ANTIOXIDANT POTENTIAL OF AN ANTHRAQUINONE METABOLITE ISOLATED FROM A MARINE FUNGUS

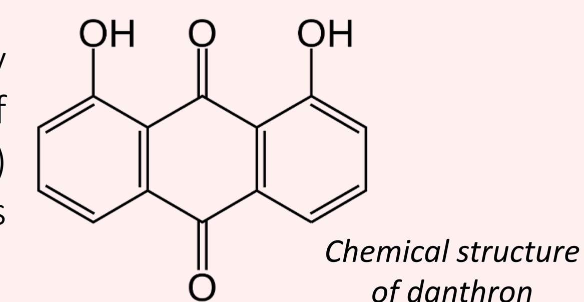
ciberer Centro de Investigación Biomédica en Red Enfermedades Raras

ibima Plataforma BIONAND

Vidal-Valenzuela, I.[1]; Torres-Vargas, J.A. [1]; García-Caballero, M. [1], Medina, M.A. [1,2] & Quesada, A.R.[1,2]

1. Department of Molecular Biology and Biochemistry, Faculty of Sciences, University of Málaga, Andalucía Tech, and IBIMA Plataforma BIONAND, E-29071 Málaga, Spain

2. Unidad 741 de CIBER "de Enfermedades Raras", E-29071 Málaga, Spain.



INTRODUCTION

The potential of marine organisms to yield bioactive molecules is vast and remains largely untapped. Notably, some marine-derived bioactive compounds have already been approved as anticancer drugs[1]. Among the numerous bioactive compounds present in marine organisms, anthraquinones represent a noteworthy class of molecules, with over 200 structurally related compounds having been isolated from diverse species of marine fungi[2]. Danthron (1,8-dihydroxy-9,10-anthraquinone) represents an exemplar of anthraquinones, with anti-tumoral and anti-angiogenic properties that have yet to be fully elucidated. The primary objective of this study was therefore to achieve this particular goal.

OH O OH

O

Chemical structure of danthron

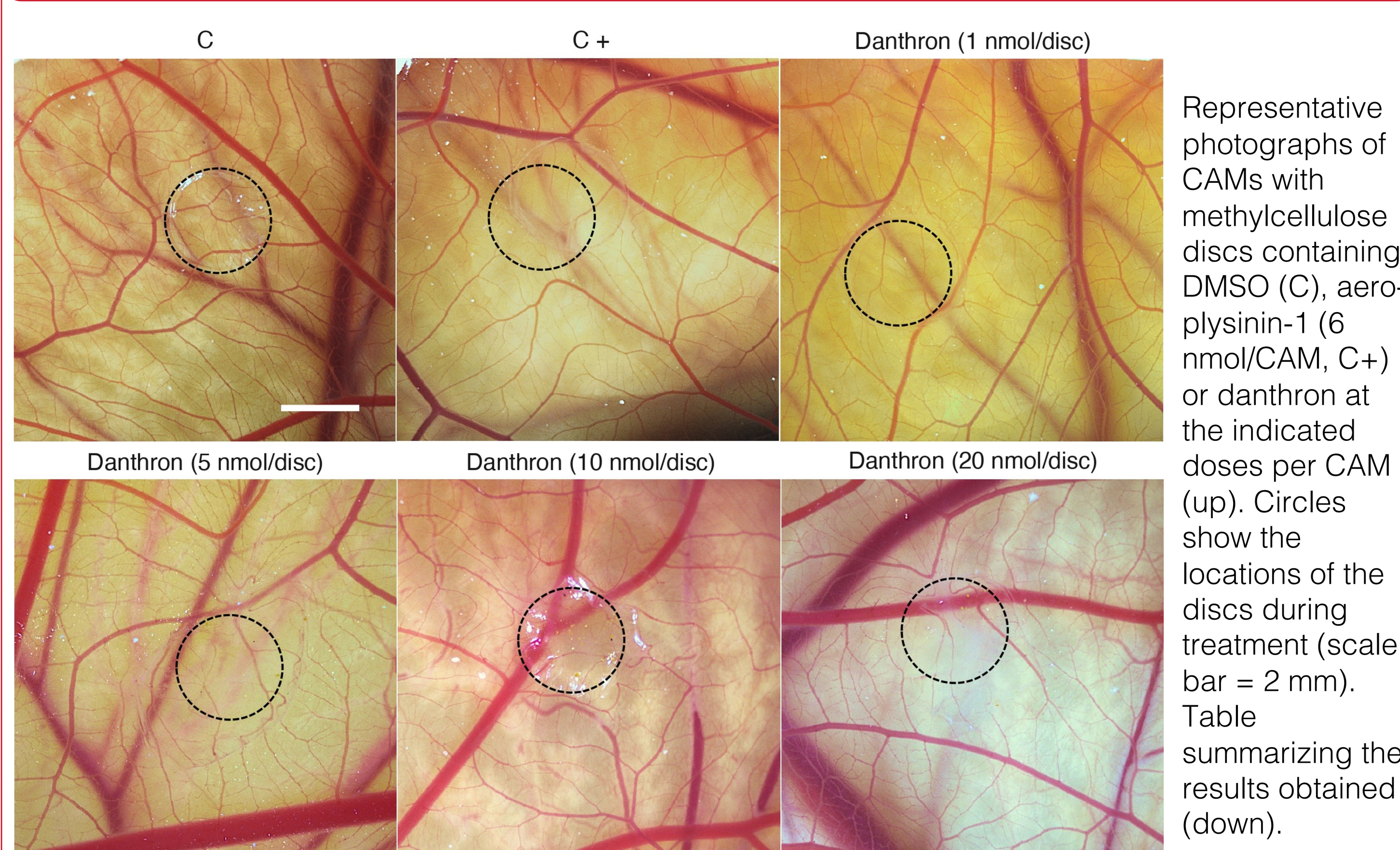
METHODS

1. **Chorioallantoic membrane (CAM) assay[3]**: eggs were incubated at 38°C in a humidified incubator with a tilting tray. After 3 days, the eggs were windowed, and at day 8, methylcellulose discs containing different amounts of danthron were carefully placed on the CAMs. After 48 h of incubation, CAMs were observed and photographed under a scope.
2. **Cell survival assay[3]**: performed under proliferative conditions; methylthiazolyldiphenyl-tetrazolium bromide (MTT) was used after 72 h of incubation of cells with different concentrations of the compound danthron and IC_{50} values were calculated from the survival curves. The MTT assay can be adapted to serve as a **cytotoxicity assay** in short-term treatments[4].
3. **Tubular-like structures formation on Matrigel[3]**: Human umbilical endothelial cells (HUVECs) were seeded in absence of serum on Matrigel layers in presence of different concentrations of danthron, and after 6 h (when tubular-like structures were formed in negative control) photos were taken.
4. **Proliferation assay (EdU)[4]**: HUVECs and cancer cells (human breast carcinoma MDA-MB231 and fibrosarcoma HT1080 cell lines) were seeded and treated for 48 h with danthron. Cell proliferation was then measured using the *baseclick EdU Flow Cytometry Kit*, according to manufacturer's specifications.
5. **Cell cycle analysis[3]**: Cell cycle analysis using flow cytometry was performed in endothelial and tumor cells that were treated with danthron for 48 h.
6. **Hoechst staining[4]**: Cells were grown on gelatin-coated cover slides, treated for 24 h with danthron, fixed and stained with Hoechst. The percentage of cells with chromatin condensation was calculated from five fields of vision across three experiments.
7. **ROS[4]**: HUVECs and tumor cells, seeded in a 24-well plate, were stained with DCFH-DA prior to their treatment with danthron and/or hydrogen peroxide. Once the treatments were added, measurements were obtained with a fluorescence plate reader every 2 h for 8 h (Ex/em: ~492–495/517–527 nm).
8. **SH[4]**: To determine cell redox capacity, endothelial and tumor cells were treated with danthron for 24 or 48 h. After washing, reaction buffer (PBS supplemented with $CaCl_2$, $MgCl_2$, glucose, lipoic acid and DTNB) was added and incubated for 1 h. The absorbance of the supernatants were measured at 412 nm and normalized by the number of cells.

Results are expressed as mean ± SD of at least three independent experiments. Statistical significance was determined using the two-sided unpaired Student t-test. Values of $p < 0.05$ were considered to be statistically significant. Significance is indicated as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

RESULTS

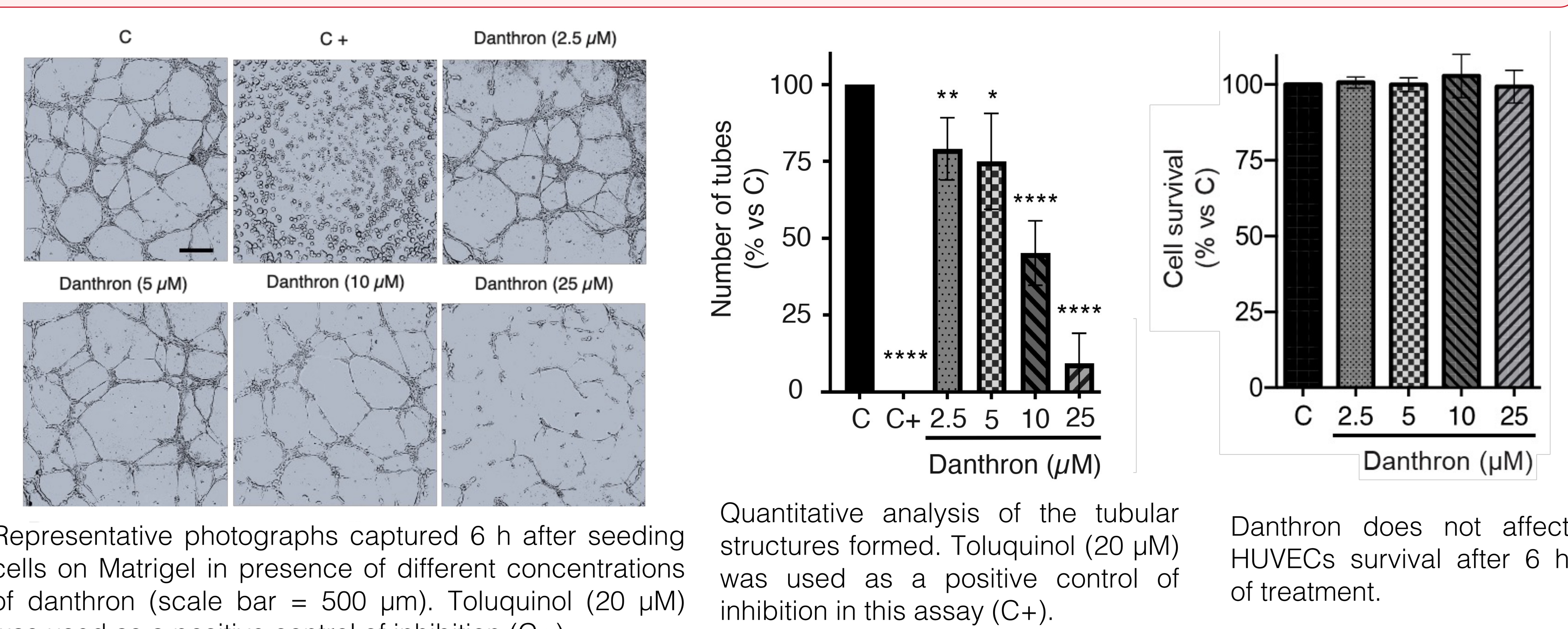
1. Danthron inhibits angiogenesis *in vivo* in a dose-dependent manner



Representative photographs of CAMs with methylcellulose discs containing DMSO (C), aeroplysinin-1 (6 nmol/CAM, C+) or danthron at the indicated doses per CAM (up). Circles show the locations of the discs during treatment (scale bar = 2 mm). Table summarizing the results obtained (down).

Condition	C	C+	Danthron (1 nmol/disc)	Danthron (5 nmol/disc)	Danthron (10 nmol/disc)	Danthron (20 nmol/disc)
% Inhibition	0	90	36	60	70	88

2. Danthron inhibits the tube formation on Matrigel by HUVECS at nontoxic doses

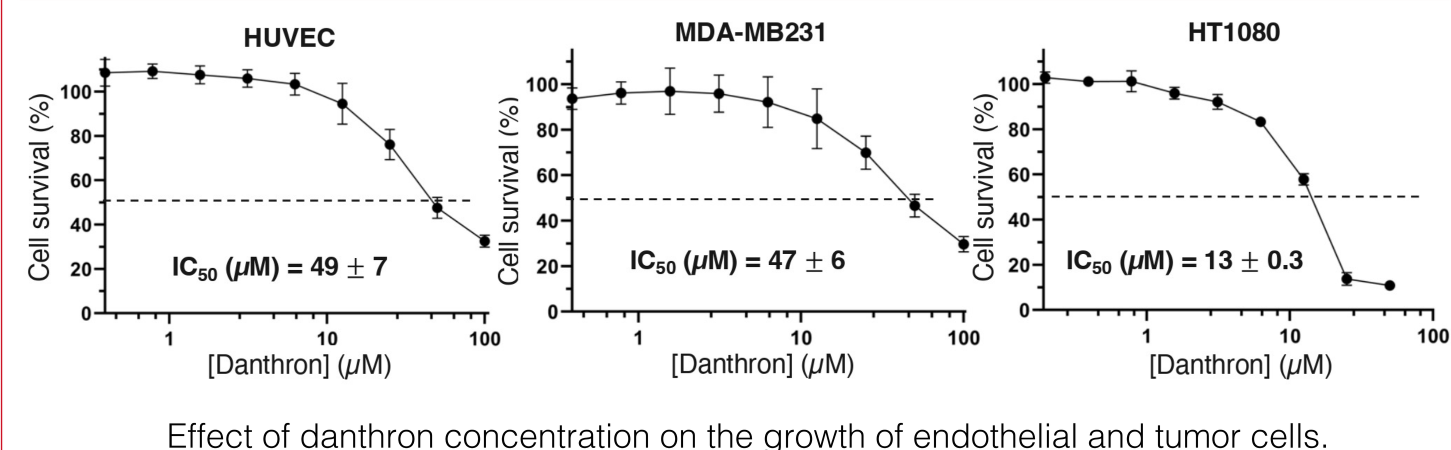


Representative photographs captured 6 h after seeding cells on Matrigel in presence of different concentrations of danthron (scale bar = 500 μm). Toluquinol (20 μM) was used as a positive control of inhibition (C+).

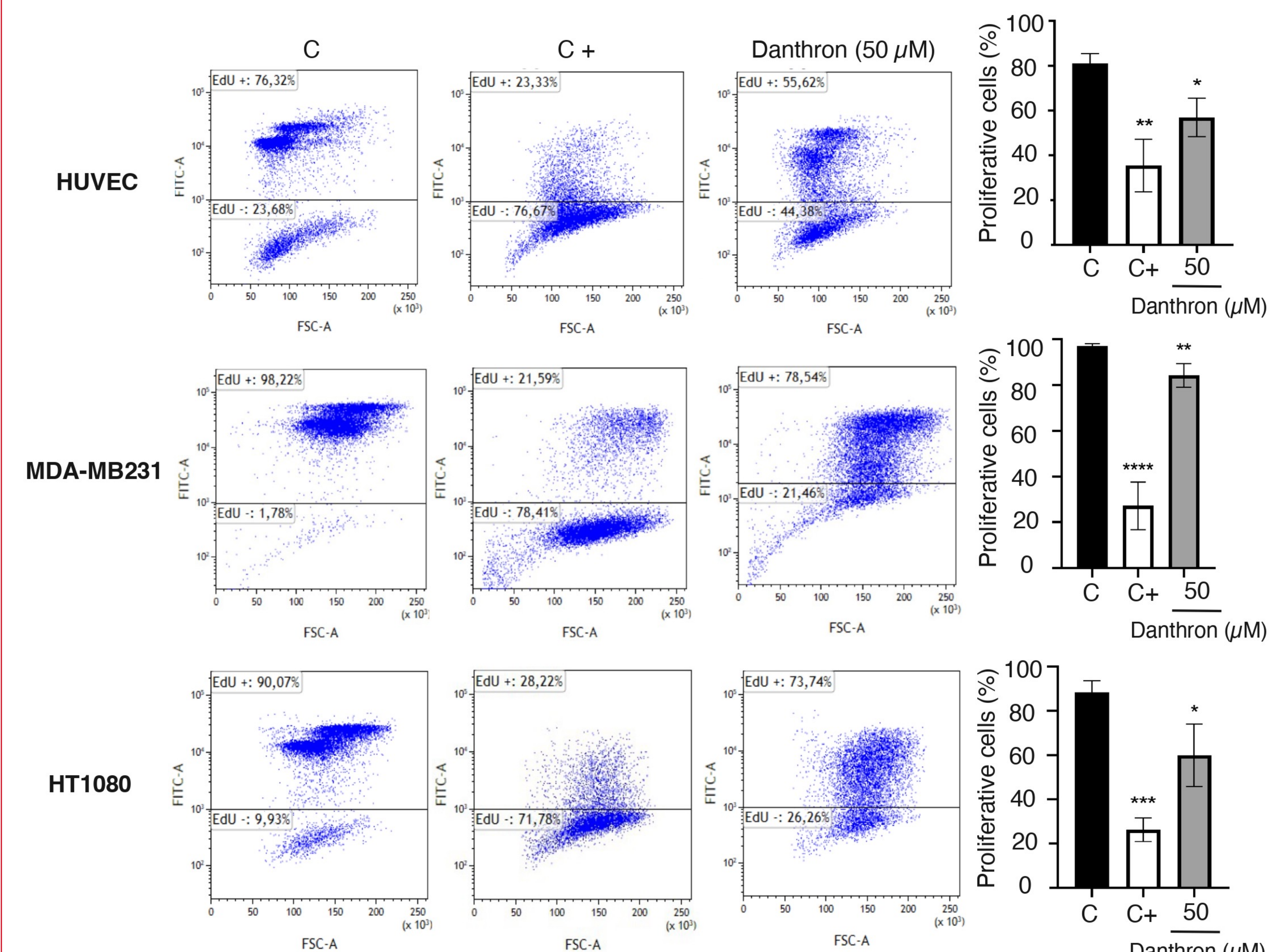
Quantitative analysis of the tubular structures formed. Toluquinol (20 μM) was used as a positive control of inhibition in this assay (C+).

Danthron does not affect HUVECs survival after 6 h of treatment.

3. Danthron inhibits the growth of endothelial and tumor cells

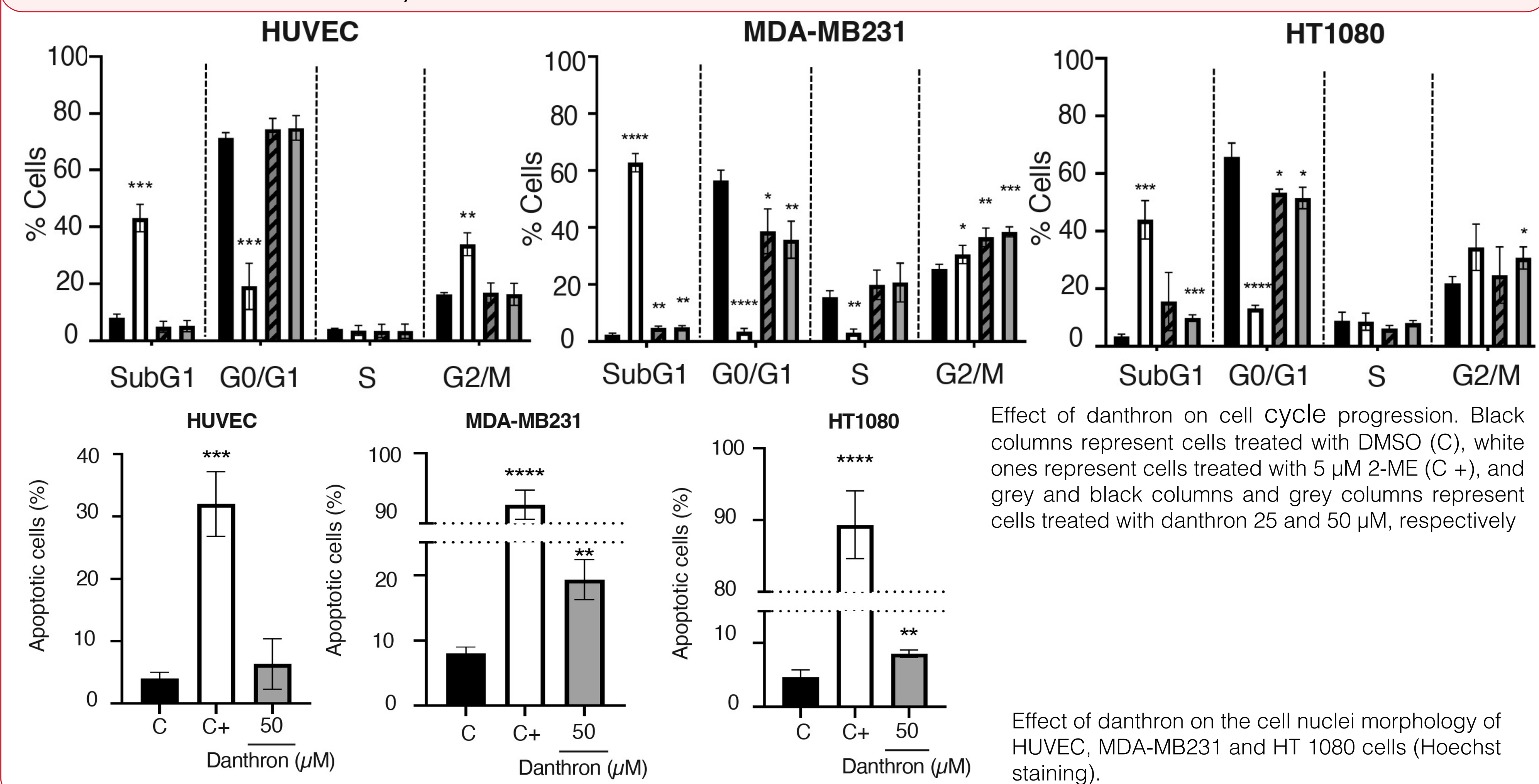


Effect of danthron concentration on the growth of endothelial and tumor cells.



Flow cytometry representative profiles of EdU-treated HUVEC, HT1080 and MDA-MB231 (Left). Cells were treated for 48 h with DMSO (C), 3 μM mitomycin C (C+) or 50 μM danthron. Quantitative analysis of EdU+ (proliferative) cells (Right).

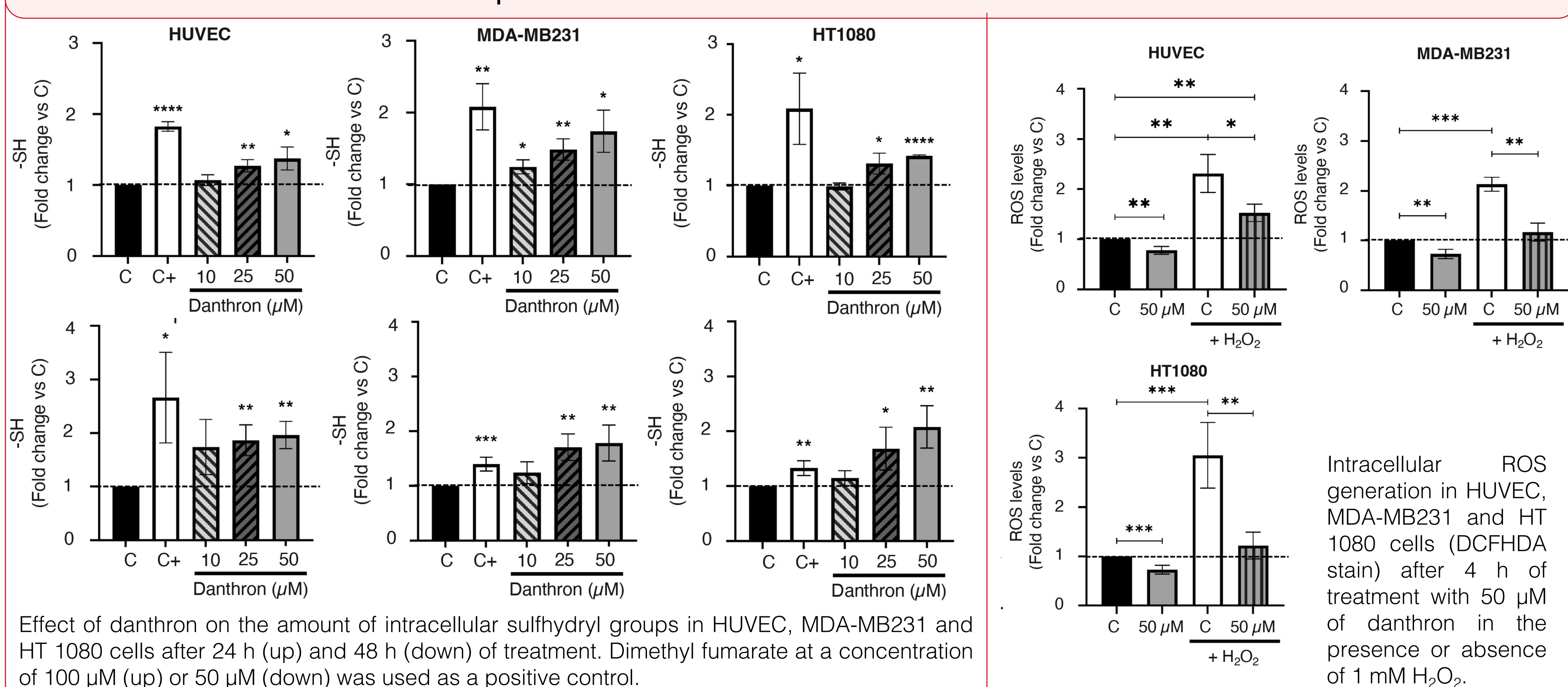
4. Danthron induces changes in the cell cycle subpopulations and apoptosis in MDA-MB231 and HT1080 cells, whereas these effects has not been detected in HUVECs



Effect of danthron on cell cycle progression. Black columns represent cells treated with DMSO (C), white ones represent cells treated with 5 μM 2-ME (C +), and grey and black columns and grey columns represent cells treated with danthron 25 and 50 μM, respectively

Effect of danthron on the cell nuclei morphology of HUVEC, MDA-MB231 and HT 1080 cells (Hoechst staining).

5. Danthron increases the amount of intracellular sulfhydryl groups and reduces intracellular ROS production in endothelial and tumor cells



Effect of danthron on the amount of intracellular sulfhydryl groups in HUVEC, MDA-MB231 and HT 1080 cells after 24 h (up) and 48 h (down) of treatment. Dimethyl fumarate at a concentration of 100 μM (up) or 50 μM (down) was used as a positive control.

Intracellular ROS generation in HUVEC, MDA-MB231 and HT 1080 cells (DCFHDA stain) after 4 h of treatment with 50 μM of danthron in the presence or absence of 1 mM H_2O_2.

CONCLUSIONS

- As suggested by the *in vivo* and the *in vitro* tube-formation assay, danthron could serve as a promising new antiangiogenic drug for treating and preventing cancer and other diseases that rely on angiogenesis.
- The results obtained in this work reveal that danthron is able to inhibit cell survival and proliferation of endothelial cells (HUVECs) and tumor cells (MDA-MB231 and HT1080). However, Its apoptosis-inducing effect seems to be selective for tumor cells.
- The antioxidant effects of danthron are evidenced by the increase in intracellular sulfhydryl groups and the decrease in intracellular ROS production in both endothelial and tumor cells.

1. Dyshlovoy, S.A., & Honecker, F. (2022). Marine Compounds and Cancer: Updates 2022. *Mar. Drugs, 20*, 759.
2. Sebak, M., Molham, F., Greco, C., Tammam, M.A., Sobeh, M., & El-Demerdash, A. (2022). Chemical diversity, medicinal potentialities, biosynthesis, and pharmacokinetics of anthraquinones and their congeners derived from marine fungi: A comprehensive up-date. *RSC Adv., 12*, 24887–24921.
3. Marrero, A. D., Castilla, L., Espartero, J. L., Madrona, A., R Quesada, A., Medina, M. Á., & Martínez-Poveda, B. (2020). A comparative study of the antiangiogenic activity of hydroxytyrosyl alkyl ethers. *Food Chem., 333*, 127476.
4. Torres-Vargas, J.A., Cheng-Sánchez, I., Martínez-Poveda, B., Medina, M.Á., Sarabia, F., García-Caballero, M., & Quesada, A.R. (2022). Characterization of the activity and the mechanism of action of a new toluquinol derivative with improved potential as an antiangiogenic drug. *Biomed. Pharmacother., 155*, 113759.

Funding: Projects PID2019-105010RB-I00 (Ministry of Science and Innovation, Government of Spain), UMA18-FEDERJA-220 and P20_00257 (Junta de Andalucía-FEDER)