Nitric Oxide Donor Doxorubicins Accumulate into Doxorubicin-Resistant Human Colon Cancer Cells Inducing Cytotoxicity

Nitric oxide donor – doxorubicin conjugates accumulate into doxorubicin resistant human colon cancer cells inducing cytotoxicity

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[a] Abbreviations: DOXO, doxorubicin; NO, nitric oxide; MDR, multidrug resistance; P-gp, P-glycoprotein; ABC, ATP binding cassette; SNAP, *S*-nitrosopenicillamine; SNP, sodium nitroprusside; GSNO, *S*-nitrosoglutathione; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one; sGC, soluble guanylate cyclase; Rp-8-Br-cGMPS, 8-bromoguanosine-3',5'-cyclic monophosphorothioate; 8-Br-cGMP, 8-bromoguanosine cyclic monophhosphate; cGMP, guanosine-3',5'-cyclicmonophosphate; MDCK cells, Madin-Darby canine kidney cells; mtALDH, mitochondrial aldehyde dehydrogenase.

ABSTRACT. Products **4** and **5** obtained by conjugation of doxorubicin (DOXO) with nitric oxide (NO) donor nitrooxy and phenylsulfonyl furoxan moiety respectively, are able to accumulate into doxorubicin resistant human colon cancer cells (HT29-dx) inducing high cytotoxicity. This behavior parallels the ability of compounds to generate NO, detected as nitrite, in these cells. Preliminary immunoblotting studies suggest that the mechanism which underlies the cytotoxic effect could involve inhibition of cellular drug efflux due to nitration of tyrosine residues of MRP3 protein pump.

KEYWORDS. Multidrug resistance, doxorubicin, nitric oxide, P-glycoprotein.

Introduction

Multidrug resistance (MDR) is one of the major reasons for the failure of cancer chemotherapy. Among the mechanisms which underlie this phenomenon, the increased capacity of the cancer cells to efflux anticancer drugs, thus limiting their cellular accumulation with consequent reduction of toxicity, is one of the most studied in cancer cell models.[1,2] Indeed, cancer cells are able to overexpress ATP binding cassette (ABC) transporter proteins which are the products of a family of 49 genes. ABC B1, better known as P-glycoprotein (P-gp) or MDR1, and ABC C1-6, better known as multidrug resistance associated proteins MRP1-6, are the most representative pumps involved in MDR.[3] One of the strategy followed to reverse MDR is the co-administration of an anticancer agent with an inhibitor of these efflux pumps. Elacridar, Tariquidar, Laniquidar, and other inhibitors of third generation are currently studied in clinical trials in association with a number of antitumoral drugs.[4] The identification of new MDR-reversing agents selectively targeting drug-resistance cells, is a field of active investigation.[5]

Recent experimental evidence shows that in doxorubicin resistant human epithelial colon cell line HT29-dx, there is a relationship between the reduced endogenous nitric oxide (NO) production and the resistance of these cells to the antibiotic.[6] This resistance could be partly reversed when the cells were incubated with inducers of NO synthesis, such as a mix of cytokines or Atorvastatin. This same result was obtained working with other two doxorubicin resistent cell populations obtained from human lung epithelial cells A549, and human myelogenous leukemic cells K562. Also classic NO-donors, such as *S*-

nitrosopenicillamine (SNAP), sodium nitroprusside (SNP), and *S*-nitrosoglutathione (GSNO), were able to reduce the efflux of doxorubicin from HT29-dx cells.[6] This effect was neither inhibited by ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC), or by the protein-G kinase inhibitor 8-bromoguanosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cGMPS), nor could be mimicked by 8-bromoguanosine cyclic monophosphate (8-Br-cGMP), and consequently it has to be guanosine-3′,5′-cyclicmonophosphate-independent (cGMP). Interestingly, experiments carried out with SNAP showed that in HT29-dx cells the nitration of tyrosine residues of MRP3 occurred, thus suggesting that nitration of the transporter is a possible mechanism for resistance inversion. Also furoxans derivatives (1,2,5-oxadiazole 2-oxides), which are known to be able to release NO under the action of thiol cofactors,[7] were found capable of inhibiting P-gp and MRP1 transporters in MDK (Madin-Darby canine kidney) -MDR1 and -MDRP1 cell lines, respectively with consequent increase of cellular accumulation of DOXO when co-administered with the antibiotic.[8] Experiments carried out with 3-phenylsulfonylfuroxan derivatives, the most potent inhibitors of the series, showed that these products were able of nitrating tyrosine residues of P-gp, which in this form is likely unable to efflux the antibiotic. Other studies report that reduced supply of oxygen could induce MDR in solid cancers, and that hypoxia induced MDR could be reversed by low concentrations of NO mimetics.[9,10]

Starting from these bases, we decided to synthesize new DOXO semisynthetic derivatives in which the antibiotic was joined through an ester linkage to moieties containing either 3-phenylsulfonylfuroxan or nitrooxy substructures, in view of the potential ability of these products to trigger anticancer action combined with reduced capacity of inducing resistance. As aforementioned, furoxan system can release NO under the action of endogenous thiols. By contrast, NO-release from organic nitrates occurs through enzymatic catalysis. A number of enzymes have been proposed for this conversion, in particular the role of mitochondrial aldehyde dehydrogenase (mtALDH) and P-450 enzyme(s) was enphasised.[11,12] The preliminary results obtained by studying the products **4**, **5** (NO-DOXO) on HT29-dx cell populations are here reported and discussed.

Results and Discussion

Compounds **4**, **5** were prepared from a mixture of 14-bromo and 14-chlorodaunorubicine hydrobromide **1** by reaction with 4-(2,3-dinitrooxypropyl)benzoic acid **2** (potassium salt) and 3-[(3-phenylsulfonyl)furoxan-4-yloxy]propanoic acid **3** (sodium salt) in acetone, respectively. After purification by flash chromatography, the products were isolated as hydrochlorides. The simple methyl esters **6** and **7**, used for a comparison, were prepared treating **2** and **3** in refluxing methanol, in the presence of H_2SO_4 (Scheme 1).

HT29-dx cells were incubated in RPMI 1640-medium with DOXO and NO-DOXO (**4**, **5**) at different concentrations (0.5÷25 μM) for 24 h. At the end of this period, the antibiotics were dosed on the cells lysate, using a spectrofluorimeter method with λ emission 475 nm, λ excitation 553 nm. At these wavelengths the spectra of DOXO, compound **4** and compound **5** were superimposable (data not shown). The results, collected in Figure 1, show that NO-DOXOs accumulate into the cells in a concentration dependent manner, unlike DOXO, whose intracellular concentration does not significantly increases, except at the highest concentration (25 μM). This effect was more accentuated for the furoxan substituted derivative **5** then for the nitrooxy substituted one **4,** and was reversed (Figure 3A) by the co-incubation with 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) and red blood cells, chosen as effective NO scavengers (Figure 3C).

Similarly NO-DOXOs increase their accumulation in a time dependent manner: the raise of the intracellular content of 5 μM antibiotics became significant after 1 h for compound **5**, after 3 h for compound **4** and was never significant between 1 and 24 h for DOXO (data not shown).

This behavior parallels the drug efflux kinetics from H29-dx cells. Indeed, when cell monolayers were loaded with different amount of the three drugs (1÷200 μM solutions) for 10 minutes and then the antibiotic intracellular content was examined spectrofluorimetrically on cell lysates collected after 10 and 20 minutes, the results outlined in Figure 2 were obtained. The measured V_{max}s rank the order DOXO (8.38 ± 0.38 nmoles of the drug transported/min/mg prot) > **4** (4.23 ± 0.18 nmoles of the drug transported/min/mg prot) > **5** (2.65 ± 0.09 nmoles of the drug transported/min/mg prot), showing the effluxes of NO-DOXOs decrease dramatically with respect to the simple antibiotic.

As consequence of this behavior the toxicity of NO-DOXOs were found definitively higher than the DOXOs one. Indeed the cells, when incubated with 5 μM solutions of NO-DOXOs, exhibited a significant increase in the release of lactate dehydrogenase (LDH), a sensitive index of drug's toxicity, with respect to DOXO alone (Figure 3B). The level of LDH was higher for the furoxan derivative **5** than for the nitrooxy substituted product **4**. In both the cases the toxicity was reduced in the presence of the NO scavengers PTIO and red blood cells. By contrast, no significant increase in LDH levels was observed with the simple acids **2**, **3**, used to obtain the two co-drugs **4**, **5**, and the related methyl esters **6**, **7**. This finding indicates that the cytotoxic effect is due to the anthracycline pharmacophore rather than to the NO-releasing moieties.

In order to evidence if NO-DOXOs were able to restore NO-production in HT29-dx cells, solutions of **4** and **5** of different concentration (0.5÷25 μM) were incubated with cell mono layers for 24 h, and then nitrite in the culture medium was quantified by Griess reaction. The results reported in Figure 4 show that nitrite production is concentration dependent and that it is maximised for furoxan derivative **5**. The nitrite release was time dependent: using a 5 μM solution of the antibiotics, a significant increase of nitrite was detected after 1 h with compound **5**, after 3 h with compound **4,** but remained not significant between 1 and 24 h with DOXO (data not shown).

It is known that drug efflux pumps P-gp and MRP3 are overexpressed in HT29-dx cells and that their nitration reduce the efflux activity.[6] In order to check whether the capacity of **4** and **5** to accumulate in these cells could be due to nitration of tyrosine residues of these efflux pumps, following NO release by the products, HT29-dx cells were incubated for 24 hours with the two antibiotics or with DOXO. The whole cellular lysate was immunoprecipitated with a specific anti-nitrotyrosine antibody and the immunoprecipitated proteins were subjected to western blotting, using anti-P-gp and anti-MRP3 antibodies (Figure 5). The presence of nitrotyrosine residues were detectable on MRP3 protein in HT29-dx cells incubated with compounds **4** and **5**, but not with DOXO alone. The amount of nitro tyrosine produced by **5** was greater than that produced by **4**. This result is in keeping with the higher levels of nitrite detected with the former compound. Also the intracellular accumulation of doxorubicin

with compound **5** was higher than with compound **4**. It is known that doxorubicin up-regulates the inducible isoform of NO synthase (iNOS),[13] thereby increasing the endogenous synthesis of NO in mammalian cells. Thus it is possible that the nitration of MRP3 elicited by compound **4**, **5** is due in part to the release of NO by the products and in part to the increased production of endogenous NO via the induction of iNOS enzyme. No nitration occurred in P-gp protein. This difference is probably due to the higher number of tyrosine residues in the former pump with respect to the latter.[6]

The preliminary results disclosed in the present work show that the design of hybrid NO-donor antitumor drugs could be a useful strategy to address the problem of MDR in cancer therapy.

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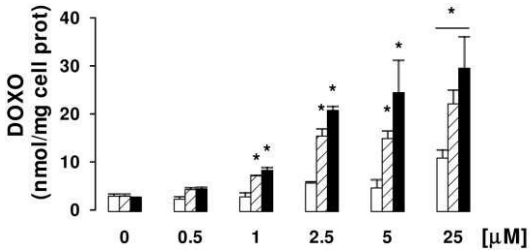


Figure 1. Dose-dependent accumulation of DOXO and NO-DOXOs in HT29-dx cells.

Cells were incubated for 24 h in the absence or presence of different concentrations (0.5, 1, 2.5, 5 and 25 μM) of DOXO (*open columns*), compound **4** (*hatched columns*) or compound **5** (*solid columns*), then lysed and checked for the intracellular drug content, as reported in Supporting information. Data are presented as means ± SD (n = 3). Vs untreated cells(*0*): * $p < 0.005$.

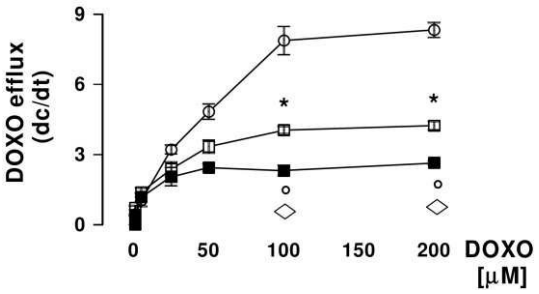


Figure 2. Efflux kinetics of DOXO and NO-DOXOs from HT29-dx cells.

HT29-dx cells were loaded in PBS buffer for 10 minutes at 37 °C with different amounts (1-200 μM) of doxorubicin (*open circle*), compound **4** (*open square*) or compound **5** (*solid square*), then washed: one aliquot of each sample was checked immediately for the content of drug (time 0), whereas a second aliquot was further incubated in fresh PBS for further 10 minutes, washed and tested for the drug content as previously described (for details see Supporting information). The rate of doxorubicin efflux was calculated from the difference between the drug concentration at time 0 and at 10 minutes, respectively. Measurements (n = 3) were done in duplicate and data are presented (mean ± SD) as the rate of efflux versus the intracellular drug concentration at time 0. Significance of compound **4** versus DOXO: ***** $p < 0.01$; significance of compound **5** versus DOXO: ° $p < 0.002$; significance of compound **5** versus compound **4**: $^{\diamond}$ $p < 0.001$.

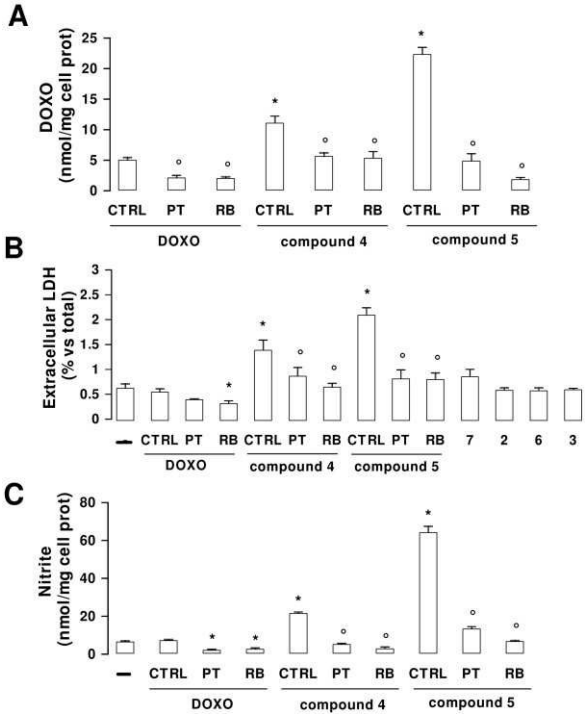


Figure 3. Effects of NO scavengers on DOXO and NO-DOXO accumulation and toxicities.

HT29-dx cells were incubated in the absence (-) or presence (CTRL) of 5 μM DOXO, compound **4** or compound **5** for 24 h; when indicated, 100 μM PTIO (PT) or 10 μl/ml of packed red blood cells were co-incubated. The intracellular drug content (panel A), the extracellular activity of LDH (panel B) and the nitrite amount in the culture supernatant (panel C) were measured in duplicate as described in the Supporting information (n = 3). Significance for panel A: vs CTRL DOXO: * $p < 0.002$; vs DOXO or compound **4** or compound **5**: ° $p < 0.05$; for panel B and C: vs CTR DOXOL: * $p < 0.05$; vs DOXO or compound **4** or compound **5**: ° $p < 0.05$.

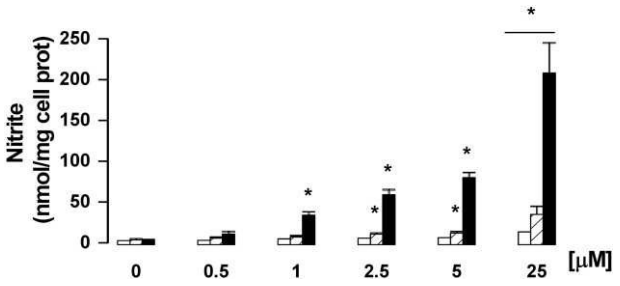


Figure 4. Dose-dependent production of nitrite in HT29-dx cells treated with DOXO and NO-DOXOs. Cells were incubated for 24 h in the absence or presence of different concentrations (0.5, 1, 2.5, 5 and 25 μM) of DOXO (*open columns*), compound **4** (*hatched columns*) or compound **5** (*solid columns*), then the levels of nitrite in the cell medium was evaluated by the Griess method. Data are presented as means ± SD (n = 3). Vs untreated cells (*0*): * $p < 0.001$.

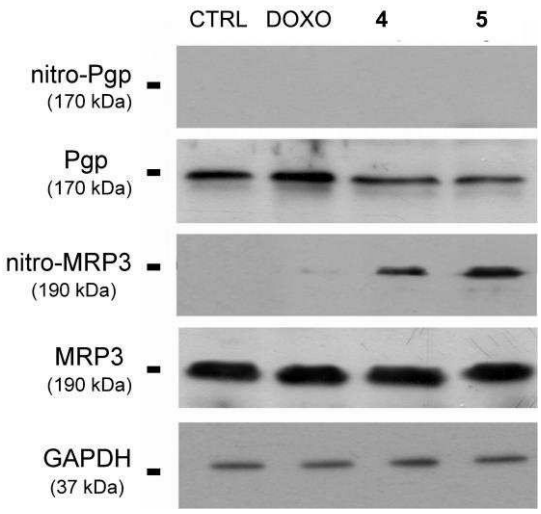
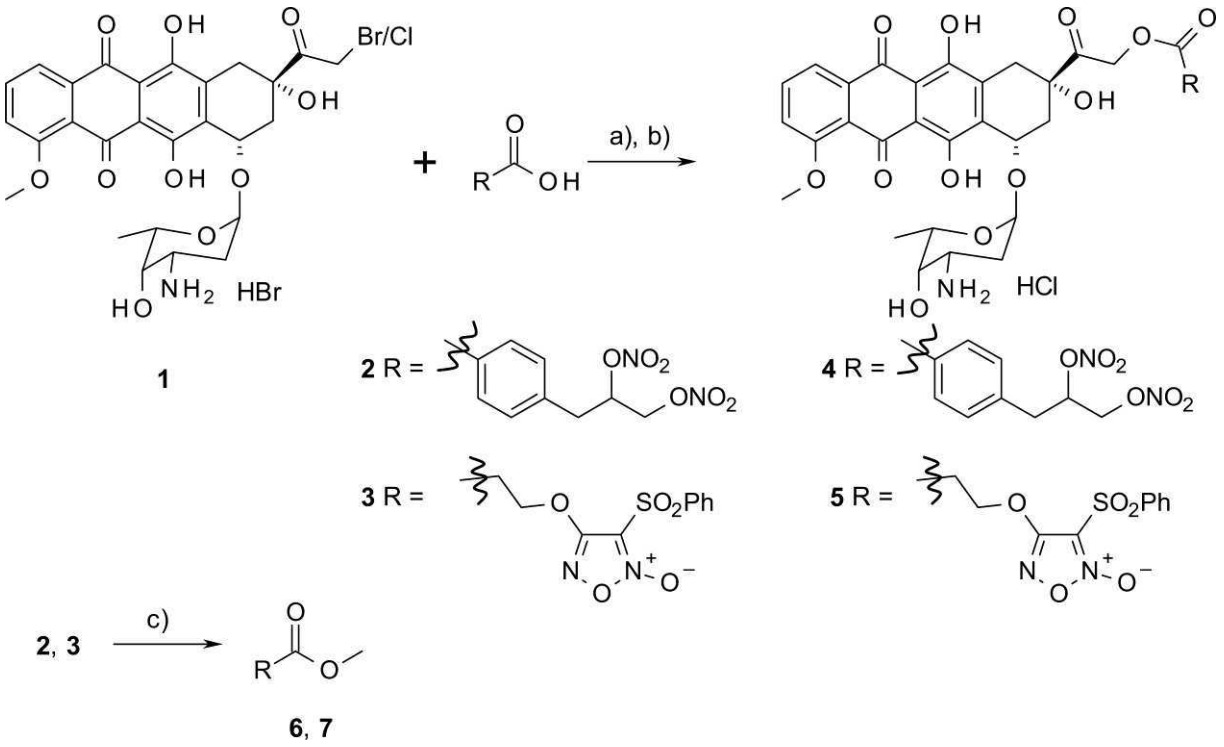


Figure 5. Nitration of drug efflux pumps by DOXO and NO-DOXOs in HT29-dx cells.

HT29-dx cells were incubated for 24 h with 5 μM DOXO, compound **4** or compound **5**. Afterwards, cells were lysed and the whole cellular lysate was immunoprecipitated with an anti-nitrotyrosine polyclonal antibody. The immunoprecipitated proteins were subjected to Western blotting, using an anti-Pgp or an anti-MRP3 antibody (see Supporting information). An aliquot of cells lysate before immunoprecipitation was analyzed for total P-gp and MRP3. The expression of the housekeeping protein GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase) was measured as a control of equal loading. The experiment is representative of three similar experiments.

Scheme 1.[a]



[a] Conditions: (a) **2**, K_2CO_3, acetone, r.t. for **4**; **3** sodium salt, acetone, reflux for **5**; (b) THF dry, HCl in dioxane dry; (c) H_2SO_4, MeOH, reflux.

SYNOPSIS TOC

NITRIC OXIDE DONOR – DOXORUBICIN CONJUGATES ACCUMULATE INTO DOXORUBICIN RESISTANT HUMAN COLON CANCER CELLS INDUCING CYTOTOXICITY

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