ANTIANGIOGENIC POTENTIAL OF AN ANTHRAQUINONE METABOLITE ISOLATED FROM A MARINE FUNGUS

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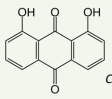
INTRODUCTION

The potential of marine organisms to yield bioactive molecules is vast and largely unexplored. Significantly, certain bioactive compounds derived from the marine environment have already received approval as anticancer drugs[1]. Among the multitude of bioactive compounds found in marine organisms, anthraquinones represent a notable 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) serves as an exemplary member of anthraquinones, possessing anti-tumoral and anti-angiogenic properties that have yet to be fully elucidated. Therefore, the primary objective of this study was to comprehensively investigate and understand these specific properties.



Chemical structure of danthron

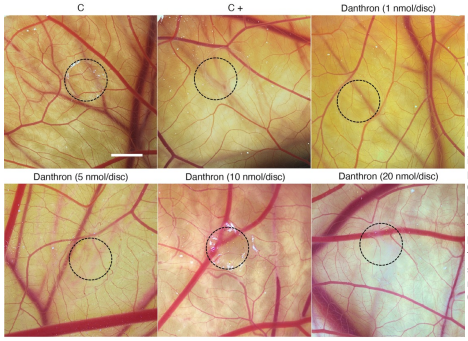
METHODS

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. **Determination of MMP activity[5]**: gelatin zymography was performed with cell lysates and conditioned media of HUVECs, MDA-MB231 and HT1080 treated 24 h with different concentrations of danthron.
8. **Invasion assay[5]**: HUVEC, MDA-MB231 and HT1080, that invade the Boyden's chamber coated with Matrigel, were photographed after 24 -34 h of incubation in presence of danthron.
9. **Migration assay[3]:** wound-healing assay was conducted in monolayers of HUVEC, MDA-MB231 and HT1080 in presence of danthron. Photos were captured at time 0, 4 and 8 h and analyzed by *Fiji software*.

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

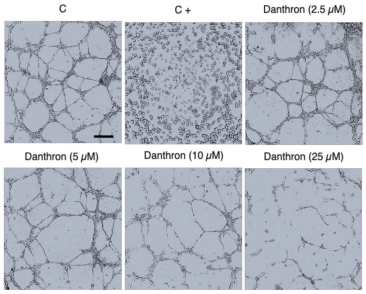


Representative photographs of CAMs with methylcellulose discs containing DMSO (C), aeroplysinin-1 (6 nmol/CAM, C+) or danthron at the indicated doses per CAM (up). Circles show the locations of the discs during treatment (scale bar = 2 mm).

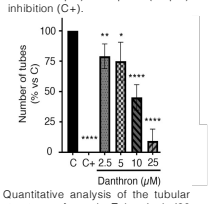
Table summarizing the results obtained (down).

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

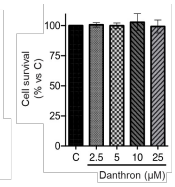
2. Danthron inhibits the tube formation on Matrigel by HUVECS at nontoxic doses



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+).

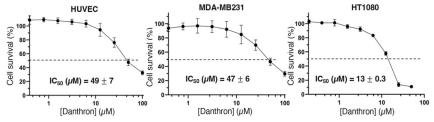


Quantitative analysis of the tubular structures formed. Toluquinol (20 μM) was used as a positive control of inhibition in this assay (C+).

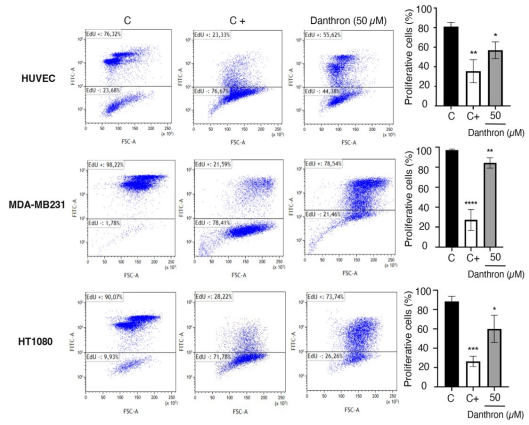


Danthron does not affect HUVECs survival after 6 h of treatment.

3. Danthron inhibits the growth of endothelial and tumor cells

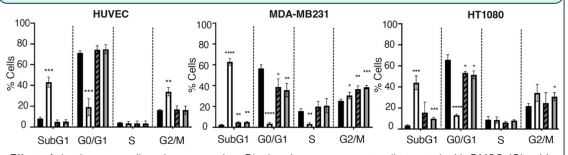


Effect of danthron concentration on the growth of endothelial and tumor cells.

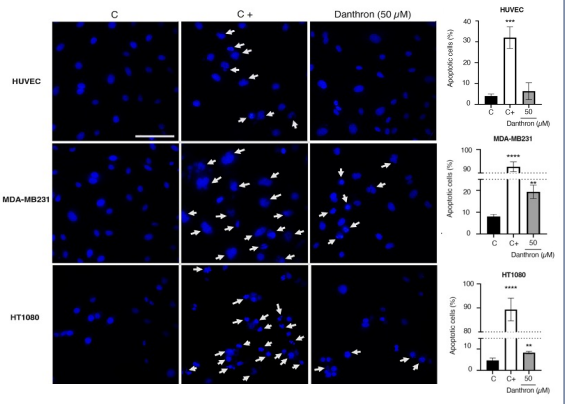


Flow cytometry representative profiles of EdU-treated HUVEC, HT1080 and MDA-MB231 (Left). Cells were treated for 48 h with DMSO (C), 3 μM mitomycin C (C+) or 50 μM danthron. Quantitative analysis of EdU+ (proliferative) cells (Right).

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

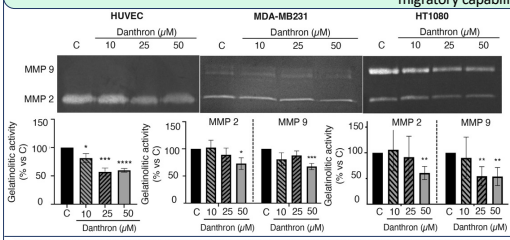


Effect of danthron on cell cycle progression. Black columns represent cells treated with DMSO (C), white ones represent cells treated with 5 μM 2-ME (C +), and grey and black columns and grey columns represent cells treated with danthron 25 and 50 μM, respectively

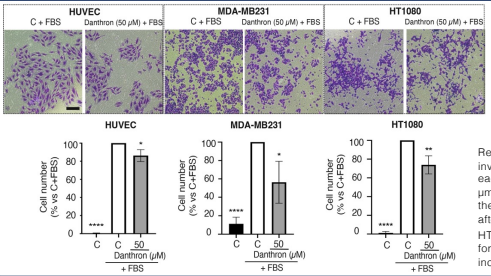


Effect of danthron on the cell nuclei morphology of HUVEC, MDA-MB231 and HT 1080 cells (Hoechst staining). (Left) Representative images are illustrated for each cell line. Cells with condensed chromatin are highlighted by white arrows (scale bar = 50 μm). (Right) Quantification of the percentage of cells with chromatin condensation.

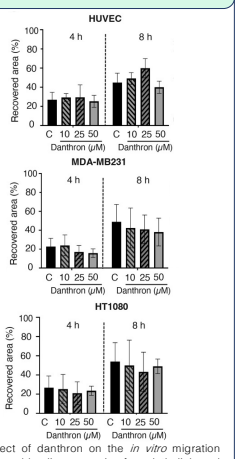
5. Danthron inhibits the proteolytic and invasive capabilities of endothelial (HUVEC) and tumor (MDA-MB231 and HT1080) cells, but not their migratory capabilities



Representative image of the conditioned media from HUVEC, MDA-MB231 and HT 1080 cells analyzed by gelatin zymography (top), quantification of the zymographic bands of gelatinases (bottom).



Representative images of invading cells are illustrated for each cell line (scale bar = 200 μm). Quantitative analysis of the number of invading cells after a treatment of 24 h for HT1080 and HUVEC, and 34 h for MDA-MB231 is also included.



Effect of danthron on the *in vitro* migration (wound-healing assay) of endothelial and tumor cells. Quantitative analysis of the recovered area after 4 and 8 h of incubation in the wound-healing assay.

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.
- Although danthron does not seem to have an anti-migratory effect on the endothelial (HUVEC) and tumoral (MDA-MB231 and HT1080) cells used in these experiments, it could present an inhibitory effect on the invasiveness of these cells, as it has been demonstrated with the reduction in the metalloproteinase activity secreted by these cells and the *in vitro* invasion assay.

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