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Brief communication

Digitoxin, at concentrations commonly found in the plasma of cardiac patients, antagonizes etoposide and idarubicin activity in K562 leukemia cells

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Abstract

Digitoxin is used in the treatment of cardiac congestion and some types of cardiac arrhythmias. The mechanism of action of this cardiac glycoside suggested that it might antagonize the anticancer activity of topoisomerase II poisons. The present report shows that digitoxin, at concentrations commonly found in the plasma of cardiac patients, significantly reduced etoposide and idarubicin-induced topoisomerase II cleavable complexes in K562 leukemia cells. This may lead to a reduction in the anticancer effect of these two topoisomerase II poisons when they are used in the clinic concurrently with digitoxin.
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1. Introduction

Digitoxin is a steroidal glycoside obtained from the leaves of *Digitalis purpurea*. This natural product has been used for many years in the clinic, mainly for the treatment of cardiac congestion and some types of cardiac arrhythmias. Its mechanism of action in the heart is well known and involves inhibition of the plasma membrane Na^+/K^+-ATPase; this results in a rise in intracellular Na^+ and Ca^{2+} and a decrease in intracellular K^+. The increased intracellular Ca^{2+} promotes muscle contraction and cardiac contractile force [1].

DNA topoisomerases (topos) are essential enzymes that govern DNA topology. During the normal catalytic cycle of these enzymes, transient enzyme-bridged DNA strand breaks are formed; this permits the enzyme to alter DNA topology allowing cellular processes such as replication, transcription, recombination and chromatin remodeling. Topo II poisons represent a group of clinically important anticancer drugs. These drugs stabilize the normally transient DNA breaks; then cellular processing can convert these protein-bridged breaks into permanent strand breaks that trigger cell death. Catalytic inhibitors of topo II, on the other hand, prevent the formation of such DNA strand breaks and can therefore reduce the activity of topo II poisons [2].

It is known that intracellular Ca^{2+} plays a role in topo II activity. It has been shown that Ca^{2+} directly binds to topo II (topo IIα) and inhibits the catalytic activity of this enzyme [3]. Therefore, since digitoxin is known to increase intracellular Ca^{2+}, this cardiac drug might reduce the catalytic activity of topo II. This would prevent to some extent the formation of transient DNA-topo II strand breaks and their subsequent stabilization by topo II poisons, therefore reducing their activity.

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The aim of this work was to evaluate if digitoxin antagonized the effect of topo II poisons in K562 leukemia cells. Here, we report that concentrations of digitoxin commonly found in the plasma of cardiac patients significantly reduce the topo II poison activity of etoposide and idarubicin in K562 leukemia cells.

2. Methods

2.1. Cell culture, drugs, and antibodies

K562, a cell line derived from a patient with chronic myelogenous leukemia, was maintained as a suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (50 U/mL)/streptomycin (50 μg/mL). This cell line was maintained at 37 °C (5% CO_2) at a density between 1×10^5 and 1×10^6 cells/mL. Cell culture reagents were obtained from Life Technologies. Idarubicin was kindly provided by Pharmacia-UpJohn (UK). The rest of the drugs used in this study were purchased from Sigma. Stock solutions of drugs were prepared in DMSO (etoposide in methanol) and they were stored at −20 °C. Anti-topo II polyclonal antibody (αCT) was raised in rabbits to recombinant human topo IIα C-terminal fragment that detected both isoforms of topo II (α and β). This antibody was diluted in PBS containing 0.1% Tween 20 and 1% BSA at a 1:50 dilution. The anti-rabbit FITC-conjugated second antibody (1262) was purchased from Sigma and was used at 1:200 dilution.

2.2. Cell viability assay

This is a colorimetric assay, which allows the quantitative determination of cell viability. It is based on the capability of metabolically active mitochondria of viable cells to transform the colorless tetrazolium salt XTT into a formazan dye. Exponentially growing cells were seeded (2×10^3/well in 100 μL) into 96-well plates. Drugs were added to plates 24 h later. Following the incubation periods, cell viability was quantified using an XTT cell proliferation kit assay (Roche, Mannheim, Germany). Plates were finally incubated for 4 h with XTT before reading them on a Bio-Rad 550 plate reader at 450 nm. Cell viability was expressed as percentage in relation to controls. All data were averaged from at least three independent experiments ± S.E.M.

2.3. TARDIS assay

The Trapped in AgaRose DNA Immuno Staining (TARDIS) assay is an in vivo immunofluorescence assay that allows measurement of drug-induced topo I and topo II (α and β) cleavable complexes in individual cells, and has been described in detail previously [4]. Briefly, cells were treated with drugs at the concentrations and for the times specified in the figure legends, and embedded in agarose on microscope slides. After placing the slides in a lysis buffer (10% SDS, 80 mM phosphate, pH 6.8, 10 mM EDTA plus protease inhibitors), proteins that were not covalently bound to the DNA were removed by placing the slides in 1 M NaCl plus protease inhibitors. Slides were incubated with primary topo II antibody and then a fluorescein isothiocyanate (FITC)-conjugated (secondary antibody) before being counter-stained with Hoechst 33258. Images were captured using an epi-fluorescence microscope attached to a cooled slow scan charge-coupled device camera that separately visualizes blue (Hoescht-stained DNA) and green (FITC-stained topo) fluorescence. For each of the eight randomly chosen fields of view, images of blue and green fluorescence were captured to give a total of approximately 100 cells/dose. Imager 2 software (Astrocam, Cambridge, UK.) was used to analyze and quantify levels of blue and green fluorescence. Figures show integrated green fluorescence values, which indicates drug-stabilized topo-DNA cleavable complexes.

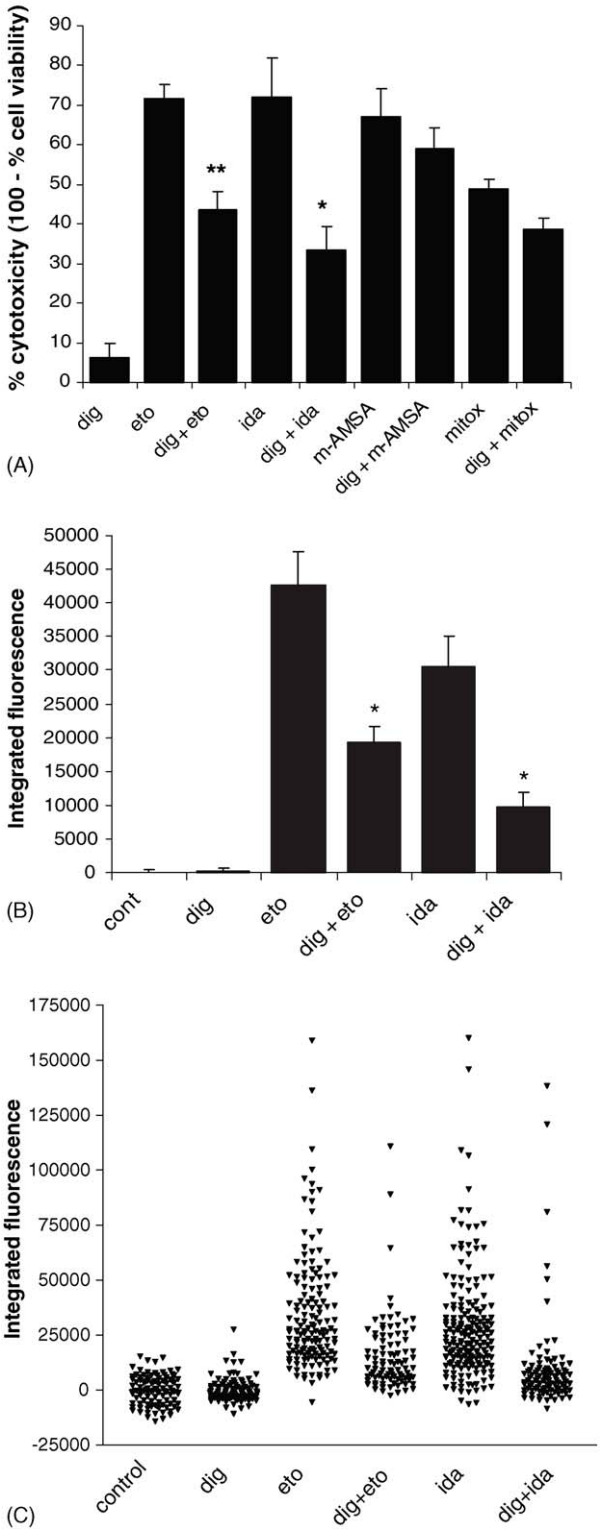
3. Results

Inhibition of the catalytic activity of topo II results in reduction of the activity of topo II poisons. Since Ca^{2+} has been shown to inhibit the catalytic activity of topo II and given that digitoxin increases the intracellular levels of Ca^{2+}, we considered the possibility that digitoxin might antagonize the activity of topo II poisons in cells. To test this hypothesis, we evaluated if digitoxin could decrease the cytotoxicity and the topo II poison activity of four representative topo II poisons in K562 leukemia cells.

The effect of digitoxin on the growth inhibition activity of the topo II poisons etoposide, idarubicin, m-AMSA, and mitoxantrone was analyzed. As described in Fig. 1A, K562 cells were treated with each topo II poison, alone and in combination with digitoxin. After drug removal and further incubation in drug free medium, cell viability was assessed using the XTT assay. The histogram in Fig. 1A shows that the cytotoxicity of etoposide, idarubicin, m-AMSA and mitoxantrone decreased in cells pre-incubated with digitoxin. These data suggest that digitoxin is antagonistic towards topo II poisons.

To assess this further, we used the TARDIS assay to evaluate if digitoxin could decrease the level of topo II cleavable complexes induced by etoposide, idarubicin, mitoxantrone and m-AMSA in K562 leukemia cells. The TARDIS assay is a sensitive method that uses specific antibodies to allow the detection of topo-DNA cleavable complexes in individual cells [4]. As described in Fig. 1B, K562 cells were treated with each topo II poison, alone and in combination with digitoxin. K562 cells were pretreated for 24 h with digitoxin at concentrations commonly found in cardiac patients (30 nM) before adding each topo II poison. Fig. 1B and C show that digitoxin decreased the levels of topo II cleavable complexes induced by idarubicin and

etoposide. In contrast, the levels of cleavable complexes stabilized by mitoxantrone and m-AMSA were not decreased by digitoxin pre-treatment at this concentration (results not shown).



4. Discussion

Previous reports have shown that the cardiac glycoside digitoxin may have anticancer effects at concentrations commonly found in cardiac patients (20–33 nM) [5,6]. For instance, a study with 9271 patients showed a relationship between high plasma concentration of digitoxin and a lower risk for leukaemia/lymphoma [5]. In a recent report we showed that digitoxin inhibited the growth of several cancer cell lines in the 3–33 nM range; the IC_{50} value against the K562 leukemia cell line was 6.4 ± 0.4 nM [6]. In that report, we discussed that digitoxin might affect topo II [6].

The present data suggest that concentrations of digitoxin commonly found in the plasma of cardiac patients may inhibit the catalytic activity of topo II. We have carried out in vitro experiments to find out if digitoxin could inhibit directly the catalytic activity of topo II and we observed that digitoxin did not prevent topo II-induced decatenation of catenated kinetoplast DNA (results not shown). This lack of activity in vitro suggests that digitoxin inhibits the activity of topo II in cells via an indirect mechanism, which might be mediated by an increase in intracellular Ca^{2+}. However, the mechanism by which digitoxin antagonizes etoposide and idarubicin activity remains to be elucidated. Thus, we cannot rule out the possibility that digitoxin can potentiate the efflux of these topo II poisons out of the cell (i.e. via activation of P-glycoprotein); this would decrease their cytotoxicity and topo II poison activity. Besides, it is not clear why digitoxin reduces the topo II poison activity of etoposide and idarubicin but not that of m-AMSA and mitoxantrone. As mentioned before Ca^{2+} inhibits the catalytic activity of topo IIα [3]; this may reduce the activity of topo poisons that target this isoenzyme. There is experimental evidence that suggests that etoposide and idarubicin are topo II poisons that mainly target topo

Fig. 1. Digitoxin antagonism on topo II poisons in K562 cells. (A) Cells were treated with digitoxin 300 nM for 4 h and with each topo II poisons for 3 h (etoposide 10 μM, idarubicin 0.025 μM, m-AMSA 1 μM and mitoxantrone 0.1 μM), alone or in combination (digitoxin 1 h prior to the topo II poison). After drug removal, cells were placed in drug-free medium for 116 h (to complete 5 days). Then the percentage of cell viability in relation to untreated cells was estimated using the XTT assay. For clarity when comparing graphs (A) and (B), we plot % of cytotoxicity, which represents 100-% cell viability. (B) K562 cells were treated with digitoxin 30 nM for 24 h and with etoposide (10 μM, 2 h) or idarubicin (1 μM, 2 h), alone or in combination (digitoxin 22 h prior to the topo II poison). Then the levels of in vivo DNA-topo II cleavable complexes were visualized and quantified in situ by the immunofluorescence assay TARDIS. Data were averaged from at least three independent experiments and are expressed as mean ± S.E.M. (C) Representative experiment in which the distribution of cleavable complexes in individual cells can be observed. Cells were treated as in (B). For statistical analysis we used *t*-test (paired, two-tailed). A *P*-value <0.05 is considered statistically significant and is indicated with an asterisk (*), a *P*-value <0.01 is represented with a double asterisk (**). Comparison of (A) and (B) suggests that digitoxin antagonizes the cytotoxicity of etoposide and idarubicin because it reduces their formation of topo II cleavable complexes.

IIα while m-AMSA and mitoxantrone mainly act on topo IIβ [7]. This suggests that etoposide and idarubicin activity might be more affected by a possible digitoxin-mediated intracellular Ca^{2+} increase than the activity of m-AMSA and mitoxantrone.

The topo II poisons etoposide and idarubicin are commonly used in the treatment of leukemia, especially in the elderly [8]. Since aged patients may have both cancer and cardiac disease, some of these patients may be treated concurrently with these topo II poisons and digitoxin. The plasma half-life of digitoxin in humans has been reported to be 77–234 h, and 30 nM is a concentration commonly found in patients treated with this drug [9]. Therefore, the statistically significant reduction of topo II poison activity of etoposide and idarubicin by pre-treatment with digitoxin 30 nM for 24 h (Fig. 1B and C) may lead to a reduction in the anticancer effect of these topo II poisons when they are used concurrently with digitoxin. Although digoxin is used more frequently than digitoxin in the treatment of cardiac disease, digitoxin is still prescribed and more attention is again being paid to this compound especially in the elderly [10].

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