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Original Article

Homoharringtonine, an approved anti-leukemia drug, suppresses triple negative breast cancer growth through a rapid reduction of anti-apoptotic protein abundance

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Abstract: Triple negative breast cancers (TNBC) without *BRCA1/2* gene mutation or BRCAness are nowadays the breast malignancies most difficult to treat. Improvement of their treatment, for all phases of the disease, is an important unmet medical need. We analyzed the effect of homoharringtonine (HHT), a natural protein synthesis inhibitor approved for treatment of chronic myeloid leukemia, on four cell lines representing aggressive, *BRCA1/2* non-mutated, TNBC genomic categories. We show that HHT inhibits *in vitro* growth of all cell lines for more than 80%, after 48-72 h exposure to 20-100 ng/mL, the concentrations achievable in human plasma after subcutaneous administration of the drug. HHT, at 100 ng/mL, strongly reduced levels of a major TNBC survival factor, anti-apoptotic protein Mcl-1, after only 2 h of exposure, in all cell lines except MDA-MB-231. Other anti-apoptotic proteins, Bcl-2, survivin and XIAP, were also strongly downregulated. Moreover, *in vivo* growth of the least sensitive cell line to HHT *in vitro*, MDA-MB-231, was inhibited for 36.5% in mice, by 1 mg/kg of the drug, given subcutaneously, bi-daily, over 7 days. These results demonstrate marked antineoplastic activity of homoharringtonine in TNBC, making further development of the drug in this disease highly warranted.

Keywords: Homoharringtonine, breast cancer, protein synthesis, translation inhibitor, Mcl-1

Introduction

Triple-negative breast cancer (TNBC) is today the breast cancer subtype most difficult to treat, due to exquisite heterogeneity at the clinical, phenotypic, genotypic and microenvironmental level [1]. Some TNBC subtypes are orphan diseases [2], whereas, in certain regions of the world, TNBC is a frequent, devastating cancer of young women, affecting reproductive and economic power of entire countries [3]. Current standard treatment for most TNBC patients is neoadjuvant chemotherapy (NACT), followed by breast surgery [4]. NACT leaves around two thirds of patients with residual disease, carrying a risk for metastatic recurrences [5]. The armamentarium for systemic treatment of metastatic TNBC being very limited [6], patients in this phase of the disease have the worst prognosis among all breast cancer patients, with

overall survival often shorter than 12 months [7].

Therefore, improvement of TNBC treatment, for all disease phases, is still a big unmet medical need. However, after the initial success of an anticancer agent or regimen, TNBC, as many other cancers, frequently develops resistance. Efficacious, rapid and affordable ways to prevent or/and combat that resistance need to be found. In such a situation, repositioning into TNBC treatment the drugs which have shown capacity to suppress other cancers, even after several prior treatments, is an approach that should not be neglected.

Homoharringtonine (HHT), also named omacetaxine mepesuccinate (international non-proprietary name), is approved by the US FDA for treatment of patients with chronic myeloid leu-

kemia (CML) resistant or intolerant to two or more tyrosine kinase inhibitors (TKI) [8]. HHT has been used in China for more than 50 years in treatment of pts with myeloid leukemias. Current regimens, which combine HHT with cytarabine and daunorubicin or aclarubicin, induce up to 85% of complete response in acute myeloid leukemia (AML), after only two cycles [9]. So, HHT is a potent anticancer agent, efficacious in several aggressive blood malignancies, even in cases of resistance to specific, targeted therapy like TKI. HHT has recently been confirmed to efficaciously kill also the lung cancer cells with primary or TKI-induced resistance [10, 11].

HHT fixes to the ribosomes, disabling elongation of the nascent peptide chain and further protein synthesis [12]. The antileukemic actions of HHT are considered to be exerted principally by this mechanism, which occurs downstream all other ways of RNA translation inhibition [13]. Inhibition of protein synthesis by HHT has been shown to strongly reduce abundance of onco-drivers (like Bcr-abl protein in CML) or other proteins crucial for cancer cell survival [14].

Increased protein synthesis is present in cancers, both as a part of increased global anabolic activity and as local adaptative event [15-17]. In particular, resistance of cancer cell to death is enabled by increased abundance of anti-apoptotic proteins [18, 19]. One of them, Mcl-1, is a crucial determinant of breast cancer survival [20, 21]. Most TNBC have increased level of Mcl-1 protein, either due to the amplification of *MCL1* gene [22], or to hyperactivation of the PI3K-mTOR axis, found in up to 70% of cases [23]. An efficacious reduction of Mcl-1 levels highly facilitates induction of cancer cell death by other anti-neoplastic agents [24]. This has encouraged development of several Mcl-1 inhibitors [25-27], however none of them is approved yet for clinical use.

As several studies have shown rapid and marked reduction of Mcl-1 protein level by HHT in blood malignancies [28-30], we evaluated efficacy of this agent on the models of aggressive TNBC. Here we are showing that HHT induces the same effect on TNBC in vitro as the one reported in blood cancers (leukemias, myeloma). In addition, HHT alone inhibits TNBC growth in mice after a short and easy to administer treatment (7 days, subcutaneous). These results support development of HHT in the

treatment of TNBC patients, thus enlarging the area of exploitation of protein synthesis inhibition as an efficacious anticancer approach.

Material and methods

Cell lines and culture conditions

We used MDA-MB-231 (ATCC® HTB-26™), MDA-MB-468 (ATCC® HTB-132™), MDA-MB-157 (ATCC® HTB-24™) and CAL-51, chosen to represent the *BRCA1/2* non-mutated TNBC transcriptomic subtypes [31]. The MDA lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). CAL-51 was kindly provided by Dr Gerard Milano, from the Centre Antoine Lacassagne, Nice, France, where the line has been established [32]. The cell line stock vials were conserved at the Biological Resources Facility of Centre Jean Perrin (biobank ID BB-0033-00075, Clermont-Ferrand, France).

The MDA cell lines were cultured as previously described [33]. CAL-51 was maintained in DMEM, supplemented with 10% heat-inactivated fetal bovine serum and 20 mg/mL gentamicin (both media from Invitrogen Life Technologies, Carlsbad, CA, USA), in a humidified atmosphere with 5% CO₂.

HHT solution preparation

Highly purified HHT of natural origin was supplied by LeukePharma Inc. (Houston, TX, USA) in the form of water-soluble, crystalline tartaric acid salt. HHT-hydrogen (2R,3R)-(+)-tartrate (named below "HHT tartrate"; empirical formula C₃₃H₄₅N₀O₁₅; extended formula C₂₉H₄₀N₀O₉, C₄H₅O₆; molecular weight 695.7 g/mol) was prepared from natural HHT by fractional crystallization which yields HHT of the same purity level as the semi-synthetic form (US Patent WO 2015101628 (2018) and related US patents).

Initial stock solution of HHT tartrate was prepared at 10 mM in sterile deionized water, aliquoted and kept at -80°C. For each experiment, a fresh HHT tartrate solution was prepared by dissolving the HHT tartrate stock in sterile phosphate-buffered saline.

Cell viability assay

Cell viability was assessed using the sulforhodamine B (SRB) assay (Sigma-Aldrich, St Louis, MO, USA), as previously described [33]. Cells

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were seeded in hexaplicates, in 96-well plates at 5×10^3 (MDA cell lines) or 1×10^3 cells/well (CAL-51).

Cell cycle analysis

Cells were seeded into 6-well plates at 5×10^4 cells per well (each cell line), left overnight to adhere, and then treated or not by 20-50 ng/mL HHT for 48 h. The assay was further performed as previously described [33].

Apoptosis assay

Cell preparation was performed as in the cell cycle assays. The cells were treated by 20-100 ng/mL of HHT for 6, 24, 48 or 72 hours. Apoptosis measurement was performed by flow-cytometry, as previously described [33].

Western blotting

Cells were plated in 10 cm Petri dishes at 5×10^5 cells per dish, left overnight to adhere and treated, the following day, with 100 ng/mL of HHT over 2-48 h. Further processing, ending with the signal revelation was done as previously described [33]. The following primary antibodies were used: anti-Mcl-1 (clone D35A5), anti-Bcl-2 (50E3), anti-XIAP (D2Z8W), anti-survivin (71G4B7), anti-caspase 3 (8G10), anti-PARP (rabbit polyclonal, cat. number 9542), anti-Myc (D84C12), anti-Akt (C67E7), anti-phospho-Akt (D9E), anti-p44/p42 MAPK (rabbit polyclonal, cat. number 9102), anti-phospho-p44/p42 MAPK (D13134E), anti-S6 ribosomal protein (5G10), anti-phospho-S6 ribosomal protein (D57.2.2E), anti- β -actin (D6A8), all from Cell Signaling Technology (Danvers, MA, USA). All antibodies were used at final concentration 1:1000. The chemiluminescent signal detection was performed using a ChemiDoc XRS + gel imager (Bio-Rad).

siRNA experiments

MDA-MB-468 and CAL-51 cells were seeded in 6-well plates, at 2×10^5 cells and 2.5 mL of DMEM per well. After 24 h, the cells were transfected by *MCL1* siRNA and mock siRNA using Lipofectamine® (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). Briefly, the solution of *MCL1* siRNA or mock siRNA were prepared by dissolving 3 μ l of the respective reagents (ON-TARGETplus Human *MCL1* siRNA and ON-

TARGETplus Non-targeting siRNA, both from Dharmacon, Lafayette, CO, USA) in 150 μ l of medium (Gibco®Opti-MEM™, Life Technologies, Carlsbad, CA, USA). Lipofectamine® RNAiMAX medium (9 μ l) was also diluted in Opti-MEM™ (150 μ l). Finally, the solution of each siRNA was added to the Lipofectamine solution, mixed well, and 250 μ l of the mixture was added to the cells. The cells were then treated by 100 ng/mL HHT for 24 h and 48 h, lysed, proteins extracted and western blots performed.

Animal studies

The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication n°85-23, revised 1996) and approved by the Ethic Committee of Clermont-Ferrand, France. All mouse studies were conducted in the animal facility of the INSERM U1240. Six weeks old Swiss nu/nu female mice were purchased from Charles River Laboratories France and housed in standard conditions (n = 5 per cage on ventilated racks, at 21-24°C, 60% humidity, 12 h light/12 h dark cycle) with ad libitum access to irradiated food and water.

For establishment of xenografts, groups of 10 mice were injected subcutaneously, in the right flank area, with 1×10^7 MDA-MB-231 or 5×10^6 MDA-MB-468 cells suspended in 150 μ l of culture medium/Matrigel® (BD Biosciences, San Jose, CA, USA) in a 4:1 ratio.

For assessment of HHT toxicity, groups of 6 mice were injected with sterile saline water (0.9% NaCl) or 1, 2, 4 or 8 mg/kg of the drug, subcutaneously, bi-daily, over 7 days. The animals were left to rest 2 more days and sacrificed at day 10. The solution for injection were freshly prepared by serial dilutions of the stock HHT-tartrate in sterile saline water and filtered through sterile filters having 0.25 μ m pore size (Merck/Millipore, Burlington, MA, USA). The number of live animals was determined at days 4, 8 and 10, after treatment initiation.

For evaluation of HHT efficacy in vivo, mice were randomized into the control or the treated group when xenografts reached a volume of 200 ± 20 mm³. The MDA-MB-231 xenografts were treated by 1 mg/kg of HHT, using the same protocol as for determination of HHT tox-

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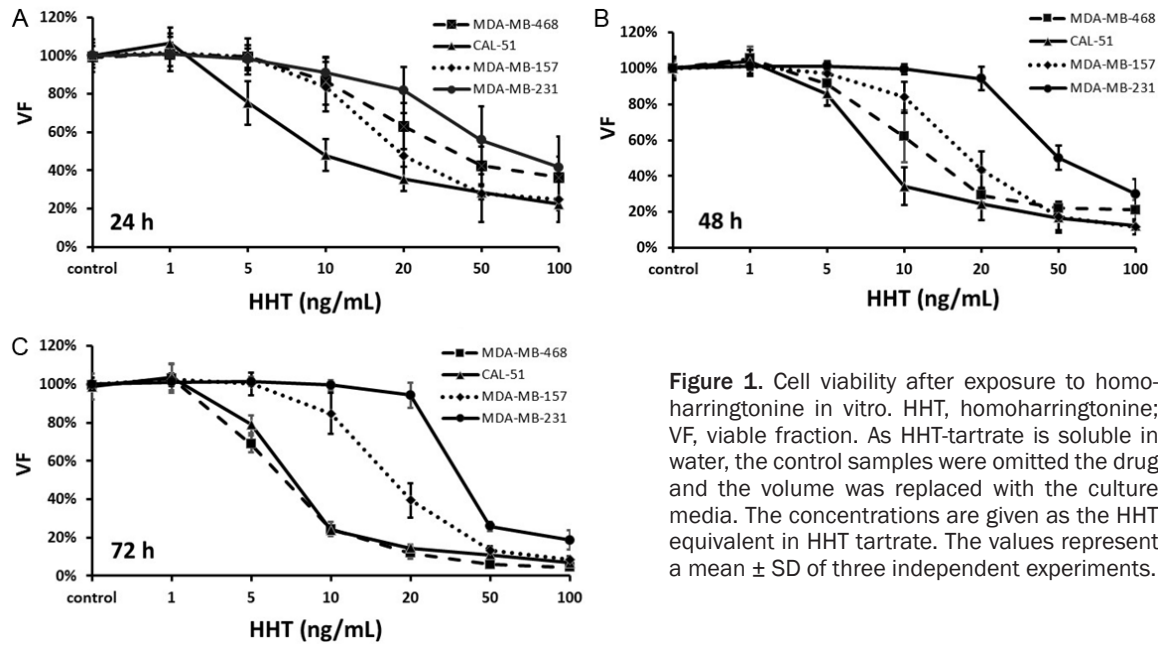


Figure 1. Cell viability after exposure to homoharringtonine in vitro. HHT, homoharringtonine; VF, viable fraction. As HHT-tartrate is soluble in water, the control samples were omitted the drug and the volume was replaced with the culture media. The concentrations are given as the HHT equivalent in HHT tartrate. The values represent a mean \pm SD of three independent experiments.

icity. The MDA-MB-468 xenografts were treated by 0.5 mg/kg, as 1 mg/kg was too toxic for the animals (all mice died after only a few days, in a preliminary experiment; data not shown). Tumor size was non-invasively assessed using a digital caliper on days 3, 6, 8 and 10 after treatment initiation. Tumor volume was calculated using the following formula: $V = (\text{length} \times \text{width}^2)/2$.

The drug was evaluated in a total of 21 mice (2 independent experiments on MDA-MB-231 tumors, with 8 or 9 mice per experiment, as well as in one experiment on MDA-MB-468 tumors, with 4 mice). Xenografting of CAL-51 cells was not successful, after several attempts.

Statistical analysis

The IC₅₀ of HHT after 24, 48 or 72 h were determined by the Chou-Talalay method [34], using Compusyn software (freely downloadable from www.combosyn.com). Mean \pm standard deviation was used for presentation of all values. Variations of the viable or apoptotic cell fraction according to cell type, exposure time or drug concentration were studied using two-way ANOVA. Comparison of mean values between two time-points was tested by Kruskal-Wallis H-test because distributions were not Gaussian or heteroscedastic. Tests were two-sided and the standard level $P \leq 0.05$ was considered statistically significant.

Results

HHT inhibits growth of TNBC cell lines, in concentrations achievable in patients' plasma

As shown on **Figure 1A**, inhibitory effect of HHT on TNBC cell growth in vitro was observed already after 24 h. At that time, IC₅₀ were 15.7 ng/mL, 19.9 ng/mL, 23.1 ng/mL and 80.5 ng/mL, for MDA-MB-157, MDA-MB-468, CAL-51 and MDA-MB-231, respectively. IC₅₀ for the first three cell lines are below the lowest concentration of HHT (31.3 ng/mL), found 5 days after the last injection, in plasma of AML patients treated subcutaneously, bi-daily, by 5 mg/m² for 9 days [35]. IC₅₀ for MDA-MB-231 is comparable to the maximal plasmatic concentration of HHT found in patients, at the same time point, after the same regimen.

HHT effect on cell line growth was dose- but not time-dependent in MDA-MB-157, MDA-MB-468 and CAL-51 (no significant change of IC₅₀ after 48 h or 72 h, $P > 0.05$, **Figure 1B** and **1C**). However, growth inhibition on MDA-MB-231 cells was both dose- and time-dependent: concentrations below 50 ng/mL could not significantly reduce cell growth, even after 72 h. However, when MDA-MB-231 was exposed to higher concentrations of HHT, the viable fraction (VF) was significantly lower after 72 h, compared to 24 h (at 50 ng/mL, VF: $25.9 \pm 2.4\%$ vs $58.1 \pm 14.5\%$, 72 h vs 24 h resp., $P < 0.01$; at 100 ng/mL, VF:

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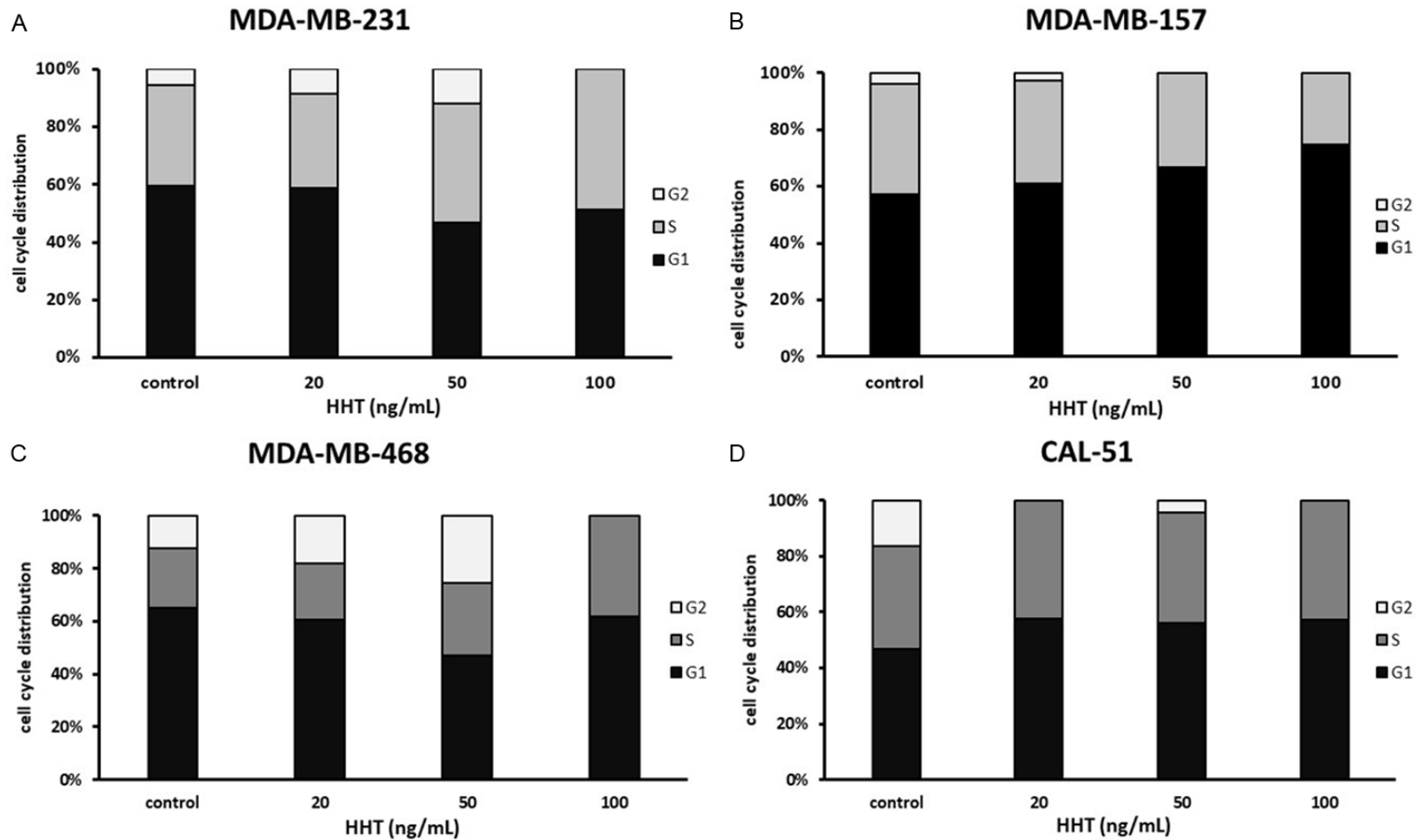
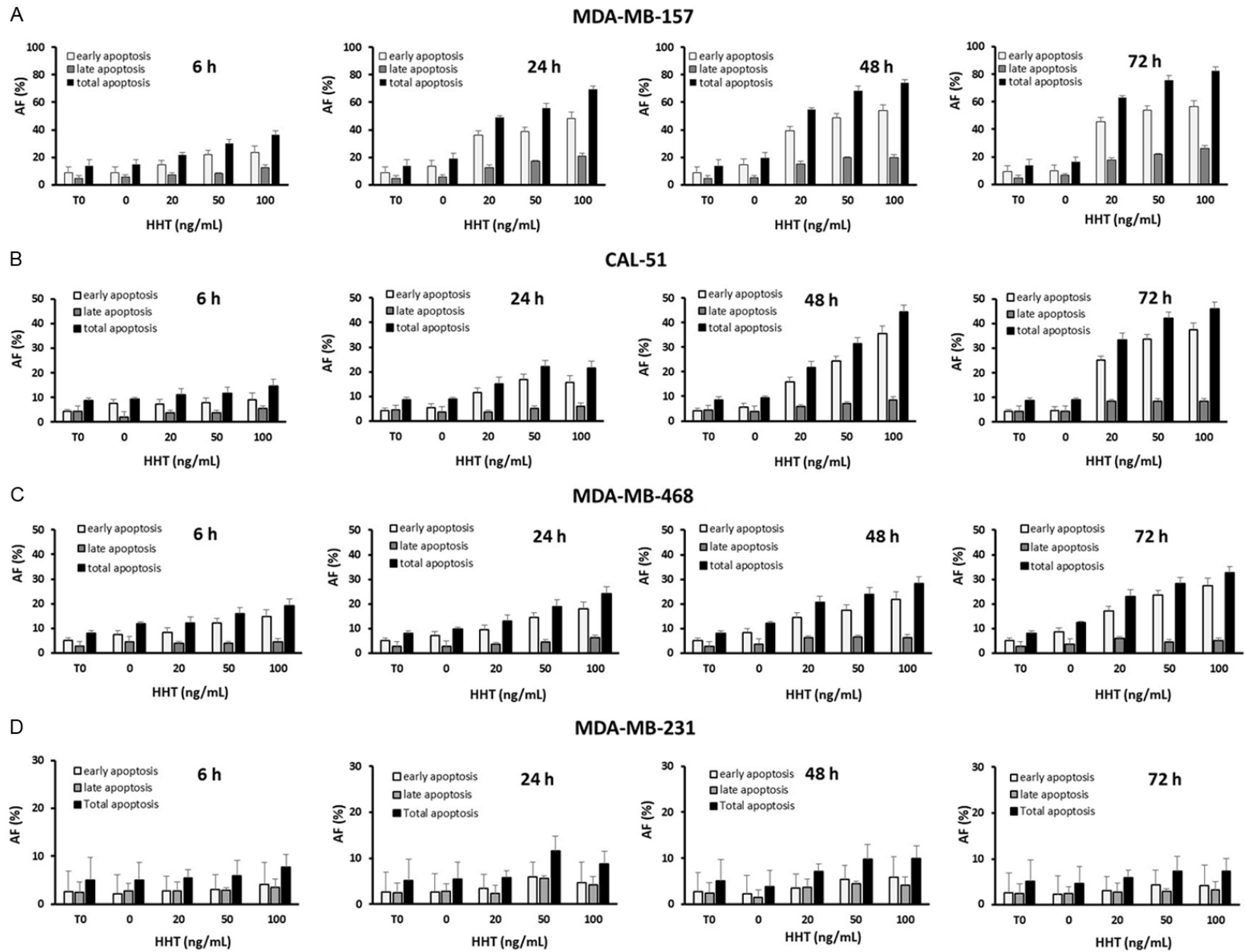


Figure 2. Distribution of cells in the cell cycle phases after exposure to homoharringtonine for 48 h. HHT, homoharringtonine. As HHT-tartrate is soluble in water, the control samples were omitted the drug and the volume was replaced with the culture media. The concentrations are given as the HHT equivalent in HHT tartrate. The figure represents the most representative of three independent experiments.

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Figure 3. Apoptotic fractions in the cell lines exposed to homoharringtonine. HHT, homoharringtonine; AF, apoptotic fraction; T0, beginning of the experiment (before adding the drug). As HHT tartrate is soluble in water, the control samples were omitted the drug and the volume was replaced with the culture media. The concentrations are given as the HHT equivalent in HHT tartrate. The values represent a mean \pm SD of three independent experiments.

18.7 \pm 4.8% vs 41.7 \pm 13.3%, 72 h vs 24 h, $P < 0.01$, **Figure 1A** and **1C**). Thus, the viable fraction of MDA-MB-231 cells, after 72 h of exposure to high concentrations of HHT, was similar to the viable fractions in other cell lines (**Figure 1C**).

Taken together, these results indicate that HHT, in vitro, strongly inhibits (more than 80%) growth of TNBC cells with different molecular characteristics (3 transcriptomic subtypes: mesenchymal (CAL-51), mesenchymal stem-like (MDA-MB-157 and MDA-MB-231, basal-like 1 (MDA-MB-468) [31]).

To get a better insight into cell growth-inhibitory action of HHT, we first analyzed changes in the size of cell populations engaged in different phases of the cell cycle, after 48 h-exposure to 20-100 ng/mL of the drug. As shown on **Figure 2B** and **2D**, HHT, already at 20 ng/mL, induced accumulation in the G1 (MDA-MB-157) or/and in the S- and G1-phase (CAL-51). Under such conditions, MDA-MB-468 and MDA-MB-231 cells were first arrested in the G2/M phase; a stronger arrest occurred at 50 ng/mL (**Figure 2A** and **2C**). The maximal concentration of HHT, 100 ng/mL, arrested both MDA-MB-468 and MDA-MB-231 cells in the S- and G1-phase of the cell cycle (**Figure 2A** and **2C**).

HHT induces apoptosis of TNBC cells, in cell-, dose- and time-dependent manner

We next evaluated the capacity of HHT to induce apoptosis in TNBC cell lines, as well as the kinetics of that phenomenon, knowing that in blood malignancies HHT can induce cell death as early as after 3-4 hours of exposure [28-30].

As shown on **Figure 3**, the four analyzed cell lines showed different levels of apoptosis after exposure to 20-100 ng/mL of the drug, over a time course from 6 to 72 h. MDA-MB-231 cells were most resistant to apoptosis induction: the total apoptotic fraction (AF) did not exceed 11.6 \pm 3.6% at any concentration and at any time point (**Table 1**). In other three cell lines (MDA-MB-157, MDA-MB-468 and CAL-51) the

AF was cell type-dependent ($P = 0.0016$): MDA-MB-157 cells were markedly more sensitive to the apoptosis induction than MDA-MB-468 and CAL-51 cells, as the mean value of AF obtained by all drug concentrations, at all time-points, was twice higher in MDA-MB-157 cells (44.9%) than the corresponding mean value obtained either in MDA-MB-468 (18.4%) or in CAL-51 cells (21.2%) ($P = 6.5 \times 10^{-5}$ for MDA-MB-157 vs MDA-MB-468 and $P = 1.4 \times 10^{-3}$ for MDA-MB-157 vs CAL-51).

The apoptotic effect of HHT was dose- and time-dependent in cells other than MDA-MB-231 (**Figure 3**). The total AF in MDA-MB-157 cells, after the lowest drug concentration tested, 20 ng/mL, was 21.9 \pm 1.6% already after 6 h of exposure, and reached the maximum, 82.4 \pm 2.5%, after 72 h of exposure to 100 ng/mL. In CAL-51 and MDA-MB-468 cells the total AF was 11.0 \pm 2.6% and 12.1 \pm 2.3%, respectively, after the lowest concentration and the shortest exposure, and reached 46.0 \pm 3.4% and 32.5 \pm 5.7% after maximal concentration and exposure time (**Table 1**).

The cell lines other than MDA-MB-231 also differed in kinetics of apoptosis induction. Indeed, apoptosis occurred most rapidly in MDA-MB-157 cells, in which AF more than doubled from 6 h to 24 h of exposure to any concentration (**Figure 3A**; **Table 1**). When incubated with 20-50 ng/mL of HHT, CAL-51 cells showed a steady rise in AF over 72 h, however, after exposure to 100 ng/mL, the AF reached its maximum already after 48 h and did not exceed that level during next 24 h (**Figure 3B**; **Table 1**). Similar kinetics of apoptosis induction was observed in MDA-MB-468 cells (**Figure 3C**; **Table 1**).

The difference in AF size between MDA-MB-157 and CAL-51 cells, after exposure to 100 ng/mL of HHT during 48 h (73.9 \pm 4.6% vs 44.2 \pm 3.5%, respectively; **Table 1**) corresponded to the difference in size of the G0/G1 fraction in those cells (74.6% vs 57.4%, respectively/data not shown, **Figure 2B** and **2D**). The MD-MB-468 cells were more arrested in the G0/G1 phase

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Table 1. Apoptotic fractions in TNBC cell lines exposed to homoharringtonine

HHT (ng/ml)	AF	T0	T6				T24			
		0	0	20	50	100	0	20	50	100
MDA-MB-157										
	Early	9.1 ± 4.2	8.9 ± 4.0	14.7 ± 3.0	21.8 ± 3.1	23.7 ± 4.5	13.6 ± 2.1	36.2 ± 3.3	38.6 ± 2.8	48.2 ± 3.5
	Late	4.7 ± 2.2	5.9 ± 1.6	7.2 ± 1.9	8.2 ± 0.6	12.6 ± 1.8	5.5 ± 1.3	12.7 ± 1.5	17.3 ± 3.3	21.1 ± 1.6
	Total	13.7 ± 4.7	14.8 ± 3.7	21.9 ± 1.6	30.0 ± 3.2	36.3 ± 2.7	19.1 ± 3.3	48.9 ± 4.8	55.9 ± 0.5	69.3 ± 4.1
MDA-MB-468										
	Early	5.2 ± 1.7	7.4 ± 1.2	8.3 ± 1.4	12.1 ± 4.1	14.7 ± 4.5	7.2 ± 0.8	9.5 ± 1.3	14.4 ± 4.9	18.1 ± 5.5
	Late	2.7 ± 1.2	4.4 ± 1.1	3.8 ± 0.9	3.8 ± 0.5	4.6 ± 0.9	2.7 ± 0.9	3.5 ± 1.7	4.5 ± 0.7	6.2 ± 0.6
	Total	7.9 ± 2.8	11.8 ± 1.7	12.1 ± 2.3	15.9 ± 4.2	19.2 ± 3.8	9.9 ± 1.5	13.0 ± 2.8	19.0 ± 5.4	24.3 ± 5.7
MDA-MB-231										
	Early	2.7 ± 0.5	2.2 ± 0.5	2.8 ± 1.2	3.0 ± 0.7	4.2 ± 1.7	2.6 ± 0.7	3.4 ± 1.0	6.0 ± 1.6	4.6 ± 1.3
	Late	2.4 ± 1.0	2.8 ± 1.0	2.7 ± 0.5	2.9 ± 0.8	3.5 ± 1.1	2.8 ± 0.8	2.3 ± 0.2	5.6 ± 2.3	4.2 ± 1.8
	Total	5.1 ± 1.4	5.0 ± 1.4	5.5 ± 1.7	6.0 ± 0.9	7.7 ± 2.1	5.5 ± 1.2	5.7 ± 1.0	11.6 ± 3.6	8.8 ± 1.8
CAL-51										
	Early	4.2 ± 1.0	7.4 ± 1.6	7.2 ± 1.8	7.8 ± 2.0	9.1 ± 2.8	5.3 ± 1.5	11.6 ± 2.8	16.9 ± 0.7	15.5 ± 1.3
	Late	4.4 ± 2.0	1.9 ± 2.2	3.8 ± 0.9	3.9 ± 0.9	5.4 ± 1.2	3.7 ± 1.2	3.6 ± 0.1	5.1 ± 0.6	6.1 ± 1.0
	Total	8.6 ± 1.1	9.3 ± 0.7	11.0 ± 2.6	11.7 ± 2.6	14.5 ± 2.8	9.0 ± 1.2	15.2 ± 2.8	22.0 ± 0.2	21.6 ± 1.1
HHT (ng/ml)	AF	T48				T72				
		0	20	50	100	0	20	50	100	
MDA-MB-157										
	Early	14.6 ± 3.8	39.4 ± 2.7	48.8 ± 2.8	53.9 ± 4.2	9.9 ± 2.7	45.5 ± 3.9	53.7 ± 4.6	56.3 ± 3.0	
	Late	5.1 ± 1.3	15.2 ± 1.9	19.7 ± 0.6	20.0 ± 4.6	6.4 ± 2.5	17.5 ± 2.8	22.0 ± 4.6	26.1 ± 1.2	
	Total	19.7 ± 3.7	54.6 ± 3.9	68.5 ± 2.8	73.9 ± 4.6	16.3 ± 4.7	62.9 ± 1.1	75.7 ± 4.0	82.4 ± 2.5	
MDA-MB-468										
	Early	8.3 ± 1.2	14.5 ± 3.3	17.6 ± 4.1	21.9 ± 5.8	8.7 ± 1.2	17.1 ± 4.1	23.6 ± 5.5	27.5 ± 4.5	
	Late	3.8 ± 1.3	6.3 ± 0.2	6.4 ± 0.9	6.3 ± 1.5	3.6 ± 0.5	6.0 ± 0.2	4.6 ± 1.8	5.0 ± 1.3	
	Total	12.1 ± 1.7	20.7 ± 3.5	24.0 ± 3.2	28.3 ± 6.0	12.3 ± 0.8	23.1 ± 4.1	28.2 ± 6.1	32.5 ± 5.7	
MDA-MB-231										
	Early	2.2 ± 0.9	3.5 ± 1.7	5.4 ± 1.4	5.8 ± 1.3	2.3 ± 1.0	3.2 ± 2.1	4.4 ± 2.2	4.1 ± 1.3	
	Late	1.5 ± 0.4	3.7 ± 2.2	4.4 ± 1.0	4.2 ± 1.8	2.4 ± 1.2	2.8 ± 1.0	2.9 ± 0.7	3.2 ± 0.2	
	Total	3.7 ± 0.5	7.2 ± 3.1	9.8 ± 1.5	10.0 ± 0.5	4.7 ± 1.7	6.0 ± 2.8	7.3 ± 2.9	7.4 ± 1.4	
CAL-51										
	Early	5.5 ± 0.8	16.0 ± 2.8	24.4 ± 2.7	35.6 ± 4.2	4.7 ± 1.4	25.0 ± 2.9	33.6 ± 4.4	37.6 ± 4.0	
	Late	3.9 ± 0.3	5.8 ± 0.5	6.9 ± 0.4	8.6 ± 1.1	4.3 ± 1.7	8.4 ± 0.9	8.5 ± 1.4	8.4 ± 1.2	
	Total	9.4 ± 0.7	21.8 ± 2.6	31.3 ± 3.1	44.2 ± 3.5	9.1 ± 2.5	33.5 ± 2.1	42.1 ± 2.9	46.0 ± 3.4	

AF, apoptotic fraction; HHT, homoharringtonine; T0, start of the experiment; T6, after 6 h of exposure; T24, after 24 h of exposure; T48, after 48 h of exposure; T72, after 72 h of exposure. All values are given in percentages (%) and represent mean ± SD of three independent experiments.

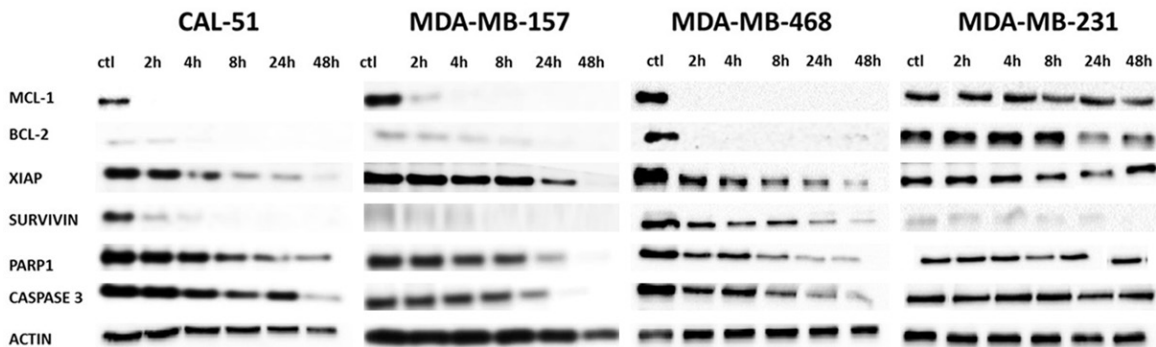


Figure 4. Levels of anti-apoptotic proteins in cell lines exposed to homoharringtonine. ctl, control, beginning of the experiment (before adding the drug). All cells were incubated with 100 ng/ml of HHT. The figure represents the most representative of three independent experiments.

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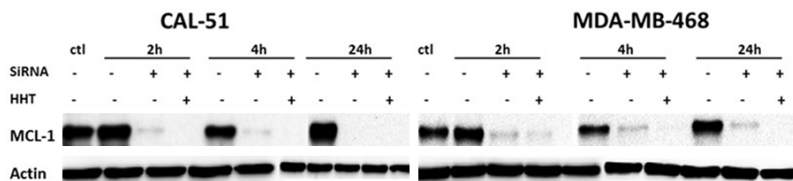


Figure 5. Mcl-1 protein level after exposure of cell lines to *MCL1* siRNA, to homoharringtonine or to both components. ctl, control, beginning of the experiment (before adding the drug); HHT, homoharringtonine. All cells were incubated with 100 ng/ml of HHT. The figure represents the most representative of two independent experiments.

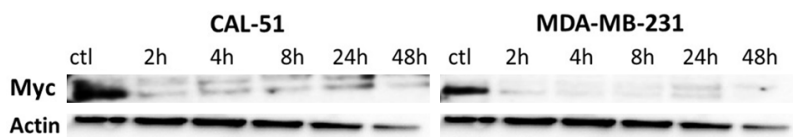


Figure 6. Myc protein level after exposure of cell lines to homoharringtonine. ctl, control, beginning of the experiment (before adding the drug). All cells were incubated with 100 ng/ml of HHT. The figure represents the most representative of two independent experiments.

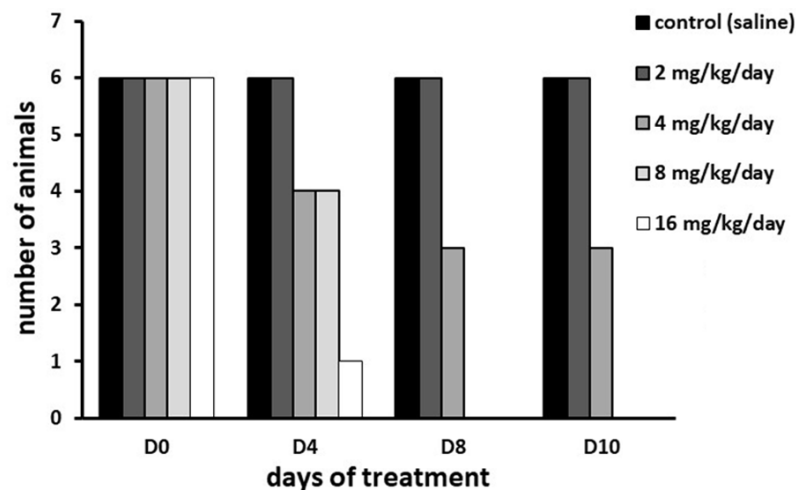


Figure 7. Determination of Maximal Tolerated Dose of homoharringtonine in mice after subcutaneous bi-daily administration over 7 days. D0, beginning of the experiment; D4, D8, D10, days 4, 8 and 10 after the treatment start.

(61.9%, data not shown) than apoptotic (AF = $28.3 \pm 6.0\%$, **Table 1**). In MDA-MB-231 cells, despite a similar cell cycle arrest like in CAL-51 cells after 100 ng/mL for 48 h (**Figure 2A**), apoptotic fraction was much smaller (**Figure 3D**). On that basis, we conclude that, in MDA-MB-157, CAL-51 and MDA-MB-468 cells, apoptosis likely takes place in the G1/G0-phase of the cell cycle. However, HHT effect on MDA-MB-231 cells is mostly cytostatic (arrest in the S-phase) and not cytotoxic.

HHT rapidly reduces abundance of several anti-apoptotic proteins, in particular of Mcl-1, which precedes apoptosis onset

As previous reports demonstrated that HHT most impacts the short-lived cellular proteins [28-30], we investigated how exposure to this drug affects abundance of several anti-apoptotic proteins, known to have a short half-life [36].

As shown on **Figure 4**, HHT provoked an extremely rapid and strong reduction of Mcl-1 levels (almost to total loss, after only 2 h of exposure) in all cell lines except in MDA-MB-231. Similar reduction of Bcl-2 was observed in MDA-MB-468 cells. In addition, survivin level was strongly and rapidly reduced in CAL-51 and MDA-MB-468 cells. XIAP abundance diminished as well, in all cells except MDA-MB-231, however the reduction was visible after a longer exposure to HHT.

Levels of caspase 3, the main apoptosis effector protein, started to lower several hours after the moment when Mcl-1 was almost undetectable. PARP1 change had similar kinetics (**Figure 4**).

We next compared Mcl-1 abundance reduction obtained by exposure to HHT or to *MCL1* small inhibiting RNA (siRNA). As shown on **Figure 5**, in both CAL-51 and MDA-MB-468 cell line, the exposure to either siRNA or to 100 ng/mL of HHT resulted in a strong reduction of Mcl-1 levels, already after 2 h. Addition of HHT, after 24 h incubation with *MCL1* siRNA, further lowered Mcl-1 levels, in both cell lines, and already after 2 h of incubation (**Figure 5**). In CAL-51 cells, level of Mcl-1 was reduced practically to zero,

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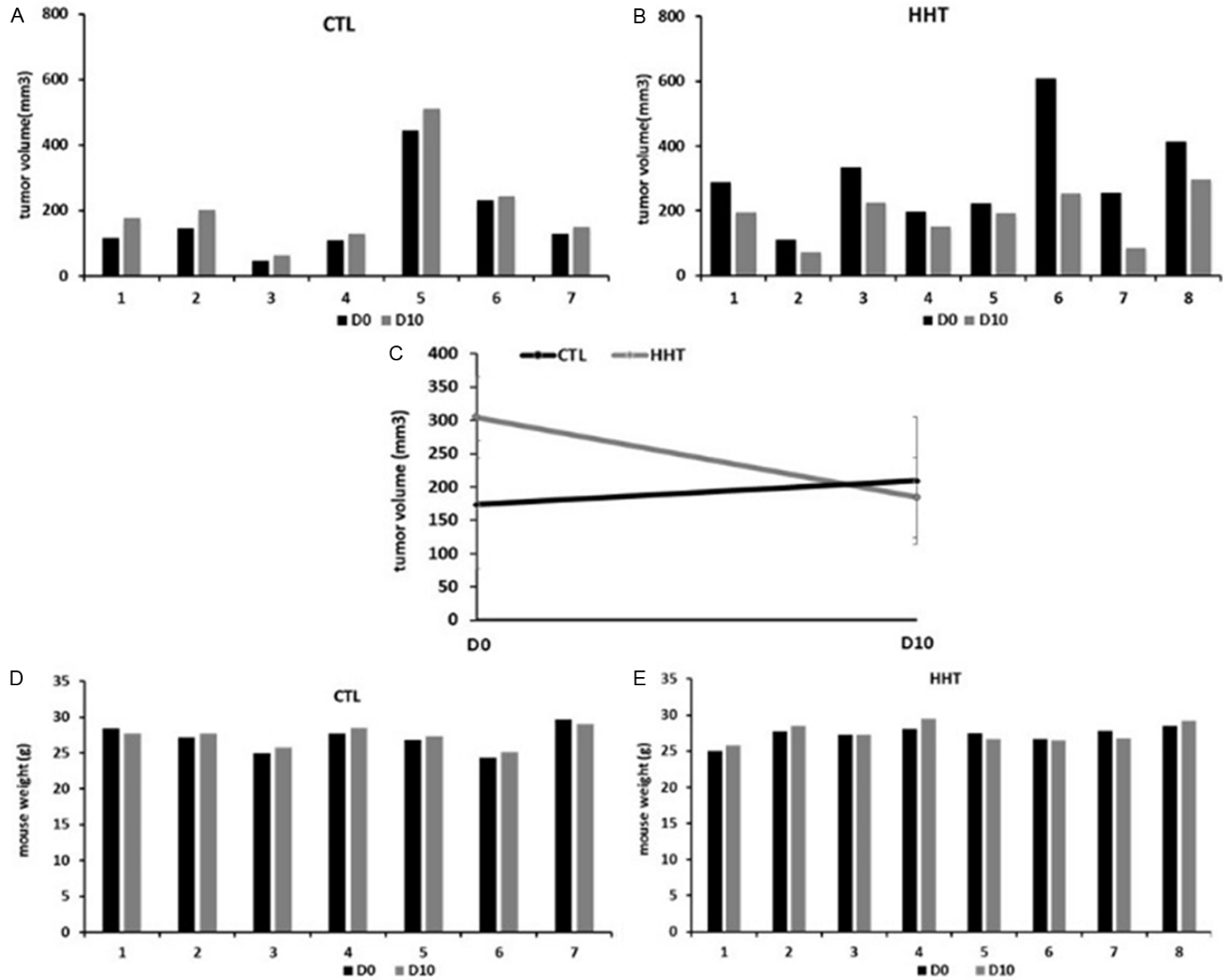


Figure 8. Effect of bi-daily subcutaneous administration of 1 mg/kg homoharringtonine over 7 days on MDA-MB-231 xenografts in mice (experiment 1). A and B: Tumor volumes, absolute values. C: Tumor volume before and after treatment, mean \pm SD. D and E: Mouse weight, absolute values. CTL, control, mice treated with saline water only; HHT, homoharringtonine; D0, beginning of the experiment; D10, day 10 after the treatment start.

either by 100 ng/mL of HHT alone, by siRNA or by both agents together, after 24 h of exposure (**Figure 5**). Interestingly, under identical conditions, in MDA-MB-468 cells there is still a residual quantity of Mcl-1 protein after 24 h of treatment with *MCL1* siRNA only (**Figure 5**).

These results demonstrate that exposure to HHT in vitro reduces Mcl-1 protein level more effectively than one of the most powerful ways to achieve that effect, inhibition by siRNA of the gene which encodes for the protein (*MCL1*).

HHT rapidly and strongly reduces Myc levels, even in MDA-MB-231 cells

After observation that HHT reduced abundance of major anti-apoptotic proteins in all cells but MDA-MB-231, we wanted to see whether it will reduce abundance of a short-lived protein not from the anti-apoptotic class. For example, Myc protein has a very short half-life [37] and an important role in TNBC resistance to therapy [22, 38].

As shown on **Figure 6**, in each MDA-MB-231 and CAL-51 cells HHT strongly reduced Myc levels already after 2 h. At that time, in MDA-MB-231 cells, Myc levels were reduced almost to zero and did not increase after (**Figure 6**).

HHT inhibits growth of MDA-MB-231 and MDA-MB-468 in vivo

As shown on **Figure 7**, the Maximal Tolerated Dose (MTD) of HHT, in a 7-day regimen, was 2 mg/kg (two subcutaneous injections, each with 1 mg/kg) per day, for the mice carrying MDA-MB-231 xenografts.

HHT had a growth-suppressive effect on MDA-MB-231 xenografts, in two experiments. In the first (**Figure 8**), the mean volume of the tumors in the treated animals was, at the end of observation (day 10), for 12% smaller than in the untreated animals (184.4 mm³ vs 209.5 mm³, treated vs untreated, respectively, **Figure 8E**). Moreover, the mean tumor volume before the initiation of the treatment by was almost twice bigger in the group that will be treated by HHT

than in the group planned for treatment with saline only (304.5 mm³ vs 173.3 mm³, respectively). In the second experiment (**Figure 9**), with less difference in tumor volume before initiation of the treatment, the mean tumor volume, at day 10, was reduced for 36.5% in the treated animals, compared to the untreated group (146.2 \pm 15.5 mm³ vs 230.4 \pm 27.7 mm³, respectively, **Figure 9E**).

In the experiment on MDA-MB-468 xenografts (**Figure 10**), the mean tumor volume, at day 10, was for almost half-smaller in the treated mice than in the untreated ones (142.8 \pm 22.5 mm³ vs 273.8 \pm 110.5 mm³, respectively, **Figure 10E**).

The difference in animal weight after treatment, in comparison to the weight before treatment, was minimal (**Figures 9 and 10**, panels C and D on each).

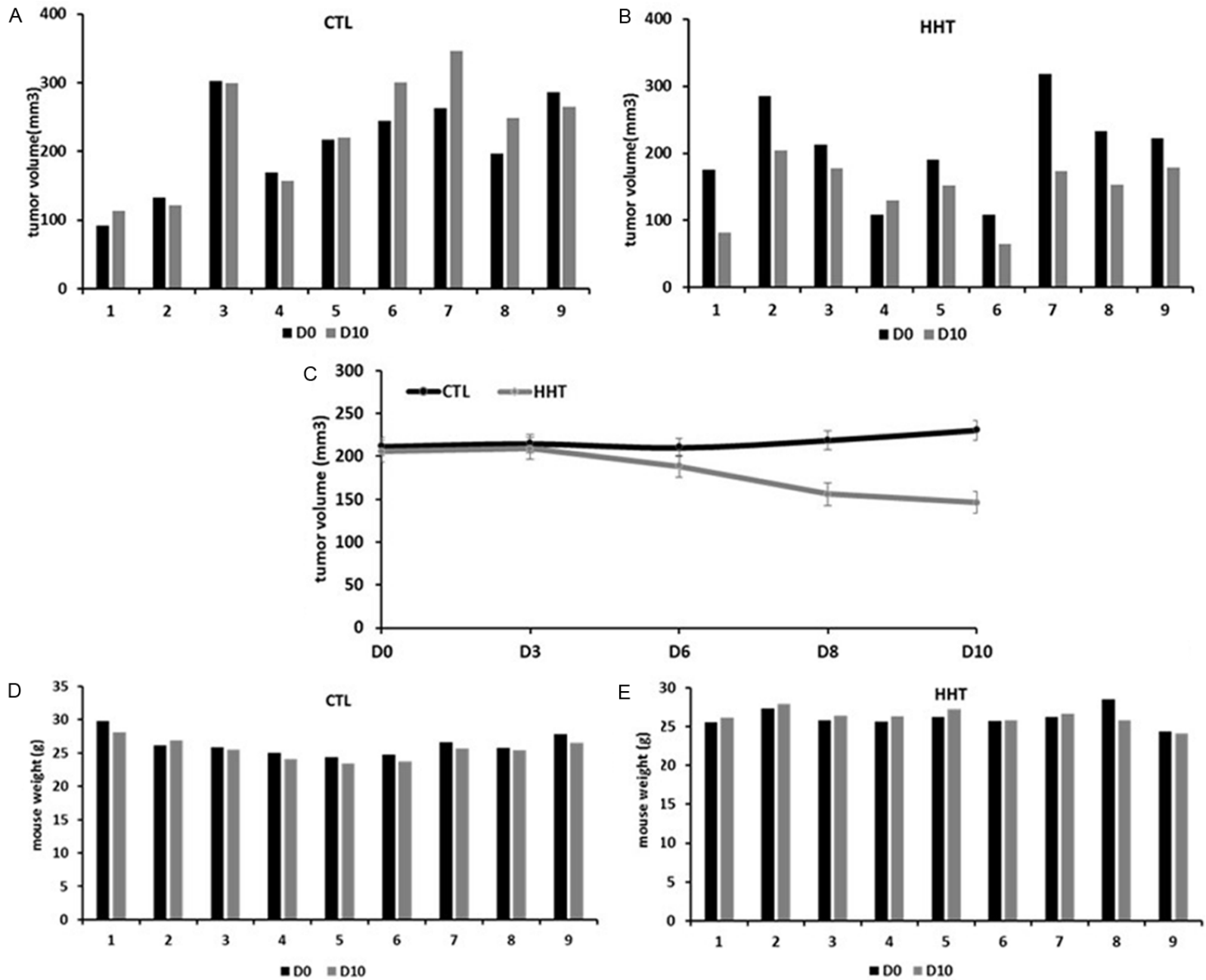
Taken together, these results indicate that HHT inhibits in vivo growth of two different models of aggressive TNBC, without general toxicity for the animals, when administered in a 7-day regimen.

Discussion

Here we report that an approved anti-leukemic agent, HHT/omacetaxine [8], has a growth-reducing effect on one of the most aggressive solid cancers, TNBC. This is the first study of HHT alone using transcriptomically defined, *BRCA1/2* non-mutated TNBC models. These models represent the TNBC subgroups nowadays most in need of new treatment approaches, as the currently available standard chemotherapy, as well as the most promising new agents, PARP or immune checkpoint inhibitors, all work best in TNBC with defects in DNA damage repair (*BRCA1/2*-mutated or with *BRC-Aness*) [4].

HHT has been principally evaluated in blood malignancies and not in solid tumors. A couple of early clinical trials, which tested HHT in the form of leaf extract, reported no effect of this molecule on breast cancer [39, 40]. Very re-

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Figure 9. Effect of bi-daily subcutaneous administration of 1 mg/kg homoharringtonine over 7 days on MDA-MB-231 xenografts in mice (experiment 2). A and B: Tumor volumes, absolute values. C: Tumor volume before and after treatment, mean \pm SD. D and E: Mouse weight, absolute values. CTL, control, mice treated with saline water only; HHT, homoharringtonine; D0, beginning of the experiment; D3, D6, D8, D10, day 3, 6, 8 and 10 after the treatment start.

cently, a natural analog of HHT, isoharringtonine, was reported to inhibit growth and migration of two TNBC cell lines, one *BRCA1*-mutated, and another highly sensitive to a standard chemotherapeutic component, paclitaxel [41]. That indicates inhibitory action of the harringtonine class components on the TNBC already well responding to the classical chemotherapy. Our study demonstrates that HHT also suppresses growth of the TNBC strains quite resistant to the available chemotherapy drugs [31]. In addition, IC₅₀ of HHT were at the low-medium nanomolar level (after 72 h of incubation, from 7 to 45 ng/mL /data not shown/, which is equivalent to 13 to 82.5 nM, molecular weight of HHT being 545.6 g/mol). Similar IC₅₀ have been seen in myeloid leukemia cell lines [28], indicating that HHT is a potent anti-neoplastic agent.

We showed that HHT very rapidly induces significant growth inhibition and cell death in 3 out of 4 cell TNBC cell lines tested. This is in concordance with studies performed on AML and myeloma cell lines, in which HHT induced apoptosis of malignant cells as early as after 4-6 h of incubation [28, 29, 42, 43]. In our study, HHT exerted the maximal apoptotic effect after a continuous exposure for 48-72 h, depending on the cell type. HHT is highly stable in cell cultures [44], so the experimental conditions we used are an example of continuous exposure to the drug. In patients, HHT gets progressively degraded; its mean steady-state half-life is around 7 h [45], which, in the bi-daily regimens, can assure exposure of cancer cells to sufficient concentration of the drug to permit its anti-cancer action.

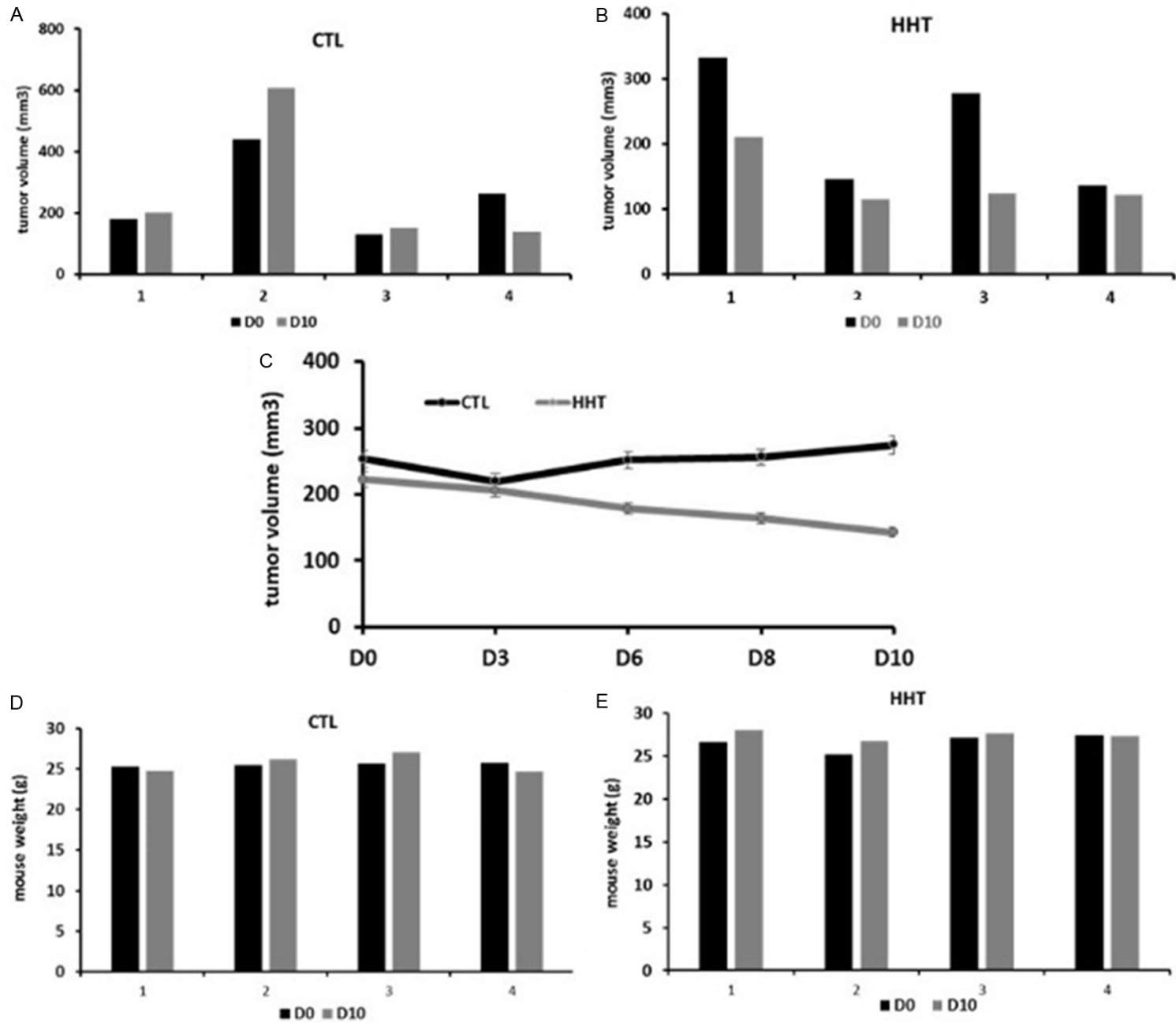
Induction of apoptosis by HHT occurred after an arrest of TNBC cells in the cell cycle. In one of the TNBC cell lines we tested, MDA-MB-231, HHT dominantly induced an S-phase arrest, without much apoptosis. However, we observed a significant reduction in MDA-MB-231 tumor volume after 7 days of HHT treatment in vivo. This discrepancy between in vitro and in vivo data could be explained by experimental design: in our in vitro studies, the maximal expo-

sure time to HHT was 48 h (cell cycle) or 72 h (apoptosis), whereas in vivo it was 7 days, plus additional 2 days, without the treatment, before the measure of tumor volume. That way the tumor cells were, in vivo, exposed to HHT for almost 5-fold longer time than in vitro. Even during the 2 days without the drug, in vivo, HHT which has already entered the cells and fixed to ribosomes, could continue inhibiting the protein synthesis and tumor growth. Indeed, there was no difference in the MDA-MB-231 tumor volume after 3 days of HHT injection, but it became visible after 6 days and was maximal after 10 days (Figure 7).

We could not test HHT on sufficient number of mice carrying xenografts of MDA-MB-468 and CAL-51 cell line, so we cannot make a strong conclusion about its efficiency in those models. Anyway, a remarkable growth-inhibitory effect of HHT was demonstrated in 4 mice carrying MDA-MB-468 xenografts. As we showed that HHT does reduce in vivo growth of the cell line the least sensitive in vitro (MDA-MB-231), it seems plausible to hypothesize that it would inhibit in vivo growth of the cell lines it most inhibited in vitro (CAL-51, MDA-MB-157, MDA-MB-468). However, that remains to be proven in future studies.

MDA-MB-468 and CAL-51 cell lines have in common hyperactivated PI3K-AKT-mTOR pathway, due to PTEN absence in the former and *PIK3CA* activating mutation in the latter [31]. These cell lines may have an increased RNA translation/increased protein synthesis so its inhibition may have a deleterious effect on their growth. We observed, on a luminal breast cancer cell line MCF-7, a growth-inhibitory effect of HHT similar to the one observed on MDA-MB-468 cells (in vitro data, not shown). MCF-7 also carries an activating mutation of *PIK3CA*, and the increased sensitivity of that cell line to HHT indicates that the drug could be efficacious in suppression of all breast cancers having the PI3K-AKT-mTOR pathway activated, at least if the activation is caused by a PI3K activating mutation or PTEN loss, irrespective of molecular subtype.

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Figure 10. Effect of bi-daily subcutaneous administration of 0.5 mg/kg homoharringtonine over 7 days on MDA-MB-468 xenografts in mice. A and B: Tumor volumes, absolute values. C: Tumor volume before and after treatment, mean \pm SD. D and E: Mouse weight, absolute values. CTL, control, mice treated with saline water only; HHT, homoharringtonine; D0, beginning of the experiment; D3, D6, D8, D10, day 3, 6, 8 and 10 after the treatment start.

We hypothesize that the strong and rapid Mcl-1 protein level reduction, is the main event which induces apoptosis of TNBC after exposure to HHT. Our experiments showed that a significant reduction of Mcl-1 protein level, either by HHT or by siRNA, is rapidly followed by apoptosis induction. Mcl-1 protein has been demonstrated to have crucial importance for TNBC survival [21]. In addition, overexpression of Mcl-1 is associated with worse survival of pts with all breast cancer subtypes [20]. Finally, downregulation of Mcl-1 levels enables sensitization of breast cancer cells to the current chemotherapy [46]. However, all reported Mcl-1 inhibitors are far earlier in the clinical development than HHT, which has already been approved in one malignant disease [8].

HHT is a universal inhibitor of protein synthesis in eukaryotes, and, as many natural products, can be considered as a multi-target agent, although all its action targets are now known. It has been demonstrated that HHT inhibits stem cells of chronic myeloid leukemia and multiple myeloma [47, 48]. In TNBC, HHT-induced downregulation of Myc protein may also have an important role in stem cell suppression [49]. We demonstrated that HHT rapidly reduces Myc abundance to very low levels even in a highly metastatic TNBC cell line, MDA-MB-231. This implies that HHT might be efficacious in suppression of TNBC metastatic progression. We showed, in two independent experiments, that HHT markedly suppresses *in vivo* growth of MDA-MB-231. In additional experiments, which need verification, we also observed that migration of this line was strongly inhibited by HHT (data not shown), indicating possible anti-metastatic properties of the drug.

In conclusion, our results show that HHT effectively suppresses an important mechanism of cancer cell resistance to death, the increased levels of Mcl-1 and other anti-apoptotic proteins. As it has been shown that Mcl-1 downregulation is of primordial importance for sensitization to many anti-cancer therapies, HHT is worth developing in TNBC, as a single agent in the metastatic phase of the disease and as a sensitizer of standard chemo- or targeted ther-

apy. Development of another natural product (HHT) for breast cancer therapy should be encouraged by the eribulin case, recently approved as a single agent in metastatic breast cancer and one of the most efficacious treatments in this setting. In addition, new purification procedures (like the one exploited for production of HHT used in this study) which yield a highly purified HHT form at a several-fold lower cost compared to semi-synthesis used for production of the currently approved omacetaxine (personal communication, LeukePharma Inc.). This will render HHT and translation/protein synthesis inhibition more available for cancer treatment, and in particular, in the regions where TNBC and other cancers are devastating the population but numerous patients cannot afford even the standard chemotherapy.

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Disclosure of conflict of interest

None.

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