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Two repeated low doses of doxorubicin are more effective than a single high dose against tumors overexpressing P-glycoprotein

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Abstract

Standard chemotherapeutic protocols, based on maximum tolerated doses, do not prevent nor overcome chemoresistance caused by the efflux transporter P-glycoprotein (Pgp). We compared the effects of two consecutive low doses versus a single high dose of doxorubicin in drug-sensitive Pgp-negative and drug-resistant Pgp-positive human and murine cancer cells.

Two consecutive low doses were significantly more cytotoxic *in vitro* and *in vivo* against drug-resistant tumors, while a single high dose failed to do so. The greater efficacy of two consecutive low doses of doxorubicin could be linked to increased levels of intracellular reactive oxygen species. These levels were produced by high electron flux from complex I to complex III of the mitochondrial respiratory chain, unrelated to the synthesis of ATP. This process induced mitochondrial oxidative damage, loss of mitochondrial potential and activation of the cytochrome c/caspase 9/caspase 3 pro-apoptotic axis in drug-resistant cells.

Our work shows that the “apparent” ineffectiveness of doxorubicin against drug-resistant tumors overexpressing Pgp can be overcome by changing the timing of its administration and its doses.

Keywords

Doxorubicin, P-glycoprotein, mitochondria, reactive oxygen species

Abbreviations

Pgp, P-glycoprotein; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase; ROS, reactive oxygen species; DCFDA-AM, 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester; TBARS,

thiobarbituric acid reactive substances; RLU, relative light unit; RFU, relative fluorescence unit; NO, nitric oxide; NOS, nitric oxide synthase; ANOVA, analysis of variance.

1. Introduction

Standard chemotherapeutic protocols are based on maximum tolerated doses of chemotherapeutic drugs: despite often good therapeutic efficacy, this approach causes side effects and will not prevent the development of chemoresistance [1]. Alternative approaches are based on the repeated administration and/or continuous infusion of low doses of chemotherapeutic drugs ("metronomic chemotherapy"). The advantages of these approaches include: more favorable pharmacokinetic and pharmacodynamic profile [2], as specific target enzymes of chemotherapeutic drugs (e.g. topoisomerase II [3] and proteins of cell cycle check-points and DNA repair [4]) possess circadian rhythms; stronger anti-angiogenic effects [5, 6]; more effective activation of the host immune system against the tumor [1].

The efficacy of these alternative protocols against chemoresistant tumors is still a matter of debate. For instance, metronomic regimens are effective in patients with resistant metastatic colon cancer [7, 8]. Low nontoxic doses of sorafenib, administered with uracil and tegafur, delay the onset of resistance to sorafenib in preclinical models of human hepatocellular carcinoma [9]. Moreover, tumors treated with metronomic chemotherapy remain sensitive to one single high dose of the same agent [10], suggesting that metronomic chemotherapy may overcome specific mechanisms of drug resistance. Opposite findings, however, have demonstrated that the prolonged administration of low doses of chemotherapeutic drugs does not protect from drug resistance, because the continuous exposure to chemotherapeutic agents up-regulates specific chemoprotective genes [11, 12] and does not destroy chemoresistant cancer stem cells [13].

A major mechanism of drug resistance is the overexpression of ATP binding cassette transporters, such as P-glycoprotein (Pgp), which pumps chemotherapeutic drugs outside the cell via ATP

hydrolysis. By doing that, Pgp limits the intracellular retention and cytotoxicity of chemotherapeutic drugs and confers a multidrug resistant phenotype to the tumor. Doxorubicin, a widely used drug in solid and hematological malignancies, is a substrate of Pgp [14].

The efficacy of alternative chemotherapeutic regimens different from standard ones based on the maximum tolerated dose of doxorubicin, is not known in Pgp-overexpressing tumors. We addressed this issue in doxorubicin-sensitive Pgp-negative and doxorubicin-resistant Pgp-positive cancer cells and found that the treatment with two repeated low doses of doxorubicin kills drug-resistant cells, by altering their redox balance and mitochondrial energy metabolism, in a different way than the treatment with a single high dose of the drug.

2. Materials and methods

2.1 Materials

The plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ). The electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). The protein content of cell lysates was assessed with the BCA kit from Sigma Chemicals Co. (St. Louis, MO). When not otherwise specified, all the other reagents were purchased from Sigma Chemicals Co.

2.2 Cells

Doxorubicin-sensitive human colon cancer HT29 cells and their doxorubicin-resistant counterpart HT29-dx cells, doxorubicin-sensitive human lung cancer A549 cells and their doxorubicin-resistant counterpart A549-dx cells were obtained as reported in [15]. HT29 and A549 cells have low/undetectable levels of Pgp, which is instead expressed in HT29-dx and A549-dx cells [15]. The mammary cancer JC cells, syngeneic with BALB/c mice, are constitutively resistant to doxorubicin and overexpress Pgp [16]. All the parental cell lines were from ATCC (Manassas, VA). HT29 and JC cells were cultured in RPMI-1640 medium, A549 cells in Ham's F12 medium, supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin-streptomycin, 1% v/v L-glutamine.

2.3 Cytotoxicity and genotoxicity assays

The extracellular release of lactate dehydrogenase (LDH), an indicator of cell damage and necrosis, was measured as detailed in [17]. The results were expressed as the percentage of extracellular LDH versus total (intracellular plus extracellular) LDH. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue) assay was used as a second test of cytotoxicity: 100,000 cells (HT29, HT29-dx, A549, A549-dx cells) or 25,000 cells (JC cells) were seeded in 24-well plates and treated as reported in the Results section, then incubated with 0.5 mg/mL of MTT for 4 h at 37°C. After removing the culture medium, the dye was solubilized with 300 µL of dimethyl sulfoxide and the absorbance was read at 570 nm, using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT). The absorbance of untreated cells was considered equal to 100% cell viability; the results were expressed as the percentage of viable cells versus untreated cells.

The genotoxic damages were evaluated by the Single Cell Gel Electrophoresis assay (Comet assay), as reported previously [18]. Images were quantified by the CometScore software (TriTek Corp., Sumerduck, VA).

2.4 Intracellular doxorubicin accumulation and localization

The intracellular doxorubicin accumulation was measured spectrofluorimetrically as described in [17]; the drug localization was evaluated by fluorescence microscope, as reported in [15].

2.5 In vivo tumor growth

1 x 10^6 JC cells, mixed with 100 µL Matrigel, were injected subcutaneously in 6-8 weeks old female BALB/c mice, housed under 12 h light/dark cycle, with food and water provided ad lib. When the tumors reached a volume of 100 mm^3, the animals were randomized to three groups: a "control" group (treated with 3 i.p. injections of saline solution every 7 days), a "single high dose" group (treated with 3 i.p. injections of 5 mg/kg doxorubicin every 7 days), and a "two repeated low dose" group (treated with 3 i.p. injections of 1 mg/kg doxorubicin every 7 days, followed by a

second dose of 1 mg/kg doxorubicin after 24 h). Tumor growth was measured daily by caliper and was calculated according to the equation $(LxW^2)/2$, where L = tumor length; W = tumor width. Animals were euthanized at day 35 after randomization. Tumors were resected, photographed and fixed in 4% v/v paraformaldehyde. Paraffin sections were stained with hematoxylin/eosin or immunostained for Ki67 (Millipore, Billerica, CA), used as an indicator of tumor cell proliferation, or cleaved caspase 3 (Asp175; Cell Signaling Technology Inc., Danvers, MA), used as an indicator of apoptosis, followed by a peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark). The intratumor accumulation of doxorubicin was measured fluorimetrically according to [19]. The mean value obtained in tumors of untreated animals was considered as background and was subtracted from the values obtained in the doxorubicin-treated animals. The results were expressed as μmol doxorubicin/mg tumor tissue proteins.

The hematochemical parameters LDH, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, creatine phosphokinase (CPK), creatinine were measured on 0.5 mL of blood collected immediately after mice sacrifice, using commercially available kits from Beckman Coulter Inc. (Brea, CA). Animal care and experimental procedures were approved by the Bio-Ethical Committee of the University of Torino, Italy.

2.6 Reactive oxygen species (ROS) measurement

1×10^6 whole cells or mitochondria, extracted as reported in [20], were re-suspended in a final volume of 0.5 mL PBS, incubated for 30 min at 37°C with 5 μmol/L of the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester (DCFDA-AM), centrifuged at 13,000 x g at 37°C and re-suspended in 0.5 mL PBS. The fluorescence of each sample, an indicator of ROS levels, was read at 492 nm (λ excitation) and 517 nm (λ emission). The results were expressed as nmol/mg cell proteins or mitochondrial proteins.

2.7 Intracellular glutathione measurement

Total glutathione was measured spectrophotometrically in cell lysates with the glutathione reductase recycling method [21]; oxidized glutathione (GSSG) was obtained after derivatization with 2-vinylpyridine [21]. For each sample, reduced glutathione (GSH) was obtained by subtracting GSSG from total glutathione. The results were expressed as percentage of GSSG/GSH ratio.

2.8 Lipid peroxidation

Lipid peroxidation was evaluated in whole cells and in mitochondria by measuring the amount of thiobarbituric acid reactive substances (TBARS), as reported [21]. The results were expressed as nmol/mg cell proteins or mitochondrial proteins.

2.9 Mitochondrial respiration and ATP synthesis

The electron transport between complex I and III was measured in mitochondrial extracts as described earlier [20]. The results were expressed as nmol reduced cytochrome c/min/mg mitochondrial proteins. The amount of ATP in mitochondria was measured with the ATP Bioluminescent Assay Kit (FL-AA, Sigma), using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek). ATP was quantified as relative light units (RLU); data were converted into nmol ATP/mg mitochondrial proteins.

2.10 Mitochondrial electric potential (Δψ) measurement

1 x 10^6 cells, re-suspended in 0.5 mL PBS, were incubated for 30 min at 37°C with 2 µmol/L of the fluorescent probe JC-1 (Biotium Inc, Hayward, CA), then centrifuged at 13,000 x g for 5 min and re-suspended in 0.5 mL PBS. The fluorescence of each sample was read using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek): the red fluorescence, index of polarized mitochondria, was detected at 550 nm (λ excitation) and 600 nm (λ emission); the green fluorescence, index of depolarized and damaged mitochondria, was detected at 485 nm (λ excitation) and 535 nm (λ emission). The fluorescence units were used to calculate the percentage of green-fluorescent vs. red-fluorescent mitochondria.

2.11 Western blot analysis

Cells were rinsed with lysis buffer (50 mmol/L Tris, 10 mmol/L EDTA, 1% v/v Triton X-100), supplemented with the protease inhibitor cocktail set III (Calbiochem, San Diego, CA) and 2 mmol/L phenylmethylsulfonyl fluoride, then sonicated and centrifuged at 13,000 x g for 10 min at 4°C. 20 μg protein extracts were subjected to SDS-PAGE and probed with the following antibodies: anti-Pgp (Calbiochem); anti-Herpud (Proteintech, Chicago, IL); anti-TRB3 (Proteintech); anti-Bip/GRP78 (Abcam, Cambridge, UK); anti-beclin (Abcam); anti-ATG12 (Abcam); anti-β-tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). 10 μg of proteins from cytosolic and mitochondrial extracts, prepared as reported in [20], were probed with an anti-cytochrome c antibody (Abcam). Anti-porin/VDAC (Abcam) and anti-β-tubulin antibodies were used to check the equal protein loading in mitochondrial and cytosolic extracts, respectively. The activation of caspase 9 and 3 was measured in the cytosolic extracts using anti-caspase 9 (GeneTex, Hsinhu City, Taiwan) and anti-caspase 3 (GeneTex) antibodies, respectively.

2.12 Pgp activity

The intracellular retention of rhodamine 123, an indicator of Pgp activity, was measured on 5 x 10^5 cells, using a FACS-Calibur flow cytometer (Becton Dickinson) equipped with the Cell Quest software (Becton Dickinson), as previously described [22]. The quantitative intracellular accumulation of rhodamine 123 was measured spectrofluorimetrically as reported in [23]. The results were expressed as relative fluorescence units (RFU)/mg cell proteins.

2.13 Nitrite and nitric oxide (NO) synthase (NOS) activity

The level of nitrite, the stable derivative of NO, in cell culture supernatants, and the activity of NOS in cell lysates were measured spectrophotometrically as described earlier [17].

2.14 NADPH oxidase activity

NADPH oxidase activity was evaluated by a chemiluminescence-based assay [24]. The results were expressed as RLU/mg cell proteins.

2.15 Data analysis and statistical procedure

All data in text and figures are provided as means ± SD. The results were analyzed by one-way analysis of variance (ANOVA) and Tukey's test. $p<0.05$ was considered significant.

3. Results

3.1 Two repeated low doses of doxorubicin are more cytotoxic than a single high dose in drug-resistant tumors

We compared the cytotoxicity induced by two repeated low doses of doxorubicin (1 μmol/L with a 24 h interval between them) and a single high dose of doxorubicin (5 μmol/L for 48 h) in human drug-sensitive colon cancer HT29 cells and in their resistant counterpart HT29-dx cells (Figure 1A), in human drug-sensitive lung cancer A549 cells and in their resistant counterpart A549-dx cells (Figure 1B), and in constitutively resistant murine mammary cancer JC cells (Figure 1C). The release of LDH was measured 48 h after the first (or sole) drug administration. In doxorubicin-sensitive cells, a single high dose treatment was more cytotoxic than two repeated low dose treatments (Figure 1A-B); by contrast, in all the drug-resistant cell populations two repeated low dose treatments were significantly more cytotoxic than a single high dose-treatment (Figure 1A-C). This behavior was confirmed in the same cell lines and experimental conditions by the MTT assay (Supplementary Figure 1A-C).

Similar results were obtained by comparing two repeated doses of 1 μmol/L doxorubicin with a 24 h interval between them versus a single dose of 2.5 μmol/L for 48 h (data not shown).

The amount of intracellular doxorubicin in HT29 and A549 cells was higher after a single high dose than after two repeated low doses (Figure 1D-E). As expected, in HT29-dx and A549-dx cells the amount of doxorubicin was lower than in the corresponding drug-sensitive cells, under each experimental condition (Figure 1D-E). Similarly, the constitutively drug-resistant JC cells showed very low amount of doxorubicin (Figure 1F). The intracellular levels of doxorubicin in the resistant cells was the same after a single high dose or two repeated low doses, regardless of the drug's

cytotoxicity (Figure 1D-F), suggesting that the different degree of cytotoxicity was not related to… different levels of doxorubicin.

As shown by tumor growth and pathology (Figure 2A and Table 1), JC tumors implanted in immunocompetent BALB/c mice were insensitive to a standard chemotherapy regimen, based on the weekly administration of a single high dose of doxorubicin. As it occurred in JC cells cultures (Figure 1F), the intratumor accumulation of doxorubicin did not differ between the animals treated weekly with a single high dose and the animals treated with two repeated low doses (Figure 2B). The treatment with a single dose did not alter the percentage of Ki67-positive proliferating cells and the percentage of cells with activated caspase 3 (Figure 2C), even though we used a dose of doxorubicin higher than the one effective against mammary doxorubicin-sensitive tumors [25, 26]. Surprisingly, the treatment with two repeated low doses reduced tumor growth (Figure 2A and Table 1) and cell proliferation (Figure 2C), and strongly increased intratumor apoptosis (Figure 2C). It is worth noting that the treatment with two repeated low doses of doxorubicin was not more toxic for liver, heart and kidney than the treatment with one single high dose, as suggested by the hematochemical parameters of the animals (Table 2).

3.2 Two repeated low doses of doxorubicin induce an oxidative stress-mediated cytotoxicity in drug-resistant cancer cells

We next set out to investigate why two repeated low doses were more cytotoxic than a single high dose in drug resistant cells. We concentrated on the JC model, which has the strongest resistance to doxorubicin (Figure 1). The higher cytotoxicity of the two repeated low dose treatments was not caused by decreased expression (Supplementary Figure 2A) or activity (Supplementary Figure 2B-C) of Pgp, by different intracellular localization (Supplementary Figure 3A) or genotoxicity (Supplementary Figure 3B) of doxorubicin, by the increase of NO (Supplementary Figure 4), a mediator of doxorubicin's antitumor activity [27], or by the induction of cell death through endoplasmic reticulum stress-mediated mechanisms or autophagy (Supplementary Figure 5).

Interestingly, the two repeated low dose treatments produced significantly higher levels of ROS than a single high dose treatment (Figure 3A). The higher amount of ROS was paralleled by increased GSSG/GSH ratio (Figure 3B) and lipid peroxidation (Figure 3C). The ROS scavenger Tempol – that decreases the doxorubicin-induced ROS production – significantly reduced the release of extracellular LDH induced by the two repeated low dose treatments (Figure 3D), suggesting that the increased ROS were causing the cytotoxic effects of this regimen. In order to investigate the source of ROS produced by the two repeated low dose treatment, we treated JC cells with the inhibitor of NADPH oxidase apocynin and with the inhibitor of mitochondrial complex I rotenone. Apocynin was used at a concentration enough to reduce significantly the NADPH oxidase activity (Supplementary Figure 6); rotenone was used at a concentration enough to lower mitochondrial electron flux significantly (Supplementary Figure 7). Under our experimental conditions, both compounds did not increase the intracellular ROS and were not toxic when used alone (Figure 3D). Rotenone, but not apocynin, prevented the increase of ROS levels and the release of LDH elicited by two repeated low doses of doxorubicin (Figure 3D). This result suggests that the mitochondria are the source of the ROS produced by the two repeated low dose treatment.

3.3 Two repeated low doses of doxorubicin impair the mitochondrial metabolism and activate the cytochrome c/caspase 9/caspase 3 axis in drug-resistant cancer cells

The treatment with a single high dose of doxorubicin did not modify the electron flux between complex I and complex III of the mitochondrial respiratory chain compared to untreated JC cells (Figure 4A). By contrast, the treatment with two repeated low doses accelerated the electron flux, both in the absence of rotenone (i.e. the electron flux from complex I to complex III) and in the presence of rotenone (i.e. the electron flux from complex II to complex III; Figure 4A). Differently from a single high dose, the two repeated low doses increased mitochondrial ROS and lipid peroxidation (Figure 4B), produced a significant mitochondrial depolarization and decreased the synthesis of mitochondrial ATP (Figure 4C). As a result, this treatment induced the release of

cytochrome c into the cytosol and the cleavage of caspase 9 and caspase 3 (Figure 4D), suggesting the activation of a mitochondrial-dependent apoptotic machinery.

4. Discussion

In our research, we compared the efficacy of a single high dose versus two repeated low doses of doxorubicin in human and murine drug-resistant cancer cells expressing Pgp. We found that the two repeated low doses were significantly more cytotoxic *in vitro* and *in vivo* against resistant tumors, while standard treatment – that is the weekly administration of a single high dose – failed. The two repeated low doses did not elicit greater side effects on the liver, heart and kidney – according to the animals' hematochemical parameters – when compared to the standard protocol.

Our results are consistent with previous findings that showed that standard doxorubicin regimens had greater side effects and lower antitumor activity than metronomic protocols with appropriate doxorubicin dosage and scheduling intervals [28]. Similarly, it has been reported that low doses of liposomal doxorubicin produced greater clinical benefits in patients with breast metastatic cancers than standard chemotherapy protocols based on maximum tolerated doses [29, 30]. In partial contrast with our data, repeated low doses of doxorubicin did not significantly reduce the growth of Pgp-positive human breast tumors and were effective only if associated with a VEGF receptor II inhibitor [31]. Since we found that the VEGF receptor II inhibitor reduced the tumor growth also when used alone, it is conceivable that the inhibition of angiogenesis – more than a doxorubicin-elicited cytotoxic effect – might be the main anti-tumor mechanism.

Intriguingly, the higher efficacy of the two repeated low dose treatment was not caused by higher intracellular accumulation of doxorubicin, as drug levels both in *in vitro*-cultured JC cells and in JC tumors implanted in BALB/c mice was the same after two repeated low doses or a single high dose. In JC tumors the two repeated low dose treatment reduced cell proliferation and increased the cleavage of caspase 3, suggesting that it activated the pro-apoptotic machinery in resistant cells.

To explain the biochemical mechanisms involved in the different efficacy of the two treatments, we used JC cells, highly resistant to doxorubicin. We observed a significant increase of intracellular ROS levels after the two repeated low doses, paralleled by typical signs of oxidative damages, such as a decrease in GSH levels and increased lipid peroxidation. The greater cytotoxicity of the two repeated low dose treatment was caused by the higher production of intracellular ROS, since it was cancelled by the ROS scavenger Tempol. Doxorubicin produces ROS because of its reduction to semiquinone radicals that generate $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, the activation of NADPH oxidase, and the genesis of redox cycles through iron-catalyzed mechanisms and the complex I of the mitochondrial respiratory chain [32]. The results of our experiments with rotenone suggest that the two repeated low doses increase ROS by modulating the activity of the mitochondrial respiratory chain. Mitochondria generate ROS mainly via complexes I and III: a high proton motive force induces a high production of ROS [33]. Interestingly, two repeated low doses of doxorubicin – differently than a single high dose – accelerated the electron flux without increasing the synthesis of ATP. This process was accompanied by increased intramitochondrial ROS and lipid peroxidation. It has been reported that doxorubicin inhibits complexes I, II and III at concentrations higher than 25 µmol/L [34], while it increases the electron flux and decreases the mitochondrial ATP at lower concentrations (i.e. 0.1-0.2 µmol/L) [35]: this observation is consistent with our results in JC cells treated with two repeated low doses of the drug. Since the two repeated low dose treatment increased the electron flux from complex I to complex III, as well as from complex II to complex III, we hypothesize that redox cycles occurring at complex I or complex III are responsible for the production of ROS. The uncoupling between electron flux and ATP synthesis induced by the two repeated low dose treatment damaged mitochondria, opened the membrane permeability transition pore, released the cytochrome c in the cytosol and activated the caspases 9 and 3. These events are suggestive of a necro-apoptotic cell death [36].

Interestingly, we observed the benefits of the two repeated low dose treatment in different doxorubicin-resistant cancer cells, regardless of the species and histological origin, but not in doxorubicin-sensitive cells. Resistant tumors have lower respiratory activity, lower mitochondrial membrane potential and lower ATP synthesis than drug sensitive tumors. These metabolic features determine a lower basal level of ROS, a factor that contributes to chemoresistance [37]. By accelerating the electron flux and keeping the synthesis of ATP low, the two repeated low dose treatment widely altered this balance, increased the amount of ROS and amplified the ROS-mediated cytotoxic effects in drug resistant cells. Indeed, it has been demonstrated that multidrug resistant cells have a paradoxical sensitivity to agents increasing ROS levels and lowering cellular ATP, an event termed "collateral sensitivity" [38]. Compounds increasing ROS and lowering ATP are excellent inducers of collateral sensitivity against drug-resistant cells *in vitro*, but they are so toxic that they cannot be used *in vivo*. Our work shows that doxorubicin, which is already used in clinical practice, may induce collateral sensitivity *in vitro* and *in vivo*.

Since doxorubicin is a typical substrate of Pgp, its efficacy in tumors expressing this transporter is severely impaired [14]. Our results demonstrate that doxorubicin efficacy is rescued in Pgp-expressing tumors by changing doses and schedule of administration: by targeting the redox balance and the mitochondrial energy metabolism, this approach may hit a potential "Achille's heel" of the resistant cells and may be noteworthy of future clinical evaluation against doxorubicin unresponsive tumors.

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Figure captions

Figure 1. Two repeated low doses of doxorubicin are more cytotoxic than a single high dose in drug resistant cancer cells

A-B-C. Doxorubicin-sensitive human colon cancer HT29 cells and doxorubicin-resistant HT29-dx cells (panel **A**), doxorubicin-sensitive human lung cancer A549 cells and doxorubicin-resistant A549-dx cells (panel **B**), constitutively doxorubicin-resistant murine mammary cancer JC cells (panel **C**) were either left untreated (0) or incubated with doxorubicin (doxo) in the following conditions: 1 μmol/L followed by another dose of 1 μmol/L L after 24 h [1(x2): "two repeated low dose treatment"], or 5 μmol/L (5: "single high dose treatment"). The release of extracellular LDH, a marker of cytotoxicity, was measured in duplicate after 48 h from the first (or sole) drug administration. Data are presented as means $\pm$ SD (n=4). Significance of treated versus respective untreated (0) cells: * $p< 0.01$; doxo 5 versus 1(x2) in the same cell type: ° $p < 0.01$. **D-E-F**. Cells were incubated with doxorubicin as reported above. The intracellular drug accumulation was measured fluorimetrically in duplicate. Data are presented as means $\pm$ SD (n=4). Significance of HT29-dx versus HT29, A549-dx versus A549: * $p< 0.02$; doxo 5 versus 1(x2) in the same cell type: ° $p < 0.01$.

Figure 2. Two repeated low doses of doxorubicin reduce the growth of drug-resistant tumors

A. JC cells were inoculated subcutaneously in immunocompetent BALB/C mice; animals were randomized in three groups when tumors reached the volume of 100 mm^3: at day 14, 21 and 28 (arrows) after randomization, the animals were injected i.p. with saline buffer (ctrl group), 5 mg/kg doxorubicin (doxo 5: "single high dose treatment" group), 1 mg/kg doxorubicin followed by a second dose of 1 mg/kg doxorubicin after 24 h [i.e. at day 15, 22, 29; doxo 1 (x2): "two repeated low dose treatment" group]. Left panel: measurement of tumor growth, until the animals were sacrificed at day 35 (n=5 animals for each treatment group; the experiment was repeated 3 times).

Significance of [doxo 1 (x2)]-group versus the other groups: * p< 0.05. Right panel: representative photos of tumors from each treatment group at day 35. **B.** Intratumor amount of doxorubicin measured in 5 animals of each group. Data are presented as means ± SD. **C.** Left panels: representative histology staining with hematoxylin/eosin (HE), with the antibodies for Ki67, chosen as proliferation marker, and for cleaved caspase 3, chosen as apoptotic marker. Bars = 100 μm for hematoxylin/eosin and Ki67; 50 μm for caspase 3. Right panels: quantification of Ki67 and caspase-3 positive cells. The percentage of Ki67-positive cells was determined by calculating the ratio between Ki67-positive nuclei and hematoxylin-positive nuclei, in sections from 5 animals of each group (106-94 nuclei/field). The percentage of caspase 3-positive cells was determined by analyzing sections from 5 animals of each group (107-96 cells/field), using Photoshop program. Ctrl group intensity was considered 100%. Significance versus ctrl group: * p < 0.005.

Figure 3. Two repeated low doses of doxorubicin induce ROS-mediated cytotoxicity in drug-resistant cells

Doxorubicin resistant JC cells were grown in fresh medium (0), treated with 5 μmol/L doxorubicin (doxo 5: "single high dose treatment") or with 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h [doxo 1(x2): "two repeated low dose treatment"], then subjected to the following tests 48 h after the first drug administration. **A**. ROS levels were measured fluorimetrically in triplicate using the DCFDA-AM probe. Data are presented as means ± SD (n=3). Significance versus untreated (0) cells: * p< 0.005. **B.** GSH and GSSG were measured spectrophotometrically in triplicate in the cell lysates. Data are presented as means ± SD (n=3) of the percentage of GSSG/GSH ratio. Significance versus untreated (0) cells: * p< 0.001. **C.** The amount of TBARS, indicators of lipid peroxidation, was detected in triplicate in the cell lysates by a spectrophotometric assay. Data are presented as means ± SD (n = 3). Significance versus untreated (0) cells: * p< 0.05. **D.** The amount of ROS in the cell lysates (open bars) and the release of LDH in

the extracellular medium (hatched bars) were measured in cells cultured for 48 h in fresh medium only (ctrl), treated with 1 µmol/L doxorubicin followed by a second dose of 1 µmol/L after 24 h (doxo), with the ROS scavenger Tempol (200 µmol/L for the last 24 h; tem), with 1 µmol/L doxorubicin followed by a second dose of 1 µmol/L after 24 h plus 200 µmol/L Tempol for the last 24 h (doxo + tem), with the NADPH oxidase inhibitor apocynin (10 µmol/L for the last 24 h; apo), with 1 µmol/L doxorubicin followed by a second dose of 1 µmol/L after 24 h plus 10 µmol/L apocynin in the last 24 h (doxo + apo), with the mitochondrial complex I inhibitor rotenone (50 µmol/L for the last 24 h; rot), with 1 µmol/L doxorubicin followed by a second dose of 1 µmol/L after 24 h plus 50 µmol/L rotenone in the last 24 h (doxo + rot). Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 3). Significance versus untreated (ctrl) cells: * $p < 0.001$; doxo + tem/doxo + rot versus doxo: ° $p < 0.001$.

Figure 4. Two repeated low doses of doxorubicin uncouple mitochondrial electron flux from ATP synthesis, and activate the cytochrome c/caspase 9/caspase 3 axis in drug-resistant cells

Doxorubicin resistant JC cells were grown for 48 h in fresh medium (0), treated with 5 µmol/L doxorubicin (doxo 5: “single high dose treatment”) or with 1 µmol/L doxorubicin followed by a second dose of 1 µmol/L after 24 h [1(x2): “two repeated low dose treatment”], then cytosolic and mitochondrial extracts were prepared. **A.** The rate of electron flux from complex I to complex III (open bars) was evaluated in isolated mitochondria as described under Materials and methods. The rate of electron flux from complex II to complex III (hatched bars) was measured in the same samples, by adding rotenone (50 µmol/L) to mitochondria suspensions 1 minute after the assay began. Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 4). Significance versus untreated (0) cells: * $p < 0.05$. **B.** ROS (open bars) and TBARS (hatched bars) amounts were detected in triplicate in isolated mitochondria. Data are presented as means $\pm$ SD (n = 4). Significance versus untreated (0) cells: * $p < 0.02$. **C.** The mitochondrial membrane potential

(open bars) was assessed by the JC-1 staining method. The percentage of green versus red mitochondria was considered a marker of mitochondrial depolarization and permeability transition. ATP (hatched bars) was measured in triplicate in the mitochondrial extracts by a chemiluminescence-based assay. Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 4). Significance versus untreated (0) cells: * $p < 0.05$. **D.** Left panel: cytosolic and mitochondrial fractions were subjected to Western blotting analysis for cytochrome c. The porin and β-tubulin expression was used as controls of equal protein loading for each fraction. Each immunoblot is representative of three independent experiments. Right panel: cytosolic extracts were resolved by SDS-PAGE and immunoblotted with specific antibodies for caspase 9 and caspase 3 (recognizing both pro-caspase and cleaved active caspase), and for β-tubulin (to check the equal protein loading). The figure is representative of three independent experiments.

Table 1. Measurement of animal weight, tumor weight and tumor growth inhibition

	ctrl	single high dose treatment	two repeated low dose treatment
Final mice weight (g)	21.21 $\pm$ 1.23	21.38 $\pm$ 1.56	20.38 $\pm$ 1.09
Final tumor weight (g)	4.79 $\pm$ 0.57	4.42 $\pm$ 0.81	1.53 $\pm$ 0.51
% inhibition rate		8.37%	68.06% *

The animals (n = 15/group) were treated as reported under Materials and methods. The percentage of inhibition rate was calculated as follows: (average tumor weight of control group - average tumor weight of test group)/average tumor weight of control group×100. Significance versus ctrl group: *$p < 0.001$.

Table 2. Hematochemical parameters of animals

	ctrl	**single high dose treatment**	**two repeated low dose treatment**
LDH **(U/L)**	6342.8 ± 1892.1	7461.4 ± 2002.6	6987.1 ± 1672.9
AST (U/L)	280.7 ± 91.9	295.7 ± 91.7	302.2 ± 75.4
ALT (U/L)	21.7 ± 2.9	19.1 ± 4.0	22.3 ± 2.8
Alkaline phosphatase (U/L)	71 ± 13	61 ± 12	59 ± 14
CPK (U/L)	474 ± 166	781 ± 227	687 ± 216
creatinine (mg/L)	0.037 ± 0.002	0.035 ± 0.003	0.032 ± 0.008

The animals (n = 15/group) were treated as reported under Materials and methods, and sacrificed at day 35 after randomization. Blood was collected immediately after euthanasing the animals.

Figure 1

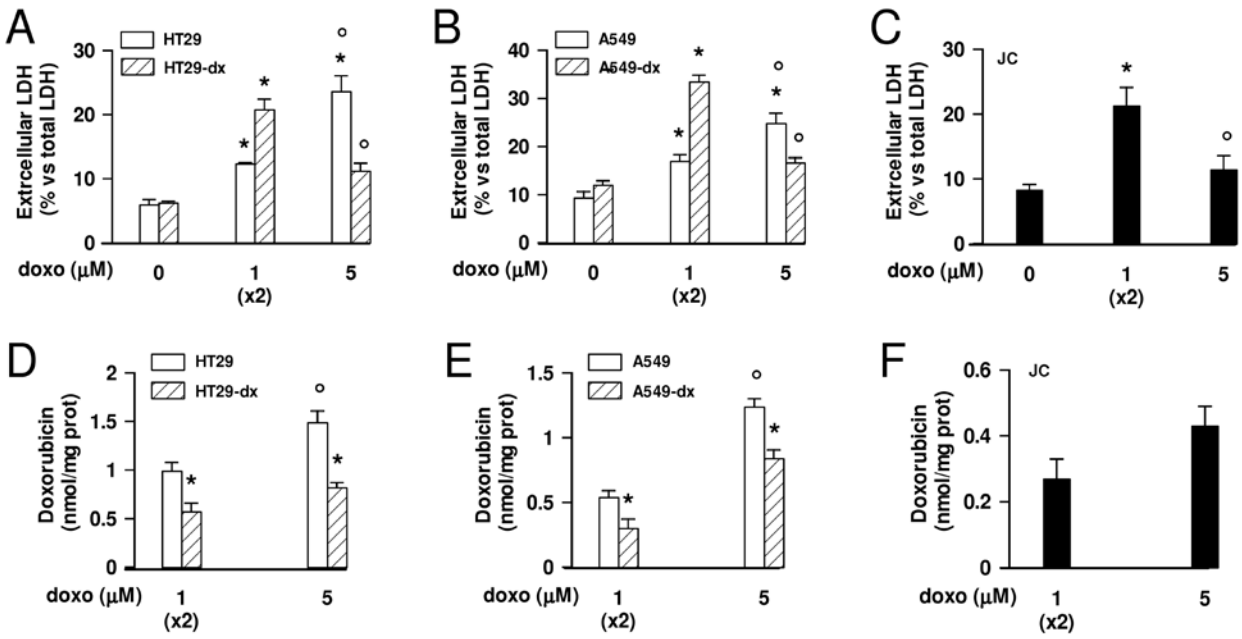

A
HT29
HT29-dx
Extrcellular LDH (% vs total LDH)
30
20
10
0
doxo (μM)
0
1
(x2)
5
B
A549
A549-dx
Extrcellular LDH (% vs total LDH)
40
30
20
10
0
doxo (μM)
0
1
(x2)
5
C
JC
Extrcellular LDH (% vs total LDH)
30
20
10
0
doxo (μM)
0
1
(x2)
5
D
HT29
HT29-dx
Doxorubicin (nmol/mg prot)
2
1.5
1
0.5
0
doxo (μM)
1
(x2)
5
E
A549
A549-dx
Doxorubicin (nmol/mg prot)
1.5
1
0.5
0
doxo (μM)
1
(x2)
5
F
JC
Doxorubicin (nmol/mg prot)
0.6
0.4
0.2
0
doxo (μM)
1
(x2)
5

Figure 2

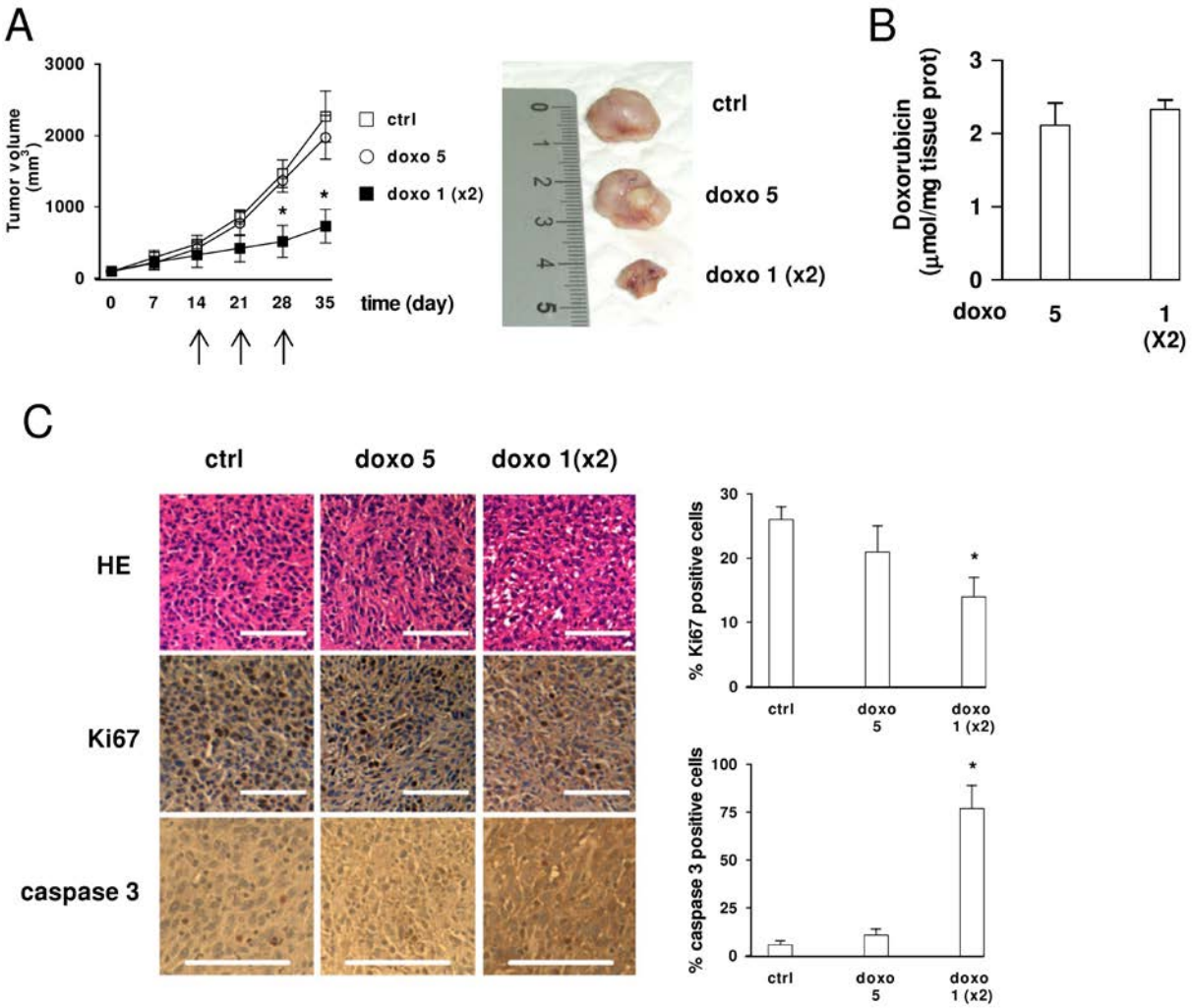

A
Tumor volume (mm3)
3000
2000
1000
0
0 7 14 21 28 35
time (day)
ctrl
doxo 5
doxo 1 (x2)
*
*
ctrl
doxo 5
doxo 1 (x2)
B
Doxorubicin (µmol/mg tissue prot)
3
2
1
0
doxo 5 1 (X2)
C
ctrl
doxo 5
doxo 1(x2)
HE
Ki67
caspase 3
% Ki67 positive cells
30
20
10
0
ctrl
doxo 5
doxo 1 (x2)
*
% caspase 3 positive cells
100
75
50
25
0
ctrl
doxo 5
doxo 1 (x2)
*

Figure 3

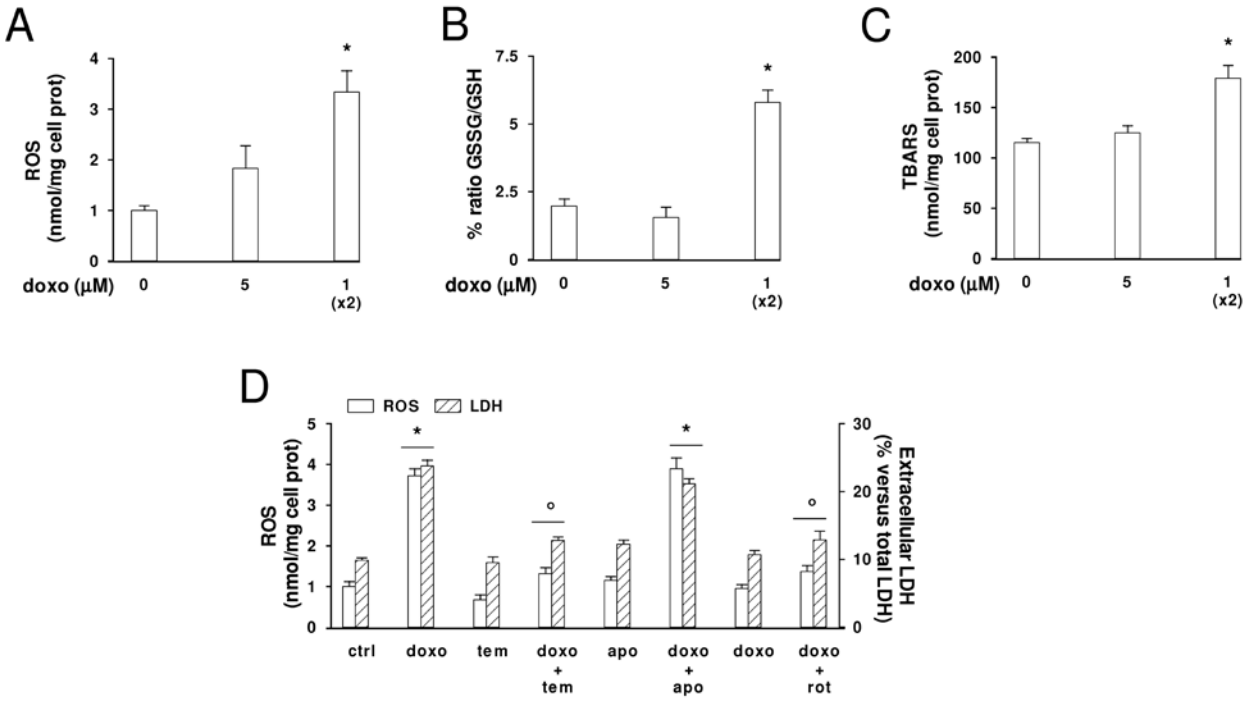
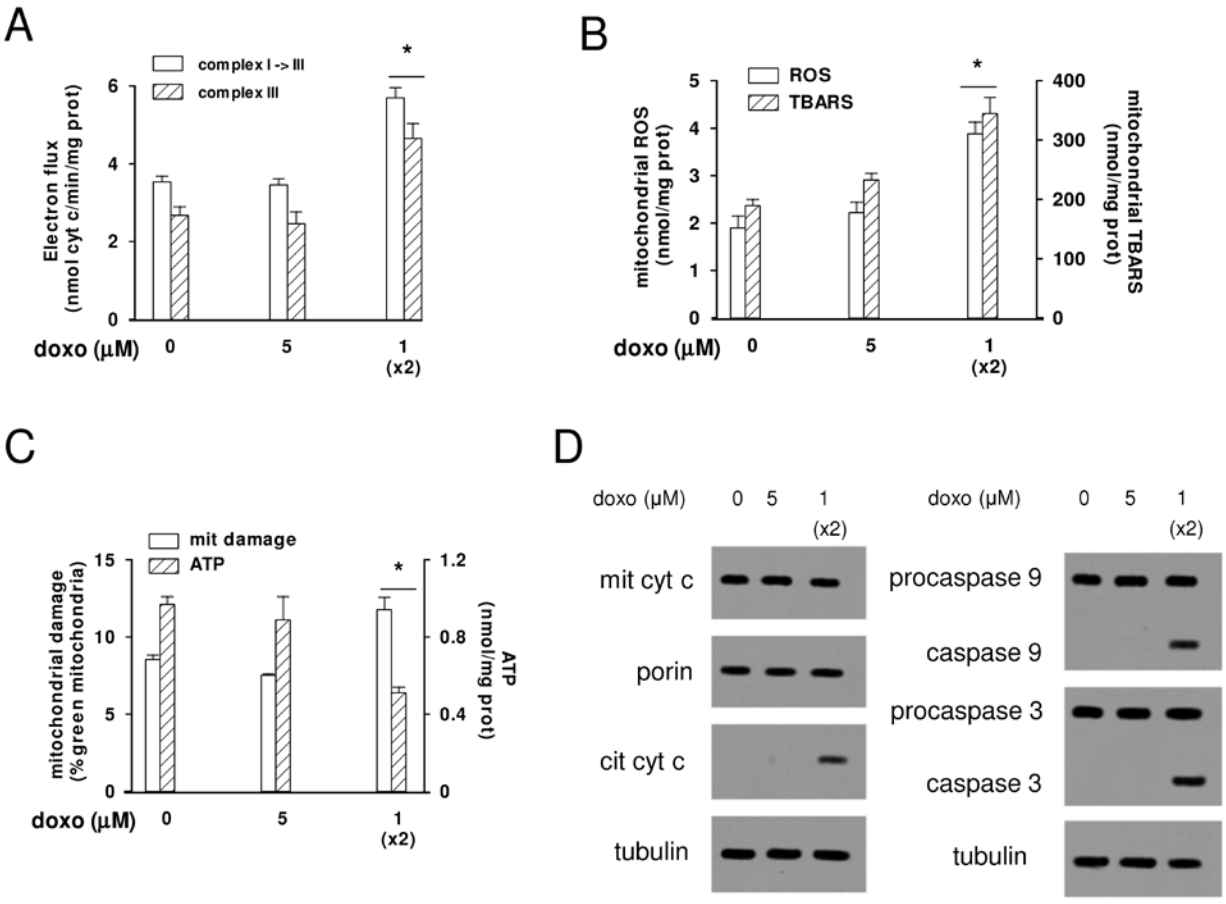
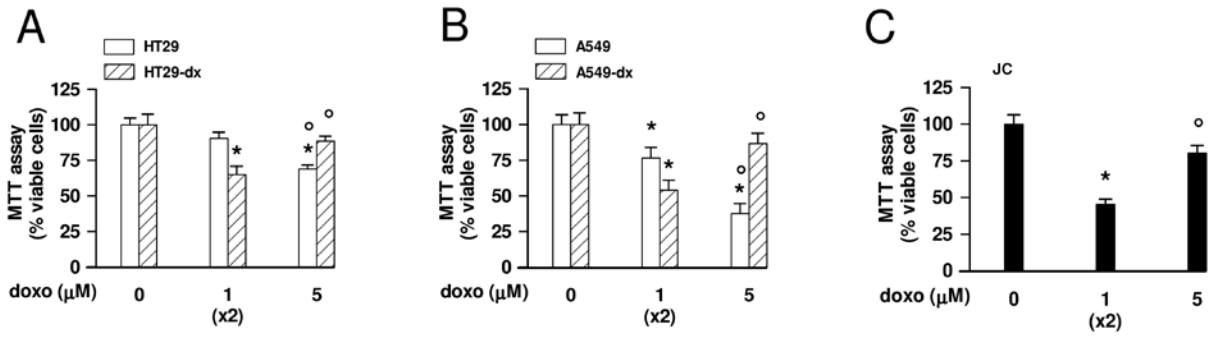

A
ROS
(nmol/mg cell prot)
4
3
2
1
0
*
doxo (µM) 0 5 1 (x2)
B
% ratio GSSG/GSH
7.5
5
2.5
0
*
doxo (µM) 0 5 1 (x2)
C
TBARS
(nmol/mg cell prot)
200
150
100
50
0
*
doxo (µM) 0 5 1 (x2)
D
ROS
LDH
ROS
(nmol/mg cell prot)
Extracellular LDH
(% versus total LDH)
ctrl
doxo
tem
doxo + tem
apo
doxo + apo
doxo
doxo + rot

Figure 4

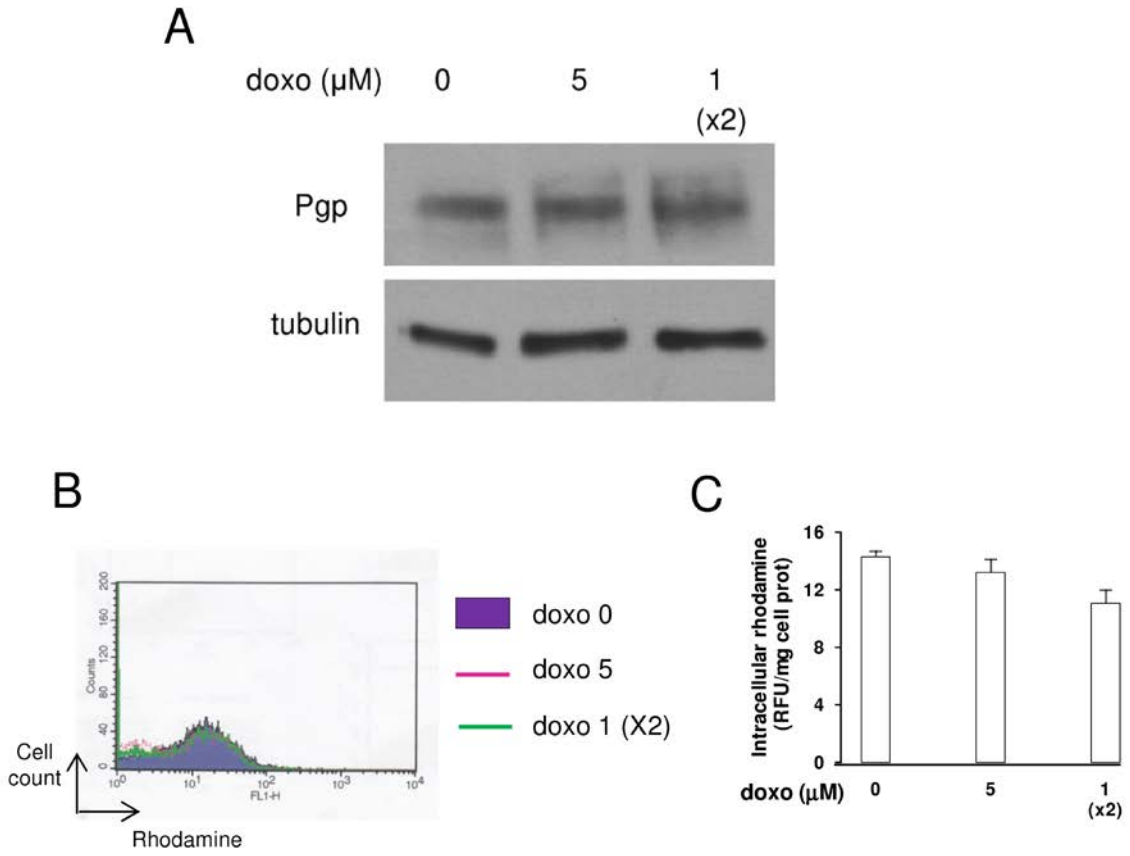

A
complex I -> III
complex III
Electron flux
(nmol cyt c/min/mg prot)
doxo (µM)
0
5
1
(x2)
*
B
ROS
TBARS
mitochondrial ROS
(nmol/mg prot)
mitochondrial TBARS
(nmol/mg prot)
C
mit damage
ATP
mitochondrial damage
(% green mitochondria)
ATP
(nmol/mg prot)
D
doxo (µM)
mit cyt c
porin
cit cyt c
tubulin
procaspase 9
caspase 9
procaspase 3
caspase 3

Supplementary Figures



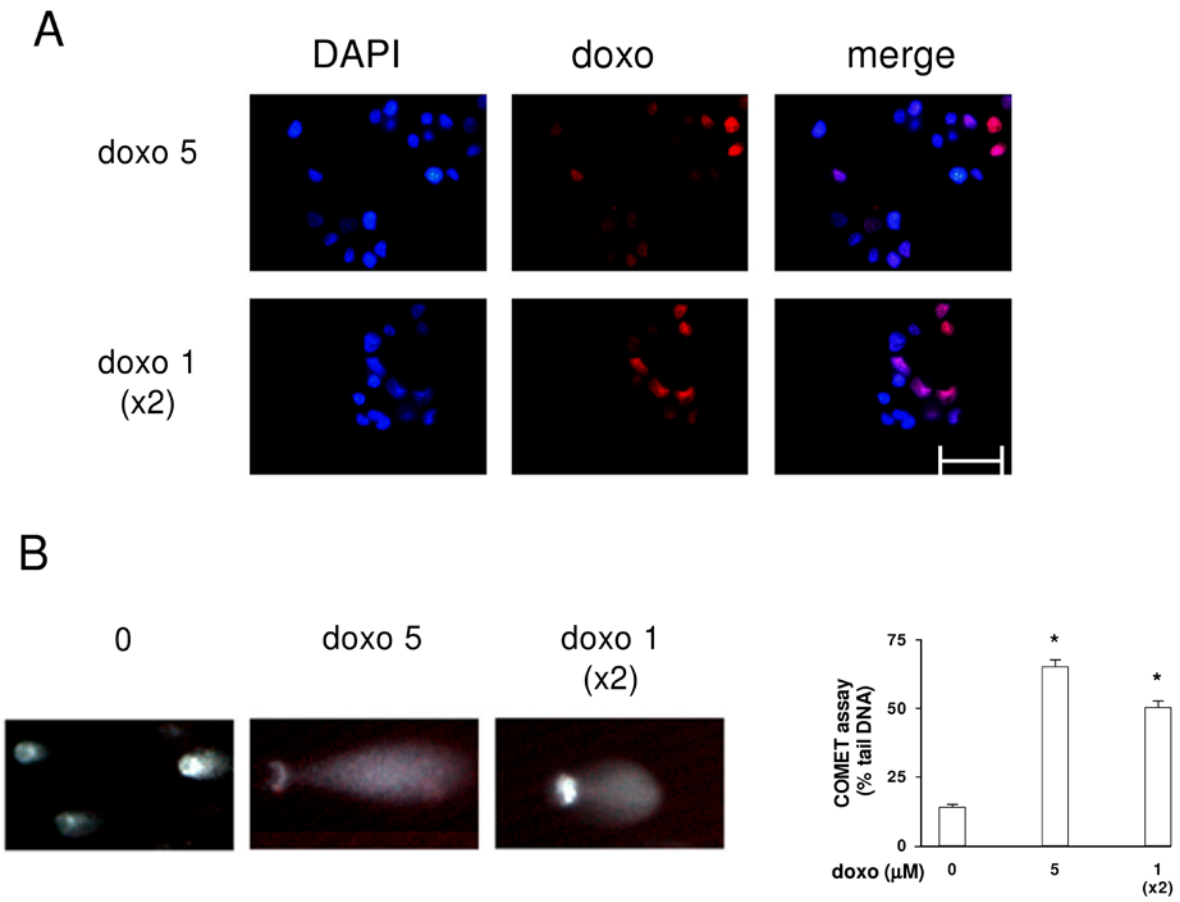
Supplementary Figure 1. Effects of two repeated low doses of doxorubicin on the viability of drug-sensitive and drug-resistant cancer cells.

A-B-C. Doxorubicin-sensitive human colon cancer HT29 cells and doxorubicin-resistant HT29-dx cells (panel **A**), doxorubicin-sensitive human lung cancer A549 cells and doxorubicin-resistant A549-dx cells (panel **B**), constitutively doxorubicin-resistant murine mammary cancer JC cells (panel **C**) were left untreated (0) or incubated with doxorubicin (doxo) in the following conditions: 1 μmol/L followed by a further dose of 1 μmol/L L after 24 h [1(x2): "two repeated low dose treatment"], or 5 μmol/L (5: "single high dose treatment"). The MTT assay, an indicator of cell viability, was performed in quadruplicate after 48 h from the first (or sole) drug administration. Data are presented as means $\pm$ SD (n=3). Significance versus respective untreated (0) cells: * $p < 0.005$; doxo 5 versus 1(x2) in the same cell type: ° $p < 0.02$.



Supplementary Figure 2. Two repeated low doses of doxorubicin do not affect Pgp expression and activity

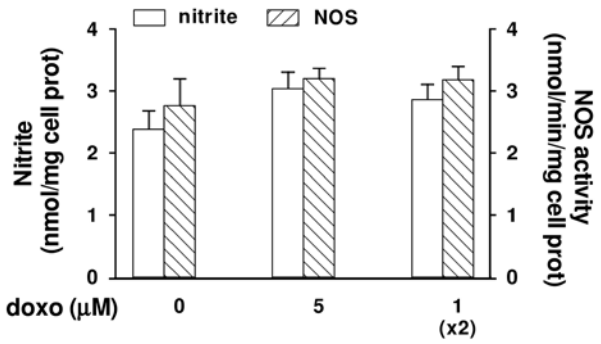
Doxorubicin-resistant JC cells were incubated without (0) or with 5 μmol/L doxorubicin (doxo 5: "single high dose treatment"), or 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h [doxo 1(x2): "two repeated low dose treatment"], then subjected to the following experiments. **A.** Western blot analysis of Pgp. The β-tubulin expression was measured to check the equal protein loading. The figure is representative of three independent experiments. **B.** The efflux of rhodamine 123, an indicator of Pgp activity, was measured by flow cytometry. The histograms are representative of three experiments with similar results. **C.** The quantification of the intracellular rhodamine 123 was measured spectrofluorimetrically in whole cells. Measurements were performed in triplicate. Data are presented as means ± SD (n=3).



Supplementary Figure 3. Two repeated low doses of doxorubicin do not produce a different intracellular distribution or genotoxicity than a single high dose

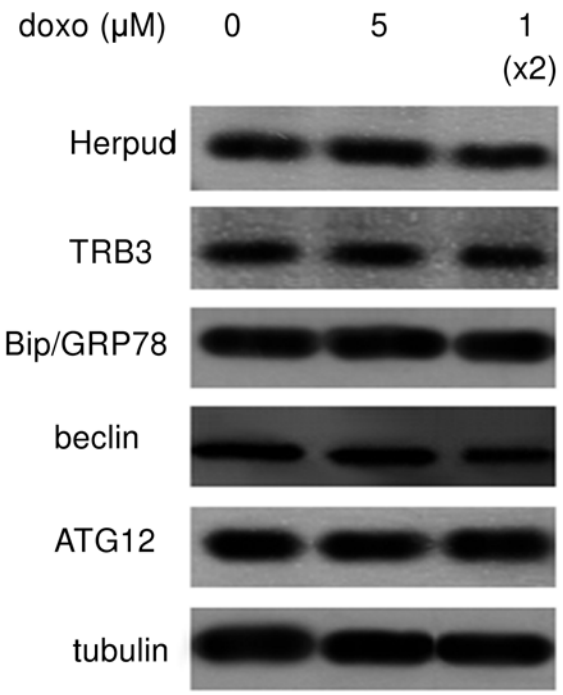
A. Fluorescence microscope analysis of doxorubicin-resistant JC cells incubated with 5 μmol/L doxorubicin (doxo 5: "single high dose treatment") or 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h [doxo 1(x2): "two repeated low dose treatment"]. The cells were counterstained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI). The photographs are representative of three independent experiments. Bars = 10 μm. **B.** Comet assay on JC cells, untreated (0) or treated as reported in **A**. Left panel: representative microphotographs of DNA comets in each experimental condition. The photographs are representative of three independent experiments. Right panel: quantitative analysis of the fluorescence microscope microphotographs:

100 tails for each experimental conditions were analyzed. Significance versus untreated (0) cells: * $p < 0.001$.



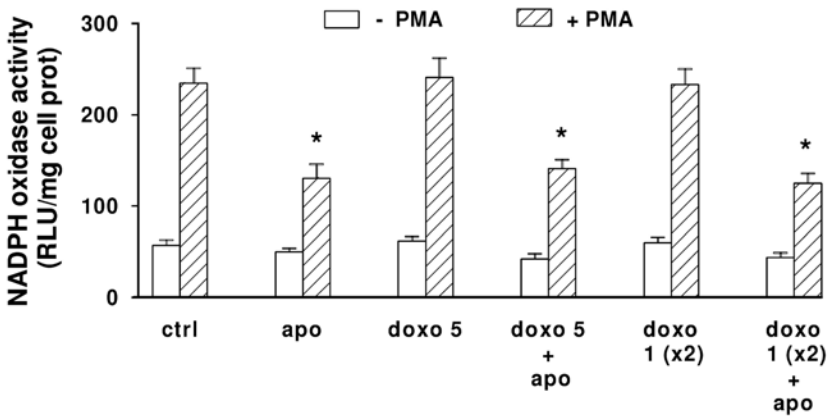
Supplementary Figure 4. Neither two repeated low doses of doxorubicin nor a single high dose increase nitric oxide synthesis

Doxorubicin-resistant JC cells were grown in fresh medium (0) for 48 h, or treated with 5 μmol/L doxorubicin (doxo 5: "single high dose treatment") or 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h [doxo 1(x2): "two repeated low dose treatment"]. Nitrite levels (open bars) were evaluated in the cell supernatants by the Griess method; the NOS activity (hatched bars) was analyzed in the cell lysates by a spectrophotometric assay. The measurements were performed in triplicate. Data are presented as means $\pm$ SD (n=3).



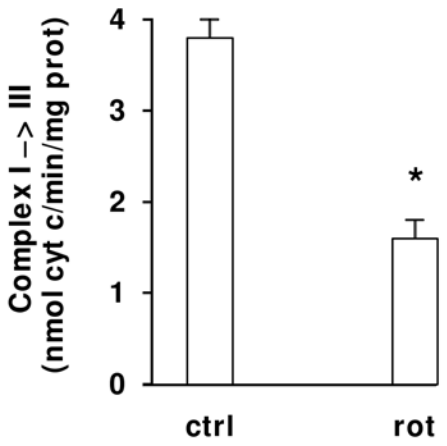
Supplementary Figure 5. Neither two repeated low doses of doxorubicin nor a single high dose change the markers of endoplasmic reticulum stress and autophagy

Doxorubicin-resistant JC cells were grown in fresh medium (0), treated with 5 μmol/L doxorubicin (doxo 5: "single high dose treatment") or with 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h [doxo 1(x2): "two repeated low dose treatment"]. Cell lysates were resolved by SDS-PAGE and immunoblotted with specific antibodies for: Herpud, TRB3, Bip/GRP78 (indexes of ER stress), beclin, ATG12 (indexes of autophagy induction), β-tubulin (to check the equal protein loading). Each of the immunoblots is representative of three independent experiments.



Supplementary Figure 6. Effects of apocynin on NADPH oxidase activity in drug-resistant cells

Doxorubicin-resistant JC cells were grown for 48 h in fresh medium (ctrl), treated with the NADPH oxidase inhibitor apocynin (10 μmol/L for the last 24 h; apo), with 5 μmol/L doxorubicin (doxo 5: "single high dose treatment"), with 5 μmol/L doxorubicin plus 10 μmol/L apocynin in the last 24 h (doxo 5 + apo), with 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h [doxo 1(x2): "two repeated low dose treatment"], or with 1 μmol/L doxorubicin followed by a second dose of 1 μmol/L after 24 h plus 10 μmol/L apocynin in the last 24 h [doxo 1(x2) + apo]. The enzymatic activity of NADPH oxidase was measured in whole cells by a chemiluminescence-based assay. When indicated, cells were pre-treated for 10 min with the NADPH oxidase activator phorbol myristate acetate (PMA, 10 nmol/L), to measure the maximal NADPH oxidase activity (hatched bars). Measurements were performed in duplicate and data are presented as means $\pm$ SD (n = 3). Significance: apo versus ctrl or doxo + apo versus doxo alone: * $p < 0.02$. Cells treated with PMA had a NADPH oxidase activity significantly higher than the corresponding untreated cells: $p < 0.001$ in all the experimental conditions (not shown in the figure).



Supplementary Figure 7. Inhibition of the electron flux from complex I to complex III by rotenone in drug-resistant cells

Doxorubicin-resistant JC cells were grown in the absence (ctrl) or in the presence of rotenone (50 μmol/L, rot) for 24 h, then the rate of electron flux from complex I to complex III was measured in isolated mitochondria as described under Materials and methods. Measurements were performed in triplicate and data are presented as means ± SD (n = 2). Significance versus untreated (ctrl) cells: * $p < 0.001$.