https://doi.org/10.31925/farmacia.2026.2.19 **ORIGINAL ARTICLE**

EXPLORING THE REPURPOSING OF CARDIOTONIC GLYCOSIDES DIGOXIN AND DIGITOXIN ON MIA PaCa-2 PANCREATIC CANCER CELL LINE AS ALTERNATIVE TREATMENTS

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a global public health issue and ranks among the top causes of death. Statistically, it is the third leading cause of cancer-related deaths in developed countries, including Europe. Given the current unsatisfactory treatment options, the concept of drug repurposing (the use of approved drugs for other purposes) represents an alternative approach of interest in pancreatic cancer. For this reason, this research proposes to explore the potential effects of digoxin and digitoxin, two digitalis compounds, on cytotoxicity, pro-apoptosis and antiproliferation in MIA PaCa-2 pancreatic cancer cells, in light of the limited and inconclusive results regarding the ability of these drugs in pancreatic cancer therapy. Towards this purpose, DGX and DGTX were evaluated on the cells using several types of preclinical experiments (MTT assay, bright-field morphological analysis, nuclear assessment through Hoechst 33342, mitochondrial *via* MitoTracker, acridine orange/propidium iodide analysis regarding the occurrence of apoptosis and necrosis and colony formation assay), as well as *in ovo* assessment of each agent's irritant capacity on the chorioallantoic membrane using the HET-CAM method.

Rezumat

Adenocarcinomul ductal pancreatic (PDAC) reprezintă o problemă de sănătate publică la nivel mondial și se numără printre principalele cauze de deces. Din punct de vedere statistic, aceasta este a treia cauză principală a deceselor cauzate de cancer în țările dezvoltate, inclusiv în Europa. Având în vedere opțiunile terapeutice actuale nesatisfăcătoare, conceptul de „*repurposing*" al medicamentelor (utilizarea medicamentelor deja aprobate în alte scopuri) reprezintă o abordare alternativă de interes în cazul cancerului pancreatic. Din acest motiv, această cercetare își propune să exploreze efectele potențiale ale digoxinei și digitoxinei, doi compuși digitalici, în ceea ce privește efectele lor citotoxice, proapoptotice și antiproliferative în celulele canceroase pancreatice MIA PaCa-2, având în vedere rezultatele limitate și neconcludente privind capacitatea acestor medicamente în terapia cancerului pancreatic. În acest scop, DGX și DGTX au fost evaluate pe celule folosind mai multe tipuri de experimente preclinice (testul MTT, analiza morfologică, evaluarea nucleară prin Hoechst 33342 și evaluarea mitocondrială prin MitoTracker, analiza cu portocaliu de acridină/iodură de propidiu privind apariția apoptozei și a necrozei, testul de formare a coloniilor), precum și evaluarea *in ovo* a capacității iritante a fiecărui agent asupra membranei corioalantoice, folosind metoda HET-CAM.

Keywords: cardiotonic glycosides, digoxin, digitoxin, pancreatic cancer, drug repurposing

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a global public health issue and ranks among the top causes of death. Statistically, it is the third leading cause of cancer-related deaths in developed countries, including Europe. In the United States, pancreatic cancers account for 8.5% of cancer-related deaths (1). Con-

sidering the spread of the disease that has been reported in recent years, and noting that about 50% of patients diagnosed with PDAC are already in advanced stages or are experiencing metastasis, where surgical resection is no longer a viable option, it is expected that by 2030, it will become the second leading cause of cancer-related death, surpassing breast and colorectal cancer (2). Today, advances in the field have led to a wide range of therapeutic strategies to support patients with PDAC; these treatments come in various forms and are generally tailored to each patient. As a result, treatment approaches are typically multidisciplinary and may include surgical techniques, multi-agent chemotherapy, radiation therapy, immunotherapy (checkpoint inhibitors, adoptive cell therapies and mRNA vaccines), and other modalities (3). Gemcitabine is the first-line drug, but its use is limited by cancer resistance, which negatively affects the prognosis (4). Unfortunately, gemcitabine is not the only obstacle to therapeutic success. Numerous other identified limitations necessitate the development of novel treatments, especially since the Human Genome Project has revealed that PDAC is a tumour type with high intertumoral genetic heterogeneity, suggesting that a single therapy may not be effective for all PDAC patients (5).

Given the current unsatisfactory treatment options, the concept of drug repurposing (the use of approved drugs for other purposes) represents an alternative approach of interest in pancreatic cancer as well, one that may lead to the discovery of promising novel medications (6). Cardiotonic glycosides have also been explored for their potential repositioning in cancer treatment (7). Several retrospective clinical studies have suggested that cardiotonic glycosides may prolong the survival of cancer patients undergoing conventional chemotherapy by modulating the immune response and triggering immunogenic cell death, an effect that most likely contributes to their clinical anticancer activity (8). This class also includes digoxin (DGX) and digitoxin (DGTX), which are commonly used to treat congestive heart failure and atrial fibrillation but have also shown potential in treating certain types of cancer (*e.g.*, digoxin in prostate cancer). However, this anticancer effect of cardiac glycosides has been studied since the 1960s, with preclinical evaluations having identified several mechanisms for their antitumor activities, one of note being their primary mechanism – namely, the inhibition of Na^+/K^+-ATPase activity, which increases intracellular Ca^{2+}, leading to tumour cell apoptosis (9). In terms of pancreatic cancer, both DGX and DGTX have proven to be potential alternatives or adjuncts to current conventional therapies. Specifically, DGX has demonstrated the ability to sensitise pancreatic cancer cells to gemcitabine treatment by inhibiting the Nrf2 signalling pathway (10). At the same time, DGTX has been found to exhibit antiproliferative effects in pancreatic cancer cell lines (11). Moreover, a 20 - 30% reduction in cancer incidence has been observed among patients receiving long-term DGX treatment (12).

Considering all of the above and in view of the growing interest in drug repurposing in cancer therapy today (13), the objective of this study was to evaluate DGX and DGTX, two well-known digitalis compounds, regarding their cytotoxic, pro-apoptotic and antiproliferative effects in MIA PaCa-2 pancreatic cancer cells, in light of the limited and inconclusive results regarding the ability of these drugs in pancreatic cancer therapy. Towards this purpose, DGX and DGTX were evaluated on the cells using several types of preclinical experiments (MTT assay, bright-field morphological analysis, nuclear assessment *via* Hoechst 33342, mitochondrial assessment *via* Mito Tracker, acridine orange/propidium iodide analysis regarding the occurrence of apoptosis and necrosis, and colony formation assay), as well as *in ovo* assessment of each agent's irritant capacity on the chorioallantoic membrane using the HET-CAM method.

Materials and Methods

Reagents and instruments

The tested compounds DGX and DGTX, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability kit, trypsin-EDTA solution, phosphate buffer saline (PBS), trypan blue solution 0.4%, acridine orange hydrochloride solution and propidium iodide were purchased from Sigma Aldrich, Merck KGaA (Darmstadt, Germany). The specific cell culture media, Dulbecco's modified eagle medium (DMEM), horse serum and foetal bovine serum (FBS), were obtained from ATCC (American Type Culture Collection, Lomianki, Poland). Paraformaldehyde 4% was delivered by Santa Cruz Biotechnology (Dallas, TX, USA). Hoechst 33342 dye and MitoTracker™ Red CMXRos were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin 100 U/mL, streptomycin 100 µg/mL and dimethyl sulfoxide (DMSO) were purchased from PanBiotech (Aidenbach, Germany). The Cytation 5 plate reader, the Lionheart FX automated microscope and the Gen5™ Microplate Data Collection and Analysis Software (v3.14) were purchased from BioTek Instruments Inc. (Winooski, VT, USA). The Stereomicroscope Discovery V8 (Zeiss, Oberkochen, Germany) was purchased from Oberkochen, Germany, equipped with an Axio CAM 105 colour camera.

Cell culture protocol

In this study, one cell line was used: the MIA PaCa-2 pancreatic carcinoma cell line. The cell line was cultured in its respective medium: DMEM supplemented with 10% FBS, 2.5% horse serum and 1% penicillin-streptomycin. The cultures were maintained in a humidified incubator at 37°C with 5% CO_2

throughout the experiments. Cell morphology was monitored daily under a microscope. For the experiments, DGX and DGTX stock solutions were prepared in DMSO, ensuring that the final DMSO concentration did not exceed 0.5%.

Cell viability assay (the MTT test)

The effects of DGX (5, 10, 25, 50 and 100 nM) and DGTX (5, 10, 25, 50 and 100 nM) on MIA PaCa-2 cells were assessed using the MTT assay to determine cell viability. Cells were plated in flat-bottom 96-well plates at a density of 1×10^4 cells *per* well and allowed to attach until reaching the appropriate confluence (approximately 70%). After attachment, the cells were exposed to the test concentrations for 24 h. The protocol was then followed as described by Grosu *et al*. (14). Cell viability was calculated as a percentage of the untreated control.

Cellular morphology and confluence assessment

The MIA PaCa-2 cells were seeded at a density of 1×10^4 cells/well in flat-bottom 96-well plates. After DGX and DGTX (5, 10, 25, 50 and 100 nM) stimulation, cell morphology was examined. Post-stimulation, the cells were imaged using the Lionheart FX automated microscope, and the resulting images were processed using Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Nuclear morphology assessment (Hoechst 33342) and mitochondrial immunofluorescence staining (MitoTracker™ Red CMXRos)

For the examination of the nuclear and mitochondrial morphology of the cells, MIA PaCa-2 cells were seeded in flat-bottom 96-well plates at a density of 1×10^4 cells/well. Cells were maintained under standard culture conditions until the desired confluence was reached. Afterwards, the cells were stimulated with DGX and DGTX (5, 10, 25, 50 and 100 nM) for 24 h. A stock solution of 1 mM was obtained by dissolving MitoTracker™ Red CMXRos in DMSO. The stock solution was diluted to a final concentration of 300 nM. Next, the protocol was followed according to the method described by (15).

Acridine orange/propidium iodide (AO/PI) assay

AO/PI double staining was used to analyse apoptotic and necrotic morphological changes in MIA PaCa-2 cells after they were treated with DGX and DGTX (5, 10, 25, 50 and 100 nM) for 24 h. Under conventional culture conditions, cells were seeded at a density of 1×10^4 cells *per* well in 96-well plates and allowed to adhere. Following treatment, 100 µL of staining solution comprising 10 µg/mL AO and 10 µg/mL PI (final combination 10%) was added to each well in the respective culture medium. The plates were incubated in the dark at room temperature for 10 minutes. A Lionheart FX automated microscope was then used to take fluorescent images at 20× magnification, 100 ms exposure time and 2048 × 2048 pixel resolution. Gen5 Microplate Data Collection and Analysis Software was used for both image acquisition and quantitative analysis.

Colony formation assay

The colony formation assay was used to determine the effects of DGX and DGTX (5, 10, 25, 50 and 100 nM) on colony formation in MIA PaCa-2 cells. After being cultivated at a density of 1×10^2 *per* well in 96-well plates, the cells were allowed to adhere. After that, the cells were exposed to the compounds for a whole day. Over the next 7 to 10 days, the cell culture medium was replaced periodically with a new one. At the end of the experiment, the cells were fixed with 4% paraformaldehyde and stained with crystal violet (0.2%) diluted in PBS for 10 minutes. The wells were rinsed with water before the representative photos were taken. After lysing the cells with 1% SLS, the absorbance was measured at 550 nm on Cytation 5.

In ovo irritation assessment (HET-CAM) assay

The irritant score of each compound was investigated using the hen's egg chorioallantoic membrane (HET-CAM) assay at 5, 25 and 100 nM. The protocol was then followed as described by Smeu *et al*. (16). By measuring the time at which changes (H – haemorrhage, L – vascular lysis and C – coagulation) occur at the vascular level, this serves as a measurement parameter that assesses the irritancy potential of test materials. The test material can be categorised as non-irritant if IS = 0 - 0.9, irritant if IS = 1 - 8.9, or severely irritant if IS = 9 - 21, based on IS results (16). The irritant potential of the samples and their combination was determined by calculating the irritation score (IS), according to the standard formula (17):

$$IS = 5 \text{ x } \frac{301-H}{300} + 7 \text{ x } \frac{301-L}{300} + 9 \text{ x } \frac{301-C}{300}.$$

Statistical analysis

GraphPad Prism software (v. 9.3.1; GraphPad Software, San Diego, CA, USA; www.graphpad.com) was used for statistical analyses. Differences between the DGX and DGTX and the untreated control cells were examined using one-way ANOVA followed by Dunnett's multiple comparison test. Statistically significant differences were indicated with asterisks as follows: * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The results were normalised to control (untreated cells, 100%). All experiments were performed in triplicate.

Results and Discussion

As pancreatic cancer is considered one of the world's deadliest forms of cancer and is often diagnosed at advanced stages, when patients may already have metastases, there is an urgent need for new treatment options. Drug repurposing is used to develop novel agents by using approved or investigational drugs outside their original clinical indications (18).

In this context, cardiotonic glycosides have also been highlighted, with numerous studies presented in the scientific literature providing data supporting their anticancer effects through various signalling pathways and target proteins. DGX and DGTX have attracted attention in anticancer therapy due to their ability to induce apoptosis by inhibiting the nuclear factor-κB (NF-κB) pathway, anti-apoptotic proteins (BCL-2 and BCL-XL) and the Akt pathway, as well as promoting the generation of reactive oxygen species (ROS) in cancer cells (19). Specifically, both DGX and DGTX have been reported to have significant implications for pancreatic cancer (10, 11). However, data concerning their precise effects on the MIA PaCa-2 pancreatic cancer cell line remain limited. Thus, the current study aimed to evaluate the cytotoxic effects of the cardiotonic glycosides DGX and DGTX on Mia Paca-2 pancreatic cancer cells following a 24-hour treatment in the concentration range of 5 - 100 nM. Furthermore, the study also focused on the *in ovo* examination of the samples' potential to cause irritation. The tests performed to achieve this objective were: (i) MTT assay to determine cell viability after treatment; (ii) observation of structural changes and the impact on cell confluence *via* automated bright-field morphological analysis; (iii) cell staining with Hoechst 33342 and MitoTracker to highlight possible nuclear abnormalities and analyse mitochondrial function; (iv) acridine orange/propidium iodide to differentiate apoptotic, necrotic and viable cells; (v) evaluation of long-term clonogenic potential using the colony formation assay; and finally, (vi) HET-CAM to determine the irritation score upon application of the samples. The concentrations selected for this study (specifically 5 - 100 nM) were based on findings reported in the literature, which indicated that DGX and DGTX exhibit anticancer activity in the nanomolar range, which extends from 1 to 100 nM, including pharmacologically relevant doses within this range, corresponding even to the therapeutic concentrations observed in patients (10, 11, 20).

Cell viability assay (the MTT test)

The MTT test is a colourimetric technique commonly used in toxicology and drug discovery studies to assess cell viability following treatment and thereby determine cellular health and metabolic activity (21). Therefore, the cell viability of MIA PaCa-2 pancreatic cancer cells was evaluated after 24 h of treatment with DGX and DGTX at concentrations of 5, 10, 25, 50 and 100 nM using the MTT assay (Figure 1). DGX treatment induced a slight stimulatory effect on cell viability at the lower concentrations tested. Specifically, exposure to 5, 10 and 25 nM DGX increased cell viability to approximately 113% compared with the untreated control. At 50 nM, cell viability decreased slightly to about 101%, even though the cells were still somewhat stimulated compared to control conditions. Only the highest DGX concentration (100 nM) produced a statistically significant reduction in cell viability, lowering it to approximately 75%. In contrast, DGTX exhibited a more pronounced and dose-dependent cytotoxic effect. While the lowest concentration (5 nM) still increased cell viability to around 104%, subsequent concentrations progressively reduced cell number. Treatment with 10 nM DGTX decreased viability to approximately 87%, while 25 and 50 nM resulted in similar reductions to about 80%. The strongest effect was observed at 100 nM, where DGTX significantly reduced cell viability to 49%, indicating a marked inhibitory effect on MIA PaCa-2 cell proliferation.

The increased cell viability observed at sub-toxic concentrations is consistent with a hormetic dose–response, in which low doses stimulate cellular activity while higher doses exert cytotoxic effects (15). From a therapeutic standpoint, this may be concerning, as sub-therapeutic levels of DGX or DGTX could facilitate tumour cell survival or proliferation, particularly in the context of pharmacokinetic variability. Nevertheless, both compounds showed clear cytotoxicity at higher concentrations, suggesting a relatively narrow therapeutic window, especially for DGX. Overall, these findings underscore the importance of precise dose optimisation to ensure effective anticancer activity while avoiding unintended stimulatory effects.

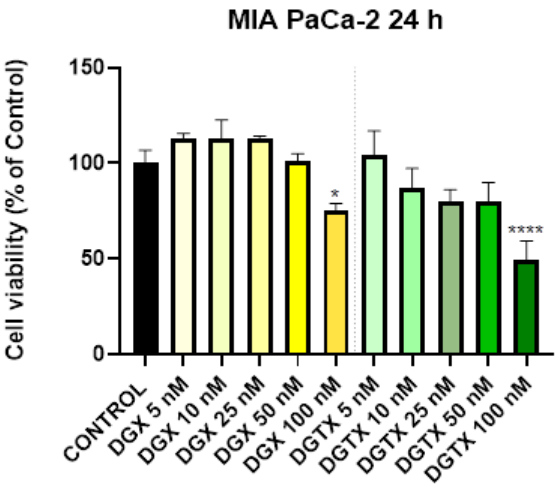

Figure 1.

The impact of DGX and DGTX on the viability of MIA PaCa-2 pancreatic carcinoma cells was assessed *in vitro* at doses of 5, 10, 25, 50 and 100 nM. Cell viability was evaluated 24 h after treatment, and results were reported as percentages relative to the untreated control group. The trials were conducted in triplicate, and the data are presented as mean ± standard deviation (SD). One-way ANOVA and Dunnett's post hoc test were used to assess statistical significance between the DGX- and DGTX-treated groups and the control (* $p < 0.1$; **** $p < 0.0001$)

DGTX treatment in MIA PaCa-2 cells showed a dose-dependent effect, whereas DGX exhibited this trend only within the 25 - 100 nM range, suggesting that MIA PaCa-2 cells are more sensitive to DGTX than to DGX under the same conditions. These observations are based on the viability percentages reported for the treatments, with only DGX at 100 nM reducing tumour cell viability to 75%. In contrast, for DGTX, reductions in viability (down to 87%) were observed even at 10 nM, progressing to 49% viable cells at 100 nM. Certain data from the literature, consistent with this study's conclusions, suggest that DGTX may have stronger cytotoxic effects than DGX. However, there are also certain differences between the two digitalis compounds, including the fact that DGX is generally considered easier to dose than DGTX and is more frequently used. Another difference is that DGX has a shorter half-life than DGTX, and from a chemical point of view, DGX has an additional hydroxyl (-OH) group compared to DGTX; *i.e.,* DGX is more hydrophilic and is excreted through the kidneys, while DGTX (lipophilic) is metabolised primarily in the liver and excreted through faeces (22). Concerning their mechanistic role, recently published papers have suggested that the target of cardiotonic glycosides may be the DNA damage response (DDR) pathway (which coordinates a number of other cellular pathways that initiate cell cycle arrest, induce apoptosis to prevent the survival of cells with DNA damage or cells carrying mutations and other functions) (23).

In another study, Yunjiang Zhou *et al*. (10) showed that DGX exerted observable cytotoxic effects at concentrations above 80 nM in pancreatic cancer cells. Furthermore, at concentrations of 20, 40 and 80 nM, DGX sensitised SW1990 and Panc-1 cells to the chemotherapeutic agent gemcitabine. Regarding the same concept of drug repurposing, DGX has been investigated for its anticancer potential in cutaneous melanoma. After 24 hours, DGX (10 - 50 nM) dose-dependently reduced cell viability in A375 cells (to approximately 43%) and in RPMI-7951 cells (to approximately 34%) (24). Numerous studies have demonstrated that DGTX affects the viability of cancer cells, and Lindholm *et al*. evaluated its cytotoxicity (1 - 100 nM) in four pancreatic cancer cell lines, BxPC-3, CFPAC-1, Panc-1 and AsPC-1, which differ with respect to KRAS/BRAF mutation status and their origin from primary tumours or metastases. Their results indicated that the cellular response varied across cell lines, and concentrations up to 10 nM did not produce significant effects. The research group also emphasised that, to achieve a maximum effect on cancer cell viability, a combined approach targeting both cancer cell metabolism and proliferation is preferable (11). In another study focusing on the impact of DGX and DGTX on cell viability, using the MTT assay, the effects were evaluated on another cancer cell line, namely SKOV3, at both 24 and 48 hours. The findings of the study showed that the cellular proliferation response was time- and dose-dependent, and the maximum inhibitory concentration (IC50) values for DGX and DGTX were 2.5×10^{-7} M and 4.0×10^{-7} M, respectively (25).

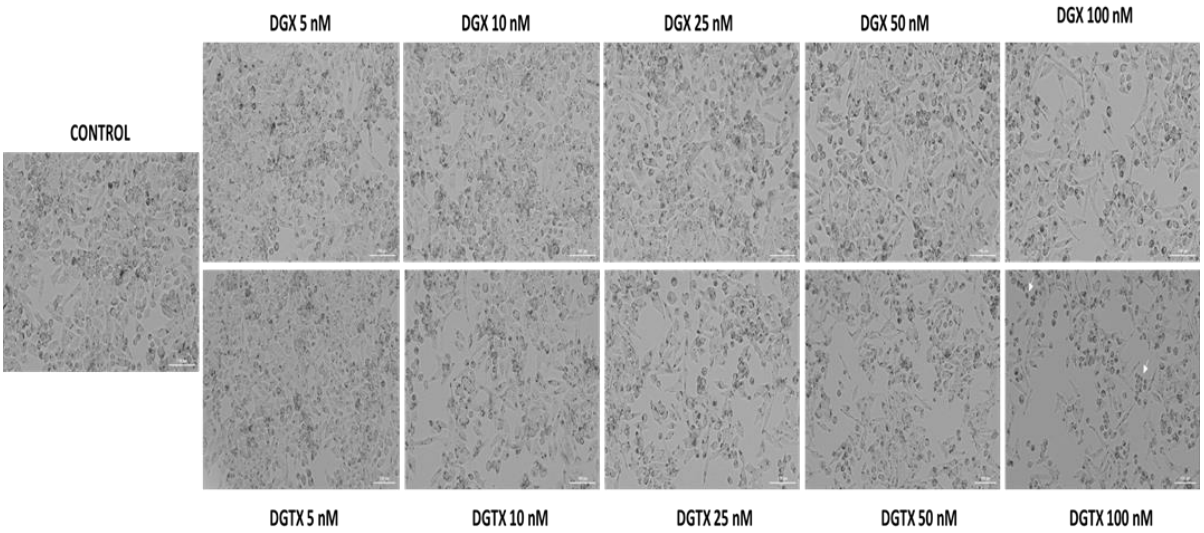

Figure 2.

MIA PaCa-2 pancreatic carcinoma cells' morphology after 24 hours of DGX and DGTX at 5, 10, 25, 50 and 100 nM treatment. White arrows indicate morphological alterations. Every picture includes a scale bar showing 100 µm

Cellular morphology and confluence assessment

After 24 hours of treatment, the test samples' cytotoxicity was assessed by imaging the MIA PaCa-2 cell line for changes in cellular shape (Figure 2). Following 24 hours of stimulation with DGX at 5, 10, 25, 50 and 100 nM, a decrease in confluence

was observed only at the highest dosage, with no apparent signs of dysmorphologies. In the DGTX treatment, a dose-dependent effect was observed. At the highest dosage examined, the cells exhibited dysmorphology, including rounding and shrinkage and a marked reduction in cell confluence.

An essential standard is to study cellular structure after treatment. This reveals cellular morphology and indicates the presence of stress conditions or other dynamic changes. Consequently, cellular morphological examination has been incorporated into various novel methods for biomedical applications, including the identification of cellular morphogenesis at different stages of the cell cycle, and is frequently employed (26). In the present work, our bright-field morphological analysis results were consistent with those obtained in the MTT assay; consequently, in the case of DGTX treatment, pancreatic cancer cells exhibited more frequent morphological abnormalities, even at lower doses.

Nuclear morphology assessment (Hoechst 33342) and mitochondrial immunofluorescence staining (MitoTracker™ Red CMXRos)

Subsequently, the DGX and DGTX (5, 25 and 100 nM) assessment focused on the cell nuclei and mitochondria 24 hours after treatment (Figure 3). This phase helped to explore the possible mechanism of cytotoxicity induced by the two cardiotonic glycosides on pancreatic carcinoma cells, using immunofluorescent staining with MitoTracker and Hoechst 33342.

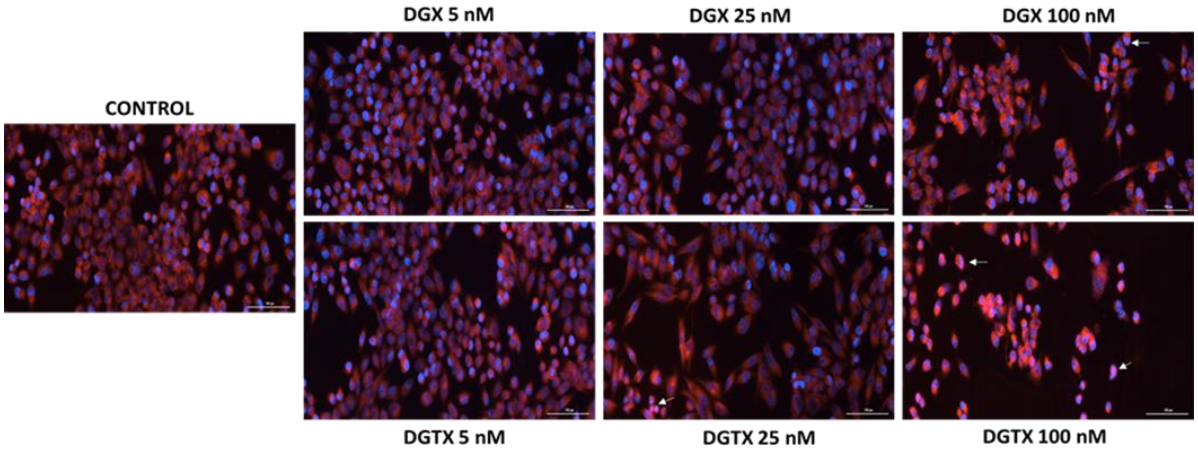

Figure 3.

MIA PaCa-2 pancreatic carcinoma cells' nuclear and mitochondrial morphology after 24 hours of DGX and DGTX at 5, 25, 100 nM treatment using the MitoTracker and Hoechst 33342 methods. White arrows indicate morphological alterations. Every picture includes a scale bar showing 100 µm

Hence, during this exploration, treatment with DGX applied to MIA PaCa-2 cells showed that only the higher dose (100 nM) induced mild alterations (minor nuclear and mitochondrial condensation) and a decrease in the number of nuclei, indicating reduced confluence. DGTX treatment produced morphological changes at this level, starting with the 25 nM dose, when slight nuclear and mitochondrial condensation, nuclear shape abnormalities and condensed nuclei were observed, along with a slight decrease in confluence. In contrast, the 5 nM dose did not alter mitochondrial or nuclear morphology. At 100 nM DGTX, these nuclear and mitochondrial dysmorphologies (nuclear condensation, nuclear shrinkage, mitochondrial condensation and decreased confluence) became more pronounced.

As versatile organelles, mitochondria fulfil many biosynthetic roles, primarily in cellular energy production, but also in maintaining intracellular calcium homeostasis, and serve as a significant source of reactive oxygen species. Apart from all this, they serve as signalling hubs that coordinate cellular responses to stress and store pro-apoptotic molecules, thereby functioning as key regulators of both cellular survival and apoptosis (27). As a result, examining mitochondrial structure – including using MitoTracker – is a technique frequently employed in research on the effects of various agents on cancer cell lines (4, 15, 27, 28). On the other hand, Hoechst dyes are among the most popular for staining DNA in both living and fixed cells. DNA content, DNA distribution and cell nucleus morphology serve as markers of cell cycle progression, allowing differentiation between distinct cell types and the identification of phenotypes induced by mutations or drugs. Thus, these objectives are most often achieved through fluorescence microscopy, which is widely used in academic research and medical diagnostics (29). The structure of the nucleus often helps identify the type of cell death, and features such as chromatin condensation or nuclear shrinkage/contraction are indicative of apoptosis (14), features also observed

when MIA PaCa-2 cells are treated with DGX and DGTX, particularly at 100 nM. Also, cardiotonic glycosides were reported in multiple *in vitro* and *in vivo* studies to directly induce apoptotic cell death in cancer cells (30). Using fluorescence microscopy, Rednic *et al*. investigated 50 nM DGX in SK-Mel-28 and RPMI-7951 melanoma cells to monitor changes at the nuclear level and in actin filaments. DGX induced slight condensation of chromatin and actin filaments in SK-Mel-28 cells, while a minimal effect was observed in RPMI-7951 cells. Furthermore, in both cell lines, DGX was tested in combination with betulinic acid, demonstrating promising synergy (31).

Programmed cell death (apoptosis) is a well-orchestrated cellular process that plays a key role in the pathogenesis of diseases. Its mechanism is complex and involves multiple pathways, and in cancer, it is a common target for many treatment strategies. One sign of apoptosis is altered nuclear morphology, which exhibits distinct features compared to a normal nucleus (notably chromatin condensation, nuclear fragmentation and cellular rounding) (32).

Acridine orange/propidium iodide (AO/PI) assay

Following the previous analyses, the next experiment involved dual staining with acridine orange and propidium iodide (AO/PI) to confirm cell viability and identify apoptotic and necrotic processes.

In this context, AO/PI staining was performed to visualise and assess the effects of DGX and DGTX treatments at 5, 25 and 100 nM on MIA PaCa-2 cell viability and apoptosis after 24 h. Cells in the untreated group exhibited uniform and strong green fluorescence following the AO/PI test, indicating intact morphology due to the penetration of acridine orange (AO) into viable cells. We observe the same pattern for Dgx at low (5 nM) and intermediate (25 nM) concentrations, where cell confluence remains similar to that of the control, and the cells retain their green fluorescence. At the highest DGX concentration (100 nM), the fluorescence shows predominantly green cells, indicating viable cells with intact membrane integrity. Only a few cells exhibit red (necrosis) and orange (late apoptosis) fluorescence, representing non-viable cells with compromised membranes. On the other hand, treatment with DGTX produced a dose-dependent effect. At a concentration of 5 nM, the cells were similar to those in the untreated group. At 25 nM, a reduction in cell confluence was observed, accompanied by signs of late apoptosis. Nevertheless, green fluorescence predominates, and cell morphology remains characteristic. At 100 nM, there is a marked reduction in the number of viable cells with green fluorescence. Also, the presence of orange and red fluorescent cells indicates late apoptosis and necrosis, respectively. Many cells appear rounded and shrunken, suggesting treatment-induced cytotoxicity and loss of normal cellular morphology.

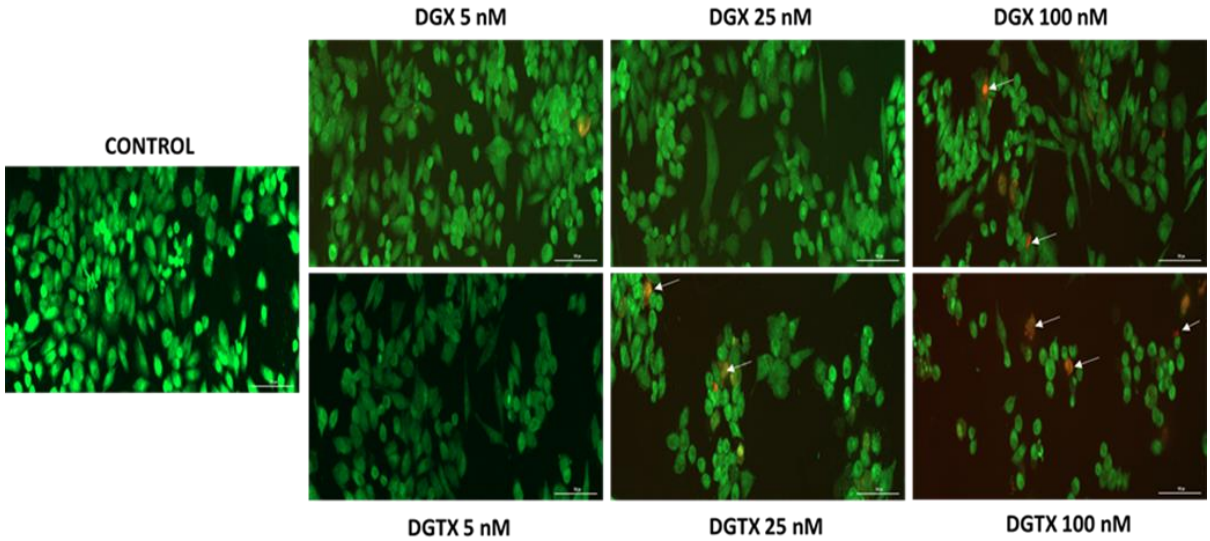

Figure 4.

Fluorescence pictures of MIA PaCa-2 pancreatic carcinoma cells stained with acridine orange/propidium iodide (AO/PI) using DGX and DGTX at 5, 25, 100 nM after 24 h. Viable cells released green fluorescence, and non-viable cells showed red fluorescence. An inverted fluorescence microscope was used to capture the images. White arrows indicate cells undergoing apoptosis or necrosis. Every picture includes a scale bar showing 100 µm

The AO/PI assay conducted in this work is a technique frequently employed to assess cell viability, in which both AO and PI are nuclear stains. AO penetrates both live and dead cells and stains all cells that have a nucleus, while PI penetrates dead cells with damaged membranes and stains all dead cells that contain a nucleus (33). Previous AO/PI analyses of melanoma cell lines (A375 and RPMI-7951) demonstrated that DGX induces dose-dependent cytotoxicity, characterised by reduced green fluorescence,

chromatin condensation and increased PI-positive cells, indicative of apoptosis and necrosis. Although in the present case, effects appear to be cell line–dependent and less pronounced in MIA PaCa-2 cells than in melanoma models (24).

Clonogenic assay

Studies have shown that cancer cells can undergo a process known as anastasis; specifically, they can recover even after reaching key stages of apoptosis (mitochondrial dysfunction, mitochondrial fragmentation and cell shrinkage). This phenomenon indicates the ability of cancer cells to survive after removal of toxic stimuli, allowing them to regain the capacity to multiply, migrate and form metastases (15). Given these reference points, the next stage of the DGX and DGTX study involved conducting a colony formation assay. DGX and DGTX treatment decreased the number of colonies and the colony-forming rate in MIA PaCa-2 cells (Figure 5) in a dose-dependent manner at all tested doses (5 - 100 nM). The colony formation rate gradually decreased after treatment with DGX, from 98% at 5 nM to 73% at 100 nM, compared with untreated controls, indicating that it inhibited clonogenic survival (Figure 5B). Colony formation was more clearly decreased by DGTX treatment, dropping from 96 at the lowest dosage (5 nM) to roughly 35% at the maximum dosage (100 nM). Alongside this decline, there were noticeably fewer and smaller colonies, suggesting reduced long-term survival and proliferative potential (Figure 5A). Although a mild stimulatory (hermetic) effect was observed at low concentrations in the MTT assay, potentially due to an adaptive stress response or transient enhancement of cellular metabolic activity, this effect was not sustained in the long-term clonogenic assay. Even at the lowest concentrations tested, both DGX and DGTX reduced colony formation capacity, indicating that the transient increase in metabolic activity does not translate into enhanced long-term proliferative potential. These results imply that the hermetic response observed at early time points may reflect short-term adaptive or metabolic effects rather than a true increase in tumorigenic capacity.

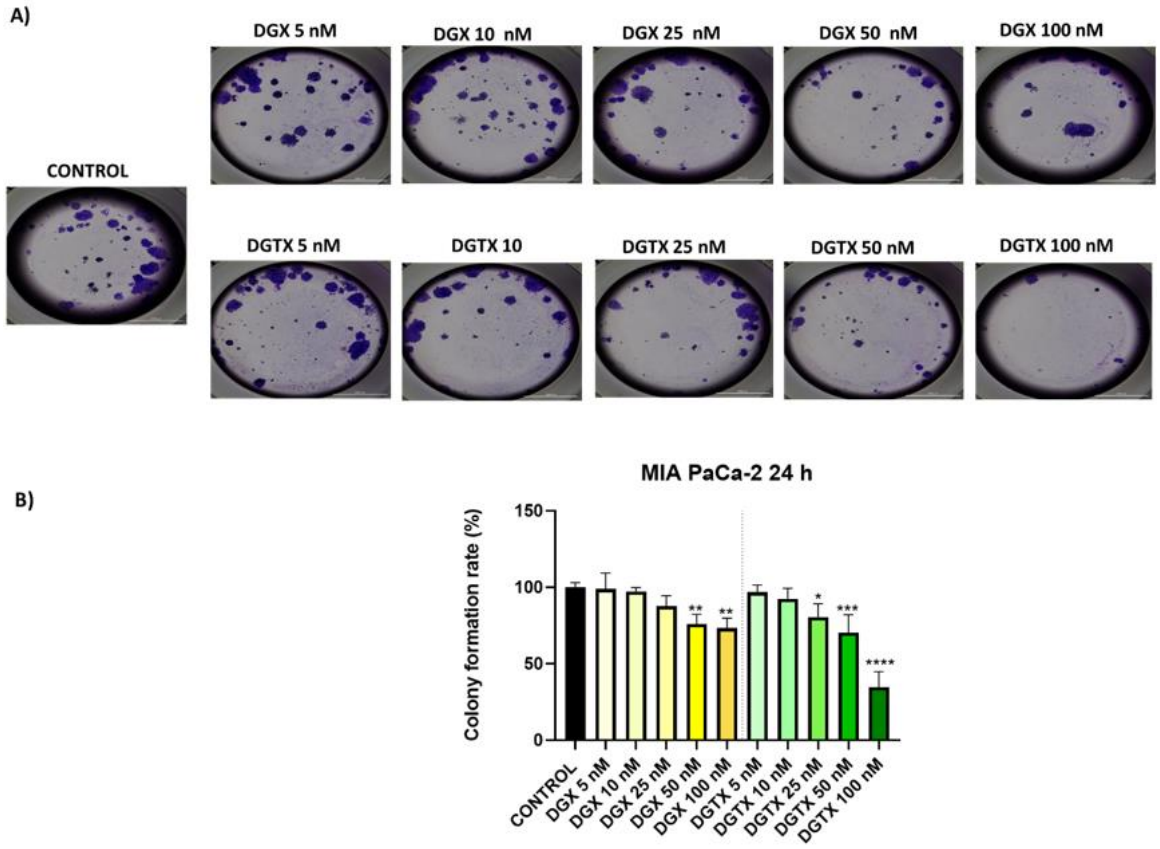

Figure 5.

A) Crystal violet-stained MIA PaCa-2 pancreatic carcinoma cell colonies are shown in representative photos after being exposed to DGX and DGTX at 5, 10, 25, 50 and 100 nM for 24 hours in comparison to untreated control cells. B) Quantitative analysis showing how DGX and DGTX affect the number of colonies overall as well as the rate of colony formation. The trials were conducted in triplicate, and the data are presented as mean ± standard deviation (SD). One-way ANOVA and Dunnett's post hoc test (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) were used to assess statistical significance between the treated groups and the control. The symbol "+" denotes statistical significance. Every picture includes a scale bar showing 2000 µm

The purpose of this method, also known as the clonogenic assay, is to measure the ability of a single cell to survive and, consequently, to form colonies over a longer period of time. This technique is used in cancer drug research to determine whether a cell possesses proliferative capacity. Wang *et al.* demonstrated that DGX inhibited the proliferation and colony-forming ability of human non-small cell lung cancer cells in the A549 and H1299 cell lines. In addition to this effect, DGX inhibited migration, invasion, adhesion and epithelial-mesenchymal transition in both cell lines (34).

As for DGTX, this digitalis compound has also been shown to inhibit colony formation in cancer cells. In H1975 cells, a human model of non-small cell lung cancer (NSCLC), a study found that DGTX reduces colony formation as the applied concentration increases, exerting a dose-dependent effect over the concentrations 0.03125, 0.0625, 0.125, 0.25 and 0.5 μM. The results for DGTX were significant, with a marked reduction in proliferation and colony formation; specifically, when cells were treated with 0.5 μM of DGTX, the percentage of cells decreased remarkably by approximately 95% compared to control cells (35).

In ovo irritation assessment (HET-CAM) assay

The final experimental stage for the two cardiotonic glycosides involved determining their effects on the vascular system of the chorioallantoic membrane of embryonated chicken eggs. For this purpose, the HET-CAM test was conducted, an alternative to the rabbit eye irritation test (36). This technique consisted, in brief, of applying DGX and DGTX at the selected concentrations (5, 25 and 100 nM) to the chorioallantoic membrane for 5 minutes, and monitoring the effects by observing changes such as lysis, haemorrhage, or coagulation. Both substances across all ranges were categorised as non-irritant, except for the positive control, SLS 1%, which was irritant.

In Table I, the irritation score (IS) for 5, 25 and 100 nM of DGX and DGTX was determined using the HET-CAM method. The positive control was SLS 1%, and the negative control was H_2O.

The chosen method is fast, efficient and cost-effective, and does not require the use of live mammals, being in good agreement with *in ovo* Draize test results for many classes of chemicals (37). Local compatibility with mucous membranes is an important biological property (38). Also, in other studies, this type of *in ovo* test has been used in several instances to determine the irritation score of agents being tested for their antitumor potential, for biosafety purposes (17, 39, 40).

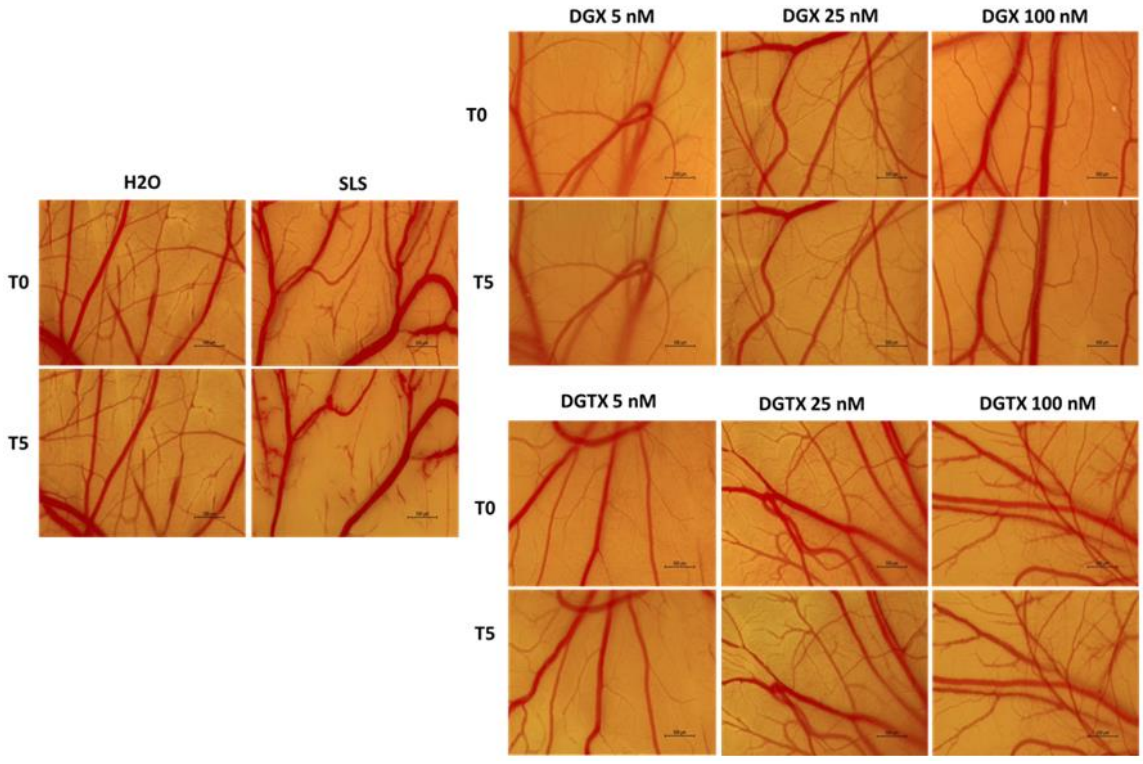

Figure 6.

Before applying the evaluated substances (T0) and 5 minutes after applying the test substances (T5) to the chorioallantoic membrane, stereomicroscopic images were taken. The groups contained the tested drugs DGX and DGTX at 5, 25 and 100 nM, a positive control (1% SLS), and a negative control (H2O). Every picture includes a scale bar showing 500 μm

Table I

Calculated IS for SLS 1% (positive control), H_2O (negative control), DGX and DGTX

Sample	Irritation Score (IS)	Irritant Potential
Positive Control (SLS 1%)	19.4	Strong irritant
Negative Control (H_2O)	0.07	Non-irritant
DGX 5 nM	0.1	Non-irritant
DGX 25 nM	0.32	Non-irritant
DGX 100 nM	0.8	Non-irritant
DGTX 5 nM	0.24	Non-irritant
DGTX 25 nM	0.41	Non-irritant
DGTX 100 nM	0.87	Non-irritant

This study has some limitations that should be noted. First, because pancreatic ductal adenocarcinoma exhibits significant genetic and phenotypic heterogeneity, the results can only be applied to a single pancreatic cancer cell line, MIA PaCa-2. Second, the selectivity of DGX and DGTX toward tumour cells could not be evaluated because no healthy pancreatic cell line was included. Third, the specific molecular pathways driving these effects were not directly examined, despite the morphological observations and fluorescence-based assays suggesting necrotic and apoptosis-associated alterations. To confirm the mechanism of action, further research should examine oxidative stress, apoptotic markers, mitochondrial dysfunction and Na^+/K^+-ATPase-related signalling pathways.

Further research into cardiotonic glycosides, especially digitoxin, as repurposed drugs used in pancreatic cancer is supported by the current findings. Although digoxin has been more extensively studied, digitoxin remains comparatively underexplored in oncology, despite evidence suggesting stronger anticancer activity in several *in vitro* models (41). Next studies should focus on determining the molecular mechanisms behind each of their anticancer actions. It would also be beneficial to investigate their therapeutic abilities in combination with conventional chemotherapy to detect a potential sensitising effect. Simultaneously, more advanced preclinical studies are needed to assess antitumor efficacy, systemic safety and the overall translational potential of these compounds in pancreatic cancer.

Conclusions

The results of the current study suggest distinct biological profiles for the two cardiotonic glycosides evaluated, digoxin and digitoxin, with digitoxin showing cytotoxicity in MIA PaCa-2 pancreatic cancer cells. Complementary analyses on the same cell line and under the same testing conditions have consistently demonstrated that digitoxin significantly reduces the viability of MIA PaCa-2 cells at the intermediate (25 nM) concentration and also induces structural and morphological changes similar to those of apoptosis. In contrast, digoxin did not exhibit cytotoxicity, as cell viability remained above the cytotoxic threshold even at the highest tested concentration (100 nM), suggesting only a limited biological effect under these conditions. In contrast, digitoxin affected nuclear integrity and mitochondrial function at lower concentrations, and AO/PI staining confirmed the clear difference in action between DGX and DGTX, with DGTX being notable for inducing both apoptosis and dose-dependent necrosis. Overall, these findings support the potential of cardiotonic glycosides, particularly digitoxin, as drug repurposing candidates for pancreatic cancer. Nevertheless, further studies are required to elucidate the underlying molecular mechanisms and to validate these effects in more complex experimental models.

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Conflict of interest

The authors declare no conflict of interest.

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