ANTI-MELANOMA AND ANTI-BREAST CANCER POTENTIAL OF NOVEL BETULONIC ACID DERIVATIVES

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BACKGROUND

Cancer, as one of the leading causes of death has a major impact on society across the world. The search for effective cancer treatments is still ongoing. One approach is to optimize natural compounds to obtain derivatives with high cytotoxicity selectively against cancer cells.

Betulonic acid (BA) belongs to pentacyclic triterpenes that exist in many plants. So far, the promising anticancer activity of these compounds has been demonstrated in both cell culture and animal models. We suggest, that chemical modifications of BA may provide compounds with optimized biological activity.

AIM OF THE STUDY

The main goal of the study was to perform screening analysis of breast cancer and melanoma cell lines for sensitivity to two new derivatives of BA with acetylenic moiety (EB171 and EB173). In the next step, the assay using zebrafish embryos and larvae was conducted to assess the toxicity of the most promising derivative.

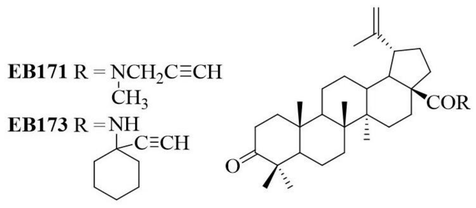

Figure 1. Chemical structure of new acetylenic derivatives of betulonic acid.

METHODS

Cell Culture

Human breast cancer MC7-7 and MDA-MB-231 cells, as well as human melanoma C32 cells and COLO 829 cells, were obtained from the American Type Culture Collection (ATCC). The base medium for MCF-7, MDA-MB-231, C32 and A375 was Dulbecco's modified Eagle's medium (DMEM). The base medium for COLO 829 was Roswell Park Memorial Institute (RPMI) 1640. To make the complete growth media, the following components were added: fetal bovine serum to a final concentration of 10%,penicillin G (final concentration: 100 U/mL), neomycin (final concentration: 10 µg/mL) and amphotericin B (final concentration: 0.25 µg/mL). Normal dermal human fibroblasts were obtained from Sigma-Aldrich and cultured in Fibroblast Growth Medium. All cells were maintained at 37◦C in humidified incubators with 5% carbon dioxide.

Cell-Based Cytotoxicity Assay

The Cell Proliferation Reagent WST-1 was used to assess the viability of the cell lines in the presence of the tested compounds. The principle of this colorimetric assay is based on the conversion of the tetrazolium salt to formazan (maximum absorption at 440 nm) by mitochondrial dehydrogenases in living cells. In brief, cells were seeded into 96-well plates (2500 cells/well) and incubated for 24 h. The compounds EB173, EB171 and betulonic acid (previously synthesized, purified and identified) were dissolved in DMSO to prepare stock solutions (20 mg/ml). The medium from the plate was replaced with EB171, EB173 or betulonic acid solutions (5, 10, 20, 50, 100 and 200 µM) at a volume of 100 µL/well and incubated for 72 h. WST-1 reagent was added (10 µL per well) 2 h before the end of the incubation period. Absorbance was read at 440 and 650 nm using the microplate reader Infinite 200 Pro.

Zebrafish Husbandry

To ensure the well-being of the zebrafish embryos, they were carefully housed in a specially controlled environment. This included maintaining a consistent temperature of 28.0 ± 1.0◦C and providing them with a light/dark cycle that is in line with the guidelines of the Research Animals Department of the esteemed RSPCA (Royal Society for the Prevention of Cruelty to Animals). According to EU Directive 2010/63/EU, the earliest life stages of zebrafish (embryo and eleutheroembryo cultures) are regarded as equivalent to an *in vitro* cell culture; therefore, they do not fall into the regulatory framework dealing with animal experiments. In our studies, we used zebrafish embryos and larvae younger than 120 hpf (hours post-fertilization); hence, ethical approval was not required. Zebrafish embryos were acquired through the process of mating adult zebrafish.

Zebrafish Toxicity Assay

The FET (Fish Embryo Toxicity) test was conducted with some modifications (Figure 2). New fertilized wildtype (WT) zebrafish embryos (0–2 hpf) exhibiting normal development or 72 hpf larvae were transferred to 6-well plates filled with a standard E3 medium and solutions of EB171 (50 and 100 µM). The control embryos were incubated in an embryo medium in the presence of 1% DMSO. The embryos were inspected under a stereomicroscope equipped with a camera at 4, 8, 12, 24, 48, 72 and 96 h of treatment. The experiments were carried out in triplicate and twenty embryos were used for each group. Every 24 h, up to four apical observations were recorded as indicators of lethality: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat. Pigmentation after 48 h and hatching rate after 48, 72 and 96 h were also observed. Additional developmental alterations (heart rate and total body length) and embryo malformations, such as pericardial edema, yolk sac edema, tail curvature, somite formation and scoliosis, were recorded at 96 h.

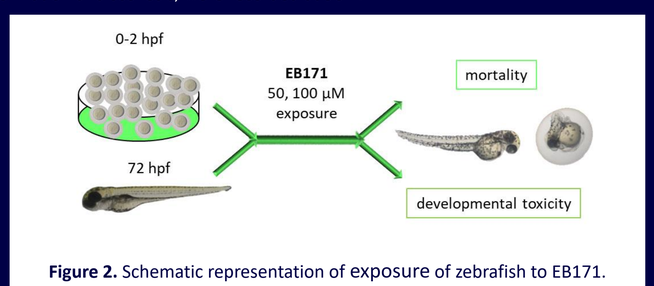

Figure 2. Schematic representation of exposure of zebrafish to EB171.

In the toxicity test with 72 hpf larvae, 3 replicates were performed. For each replicate, 20 objects were used in each concentration and 20 larvae were used as a control (1% DMSO). The larvae were monitored for 24 and 48 h after the treatment. The survival rate and morphological deformities were examined and documented using a stereomicroscope equipped with a camera. After completing the observations, all remaining embryos/larvae were euthanized using a buffered tricaine methane-sulphonate solution, as per the OECD Test Guideline 236 (Organization for Economic Co-operation and Development 2013).

Statistics

Shapiro–Wilk's W test of normality was used for data distribution analysis. The normally distributed data were analyzed using a one-way analysis of variance (ANOVA). Dunnett's test was used in the case of the cell-based cytotoxicity assay. For the zebrafish toxicity assay, the relationships between the two variables were analyzed using the Fisher independence test. The statistical analysis was conducted using the GraphPad Prism 9.4 software. The differences were deemed statistically significant when $p < 0.05$.

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RESULTS

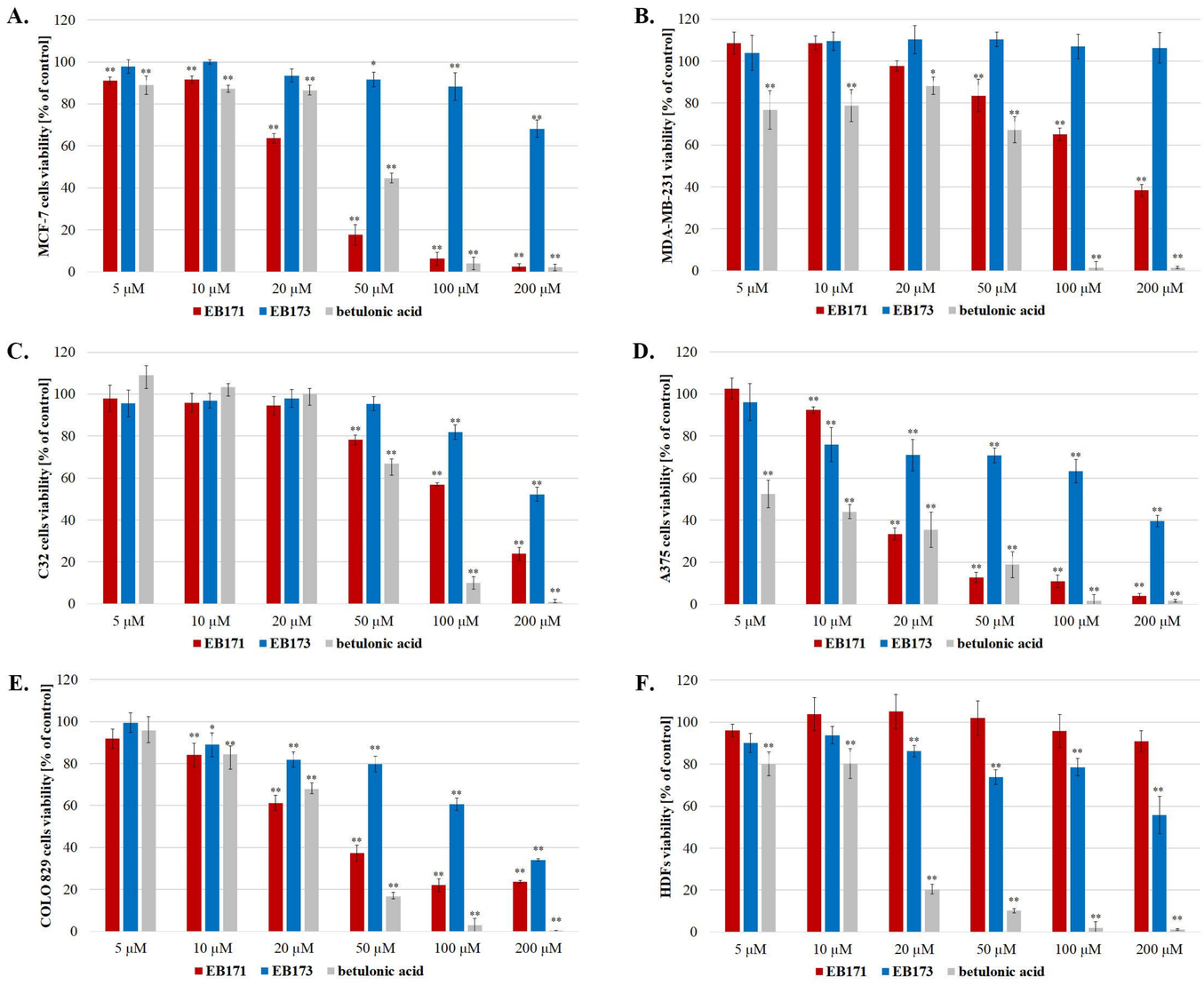

Figure 3. Figure 3. The effect of newly synthesized compounds (EB171 and EB173) and betulonic acid on viability of breast cancer cells (MCF-7 and MDA-MB-231), melanoma cells (C32, COLO 829 and A375) and normal human fibroblasts (HDFs) after 72 h of treatment. The results were expressed as a percentage of the control. Bars represent the mean ± SD of three independent experiments; * $p < 0.05$ and ** $p < 0.005$.

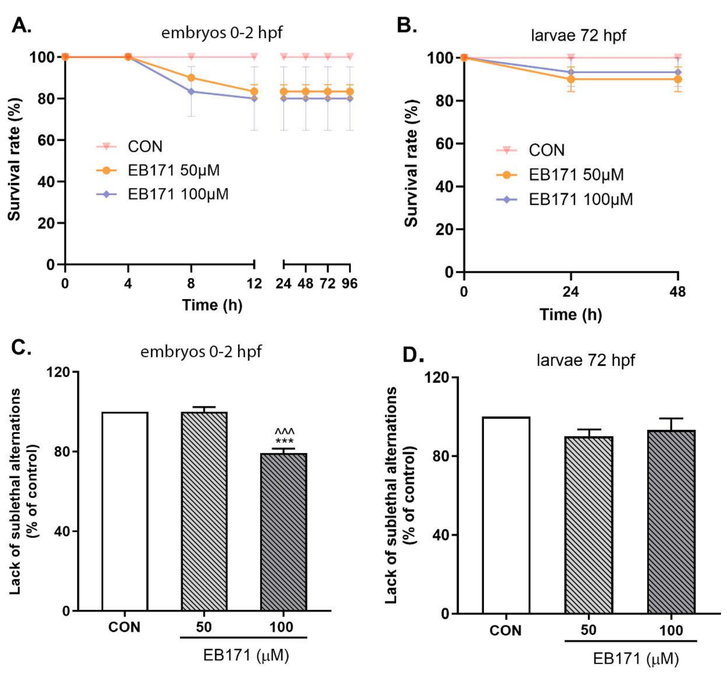

Figure 4. Survival rate (A,B) and lack of sublethal alternations (C,D) for zebrafish embryos at 0–2 hpf and larvae at 72 hpf exposed to EB171 or control (CON) for 96 h and 48 h, respectively. Lethal and sublethal alternations (E) for zebrafish embryos at 0–2 hpf exposed to EB171 concentrations or control (CON) for 24 and 96 h. Data are shown as mean ± SEM. *** $p < 0.001$ vs. CON; ^^^ $p < 0.001$ vs. EB171 50 µM, n = 60 for each concentration. Abbreviations: hpf—hours post-fertilization.

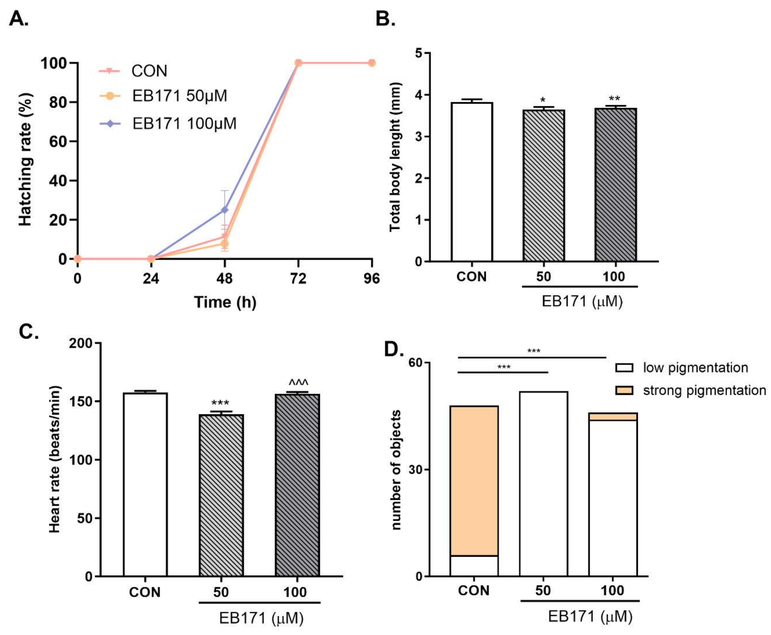

Figure 5. Hatching rate (A), total body length (B), heart rate (C) and pigmentation (D) for zebrafish embryos at 0–2 hpf exposed to EB171 concentrations or control (CON) for 96 h. Data are shown as mean ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CON; ^^^ $p < 0.001$ vs. EB171 50 µM, n = 60 for each concentration. Abbreviations: hpf—hours post-fertilization.

CONCLUSIONS:

- ✓ The study has shown that one of our derivative – N-[3-oxolup-20(29)-en-28-oyl]methylpropargilamine (EB173) – exhibits a strong cytotoxic effect on MCF-7 breast cancer cells and melanoma cell lines: A-375 and COLO 829, while it has no impact on viability of normal human fibroblasts (NHF). Analysis on *Danio rerio* have confirmed no toxicity of the compound.
- ✓ The results from the WST-1 test obtained for BA has indicated its significant cytotoxicity on the analyzed cancer cell lines, but also on NHF.
- ✓ The obtained results indicate a promising direction of chemical modification of betulonic acid in order to get non-toxic compounds with anti-melanoma and anti-breast cancer activity.