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ORIGINAL ARTICLE

Antitumor effects of ivermectin at clinically feasible concentrations support its clinical development as a repositioned cancer drug

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Abstract

Purpose Ivermectin is an antiparasitic drug that exhibits antitumor effects in preclinical studies, and as such is currently being repositioned for cancer treatment. However, divergences exist regarding its employed doses in preclinical works. Therefore, the aim of this study was to determine whether the antitumor effects of ivermectin are observable at clinically feasible drug concentrations.

Methods Twenty-eight malignant cell lines were treated with 5 µM ivermectin. Cell viability, clonogenicity, cell cycle, cell death and pharmacological interaction with common cytotoxic drugs were assessed, as well as the consequences of its use on stem cell-enriched populations. The antitumor in vivo effects of ivermectin were also evaluated.

Results The breast MDA-MB-231, MDA-MB-468, and MCF-7, and the ovarian SKOV-3, were the most sensitive cancer cell lines to ivermectin. Conversely, the prostate cancer cell line DU145 was the most resistant to its use. In the most sensitive cells, ivermectin induced cell cycle arrest at G_0–G_1 phase, with modulation of proteins associated with cell cycle control. Furthermore, ivermectin was synergistic with docetaxel, cyclophosphamide and tamoxifen. Ivermectin reduced both cell viability and colony formation capacity in the stem cell-enriched population as compared with the parental one. Finally, in tumor-bearing mice ivermectin successfully reduced both tumor size and weight.

Conclusion Our results on the antitumor effects of ivermectin support its clinical testing.

Keywords Ivermectin · Cancer · Cancer stem cells · Drug repurposing

Introduction

Avermectins are a complex of 16-membered macrocyclic lactones produced from soil fermentation of the actinomycete *Streptomyces avermitilis* [1, 2]. Eight avermectin compounds exist (A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b), and among them, the mixture of 80% B1a and 20% B1b has the highest antiparasitic activity and safety [3]. Such mix compound is known as ivermectin [3]. Ivermectin is a broad-spectrum antiparasitic agent with human therapeutic dose rang between 0.1 and 0.4 mg/kg [4–7]. As an antiparasitic, ivermectin prevents the closure of glutamate-gated chloride ion channels, leading to plasma membrane hyperpolarization, which paralyzes pharyngeal and somatic muscles of the parasite, leading to its death [8].

Ivermectin is a drug candidate for repurposing as an anticancer drug [9]. As such, it is important to determine whether its antitumor effects in vitro can be achieved at pharmacological doses. The therapeutic doses as an antiparasitic compound in human ranges between 0.1 and 0.4 mg/kg, resulting in an AUC of 1444 µg/h/mL, which translates into 1.65 µM using the calculator found in https://www.tocris.com/resources/molarity-calculator, which considers the molecular weight of ivermectin of 875.1 g/mol, a volume of 1 mL, and the mass of 1.444 µg/h/mL. Nevertheless, studies showing the in vitro antitumor effects of ivermectin use this drug at concentrations up to 100 µM, which could not

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be clinically reachable [9–20]. In a phase I pharmacokinetic study done in healthy volunteers, it was demonstrated that doses up to 2 mg/kg can be tolerable [21] using the same formula, we calculated that at this dose, which leads to an AUC of 4547 µg/h/mL [21], the in vitro concentration to be tested test would be 5 µM of ivermectin.

In this sense, we analyzed the in vitro antitumor effects of ivermectin in 28 cancer cell lines using a 5 µM concentration. We found that ivermectin differentially reduced cell viability and clonogenic capacity, through the induction of cell cycle arrest. Moreover, ivermectin synergized with docetaxel, tamoxifen and cyclophosphamide. We also identified a higher antineoplastic effect on cancer stem cells than in the parental population. Finally, we demonstrated an important antitumor effect of ivermectin in vivo.

Materials and methods

Cell lines and drugs

All the cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All the media were supplemented with fetal bovine serum and streptomycin/amphotericin solution (Invitrogen, MA, USA), for a final concentration of 10% and 1%, respectively. All the cell lines, as well as their tissue of origin and the culture medium used for each one, are found on Suppl. Table 1. Particularly, the complete medium of MCF10-A cells was supplemented as previously reported [22]. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. The drugs and their respective vehicles that were employed for this work are found in Suppl. Table 2.

Viability assays and inhibitory concentrations (ICs) with ivermectin

Each cell line was seeded with 1 mL of its respective medium, during a pre-incubation period of 24 h. Then, cells were treated during 72 h with either 5 µM ivermectin or its vehicle (0.1% absolute ethanol). After that, cells were detached with a 0.5% trypsin–1% EDTA solution (Gibco, NY, USA and Invitrogen, MA, USA, respectively) for cell counting with the trypan blue exclusion assay. Briefly, cells were gently mixed at 1:1 ratio with trypan blue stain solution (Life Technologies®), and cell viability was evaluated with a TC10™ Automated Cell Counter (BioRad®). The cytotoxic effect was expressed as the percentage of cell viability relative to control cells. The resulting data were introduced in the SigmaPlot® software version 10.0. The percentage of growth inhibition was calculated, and IC_{20}–IC_{50} values were obtained from survival curves.

Clonogenic assays of ivermectin-treated cells

After finishing cell viability assays, 1000 cells/condition were recovered and cultured in 2 mL of drug-free complete medium during 14–21 days, according to the specific cell line (Suppl. Table 1). Subsequently, colonies were fixed with a methanol/acetic acid (3:1 v/v) solution, and stained with 0.4% crystal violet (Sigma-Aldrich, Germany). Colonies on culture dish were counted with a stereo microscope and quantified using the ImageJ software (2.0 version).

Flow cytometry determination of apoptosis and necrosis of ivermectin-treated cells

DU145, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines were treated with 5 µM ivermectin or its vehicle during 72 h. After that, cells were trypsinized, collected and washed with PBS 1X. Then, cells were labelled with the annexin-V-FLUOS staining Kit (Roche, Germany), and quantified with a BD FACSCanto™ II flow cytometer. 50,000 events/sample were employed to simultaneously analyze apoptosis and necrosis, with the BC FACSDiva™ V6.1.3 software (Becton Dickinson, USA).

Cell cycle analysis of ivermectin-treated cells by flow cytometry

DU145, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines were treated with 5 µM ivermectin or its vehicle during 72 h. After that, cells were trypsinized, collected and washed with PBS 1X. Then, cells were stained with propidium iodide (Sigma®) during 20 min at room temperature in the dark. 50,000 events/sample were collected to evaluate cell cycle with a BD FACSCanto™ II flow cytometer (Becton Dickinson, USA). Cell cycle analysis was performed with the ModFit LT V.2.0 software.

Viability curves and identification of ICs of the chemotherapeutic drugs

DU145, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines were treated with increasing doses of either cyclophosphamide (0.5–10 µM), docetaxel (1–100 pM, 1–10 nM), or tamoxifen (0.5–10 µM), and compared against the corresponding vehicle, during a 72 h period. Then, cell viability and the $IC_{20–50}$ values were evaluated as stated before.

Pharmacological interactions

Increasing doses of ivermectin (IC_{20}, IC_{30}, IC_{40}, and IC_{50}) were combined with their respective increasing doses of

either docetaxel, tamoxifen or cyclophosphamide, depending on the evaluated cell line. The resulting mixes were employed during 72 h for viability assays on the DU145, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines, as previously described. The pharmacological interaction was determined using the combination index (CI) method with the Calcusyn software (Biosoft®) [23], to determine the presence of synergism, antagonism, or additive effects.

Selection of cancer stem-like cells by sorting markers of stemness

Cells from MCF-7, SKOV-3, and MDA-MB-468 cell lines (6×10^6, 6×10^6 and 4×10^6 cells, respectively) were incubated at 4 °C in the dark during 40 min with fluorophore-conjugated monoclonal antibodies against human CD44-FITC (BD Biosciences, 555742) and human CD24-PE (BioLegend, Inc, 311106), or against their respective isotype controls (FITC-IgG2bκ, BD Biosciences, 555742; PE-IgG2aκ, BD Biosciences, 555574). Labeled cells were washed twice with wash buffer. The marker populations were evaluated using BD FACSDiva version 6.1.3 (BD Biosciences), with a BD FACSAria II™ (BD Biosciences) flow cytometer (Suppl. Figure 1).

Cell viability evaluation on cancer stem-like cells

Spheres from MCF-7, MDA-MB-468 and SKOV-3 cell lines were grown in low-adherence flasks (Corning, ME, USA) under stem-cell conditions, as reported by [24, 25]. After a 15-day period of growing, spheres were harvested and centrifuged (350G, 5 min). Next, cellular viability was measured as stated before. 5×10^3 cells/well were seeded in low-adherence 24-well plates (Corning, ME, USA), and treated with either 5 µM ivermectin or its vehicle during 72 h. Finally, cells were stained with trypan blue to assess cell viability, as aforementioned. The number of colonies was counted every 24 h with an inverted optical microscope.

Quantitative reverse transcription and real-time PCR

Cancer stem-like cells from the MCF-7, MDA-MB-468 and SKOV-3 cell lines were treated with either 5 µM ivermectin or its vehicle during 72 h. RNA isolation, qPCR, and primers for *Nanog*, *Oct-4* and *Sox-2* genes were done according to our previous report [9]. The employed primers for mastermind-like transcriptional coactivator 3 (*Maml3*) were: forward, 5′-GGG CGG CAT AAA CAC CAA-3′, and reverse, 5′-GAC ACG CGC GAC ACA CA-3′, and were employed by following [9].

Western blot analysis

DU145, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines were treated with either 5 µM ivermectin or its vehicle during 72 h. Then, total protein extraction and western blot analysis were done, by following our previous report [9]. The primary antibodies anti-PCNA (1:1500; cat. no. SC56, Santa Cruz Biotechnology), anti-Cyclin E (1:1500; cat. no. SC198, Santa Cruz Biotechnology), anti-Cyclin D (1:1500; cat. no. SC753, Santa Cruz Biotechnology), and anti-p21 (1:1500; cat. no. SC-6246, Santa Cruz Biotechnology) or anti-actin peroxidase (1:20,000; cat. no. A 3854, Sigma-Aldrich, Germany) were used. For cyclins E and D, the secondary bovine anti-rabbit antibody (1:2000; cat. no. sc-2370, Santa Cruz Biotechnology) was used, and for p21 and PCNA, the secondary bovine anti-mouse antibody (1:2000; cat. no. sc-2371, Santa Cruz Biotechnology) was employed.

In vivo animal studies

All animal experiments were approved and conducted under the guidelines of the Bioethical and Scientific committees of the National Institute of Oncology (protocol numbers CEI/1145/17 and 017/016/IBI, respectively), in Mexico City, Mexico. 5×10^5 JC murine breast cancer cells were subcutaneously injected in one flank of 6-week-old female Balb/c mice ($n=36$). Each mouse was daily treated with cyclodextrin [26] carrier alone, or with ivermectin conjugated with cyclodextrin (45% final concentration), for a final dosage of 3 mg/kg, during 21 days. This dose is equivalent to 243 µg/kg in humans, according to the Reagan-Shaw formula [27]: human equivalent dose = animal dose (mg/kg) × mouse K_m/human K_m, where the human and mouse K_m was 37 and 3, respectively. Tumor volumes were calculated according to the formula: volume = (Major axis × minor axis^2) × (π/6). Tumor volumes and mice global weight were measured every 3 days until they were killed at day 21, when the tumor was also weighted.

Statistical analyses

Analyses were performed with the GraphPad Prism software (version 6.0; GraphPad Software, CA, USA). All the in vitro and in vivo experiments were conducted at least in triplicate, with three internal replicates. p values were calculated using the unpaired Student's t test followed by Welch's post hoc test for apoptosis and cell cycle assays, as well as for both spheres' viability and colony-forming capacity on the stem population, for mice weight one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test for viability and clonogenicity assay over the 30 cell lines; and two-way ANOVA followed by the Kruskal–Wallis post-hoc

test for tumor volume. Data were expressed as mean ± SD. A p value < 0.05 was considered as statistically significant.

Results

Ivermectin mainly inhibits cell viability and clonogenic capacity on breast and ovarian cancer cell lines

We screened 28 malignant and two healthy cell lines with the maximum safe human serum concentration of ivermectin (5 µM). We found that the two healthy cell lines MCF10-A and HaCaT, as well as the U-2SO and DU145 malignant cell lines, were the most resistant to ivermectin treatment. On the contrary, the MDA-MB-231, MCF-7, MDA-MB-468 and SKOV-3 cell lines were highly responsive, since the percentage of viability decreased by 58%, 67%, 67%, and 70%, respectively (Fig. 1a). Next, clonogenicity tests were performed to determine the ivermectin effect over the ability to form colonies (Fig. 1b). There was no significant effect on the clonogenic capacity in MCF10-A, DU145, A375 and U-2SO cells. Conversely, HT-1080, MDA-MB-468, MDA-MB-231 and SKOV-3 cells got reduced their ability to form colonies (Fig. 1c). To select the cell lines for further assays, we made a linear correlation between viability and clonogenicity. We selected DU145 as a resistant cell line, and MCF-7, MDA-MB-231 and MDA-MB-468 as sensitive cell lines to ivermectin (Fig. 1d).

Ivermectin does not induce cell death by apoptosis on cancer cell lines

Next, to evaluate whether the reduction on cell viability after treatment with ivermectin was due to apoptosis or necrosis, we treated DU145, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines, with 5 µM ivermectin. We observed an absence of significant effect on apoptosis and necrosis in all the evaluated cell lines (Fig. 2a–d). Therefore, we suggested that 5 µM ivermectin does not induce cell death through these pathways.

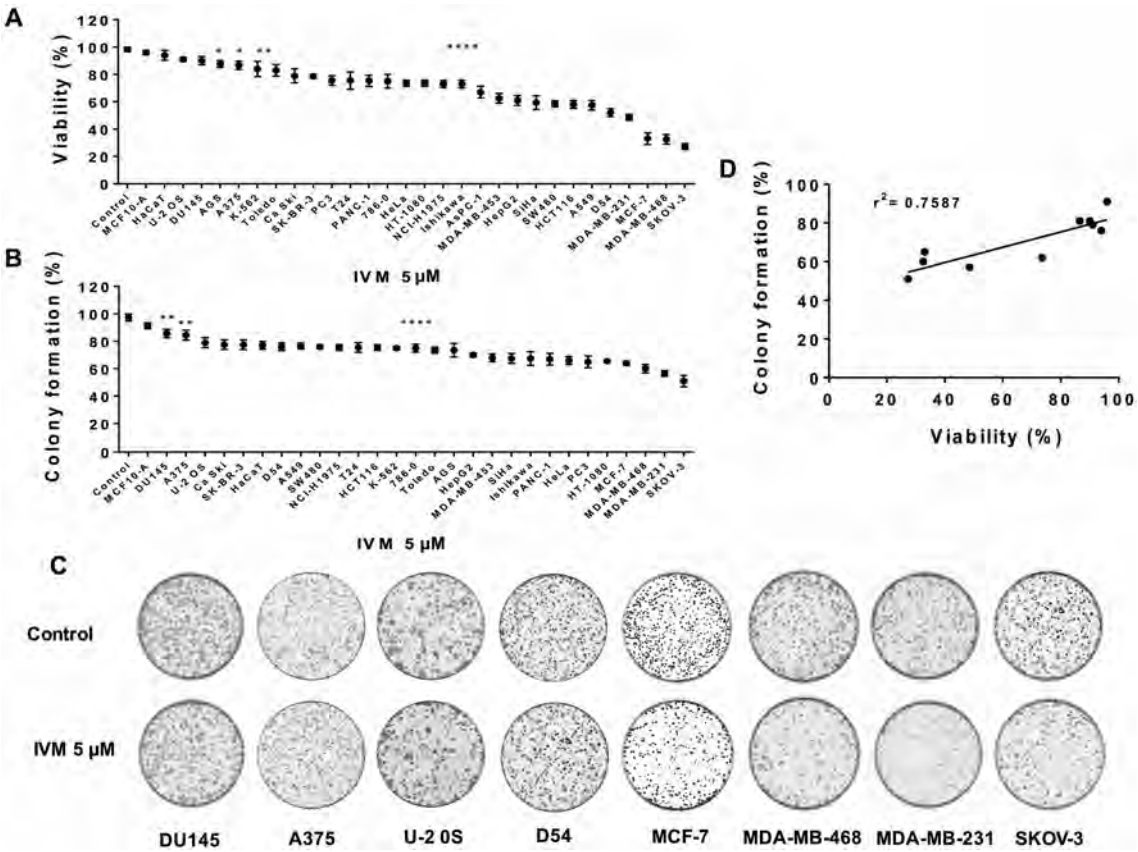

Fig. 1 Ivermectin effect on cell viability and clonogenic capacity over different tumorigenic cell lines. Viability (**a**) and clonogenic (**b**) effects on different cancer cell lines after 5 µM ivermectin. Colony formation on the most sensitive and resistant cell lines to the treatment with ivermectin (**c**). Linear correlation between viability and clonogenicity of the most resistant and sensitive cell lines to ivermectin (**d**). *IVM* Ivermectin; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$

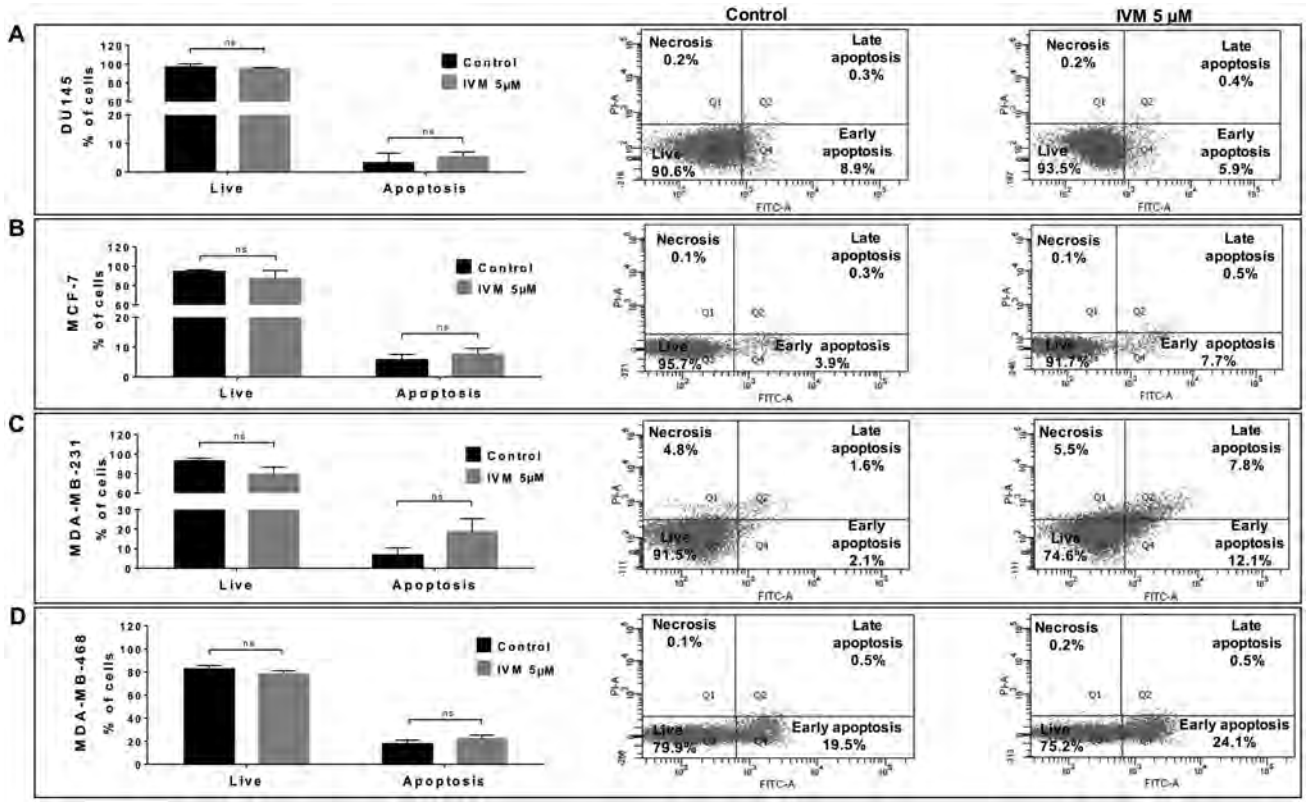

Fig. 2 Ivermectin (5 µM) does not induce apoptosis on the evaluated malignant cell lines. Apoptosis assays on the most resistant cell line DU145 (**a**) and on the most sensitive cell lines MDA-MB-231 (**b**), MCF-7 (**c**), and MDA-MB-468 (**d**). *IVM* Ivermectin, *ns* Non-significant

Ivermectin arrests cells at G_0-G_1 phase of the cell cycle

To conclude whether the effect of ivermectin on sensitive and resistant cell lines was associated with disturbances on the cell cycle phases, we performed cell cycle assays. We observed no significant changes on the resistant line DU145 (Fig. 3a). However, the sensitive cell lines MCF-7, MDA-MB-231 and MDA-MB-468 were arrested in the cell cycle phase G_0-G_1. (Fig. 3b–d). Western blot assays showed that the treatment with ivermectin on the sensitive cell lines decreases the levels of cyclin D, cyclin E and PCNA, while p21 got increased (Fig. 3e–f). These results suggest that ivermectin blockades the transition from G_0-G_1 to S phase.

The pharmacological combination of ivermectin and standard chemotherapeutics has synergistic effects

Since ivermectin could be therapeutically repositioned, we decided to evaluate a possible synergistic effect between ivermectin and some chemotherapy drugs currently used to treat breast and prostatic cancer. To this end, we performed dose–response curves with docetaxel, tamoxifen and cyclophosphamide. The inhibitory concentrations $(ICs)_{20–50}$ were calculated for both ivermectin (Table 1) and the chemotherapeutic compounds (Table 2). Subsequently, the combination index (CI) was determined by combining the ICs of ivermectin plus the chemotherapeutic drug. Interestingly, the resistant cell line DU145 had the most synergistic effect, since the IC_{20} combination of ivermectin plus docetaxel showed a strong negative CI value (Fig. 4a). Besides, we observed a synergistic effect on MCF-7, MDA-MB-231 and MDA-MB-468 cell lines, starting at IC_{30} doses of the analyzed compounds (Fig. 4b–d). This suggests that ivermectin could be combined with some standard therapies as a treatment against certain types of cancer.

Ivermectin reduces cell viability and colony percentage on the cancer stem-like malignant population

We have previously reported that ivermectin preferentially targets the cancer stem-like population on the MDA-MB-231 cell line [9]. Therefore, we wanted to determine whether ivermectin has the same effect over the cancer stem-like cells derived from MCF-7, MDA-MB-468 and SKOV-3 cell lines. After isolating the cancer stem-like population

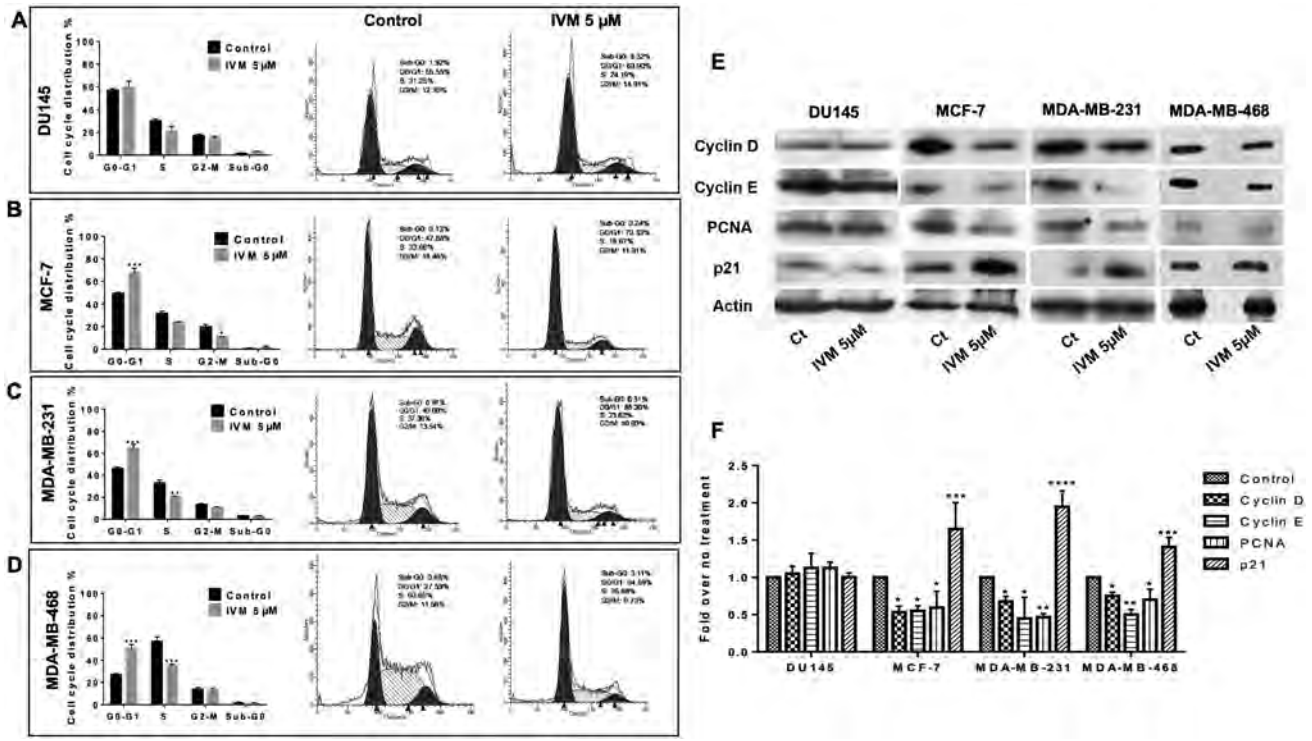

Fig. 3 Ivermectin (5 µM) arrests cell cycle at G_0-G_1 phase. Cell cycle assay on the DU145 (**a**), MCF-7 (**b**), MDA-MB-231 (**c**) and MDA-MB-468 (**d**) cell lines. Western blot assays of cyclin D, cyclin E, PCNA, p21 and actin proteins of the aforementioned cell lines (**e**) and their densitometry comparison (**f**). *IVM* Ivermectin; $*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$

Table 1 Inhibitory concentrations $(IC)_{20\text{-}50}$ of ivermectin

Cell line	IC_{20} (µM)	IC_{30} (µM)	IC_{40} (µM)	IC_{50} (µM)
DU145	10.3	11.5	12.5	13.5
MCF-7	0.42	0.69	1.09	1.66
MDA-MB-231	0.47	0.85	2.03	6
MDA-MB-468	0.31	0.55	0.93	1.52

from the parental cells (total of sorted populations 7.8×10^5, 9.4×10^5, and 1.8×10^4, respectively), the sorted populations were treated with 5 µM ivermectin. We observed that among all the evaluated cell lines, a decrease in cell viability and clonogenicity is more evident in the cancer stem-like cells than in their parental population (Fig. 5a–c). In addition, we performed qPCR experiments to measure *Nanog*, *Sox2*, *Oct4* and *Mamal3*, and it was found that the expression levels of those genes was reduced (Fig. 5d–f). This suggests that ivermectin has a preferential depletion effect on the cancer stem-like cell population.

Ivermectin reduces the tumoral volume in a syngeneic mice model

After treating the murine breast cancer cell line JC with 5 µM ivermectin, the cell viability and the number of colonies decreased by 75% and 21%, respectively (Fig. 6a, b). Next, after treating JC-bearing mice with ivermectin, we observed that tumors grew slower than those in mice from the control group (Fig. 6c). At day 21 of the assay, the average volume of tumors in treated mice was 63% smaller than in the control group (Fig. 6d–f). Interestingly, while the tumor weight was 56% lower in the treated group with respect to the control (Fig. 6e), the mice weight did not show

Table 2 Inhibitory concentrations $(IC)_{20\text{-}50}$ of docetaxel, tamoxifen and cyclophosphamide

Cell line	Drug	IC_{20} (µM)	IC_{30} (µM)	IC_{40} (µM)	IC_{50} (µM)
DU145	Docetaxel	6.25	14.34	41	111.1
MCF-7	Tamoxifen	0.57	1.16	1.86	2.8
MDA-MB-231	Docetaxel	0.00096	0.00013	0.00017	0.00022
MDA-MB-468	Cyclophosphamide	0.23	0.56	1.05	1.65

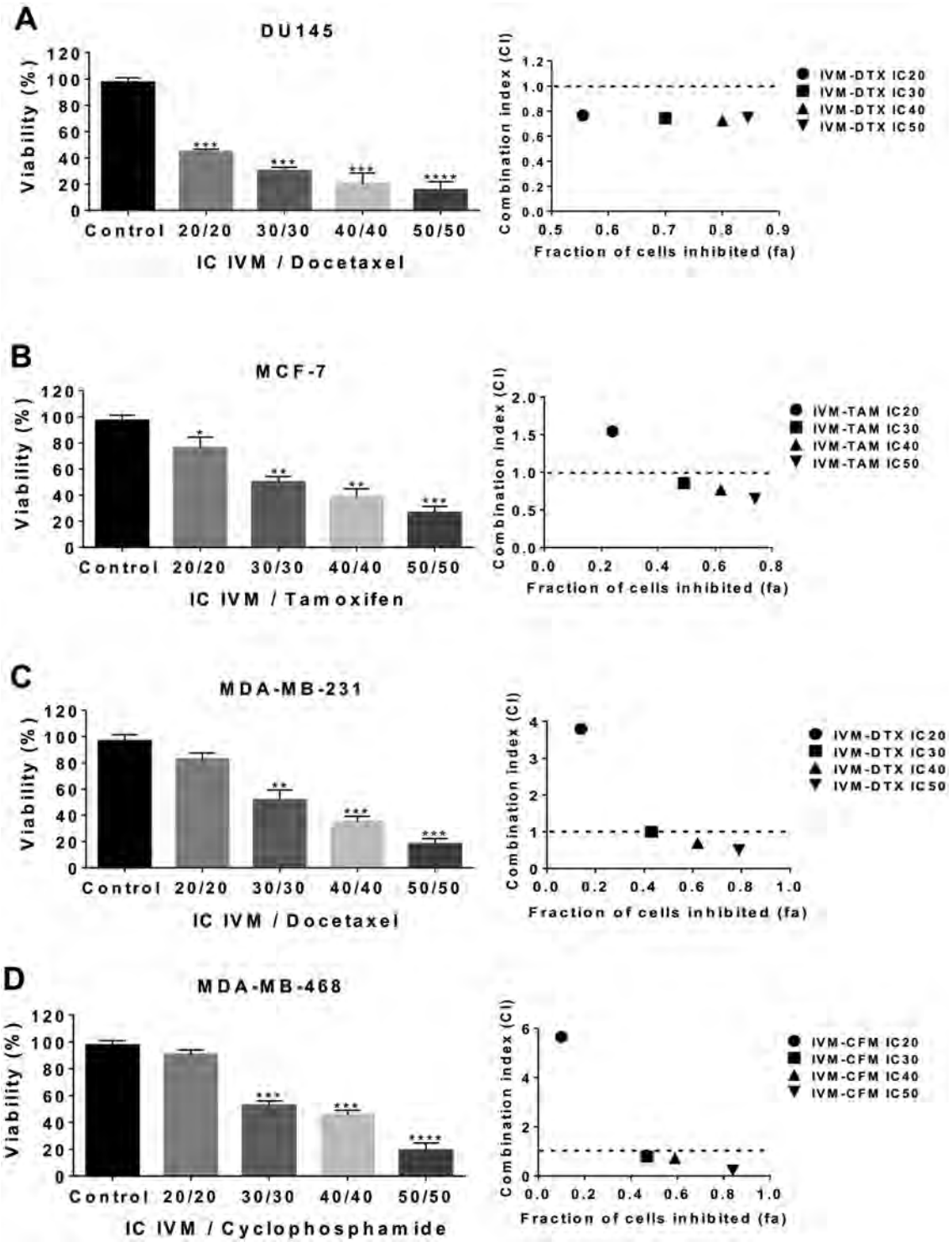

Fig. 4 The combination of ivermectin with standard chemotherapeutic compounds has synergistic effects. Effect on cellular viability and pharmacological interactions between the inhibitory concentrations $(IC)_{20–50}$ of ivermectin plus either docetaxel, tamoxifen or cyclophosphamide, on the DU145 (**a**), MCF-7 (**b**), MDA-MB-231 (**c**) and MDA-MB-468 (**d**) cell lines. *IVM* Ivermectin, *DTX* Docetaxel, *TAM* Tamoxifen, *CFM* Cyclophosphamide; $*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$

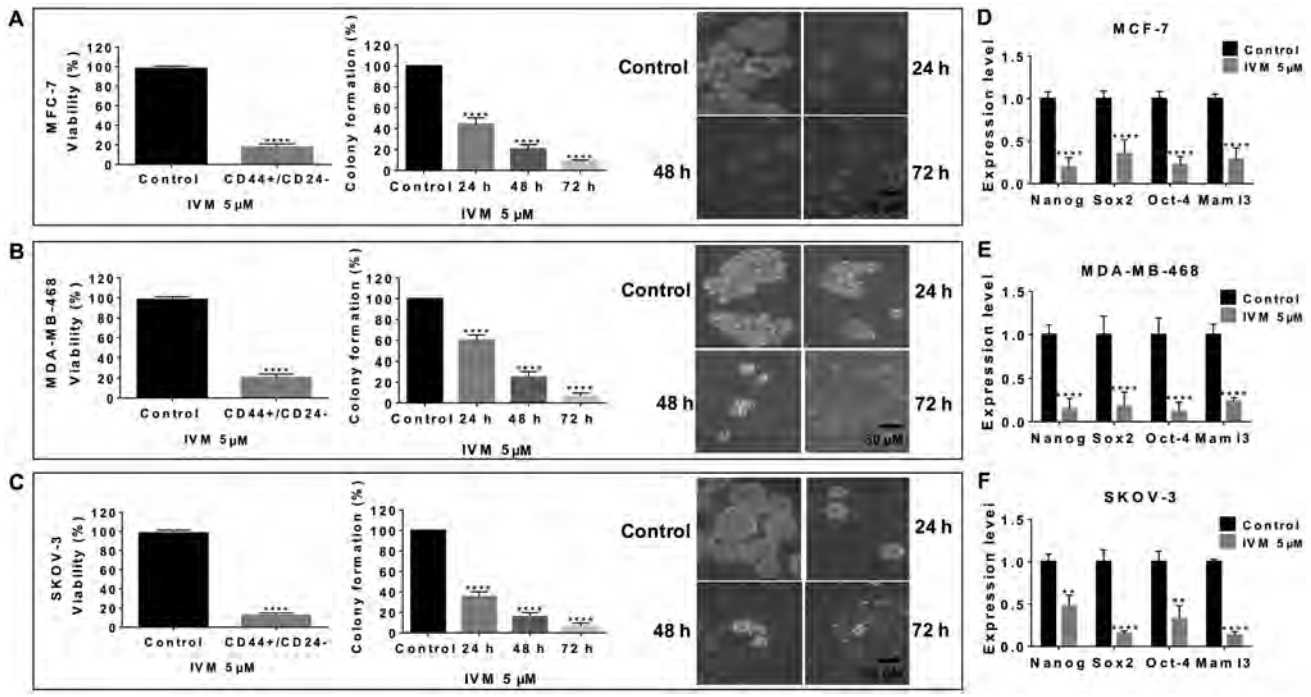

Fig. 5 Ivermectin (5 µM) reduces the viability and the colony-forming capacity on the cancer stem-like malignant population. Effect in cell viability and colony formation ability of cancer stem-like populations from the MCF-7 (**a**), MDA-MB-468 (**b**) and SKOV-3 (**c**) cell lines. Representative photography of colonies of each cell line at 24, 48 and 72 h of treatment, and of colonies from control cells after 72 h, are also provided. Relative mRNA levels of stemness genes from the MCF-7 (**d**), MDA-MB-468 (**e**) and SKOV-3 (**f**) cell lines. *IVM* Ivermectin; $^{**}p < 0.01$; $^{****}p < 0.0001$

significant differences between the groups (fig. 6g, h). Such results are consistent with our previous observations where ivermectin stops cell proliferation in vitro, suggesting that ivermectin acts the same way in vivo. A weakness of this study is that we were unable to purchase immunosuppressed mice to be injected with human cancer cells.

Discussion

Currently, affordability of safer and effective anticancer drugs is urgently needed for cancer patients. Drug repurposing can help to solve the current problem. Among many drugs in development under the repurposing approach, ivermectin is very promising because its antitumor effects are seen at concentrations achievable in patients receiving this drug, which are around 5 µM [21]. Secondly, ivermectin has been administered to millions of patients, and therefore, its safety is not a major concern. Together, our results on the growth inhibition of the human cancer cell lines plus the previous data from around 26 cancer cell lines treated with ivermectin [10] indicate that more than 50 human cell lines are inhibited in some degree by ivermectin, which supports its potential as a wide-spectrum cancer drug. Moreover, we observed a directed correlation between in vitro sensitivity with clonogenicity (Fig. 1). Regarding in vivo data, human xenografts of glioblastoma, leukemia and carcinomas of breast and colon origins in nude mice, as well as numerous murine cell lines in syngeneic models, have shown that a median dose of 5 mg/kg ivermectin has a strong antitumor effect [12, 13, 15, 17, 18]. Here we were unable to get immunosuppressed mice. Hence, we tested the JC murine breast cancer cells in Balb/c mice. At a dose of 3 mg/kg (human equivalent dose of 243 µg/kg), according with the formula by [27], we observed more than 60% reduction in tumor size, without changes in mice weight.

To further analyze the cellular effects of ivermectin, we selected a resistant (prostate DU145, inhibition of 9% only) and sensitive cell lines (breast MDA-MB-231, MCF-7 and MDA-MB-468, viability inhibition ranging between 58 and 70%). As expected, no significant effect with 5 µM ivermectin in cell cycle and apoptosis was observed in DU145, whereas in the sensitive cells, there was a statistically significant arrest in G_0-G_1, but cell death by apoptosis was not observed. These results partly agree with other works where ivermectin inhibits cell proliferation [12, 14, 15, 19, 28, 29]. A study showed that in colon carcinoma cells, ivermectin induced the expression of p21 and reduced cyclin D1 [13]. As we observed mainly a G_0-G_1, arrest, we demonstrated that ivermectin increased the expression of p21, while

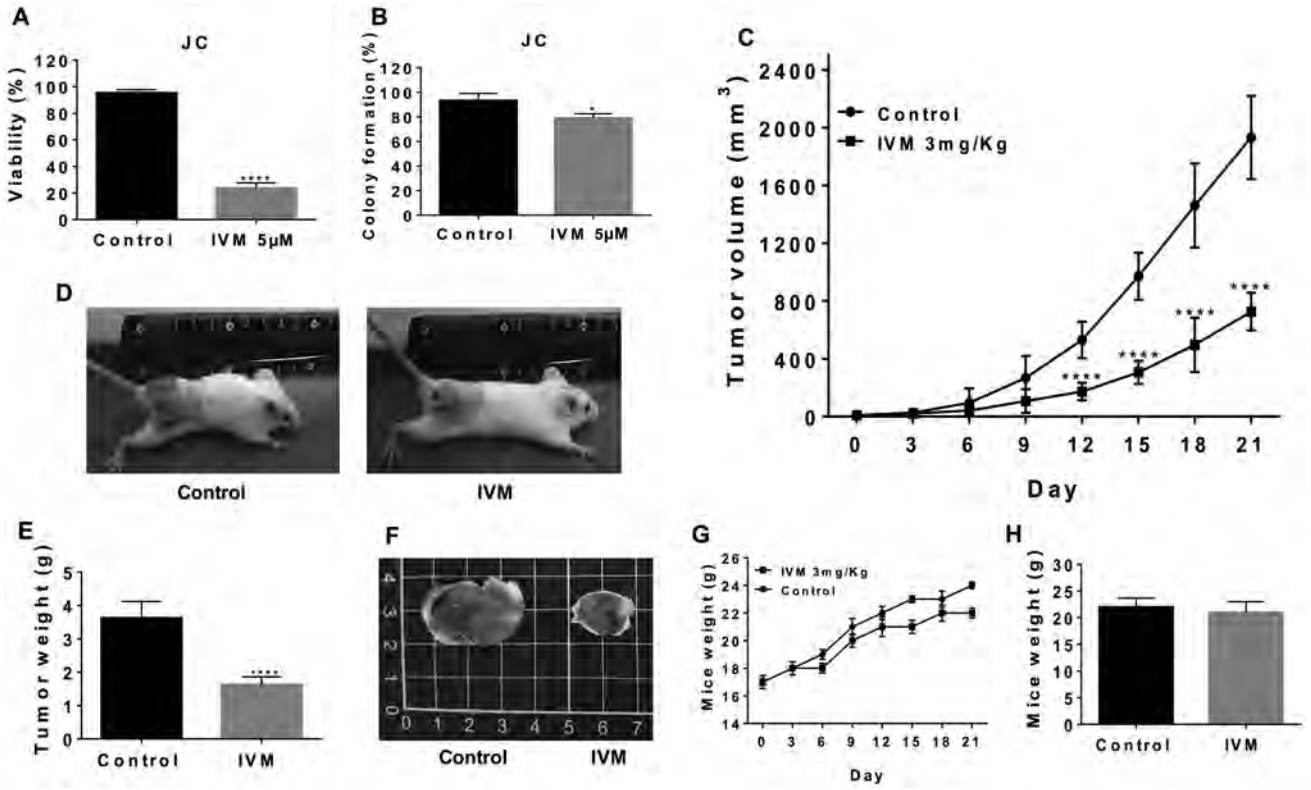

Fig. 6 Ivermectin diminishes the tumor volume in an allogenic mice model. Effect on the percentage of cell viability (**a**) and on the number of colonies (**b**) in the JC cell line after the treatment with ivermectin. Tumor volume over time on mice (**c**). Representative mice photography at day 21 of treatment (**d**). Tumor weight (**e**) and representative photography of recovered tumors (**f**) at the end of the 21-day assays. Mice weight over time (**g**). Mice weight after removing the tumor at the end of the 21-day assays (**h**). *IVM* Ivermectin; $*p < 0.05$; $****p < 0.0001$

reduced cyclin D, cyclin E and PCNA proteins. Further characterization of ivermectin regarding the expression of cdk4 and cdk6 (G1 regulatory proteins), as well as cdk2 and cyclin A (S phase regulators), are needed for better characterization of ivermectin effects upon cell cycle. Nevertheless, our results are in agreement with the data from Song et al. [29].

Ivermectin has shown to preferentially inhibit the viability of cancer stem-like cells enriched populations ($CD44^+/CD24^-$), as compared to parental cells, in MDA-MB-231 breast cancer cells [9]. To extend these observations, here we show that ivermectin preferentially inhibits the sorted $CD44^+/CD24^-$ cells, of two breast cancer and one ovarian cancer cell lines. These effects were associated with a decreased expression of the stem cell pluripotency and self-renewal genes, *Sox2* and *Nanog* [30]. The mechanism by which ivermectin has this effect on cancer stem-like cells is still unknown. However, a study showed that ivermectin inhibits the function of SIN3 [16], which is part of a complex that positively regulates *Nanog* and *Sox2* [13]. According to these observations, ivermectin has shown to reduce the growth of tumorspheres of triple-negative breast cancer [16] and colon cancer [13] cell lines.

Ivermectin can be considered as a multi-targeted drug as it directly or indirectly modulates at least nine targets or pathways [10]. Adding chemotherapy drugs may increase the antitumor actions of ivermectin. A work has shown in myeloid leukemia cells that ivermectin synergizes with daunorubicin and cytarabine [18]. Here we demonstrate a synergy between ivermectin with docetaxel or cyclophosphamide in estrogen receptor-negative breast cancer cells, and with tamoxifen in MCF-7 cells. Of note, while DU145 is minimally inhibited by docetaxel, when used together with ivermectin, a strong synergy is observed. As ivermectin is a multitargeted drug, it is difficult to determine these interacting mechanisms leading to synergy. Inhibition of MDR by ivermectin [11, 31] may underlie the synergy with tamoxifen, which itself is a MDR inhibitor [32], as well as the inhibition of Pak1 as a described mechanism of tamoxifen resistance [33]. MDR inhibition may also participate in synergy with docetaxel and cyclophosphamide [34, 35]. Ivermectin decreases expression of stem-cell like markers and exerts relative selectively upon stem-cell like populations [9]. A potential reduction on the stem cell marker ALDH [36] by ivermectin can be responsible for its synergy with

cyclophosphamide, as this is a key enzyme for its detoxification [37].

In summary, we show that ivermectin, at clinically feasible concentrations, has inhibitory actions mainly in human breast cancer cells, which are related to cell cycle arrest. It also showed a preferential effect on cancer stem cells, and synergized with several chemotherapy drugs. Finally, we showed that ivermectin inhibits the tumor growth in a breast cancer mouse model. Although this work is not mechanistic, it adds preclinical evidence to support the clinical testing of ivermectin as an anti-cancer drug.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal experiments were approved and conducted under the guidelines of the Bioethical and Scientific committees of the National Institute of Oncology (protocol numbers CEI/1145/17 and 017/016/IBI, respectively), in Mexico City, Mexico.

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