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Digoxin and ouabain induce P-glycoprotein by activating calmodulin kinase II and hypoxia-inducible factor-1α in human colon cancer cells

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A B S T R A C T

Digoxin and ouabain are cardioactive glycosides, which inhibit the Na+/K+-ATPase pump and in this way they increase the intracellular concentration of cytosolic calcium ([Ca++]). They are also strong inducers of the P-glycoprotein (Pgp), a transmembrane transporter which extrudes several drugs, including anticancer agents like doxorubicin. An increased amount of Pgp limits the absorption of drugs through epithelial cells, thus inducing resistance to chemotherapy. The mechanism by which cardioactive glycosides increase Pgp is not known and in this work we investigated whether digoxin and ouabain elicited the expression of Pgp with a calcium-driven mechanism. In human colon cancer HT29 cells both glycosides increased the [Ca++] and this event was dependent on the calcium influx via the Na+/Ca++ exchanger. The increased [Ca++] enhanced the activity of the calmodulin kinase II enzyme, which in turn activated the transcription factor hypoxia-inducible factor-1α. This one was responsible for the increased expression of Pgp, which actively extruded doxorubicin from the cells and significantly reduced the pro-apoptotic effect of the drug. All the effects of glycosides were prevented by inhibiting the Na+/Ca++ exchanger or the calmodulin kinase II. This work clarified the molecular mechanisms by which digoxin and ouabain induce Pgp and pointed out that the administration of cardioactive glycosides may widely affect the absorption of drugs in colon epithelium. Moreover, our results suggest that the efficacy of chemotherapeutic agent substrates of Pgp may be strongly reduced in patients taking digoxin.

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Introduction

P-glycoprotein (Pgp), a transmembrane ATP-dependent efflux pump, which is encoded by the mdr-1 gene, is physiologically present in the apical membrane of different epithelial cells, such as colon mucosa, hepatic bile ducts and renal proximal tubules (Takara et al., 2006). Pgp recognizes endogenous metabolites and xenobiotics as substrates, including anticancer drugs such as anthracyclines (like doxorubicin), epipodophyllotoxins and Vinca alkaloids; antibiotics; antiretroviral drugs; analgesic and anti-inflammatory drugs; immunosuppressive drugs; and anti-arrhythmic and cardioactive drugs, such as digoxin and verapamil (de Lange, 2004). Due to the broad spectrum of substrates, the amount of Pgp in critical tissues, as intestine, liver and kidney, may strongly affect the drug absorption and excretion, influencing the efficacy of therapy (Bodo et al., 2003; Geick et al., 2001). Moreover, the overexpression of Pgp in tumor cells is the main mechanism of the multidrug resistance (MDR), an intrinsic or acquired cross-resistance toward different chemotherapeutic agents, which represents the major obstacle to a successful cancer therapy (Takara et al., 2006).

Interestingly, many substrates of Pgp also act as Pgp inducers, thus enhancing their own efflux and limiting the intracellular accumulation. For instance, rifampin induces Pgp in human colon cells, increasing the mdr-1 gene transcription via the activation of the pregnane-X receptor (Geick et al., 2001; Haslam et al., 2008). Also digoxin is both substrate and inducer of Pgp, but its effect is independent from the pregnane-X receptor (Haslam et al., 2008). Notably, other two glycosides, ouabain and palitoxin, share with digoxin the property to induce Pgp (Brouillard et al., 2001). Cardioactive glycosides are strong inhibitors of the Na+/K+-ATPase pump and increase the [Ca++] concentration, by forcing the Na+/Ca++ exchanger to extrude Na+ in exchange with Ca++ (Kaplan, 2002). It has been suggested that the enhanced expression of mdr-1 gene elicited by digoxin and ouabain depends on...
the increased [Ca\(^{++}\)] levels: indeed the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) abrogates the Pgp induction exerted by ouabain, whereas thapsigargin, which increases the [Ca\(^{++}\)] by inhibiting the sarcoplasmic/endoplasmic reticulum Ca\(^{++}\)-ATPase, enhances the ouabain effect (Baudouin-Legros et al., 2003; Brouillard et al., 2001). However the mechanisms by which the increased intracellular Ca\(^{++}\) levels modulate the transcription of mdr-1 gene are not yet clarified.

An increase of [Ca\(^{++}\)] may activate the transcription factor hypoxia-inducible factor-1 (HIF-1) (Hui et al., 2006; Yuan et al., 2005), which controls several genes involved in cell growth, angiogenesis, matrix remodeling, glucose and iron metabolism, intracellular pH modulation (O’Donnell et al., 2006), and in Pgp up-regulation (Comerford et al., 2002). HIF-1 is composed of two subunits: the α subunit is constitutive, whereas the α subunit is rapidly degraded in normoxia, but becomes stable in hypoxia (O’Donnell et al., 2006).

In this work we investigated whether digoxin and ouabain induce the transcription of mdr-1 gene in human colon cancer cells by a [Ca\(^{++}\)]-driven activation of HIF-1α, thus increasing the Pgp activity and the cell resistance to doxorubicin.

Materials and methods

Materials. Foetal bovine serum (FBS) and RPMI 1640 medium were supplied by BioWhittaker (Verviers, Belgium); plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA). 2-[2-[4-(nitrobenzoxyl)]phenyl]ethyliothiourea (KB-R7943), 2-[(2-hydroxyethyl)]-N-[4-(methoxybenzenesulfonyl)]amino-N-[4-chlorocinnamyl]-N-methylbenzylamine (KN93) and YC-1 were purchased from Calbiochem (La Jolla, CA). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). When not otherwise specified, the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells. Human colon cancer HT29 cells and human liver cancer HepG2 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and maintained in a humidified atmosphere at 37 °C, 5% CO\(_2\) and 3% O\(_2\). To culture them in hypoxic conditions, cells were grown for 24 h in a humidified atmosphere at 37 °C, 5% CO\(_2\) and 3% O\(_2\).

[Ca\(^{++}\)] measurement. Cells were grown on sterile glass coverslips under the experimental conditions indicated in the Results section, washed twice with PBS and incubated for 10 min at 37 °C in Hepes–Ca buffer (10 mM Hepes, 145 mM NaCl, 1 mM Ca\(_{\text{Cl}_2}\), 5 mM KCl, 1 mM MgSO\(_4\), and 10 mM glucose, pH 7.4), with 10 μM of calcium-sensitive fluorescent probe 1-[2-5-carboxyoxazol-2-yl]-6-aminobenzofuran-3-oxyl]-2-[(2-amino-5′-methylphenoxo)-ethane-N,N,N′,N′-tetraacetic acid acetoxyethylster (FURA-AM). After FURA-AM loading, coverslips were washed with Hepes–Ca buffer and firmly positioned in a quartz cuvette (1 cm) containing 1 ml of Hepes–Ca. The cuvette holder was thermostated at 37 °C and the fluorescence of coverslips was measured in a Perkin Elmer LS-5 spectrofluorimeter (Perkin Elmer) for 30 min. Excitation and emission wavelengths were 490 and 530 nm, respectively. Calculation of [Ca\(^{++}\)] levels was performed as previously described (Hamm et al., 1984). The fluorescence of Ca\(^{++}\)-saturated dye (F\(_{\text{max}}\)), obtained by treating cells with 10 μM ionomycin in Hepes–Ca buffer, was taken as the maximal emission. 2 mM MnCl\(_2\) was then added to displace Ca\(^{++}\) from FURA and to obtain the value of FURA autofluorescence (F\(_{\text{aut}}\)) alone. To prevent the [Ca\(^{++}\)] increase, HT29 cells were pre-incubated for 3 h with 10 μM BAPTA acetoxyethylster (BAPTA-AM) in order to load them with the Ca\(^{++}\) chelator BAPTA, then washed with PBS, and subjected to the same procedure of the other experimental points. To evaluate whether the [Ca\(^{++}\)] increase may induce a pro-apoptotic response, the cytosolic release of cytochrome c was assessed: mitochondrial and cytosolic fractions were separated as described (Wibom et al., 2002), then 10 μg from each fraction was subjected to SDS-PAGE and probed with an anti-cytochrome c antibody (diluted 1:1,000 in PBS–BSA 1%, from Becton Dickinson).

Calmodulin kinase II (CaMKII) activity. CaMKII activity was measured using the Cycllex Calmodulin kinase II Assay Kit (CyClex Co., Nagano, Japan), a single site binding immunnoassay. Cells were cultured in 35-mm diameter Petri dishes, washed with ice-cold PBS and lysed in 0.2 ml lysis buffer (50 mM Tris–HCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 2 mM NaVO\(_4\), 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride PMSF and 10 mM β-mercaptoethanol, pH 8.0). Samples were sonicated on crushed ice with two 10 s bursts and centrifuged at 13,000 × g for 5 min at 4 °C. Supernatants were treated following the manufacturer’s instructions and the protein content was measured. Samples were diluted 1:10 in the kinase buffer provided with the kit, containing 20 mM ATP, and incubated for 60 min at 30 °C in 96-well plates, pre-coated with the recombinant peptide Syntide-2, which contains a unique target sequence for CaMKII. Wells were washed 5 times with 2% (v/v) Tween-20, and 100 μl of the horseradish peroxidase-conjugated anti-phospho Syntide-2 monoclonal antibody was added. After 60 min of incubation at room temperature, samples were washed again, and 100 μl of the chromogenic substrate tetramethylbenzidine was added. After 15 min of incubation at room temperature, the reaction was stopped with 100 μl of 0.5 N H\(_2\)SO\(_4\) and the absorbance was read at 450 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). For each set of experiments, a titration curve was prepared, using serial dilution of recombinant CaMKII enzyme (CyClex Co.) in kinase buffer. Data were expressed as mU absorbance/mg cell proteins.

Western blot analysis. Cells were solubilized in boiling lysis buffer (125 mM Tris, 135 mM NaCl, 20% SDS and 10% glycerol, pH 7.4) and whole-cell extracts were supplemented with the protease inhibitor cocktail set III (Calbiochem), 2 mM PMSF, 2.5 mM NaF and 1 mM NaVO\(_4\). 30 μg of proteins was separated by SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore, Bedford, MA) and probed with the following antibodies: anti-HIF-1α (from mouse, diluted 1:500 in PBS–BSA 1%, Becton Dickinson), anti-Pgp (from rabbit, diluted 1:250 in PBS–BSA 1%, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, from rabbit, diluted 1:500 in PBS–BSA 1%, Santa Cruz Biotechnology). After 1 h of incubation, the membrane was washed with PBS–Tween 0.1% and subjected for 1 h to a peroxidase-conjugated anti-mouse or anti-rabbit antibody (diluted 1:3000 in PBS–Tween with Blocker Not-Fat Dry Milk 5%, Bio-Rad). The membrane was washed again with PBS–Twee, and proteins were detected by enhanced chemiluminescence (Immun-Star, Bio-Rad). To assess HIF-1α phosphorylation, the whole-cell lysate was immunoprecipitated overnight with the rabbit polyclonal anti-HIF-1α antibody (diluted 1: 250, Santa Cruz Biotechnology) and the immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane sheets and probed with a biotin-conjugated anti-phosphoserine antibody (diluted 1:1000 in TBS–BSA 3%, Sigma Chemical Co.) for 1 h. The membrane was washed in TBS–Twee 0.1% and subjected for 1 h to a streptavidin- and horseradish peroxidase-conjugated polimer (diluted 1:10,000 in TBS–BSA 3%, Sigma Chemical Co.). The membrane was washed again with TBS–Twee and proteins were detected by enhanced chemiluminescence, as reported above.

Electrophoretic mobility shift assay (EMSA). Cells were plated in 60-mm diameter dishes at confluence and all the procedures for nuclear protein extraction were performed at 4 °C using ice-cold reagents. Cells were mechanically scraped in PBS, washed and re-suspended in lysis buffer A (10 mM Hepes, 15 mM KCl, 2 mM MgCl\(_2\), 0.1 mM EDTA,
1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1% NP-40, pH 7.6). This suspension was incubated for 10 min on ice with occasional vortexing, and centrifuged for 30 s at 13,000 × g to pellet nuclei, which were rinsed with 0.2 ml of wash buffer B (25 mM Hepes, 2 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, and 2 μg/ml leupeptin, pH 7.6) and incubated at 4 °C for 20 min. An equal volume of buffer C (25 mM Hepes, 0.1 mM EDTA, and 20% glycerol, pH 7.6) was added, the mix was centrifuged at 20,000 × g and the supernatant was stored at −80 °C until used for EMSA. The detection of HIF-1α was performed on 10 μg nuclear extracts as previously described (Riganti et al., 2008).

Real time polymerase chain reaction (RT-PCR). Total RNA was obtained as previously described (Chomczynski and Sacchi, 1987). 5 μg of RNA was reverse-transcribed by 200 U of M-MLV reverse transcriptase (Invitrogen, Milan, Italy), in the presence of 40 U/μl RNaseOUT (Invitrogen). Quantitative RT-PCR was carried out using IQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer’s instructions. The same cDNA preparation was used for the quantitation of Pgp and GAPDH, used as a housekeeping gene. The sequences of Pgp primers for quantitative RT-PCR were: 5′-TGCTGAGCCGGTCTC-ACC-3′, 5′-ATAACGAAATGCTTCAGAATG-3′ (Invitrogen). Cycling for Pgp was: 1 cycle at 94 °C for 2 min, followed by 45 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The sequences of GAPDH primers were 5′-GAAGGTGAAGGTCGGAGT-3′, 5′-CATGGTGGAATCATATTGGAA-3′ (Invitrogen). Cycling for GAPDH was: 1 cycle at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The relative quantification of each sample was performed comparing the Pgp PCR product with the GAPDH product, using the Bio-Rad Software Gene Expression Quantitation (Bio-Rad).

Doxorubicin accumulation. Intracellular doxorubicin accumulation was measured by a fluorometric assay as described (Riganti et al., 2005). Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was prepared in the absence of cells in each set of experiments and its fluorescence was subtracted from that measured in the samples. Fluorescence was converted in ng doxorubicin/mg cell proteins, using a calibration curve prepared previously.

Annexin V assay. Cells were incubated in the experimental conditions described under the Results section, then they were rinsed twice with fresh PBS, detached with the Cell Dissociation Solution (Sigma Chemical Co.) and incubated for 10 min at room temperature in a solution of 1 ml of binding buffer (100 mM Hepes, 140 mM NaCl, and 25 mM CaCl₂, pH 7.5) containing 10 μM annexin V-fluorescein isothiocyanate conjugate (FITC). The cell suspensions were washed three times with fresh PBS and rinsed with 1 ml of binding buffer. The fluorescence of each sample was recorded using a FACSCalibur system (Becton Dickinson). For each analysis, 10,000 events were collected; the green fluorescence (for annexin V-FITC) was selected using a 530 nm band pass filter, the red fluorescence (for doxorubicin) was measured with a 640 nm longpass filter. The percentage of cells positive for annexin V, in the absence or presence of doxorubicin, was calculated by the Cell Quest software (Becton Dickinson).

Statistical analysis. All data in text and figures are provided as means ± SE. The results were analysed by a one-way Analysis of Variance (ANOVA) and Tukey’s test. p < 0.05 was considered significant.

Results

Digoxin and ouabain increase [Ca++], in HT29 cells

After 24 h of incubation, digoxin and ouabain (0.1–10 μM) increased the [Ca++], in HT29 cells as a function of dose (Fig. 1A). When cardioactive glycosides were used at 1 μM, the raise of [Ca++], was not detectable after 3 h, increased after 6 h and was even more evident after 24 h, whereas slightly decreased at 48 h (Fig. 1B). No changes in [Ca++], levels were detected in cells pre-incubated for 3 h with 10 μM BAPTA-AM before digoxin or ouabain (data not shown). To assess whether the increase of [Ca++], elicited by cardioactive glycosides induced the activation of an apoptotic process in HT29 cells, we measured the release of cytochrome c from mitochondria into the cytosol: after 24 h of incubation with 1 μM digoxin or ouabain, the cytochrome c was not released from mitochondria (data not shown). At the lights of these results, 1 μM digoxin or ouabain for 24 h was the minimal dose able to induce a significant increase of the [Ca++], in HT29 cells, without exerting any pro-apoptotic effect. Therefore we chose these experimental conditions in all the subsequent experiments.

In many mammalian tissues the increase of [Ca++], exerted by digoxin or ouabain was due to the enhanced Ca++ influx through the Na+/Ca++ exchanger, driven by the increased [Na+], (Kaplan, 2002). When the Na+/Ca++ exchanger inhibitor KB-R7943 was co-incubated with digoxin or ouabain, the cardioactive glycosides were not able to elicit any [Ca++], increase (Fig. 2). This result suggested that the peak of [Ca++], observed in HT29 cells treated with digoxin and ouabain was due to the reverse activity of the Na+/Ca++ exchanger.
Digoxin and ouabain enhance the CaMKII activity in HT29 cells, whereas KB-R7943 reverts their effect

Digoxin and ouabain increased the activity of CaMKII (Fig. 3) and this effect was prevented by 3 h of pre-incubation with 10 μM BAPTA-AM (data not shown). When co-incubated with digoxin or ouabain, KB-R7943, which abolished the [Ca ++]i peak induced by the cardioactive glycosides (Fig. 2), also prevented the increase of CaMKII activity. The KB-R7943 effect was superimposable to the effect of the CaMKII inhibitor KN93 (Fig. 3).

Digoxin and ouabain induce HIF-1α phosphorylation and activity in a Na+/Ca++ exchanger- and CaMKII-dependent way

The increase of [Ca ++]i and CaMKII activity have been related to the activation of the transcription factor HIF-1α in several cell lines (Hui et al., 2006; Yuan et al., 2005). Untreated HT29 cells showed an undetectable amount of HIF-1α protein (Fig. 4A) and activity (Fig. 4B). Both digoxin and ouabain induced the phosphorylation on serine of HIF-1α (Fig. 4A), which was accompanied by an increased amount (Fig. 4A) and a clear nuclear translocation of the protein (Fig. 4B). When used alone, the Na+/Ca++ exchanger inhibitor KB-R7943 and the CaMKII inhibitor KN93 did not affect the HIF-1α phosphorylation, amount and activity. However, they both prevented the phosphorylation and the nuclear translocation of HIF-1α elicited by digoxin and ouabain, and reduced the total amount of HIF-1α to control level (Fig. 4).

Fig. 2. Effect of digoxin, ouabain and KB-R7943 on [Ca ++]i. HT29 cells were cultured on sterile glass coverslips for 24 h in the absence (CTRL) or in the presence of digoxin (1 μM, DGX), ouabain (1 μM, OUA) and KB-R7943 (1 μM, KB), in different combinations. Subsequently the [Ca ++]i was measured with FURA-AM in duplicate as reported under Materials and methods. Data are presented as means ± SE (n = 4) vs CTRL: *p < 0.002; vs DGX or OUA: °p < 0.005.

Fig. 3. Effect of digoxin, ouabain, KB-R7943 and KN93 on CaMKII activity. HT29 cells were cultured for 24 h in the absence (CTRL) or presence of digoxin (1 μM, DGX), ouabain (1 μM, OUA), KB-R7943 (1 μM, KB) and KN93 (10 μM, KN), differently combined. The cells were lysed and an ELISA assay for the activity of CaMKII was performed, as described under Materials and methods. Measurements were performed in duplicate. Data are presented as means ± SE (n = 3) vs CTRL: *p < 0.05; vs DGX or OUA: °p < 0.05.

Fig. 4. Effect of cardioactive glycosides, KB-R7943 and KN93 on HIF-1α phosphorylation and activity. HT29 cells were incubated for 24 h in the absence (CTRL) or in the presence of either digoxin (1 μM, DGX) or ouabain (1 μM, OUA), alone or together with KB-R7943 (1 μM, KB) and KN93 (10 μM, KN), then the following investigations were performed. (A) Western blotting detection of phospho(Ser)-HIF-1α (pHIF-1α) and total HIF-1α. To assess the pHIF-1α protein, whole-cell lysates were incubated with an anti-HIF-1α antibody, subsequently the immunoprecipitated proteins were separated by SDS-PAGE and probed with an anti-phosphoserine antibody, as described under the Materials and methods section. Western blot analysis for total HIF-1α was performed on the whole-cell lysates, using an anti-HIF-1α antibody. The expression of GAPDH, the product of a housekeeping gene, was used as a control of equal protein loading. The figure is representative of three experiments with similar results. (B) EMSA detection of HIF-1α nuclear translocation. EMSA was performed on nuclear extracts as detailed under Materials and methods. The lane marked with “+” was loaded with nuclear extracts obtained from HT29 cells incubated for 24 h in a humidified hypoxic atmosphere (3% O2, 5% CO2, 37 °C). This experimental condition was chosen as a positive control for maximal HIF-1α activation. In each experiment one lane was loaded with bidistilled water (−) in place of cellular extracts. The figure is representative of three experiments with similar results.
Cardioactive glycosides, such as digoxin and ouabain, which inhibit the Na+/K+-ATPase pump and increase the intracellular [Na+]i concentration, may enhance the Ca++ influx through the Na+/Ca++ exchanger. By doing so, they increase the [Ca++], and exert a positive inotropic effect on cardiac muscle (Kaplan, 2002). Ouabain and digoxin have a similar structure and share most of the biological properties; however the former has no current clinical employment, whereas the latter is one of the most widely used drug in heart diseases (Adorisio et al., 2006).

Besides inhibiting the Na+/K+-ATPase, cardioactive glycosides have many other effects: for instance, they affect the membrane fluidity, modulate the transcription of several genes by activating NF-κB and AP1, inhibit the glycolysis, and increase the cellular synthesis of housekeeping gene, was used as a control of equal protein loading. The figure is representative of three experiments with similar results.

**Discussion**

Cardioactive glycosides, such as digoxin and ouabain, which inhibit the Na+/K+-ATPase pump and increase the intracellular [Na+]i concentration, may enhance the Ca++ influx through the Na+/Ca++ exchanger. By doing so, they increase the [Ca++], and exert a positive inotropic effect on cardiac muscle (Kaplan, 2002). Ouabain and digoxin have a similar structure and share most of the biological properties; however the former has no current clinical employment, whereas the latter is one of the most widely used drug in heart diseases (Adorisio et al., 2006).

Besides inhibiting the Na+/K+-ATPase, cardioactive glycosides have many other effects: for instance, they affect the membrane fluidity, modulate the transcription of several genes by activating NF-κB and AP1, inhibit the glycolysis, and increase the cellular synthesis of CaMKII-dependent mechanism.
of reactive oxygen species (Prassas and Diamandis, 2008). The Src kinase, some phospholipase C isoforms, the Ras/Raf/MAP kinase pathway, and the ERK1-2 kinases are downstream effectors in the Src
kinase, some phospholipase C isoforms, the Ras/Raf/MAP kinase... in our experimental conditions were not suf... of reactive oxygen species (Prassas and Diamandis, 2008). The Src kinase, some phospholipase C isoforms, the Ras/Raf/MAP kinase pathway, and the ERK1-2 kinases are downstream effectors in the digoxin and ouabain cell signaling (Prassas and Diamandis, 2008; Schoner and Scheiner-Bobis, 2007). Interestingly, cardiovascular glycosides have been reported to increase the expression of pro-apoptotic molecules, such as Fas ligand (Raghavendra et al., 2007) and caspase 3 (Winnicka et al., 2008), and to induce the cell cycle arrest (Kometiani et al., 2005; Prassas and Diamandis, 2008). Therefore they have recently been proposed as potential anticancer drugs (Prassas and Diamandis, 2008; Winnicka et al., 2008).

In mammalian tissues digoxin, ouabain and other inhibitors of Na+/K+-ATPase pump are known inducers of Pgp (Baudouin-Legros et al., 2003; Brouillard et al., 2001), an ATP-binding cassette transmembrane protein, which actively extrudes several endogenous metabolites, toxic compounds and drugs (de Lange, 2004). Anticancer drugs, such as anthracyclines, epipodophyllotoxins and Vinca alkaloids, are substrates of Pgp, whose expression induces a MDR phenotype in tumors and reduces the efficacy of chemotherapy (Takara et al., 2006). Pgp may be constitutively present in solid tissues or may be induced by the substrates themselves, like digoxin (Haslam et al., 2008). The mechanism by which cardiovascular glycosides increase the Pgp is still a matter of debate. When [Ca+++]i is increased with BAPTA, digoxin and ouabain lose their ability to increase Pgp in lung, liver and colon cancer cells (Baudouin-Legros et al., 2003; Brouillard et al., 2001), suggesting that the increased [Ca+++]i may be responsible for the enhanced Pgp expression elicited by cardiovascular glycosides.

In our work we investigated how the [Ca+++]i peak induced by digoxin and ouabain increased Pgp in human colon cancer HT29 cells and whether the cardiovascular glycosides affected the efficacy of the anticancer drug doxorubicin, which is a Pgp substrate.

Our dose-dependence experiments showed that digoxin and ouabain were able to increase the [Ca+++]i at micromolar concentrations in HT29 cells. Notably, the intraluminal concentration of digoxin after an oral administration is approximately in the micromolar range and at this concentration the drug was reported to induce the Pgp expression and activity in human colon epithelia (Haslam et al., 2008). In HT29 cells treated with 1 μM digoxin or ouabain, the [Ca+++]i levels gradually raised in the time period of 24 h, and slightly decreased at 48 h. Such a kinetics may be in keeping with the indirect mechanism by which cardiovascular glycosides increase the [Ca+++]i: it is reasonable to think that also in colon, as well as in cardiomyocytes, the raising [Ca+++]i, levels are due to the calcium influx through the Na+/Ca++ exchanger, secondary to the Na+/Ca++-ATPase inhibition.

Since the increase of [Ca+++]i may induce cells to enter in apoptosis, we measured the release of cytochrome c from mitochondria into the cytosol, taken as a sensitive index of the activation of the apoptosis intrinsic pathway. Neither digoxin nor ouabain induced the release of cytochrome c after 24 h, suggesting that the higher [Ca+++]i levels observed in our experimental conditions were not sufficient to elicit apoptosis in HT29 cells or that the mechanisms of Ca++ extrusion and sequestration limited the calcium-induced damage in these cells. Indeed the [Ca+++]i did not further increase at 48 h in the presence of digoxin and ouabain, suggesting that at this time point the calcium extrusion and sequestration mechanisms counteracted the glycoside effect. When the Na+/Ca++ exchanger was blocked by KB-R7943, which does not allow any inward Ca++ flux (Li et al., 2008), cardiovascular glycosides were not able to increase the [Ca+++]i in HT29 cells. Na+/Ca++ exchanger is expressed in several tissues (Annunziato et al., 2004), and our results suggested that it may operate also in HT29 cells. Therefore it is conceivable that the intracellular peak of calcium elicited by digoxin and ouabain was dependent on the Na+/Ca++ exchanger activity.

Interestingly, two sesquiterpene lactones, artemisinin and parthenolide, also augmented the expression of Pgp in a calcium-dependent way in human colon cancer cells (Riganti et al., 2009). The calcium increase was obtained through a different mechanism (i.e. the inhibition of the sarcoplasmic/endoplasmic reticulum Ca++-ATPase) and with a different kinetics, due to the faster and more transient peak of [Ca+++]i, induced (Riganti et al., 2009). Sesquiterpene lactones have a chemical structure unrelated to cardiovascular glycosides and have a different cellular target; the only common effect exerted by both the drug families is the raising of intracellular calcium, followed by the up-regulation of mdr-1 gene. Therefore the property of inducing Pgp seems not exclusive of cardiovascular glycosides, but rather shared with many other compounds increasing the intracellular calcium.

An intracellular increase of Ca++ has pleiotropic effects on human cells. For instance, calcium may activate calmodulin and CaMKII, which is a fine sensor of the [Ca+++]i increase and a polyedric mediator of calcium signaling (Anderson, 2005). Interestingly, digoxin and ouabain enhanced the activity of the CaMKII enzyme in HT29 cells. On the contrary, KB-R7943, which abrogated the [Ca+++]i increase, completely prevented the calcium glycoside effect, and was as
effective as the CaMII inhibitor KN93. The inhibition of Na\(^+\)/K\(^-\) ATPase pump by ouabain has been reported to modulate the gene transcription in cardiomyocytes in a Ca\(^++\)- and CaMII-dependent manner (Huang et al., 1997), but to our knowledge the molecular basis of this event is still unknown. Acting as a serine–threonine kinase, CaMII can modulate the activity of several enzymes and transcription factors, including HIF-1\(\alpha\) (Yuan et al., 2005; Zhu et al., 2003). It has been suggested that CAMKI increases the HIF-1\(\alpha\) amount with an indirect mechanism, i.e. by activating calcineurin, which is critical to prevent the degradation of HIF-1\(\alpha\) (Liu et al., 2007). In addition, when phosphorylated on serine by different kinases, HIF-1\(\alpha\) is more stable (O’Donnell et al., 2006; Sodhi et al., 2001). We thus hypothesize that CaMII may directly phosphorylate and activate HIF-1\(\alpha\) in HT29 cells, as occurred in a rat pheochromocytoma cell line (Yuan et al., 2005).

HIF-1\(\alpha\) was undetectable and not phosphorylated in untreated HT29 cells, whereas became phosphorylated on serine in the presence of digoxin and ouabain, which enhanced the CaMII activity. In parallel the amount and the nuclear translocation of HIF-1\(\alpha\) were increased. Again these events were abolished by KB-R7943, which prevented the CaMII activation by calcium, and by KN93, which directly inhibited the CaMII enzyme. Our results suggest that cardioactive glycosides activate HIF-1\(\alpha\) in human colon cells with a Na\(^+\)/Ca\(^++\) exchanger- and CaMII-dependent mechanism, and that the phosphorylation by CaMII stabilizes HIF-1\(\alpha\) and promotes its nuclear translocation. In human hepatoblastoma Hep3B cells and in human prostate cancer PC3 cells, which had a constitutively active HIF-1\(\alpha\) also under normoxia, nanomolar concentrations of digoxin and ouabain reduced the amounts of HIF-1\(\alpha\), by affecting the mRNA translation; this effect was evident in hypoxia, but less clear in normoxia and in cells transfected with an HIF-1\(\alpha\) expression vector, probably because under these conditions tumor cells had a different regulation of HIF-1\(\alpha\) synthesis (Zhang et al., 2008). The differences in the drugs concentration, in the experimental conditions (normoxia vs hypoxia) and in the cell lines (HT29 cells are devoid of a constitutive expression of HIF-1\(\alpha\)) may account for the discrepancies with our results. It is a quite common evidence that cardiac glycosides display opposite effects in different experimental models, as a consequence of their pleiotropic effects on cell metabolism (Prassas and Diamandis, 2008; Schoner and Scheiner-Bobis, 2007). Moreover HIF-1\(\alpha\) may be variably regulated at several levels and by several factors (O’Donnell et al., 2006; Sodhi et al., 2001): the prevalence of either factor accounts for the cells amount and activity of HIF-1\(\alpha\). In our experimental model, digoxin and ouabain control the intracellular calcium levels and the CaMII activity, two critical factors which promote HIF-1\(\alpha\) activation in HT29 cells (Riganiti et al., 2009).

A hypoxia responsive element, which binds HIF-1\(\alpha\), has been identified in the promoter of mdr-1 gene (Comerford et al., 2002). The activation of HIF-1\(\alpha\) may explain why the inner core of solid tumors, which are frequently hypoxic and contain high amounts of HIF-1\(\alpha\), often express Pgp and are resistant to chemotherapy (O’Donnell et al., 2006). In HT29 cells the enhanced expression of Pgp induced by digoxin and ouabain correlated with the activation of HIF-1\(\alpha\). Indeed in the presence of the HIF-1\(\alpha\) inhibitor YC-1, the cardioactive glycosides lose their ability to up-regulate the mdr-1 gene. Also when the nuclear translocation of HIF-1\(\alpha\) was prevented by the co-incubation with KB-R7943 or KN93, neither Pgp mRNA nor Pgp protein expression were detectable. When highly expressed, Pgp extrudes more actively its substrates, limiting their intracellular accumulation. Digoxin itself is more actively transported out of the human colon T84 cells, as a consequence of the increase of Pgp (Haslam et al., 2008). As an index of Pgp activity we measured the intracellular accumulation of doxorubicin, an anthracycline which is widely used in clinical protocols and often represents the first-line therapy against solid and hematological malignancies (Cortes-Funes and Coronado, 2007). Digoxin and ouabain strongly reduced the doxorubicin intracellular content, and transformed the HT29 cells, which are sensitive to the pro-apoptotic effect of doxorubicin, in cells resistant to the drug. However the resistance was corrected by KB-R7943 or KN93, which restored both the intracellular accumulation and the cytotoxicity of doxorubicin.

In summary, our work puts light on the molecular mechanism by which cardioactive glycosides increase Pgp expression, suggesting that the raising levels of [Ca\(^++\)]\(_i\), due to the inward flux through the Na\(^+\)/Ca\(^++\) exchanger, activate the CaMII enzyme. CaMII in turn may favor the activation of HIF-1\(\alpha\), which is responsible for the increasing expression and activity of Pgp. We obtained superimposable results in the human liver cancer HepG2 cell line (data not shown), suggesting that digoxin and ouabain induce Pgp expression in human colon and liver cells with a common mechanism.

Our findings might have physiopathological consequences. The number of patients taking digoxin for heart disease is high, and clinicians should take into account that the absorption and systemic distribution of other drugs may be impaired in these patients, because of the digoxin-induced expression of Pgp in colon and liver. Moreover, cardioactive glycosides have pro-apoptotic effects in several solid tumor cell lines (Raghavendra et al., 2007; Winnicka et al., 2008) and enhance the damages induced by radiotherapy in transformed cells (Nasu et al., 2002). For these reasons they have recently proposed as potential adjuvant drugs in cancer therapy (Prassas and Diamandis, 2008; Zhang et al., 2008). Unfortunately, many anticancer drugs used in chemotherapy protocols are substrates of Pgp. At the light of our results we hypothesize that the co-administration of digoxin might increase the efflux of chemotherapeutic agents from tumor cells. The effects of the interaction between doxorubicin and cardioactive glycosides on cardiomyocytes have been sometimes contradictory: for example, the chronic administration of doxorubicin plus digoxin in New Zealand rabbits reduces their overall survival when compared to the administration of doxorubicin alone (Reeves et al., 1990); on the contrary other works have shown that cardioactive glycosides prevent the acute myocardial damage in animals and humans (Somberg et al., 1978; Whittaker and Al-Ismail, 1984). Interestingly such a protective effect of digoxin owes to the reduced doxorubicin uptake in cardiomyocytes (Somberg et al., 1978), a finding in keeping with our results on colon and liver cancer cells. This variability of effects might depend on the different species evaluated, on the parameters considered to assess the cell damage and on the different protocols of drugs administration. Therefore future in vitro and in vivo studies seem mandatory to better elucidate the effects of cardioactive glycosides on chemotherapy efficacy in cancer cells, as well as on chemotherapy-dependent side effects in non transformed tissues.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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