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Digitoxin enhances the growth inhibitory effects of thapsigargin and simvastatin on ER negative human breast cancer cells

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ABSTRACT

Background: The cardiac glycoside digitoxin preferentially inhibits the growth of breast cancer cells and targets the Erk pathway. Digitoxin alters the expression of genes that mediate calcium metabolism and IAP genes.
Purpose: Since the optimal treatment for cancer involves the use of agents in combination, we assessed the growth inhibitory effects of digitoxin combined with agents that alter calcium metabolism, thapsigargin, a sarcoplasmic/ER Ca^{2+}-ATPase inhibitor, and the statin simvastatin, as well as digitoxin's effect on the IAP pathway of apoptosis.
Methods: To reveal signaling pathways, we treated human cancer cells with digitoxin, alone or combined with thapsigargin or simvastatin, and measured cell growth using the MTT and colony formation assays. We used histology and Western blot analysis of HEK293 cells to assay effects on IAPs.
Results: Digitoxin inhibited the growth of breast, colon and ovarian cancer cells. Consistent with an effect on calcium metabolism, digitoxin exhibited synergy with thapsigargin and simvastatin on ER-negative breast cancer cells. Digitoxin activates expression of Erk pathway genes and suppresses expression of IAP genes. The growth inhibitory effects on HEK293 cells are not blocked by the pancaspase inhibitor zVAD-FMK, indicating that digitoxin may act by a caspase independent pathway of apoptosis. Furthermore, digitoxin does not have an effect on XIAP protein, a major anti-apoptotic protein.
Conclusion: Digitoxin appears to act through the Erk and stress response pathways and is worthwhile to study to prevent and treat cancer. Our findings warn of possible safety issues for cardiac patients who take a combination of digitoxin and statins.

1. Introduction

The leaves of foxglove (*Digitalis purpurea*, Plantaginaceae, the plantain family) contain more than 30 cardiac glycosides, among these are digitoxin and ouabain. The traditional use of digitoxin was as a remedy for heart diseases. The therapeutic range for digitoxin is narrow, from approximately 10 ng/ml (13 nM) to 35 ng/ml (46 nM). Studies indicate that digitoxin can suppress the growth of breast cancer cells at doses within this range and can synergize with chemotherapy agents such as paclitaxel at doses below these levels [1]. Further, digitoxin appears to render malignant prostate cancer cells sensitive to anoikis, which inhibits tumor metastasis [2]. This may partially explain the findings that digitoxin reduces metastases and clinical relapse in cancer patients [3].

Studies in animals indicate that digitoxin inhibits 2-stages of mouse skin papillomas induced by 7,12-dimethylbenzathracene (DMBA) and tetradecanoylphorbol-13-acetate (TPA) as well as mouse tumors induced by 4-Nitroquinoline 1-oxide (4NQO) and glycerol [4]. Nonetheless, it is not clear whether this agent can be useful to prevent and treat breast cancer. Some studies show a suppressive effect on the development of tumors, while others show the opposite effect [5]. Recent studies disagree as to whether digitoxin is selective [5,6] or is not selective [7] for malignant compared to nonmalignant cells.

Abbreviations: DAG, diacylglycerol; D7, diospyrin diethylether; ER, endoplasmic reticulum; FAP, fibroblast activation protein; HCC, hepatocellular cancer; HMGR, HMG-CoA reductase; IAP, inhibitor of apoptosis; IP3, inositol 1,4,5-trisphosphate; 4NQO, 4-Nitroquinoline 1-oxide; PSA, prostate specific antigen; PSMA, prostate specific memory antigen; PIP2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; SERCA, sarcoplasmic/Er Ca^{2+}-ATPase inhibitor; DMBA, dimethylbenzathracene; TPA, tetradecanoylphorbol-13-acetate.

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Previous studies indicated that digitoxin (structure, Fig. 1A) inhibits the activity of the Na^+/K^+ ATPase. Cardiac glycosides bind to the α subunit of the Na^+/K^+ ATPase and inhibit the active transport of Na^+ and K^+ across cell membranes [8]. This leads to a small increase in Na^+ and a large increase in calcium. When the cardiac glycoside ouabain binds to the Na^+/K^+ ATPase, it converts the enzyme to a signal transducer by releasing Src and activating downstream pathways, in particular pathways that are instrumental in cell survival such as the Ras/Raf/ERK1/2 and phospholipase C (PLC)/protein kinase C (PKC) and mitochondrial ROS production [9]. Recent studies suggest that this mechanism may need to be revised. Most studies of cardiac glycosides have been performed with ouabain, but there are defined differences in the effects of ouabain and digitalis glycosides.

Digitoxin may mediate calcium entry into cells by forming calcium channels [10,11]; these form at or above the therapeutic level (40 nM), suggesting calcium may be responsible for the toxic effect. The influence of digitoxin on TNF-α/NF-κB signaling occurs at subnanomolar doses and probably is not related to digitoxin calcium channels.

The finding that digitoxin altered the expression of several genes involved in calcium metabolism in MDA-MB-453 breast cancer cells: EGR1, IHPK2 and NR4A1 [9] supports the idea that the primary target may relate to calcium signaling. Calcium plays a role in multiple cellular processes including growth and tumorigenesis. Elevated levels of calcium have been shown to regulate cellular growth, differentiation and survival via activation of PLC by receptor tyrosine kinases [12]. Disruption of calcium metabolism appears to induce apoptotic, necrotic or autophagic cell death [13].

Current evidence indicates that the optimal treatment of breast cancer most likely requires a combination of agents or modalities. Therefore, in the present study we examined whether digitoxin exerts synergistic effects on growth inhibition of human breast cancer cells when combined with agents that have significant anticancer potential and alter calcium metabolism, including the ER stress inducer thapsigargin and the statin simvastatin.

Following observations that digitoxin suppresses the expression of IAPs, including survivin [9], we investigated whether digitoxin alters the IAP pathway of apoptosis. The ultimate goal is to determine the anti-cancer potential of digitoxin and related compounds.

2. Materials and methods

2.1. Materials

All solvents and reagents were reagent grade; H_2O was distilled and deionized. Agents: digitoxin (Sigma, St. Louis, MO), thapsigargin (Sigma); U0126 (LC Laboratories, Woburn, MA) were dissolved in dimethylsulfoxide (DMSO) (Sigma), while heparin (Sigma) was dissolved in deionized water, prior to addition to cell cultures. The triterpene glycosides D_{11} from *Cimicifuga dahurica* and D_{13} from *Cimicifuga acerina* were the kind gift of Dr. Ye Wen-Cai (Guangzhou, China) [14,15,16]. N-benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)fluoromethyl ketone (zVAD-fmk) was obtained from Enzo Life Sciences (Farmingdale, NY).

2.2. Cell cultures

MDA-MB-453 (Her2 overexpressing) breast and HT29 (p53 positive) colon and SKOV3 (Her2 positive) ovarian human cancer and 293T human embryonic kidney cells were obtained from ATCC (Manassas, VA). MCF7 human breast cancer cells were the kind gift of Dr. Moira Suane (CUNY, New York, NY). HEK293 cells were provided by H. Steller at The Rockefeller University. MCF7, MDA-MB-453 and 293T cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco BRL Life Technologies, Inc., Rockville, MD) containing 10% (v/v) fetal bovine serum (FBS) (Gibco BRL), while HT29 and SKOV3 were maintained in McCoy's media plus 10% FBS; all cells at 37 °C, 5% CO_2, plus Pen Strep (Gibco).

2.3. Proliferation assay

2.3.1. MTT assay

The MTT (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide) cell proliferation assay system (Roche Diagnostic, Indianapolis, IN) was used to determine the sensitivity of the various cell lines to agents. Cells were seeded at 1×10^4 cells/well in 96-well plates and allowed to attach for 24 h. The medium was replaced with fresh medium containing DMSO or compound. The cells were treated for 96 h, after which they were incubated with MTT reagents and the absorbance read at 650 and 575 nm.

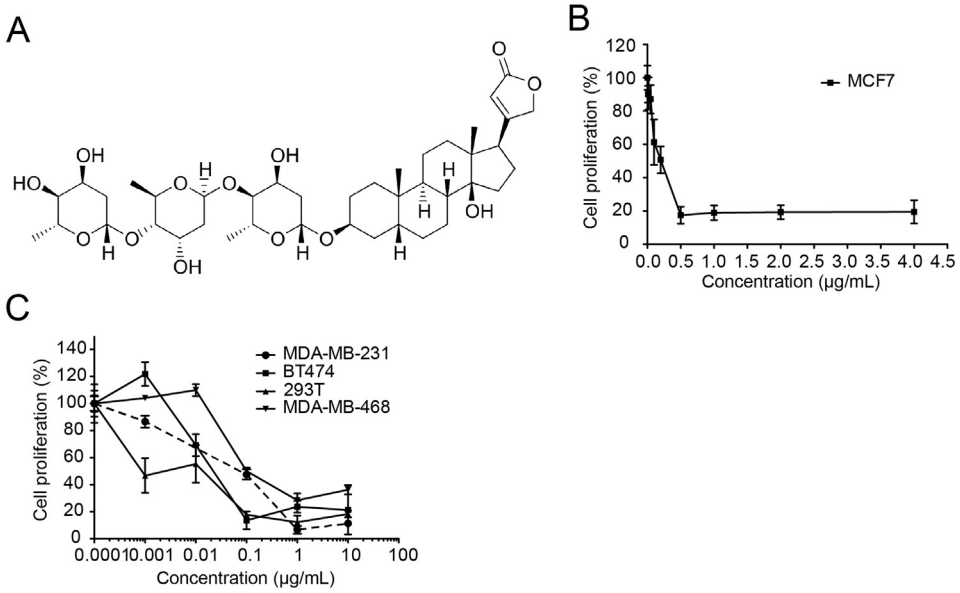

Fig. 1. Structure and growth inhibitory activity of digitoxin: A) structure of digitoxin. Growth inhibitory activity of digitoxin on: B) MCF7; C) BT474, MDA-MB-468, MDA-MB-231, 293T cells. Cells were exposed to increasing concentrations of agents for 96 h and the number of viable cells determined by the MTT assay.