides and the salvaged nucleosides reincorporated into DNA. Nucleotides and nucleosides are regenerated, contributing to DNA formation and reactivating cell proliferation and growth [36]. Therefore, we have measured intracellular Urd and observed that the significant increase in intracellular Urd may be related to the reduction of HepG2 cellular proliferation. Moreover, we have compared intracellular levels of Urd in HepG2 cells treated with both CPBMF65 and Urd. Interestingly, despite the significant difference between CPMF65 (90 μM) and Urd (10 mM), CPBMF65 increased intracellular Urd with a much

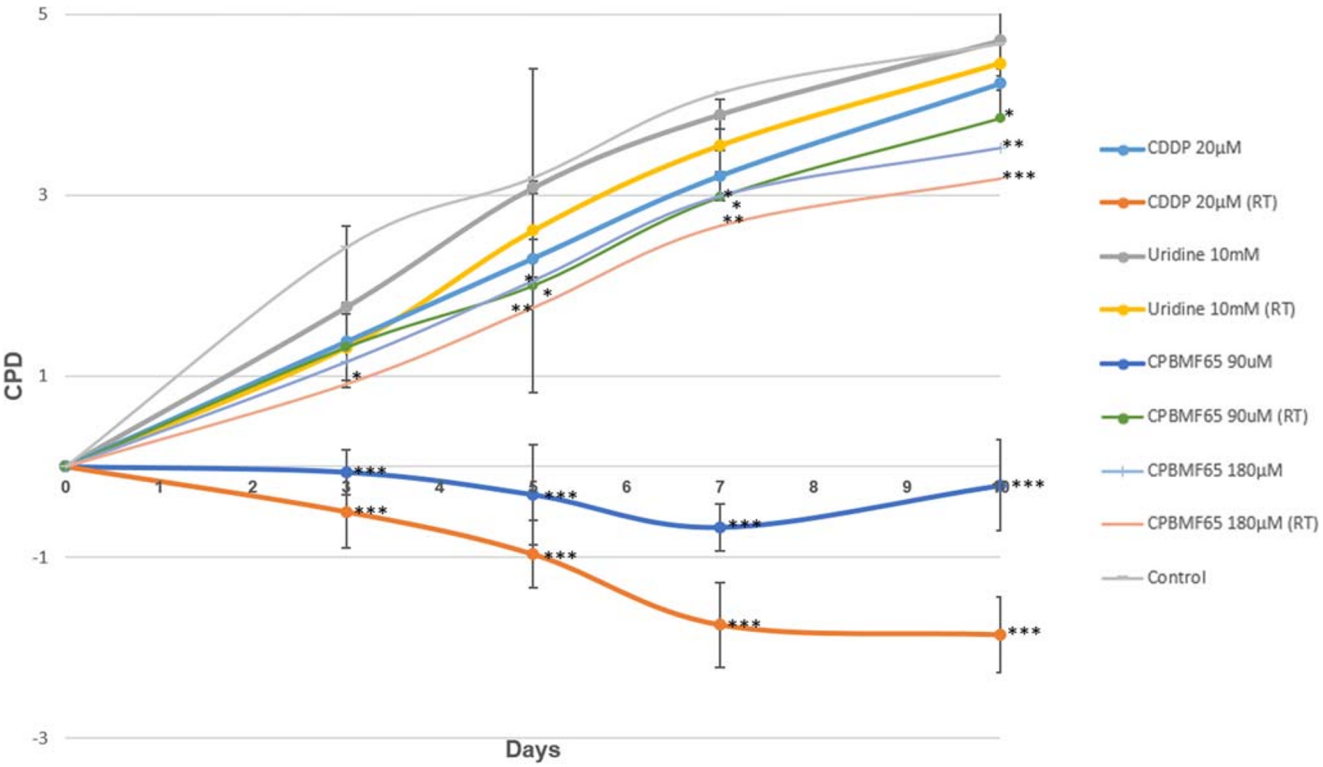

Fig. 6 CPBMF65 reduces tumor cell regrowth in HepG2 cells. Cells were exposed to uridine (10 mM), CPBMF65 (90 and 180 μM), and cisplatin (20 μM, positive control). Data represent the mean ± SD, n = 03. *p < 0.05; **p < 0.01; ***p < 0.001

lower dose, in μM, while a mM dose of Urd had to be used in order to reach a similar result. In addition, when HepG2 cells were treated with Urd, there was no induction of death by toxicity (necrosis), and there was a reduction in the cell number through the same mechanism (cell cycle arrest and senescence), demonstrated when cells were treated with CPBMF65.

In order to verify the long-term effect of the treatment, we also assessed cell viability during a longer period, both with and without retreatment with CPBMF65, Urd, and CDDP (positive control). This experiment enabled us to verify a possible long-term regrowth of tumor cells after treatments. The CPBMF65 molecule demonstrated an interesting result in maintaining the CPD, which was reduced during long-term treatment with a single dose of CPBMF65 (180 μM). CPBMF65 (90 μM) also reduced cell proliferation but only when retreated. However, cells that received treatment with Urd were not able to maintain low CPD levels, resulting in new proliferation.

In conclusion, our results highlight the effect of CPBMF65 in the increase of intracellular Urd, senescence, and cell cycle arrest in HepG2 cells. In addition, the antiproliferative effect of CPBMF65 has a long-term response. Treatment with Urd also shows antiproliferative effects, with cell cycle arrest, senescence, and increased intracellular Urd. Although, it is not able to maintain reduced cell proliferation, even after retreatment was used. Our study demonstrates, for the first time, the ability of a hUP1 inhibitor (CPBMF65) to reduce HepG2 cell proliferation through cell cycle arrest and senescence. Based on its *in vitro* antitumor activity and low toxicity in normal cells, this new compound (CPBMF65) could be considered as a future research candidate for *in vivo* treatment of HCC, considering the need for the development of effective treatments.

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Compliance with ethical standards

Conflict of interest EFGS, KGL, GCK, GVH, LP, AVC, RBG, BSB, HBD, CL, MCRG, BPC, GLA, LAB, MVFD, PM, and JRO declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals, performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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