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UNIVERSITÀ DEGLI STUDI DI TORINO

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Graphical Abstract

Doxorubicin-antioxidant co-drugs

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ABSTRACT

Doxorubicin-antioxidant multitarget compounds **6** and **7** were obtained by combining doxorubicin (DOX) with caffeic and ferulic acids through an ester linkage at C-14. The products were studied in *in vitro* models of cardiomyocytes and breast cancer cells, characterized by different degrees of resistance to DOX, due to different expressions of ATP binding cassette (ABC) transporters. Compound **7** was found to be less toxic than DOX in cardiomyocytes and to display the same possibly higher toxicity against the resistant breast cancer cells. This result shows that appropriate DOX-antioxidant co-drugs can limit the onset of cardiac damage, a significant side-effect of DOX, without impairing the antitumor activity of the parent antibiotic.

Doxorubicin (DOX) (**1**, Figure 1) also known as Adriamycin, is a potent broad-spectrum antineoplastic antibiotic belonging to the anthracycline family. It is widely used, as single agent or in combination with other anticancer drugs, in treating a variety of cancers including solid tumors, soft-tissue sarcomas, lymphomas, and leukemias.¹ DOX displays a number of clinical toxicities, of which cardiomyopathy is the most important. Two kinds of cardiomyopathies can occur: an acute form and a chronic, cumulative dose-related form. The former kind is rarely a serious problem, while the latter can lead to congestive heart failure that is unresponsive to digitalis.² The mortality rate in patients with congestive heart failure is close to 50%. The classic molecular mechanisms underlying both the anticancer and the toxic effects of DOX operate at two distinct levels: by modifying DNA, and by inducing oxidative stress. Other possible mechanisms include altered metabolism of Ca⁺⁺ ions or prostaglandins.³

Figure 1. Doxorubicin (**1**) and hybrid compounds general structure.

Oxidative stress is the consequence of a disturbed prooxidant / antioxidant intracellular balance, due to abnormal production of reactive oxygen (ROS) and nitrogen (RNS) species and/or to depletion of antioxidant defenses. Continuitive oxidative stress leads to cell damage, consequent on the alteration of enzymes, proteins, and DNA, and to lipid peroxidation, with the final formation of reactive electrophilic aldehydes.^{4,5} The heart is very sensitive to oxidative stress, because of its strongly oxidative metabolism and poor antioxidant defenses. DOX can generate free radicals in a number of different ways: it is reduced by various biological systems to a semiquinone free radical giving rise to a number of ROS, including peroxide anions (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[•]). DOX activates NAD(P)H oxidases (NPHs), which generate free radicals; it also produces ROS through a non-enzymatic mechanism involving iron. Lastly, DOX undergoes metabolic transformation to doxorubicinol, a secondary alcohol that induces the release of iron from cytoplasmic aconitase, with consequent ROS generation.^{3,6} It is known that the heart is very rich in mitochondria, which contain a phospholipid called cardiolipin, for which DOX displays great affinity;⁷ DOX undergoes redox cycling at complex I of the mitochondrial electron transport chain. The final result is cardiomyocyte damage, mainly deriving from the impairment of mitochondrial functioning.^{8,9}

The use of a variety of natural and synthetic antioxidants to prevent DOX's cardiotoxicity has been considered,^{1,3,10-12} and combinations of DOX with agent(s) capable of blocking its ROS mediated cardiotoxicity effect have been investigated. To the best of our knowledge, no study has yet reported the combination of DOX and the antioxidant in a single molecule. The use of such polyvalent molecules can show some advantages compared with a cocktail of drugs, including a lower risk of drug-drug interactions, improved compliance by the patient, and a more predictable pharmacokinetic profile. As a development of our studies on semi-synthetic doxorubicins,¹³ we here report preliminary results obtained with the first two DOX hybrids of general structure **2**, in which the antibiotic is linked with selected antioxidant moieties through appropriate spacers.

The co-drugs **6** and **7** derive from the combination of DOX, respectively, with caffeic acid, and with ferulic acid, through an ester linkage at C-14. These two acids belong to the class of polyphenols, which are important antioxidants widely distributed in the human diet, particularly in fruits, cereals vegetables and beverages (coffee, beer, wine, fruit juices). The phenolic acid are rarely present in the

free form, more frequently occurring as esters.¹⁴ The co-drugs were prepared from a mixture of 14-bromo / 14-chlorodaunorubicin **3** by reaction with the sodium salt of the appropriate acid (**4**, **5**) in boiling acetone (Scheme 1). After purification by flash chromatography, the products were isolated as hydrochlorides.

Scheme 1 i) acetone, reflux.

Compounds **6** and **7** were then evaluated in *in vitro* models of cardiomyocytes and breast cancer cells, cell lines that are characterized by different degrees of resistance to DOX, due to their different expressions of ATP binding cassette (ABC) transporters, namely P-glycoprotein (Pgp/ABCB1), multidrug resistance related protein (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2), all of which efflux DOX.

Figure 2. Cytotoxicity and intracellular accumulation of DOX, **6** and **7**.¹⁵

H9c2 cells, which are characterized by several cardiomyocyte-like properties,¹⁶ and the human breast cancer cell lines MCF7 and MDA-MB-231 were chosen to check the *in vitro* cytotoxicity of ferulic acid-DOX (**7**) and caffeic acid-DOX (**6**) conjugates. **7** was significantly less toxic than DOX in H9c2 cells and retained the same toxicity as DOX in MCF7 cells; of note, it also induced cell damage in MDA-MB-231, which were refractory to DOX-induced cytotoxicity (Figure 2A, right panel). By contrast, **6** was as toxic as DOX in H9c2 cells and MCF7 cells, but was ineffective against MDA-MB-231 (Figure 2A, left panel). Interestingly, **7** accumulated in larger amounts than did the parent drug DOX in both types of breast cancer cells, but not in cardiomyocytes (Figure 1B, right panel), whereas **6** had the same rate of accumulation as DOX in all cell lines tested (Figure 2B, left panel). Since **6** showed neither reduced toxicity/accumulation in H9c2 cells, nor increased toxicity/accumulation in MCF7 or MDA-MB-231 cells, it was not investigated further.

To clarify the differential accumulation pattern of **7** in cardiomyocytes and breast cancer cells, expression of the ABC transporters involved in DOX efflux was analyzed: as shown in Figure 3A, H9c2 cells had undetectable levels of all transporters, MCF7 cells showed very low amounts of Pgp and BCRP, whereas MDA-MB-231 had high expression levels of Pgp, MRP1 and BCRP. This pattern of expression is in agreement with the DOX accumulation found, which followed this order: H9c2 > MCF7 > MDA-MB-231 cells (Figure 2B). In keeping with these data, the $V_{\max}$ of DOX efflux was highest in MDA-MB-231 cells, followed by MCF7 and then H9c2 cells (Figure 3B, upper panel). Of note, the $V_{\max}$ of **7** was not different from that of DOX in H9c2 cells, and was reduced in MCF7 cells and MDA-MB-231 cells (Figure 3B, lower panel, Table 1). The K_m of DOX and **7** were relatively uniform in all three cell types (Figure 3B, Table 1). Overall, these results suggest that **7** was effluxed less markedly by the ABC transporters present in breast cancer cells, leading to its being accumulated to a greater extent than was DOX, in MCF7 and MDA-MB-231 cells; conversely, **7** was not retained more abundantly than DOX in cardiomyocytes, which had undetectable levels of ABC transporters.

Figure 3. A: Expression of Pgp, MRP1, and BCRP, in H9c2, MCF7 and MDA-MB-231 cells.¹⁷ B: Efflux of **7** from cardiomyocytes and from breast cancer cells.¹⁸

Table 1. Compounds efflux from cardiomyocytes and from breast cancer cells.¹⁸

	Cells	K_m	V_{max}
DOX	H9c2	1.96 ± 0.26	11.89 ± 0.53
	MCF7	1.88 ± 0.21	18.90 ± 0.29
	MDA	1.35 ± 0.53	33.11 ± 1.90
7	H9c2	2.01 ± 0.18	11.70 ± 0.61
	MCF7	1.83 ± 0.24	11.10 ± 0.55
	MDA	1.25 ± 0.31	16.53 ± 0.70

DOX and **7** had the same potency as topoisomerase II inhibitors (Supplementary, Figure S2) and a similar intracellular distribution: most of **7** was found within nuclei, a small percentage in mitochondria (Supplementary, Figure S3). In nuclear extracts of breast cancer cell lines, the amount of **7** was higher than that of DOX (Supplementary, Figure S3), in line with its lower efflux rate from whole cells.

Finally, we checked the effects of **7** on preventing oxidative stress, and analyzed intracellular levels of ROS, and of malonyldialdehyde (MDA), a marker of lipid peroxidation and oxidative damage. Compared with DOX, **7** significantly reduced ROS and MDA in H9c2 cardiomyocytes; the reduction was smaller in MCF7 cells. In MDA-MB-231 cells, DOX caused no increase in ROS or MDA, and **7** did not produce any significant changes (Figure 4). The intramitochondrial ROS level was similar for DOX and **7**, and followed the same trend of ROS in whole cells (Supplementary, Figure S4).

Figure 4. Effects of **7** on ROS production and lipid peroxidation. A: ROS levels. B: Lipoperoxidation assessment.¹⁹

In this study we show preliminary in vitro data on the efficacy of DOX conjugated with antioxidants, designed to reduce drug-induced cardiotoxicity caused by oxidative damages. Oxidative stress, however, is one of the multiple biochemical mechanisms by which DOX exerts its anticancer effects;³ thus it is mandatory to design DOX-antioxidant derivatives that reduce oxidative stress in cardiomyocytes, but do not lose their cytotoxicity against tumor cells. Ferulic acid and caffeic acid lipophilic derivatives have recently been shown to exert cytotoxic effects in breast cancer cells, including MCF7 and MDA-MB-231 cells;²⁰ moreover, both compounds are known to be antioxidant molecules.²¹ In the light of these observations, ferulic acid-DOX (**7**) and caffeic acid-DOX (**6**) derivatives could be suitable co-drugs, able on one hand to reduce oxidative stress, while on the other hand retaining DOX's cytotoxic effects against breast cancer cells. Indeed, **7** was less toxic than DOX in cardiomyocytes in vitro, whereas its toxicity against breast cancer cells was equal to or higher than that of the parent drug. Conversely, **6** maintained the same toxicity profile as DOX. This difference could be due to the different lipophilicities of these hybrid compounds, or may be explained by the different rates of intracellular accumulation of the two semisynthetic DOXs: while **6** had the same intracellular retention as DOX, **7**, surprisingly, accumulated in larger amounts in breast cancer cells.

Cytotoxicity against cardiomyocytes and tumor cells is directly related to the concentration of intracellular DOX, which in turn is dependent on the ABC transporters Pgp, MRP1 and BCRP.²² In our models, the accumulation of DOX was lower in ABC transporter-

positive cells, which showed the most marked DOX efflux. By contrast, the accumulation ratio of **7** versus DOX was higher in MDA-MB-231 cells, which have high expressions of Pgp, MRP1 and BCRP, than it was in MCF7 or H9c2 cells. The decrease in $V_{\max}$ efflux suggests that **7** is prone to accumulate markedly within ABC transporter-positive cancer cells, which are usually resistant to DOX's toxicity. The absence of Pgp, MRP1 and BCRP in H9c2 cells explains why **7** was not accumulated more markedly in cardiomyocytes, nor effluxed to a lesser extent, than was DOX. According to K_m and $V_{\max}$ values of DOX and **7**, we suggest that the latter binds to ABC transporters with the same affinity as DOX, but it is transported outside at a lower rate, probably due to the different sterical and physico-chemical properties of **7**. On the other hand, the different structure of **7** did not affect other "peculiar" properties of DOX, such as the predominant intranuclear accumulation and the effective inhibition of topoisomerase II.

Within H9c2 cells, the ferulic acid co-drug **7** was found to reduce the oxidative stress induced by DOX, a result that is in accordance with its decreased cytotoxicity. In the DOX-sensitive breast cancer MCF7 cells, **7** did not show any superior benefit compared to DOX: although **7** induced less oxidative stress, it was also effluxed to a lesser extent than was DOX, due to the lower content of Pgp and BCRP. **7** accumulated more markedly than DOX in MCF7 cells; this increased accumulation likely compensates for the decreased oxidative damage, leading to the same cytotoxicity as has DOX. The greatest benefit of **7** was seen in DOX-resistant tumor cells, i.e. MDA-MB-231: DOX was strongly effluxed from these cells, eliminating its cytotoxicity. Conversely **7**, which was effluxed less markedly and reached sufficient intracellular concentration to induce appreciable cytotoxic effects. Notably, at this concentration **7** did not elicit any cell damage on cardiomyocytes.

The most critical limitation on the use of DOX against breast tumors is the onset of cardiotoxicity.²³ This preliminary study shows that the compound **7**, obtained by combining DOX with ferulic acid, is more effective in resistant breast cancer cell, and less toxic against cardiomyocytes, making it worthy of future in vitro and in vivo investigations in DOX-resistant tumors. It may be speculated that **7** might induce the same cytotoxic effects against resistant breast tumors, even if administered at lower doses than DOX: since the chronic form of cardiotoxicity is dependent on the cumulative dose of DOX,²³ the use of **7** instead of DOX might lead to a reduced risk of cardiac damage in patients bearing resistant breast tumors.

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References and notes

- Minotti, G.; Menna, P.; Salvatorelli, E.; Cairo, G.; Gianni, L. *Pharmacol. Rev.* **2004**, *56*, 185, and references therein reported.
- Chabner, A.; Amrein, P. C.; Druker, B. J.; Michaelson, M. D.; Mitsiades, C. S.; Goss, P. E.; Ryan, D. P.; Ramachandra, S.; Richardson, P. G.; Supko, J. G.; Wilson, W. H. *Antineoplastic Agents*. In Goodman & Gilman's, *The Pharmacological Basis of Therapeutic*; Brunton, L.; Lazo, J. S.; Parker, K. L., Eds. McGraw-Hill: New York, Chicago, **2006**; 1315.
- Granados-Principal, S.; Quiles, J. L.; Ramirez-Tortosa, C. L.; Sanchez-Rovira, P.; Ramirez-Tortosa, M. *Food Chem. Toxicol.* **2010**, *48*, 1425, and references therein reported.
- Eberhardt, M. K. *Reactive Oxygen Metabolites: Chemistry and Medical Consequences*; CRC Press: Boca Raton, FL, **2000**.
- Esterbauer, H.; Schaur, R. J.; Zollner, H. *Free Radical Biol. Med.* **1991**, *11*, 81.
- Simunek, T.; Sterba, M.; Popelova, O.; Adamcova, M.; Hrdina, R.; Gersl, V. *Pharmacol. Rep.* **2009**, *61*, 154.
- Goormaghtigh, E.; Ruyschaert, J. M. *Biophys. Acta* **1984**, *779*, 271.
- Berthiaume, J. M.; Wallace, K. B. *Cell Biol. Toxicol.* **2007**, *23*, 15.
- Doroshov J. H.; Davies, K. J. A. *J. Biol. Chem.* **1986**, *261*, 3068.
- Quiles, J. L.; Huertas, J. R.; Battino, M.; Mataix, J.; Ramirez-Tortosa, M. C. *Toxicology* **2002**, *180*, 79, and references therein reported.
- DeAtley, S. M.; Aksenov, M. Y.; Aksenova, M. V.; Harris, B.; Hadley, R.; Harper, P. C.; Carney, J. M.; Butterfield, D. A. *Cancer Lett.* **1999**, *136*, 41.
- Hideg, K.; Kalai, T. *Cardiovasc. Toxicol.* **2007**, *7*, 160.
- a) Chegaev, K.; Riganti, C.; Lazzarato, L.; Rolando, B.; Guglielmo, S.; Campia, I.; Fruttero, R.; Bosia, A.; Gasco, A. *ACS Med. Chem. Lett.* **2011**, *2*(7), 494; b) Riganti, C.; Rolando, B.; Kopecka, J.; Campia, I.; Chegaev, K.; Lazzarato, L.; Federico, A.; Fruttero, R.; Ghigo, D. *Mol. Pharmaceutics* **2012**, *10*(1), 161.
- Poquet, L.; Clifford, M. N.; Williamson, G. *Bioavailability of flavanols and phenolic acids*. In *Plant Phenolics and Human Health*; Fraga C.G., Ed.; Wiley: New Jersey, **2010**, pp 51.
- Cells were incubated for 24 h in fresh medium (ctrl) or with 5 $\mu\text{mol/L}$ DOX, **6** and **7**. **A**: LDH release was evaluated in duplicate in the cell culture supernatant and in the cell lysate, and the former was expressed as percentage of the total LDH activity (extracellular + intracellular). Data are presented as means $\pm$ SD ($n = 4$). Vs. ctrl: * $p < 0.01$; **7** vs. DOX: ° $p < 0.005$. **B**: The amount of intracellular DOX was measured in duplicate in cell lysates. Data are presented as means $\pm$ SD ($n = 3$). **7** vs. DOX: * $p < 0.05$.
- Hescheler, J.; Meyer, R.; Plant, S.; Krautwurst, D.; Rosenthal, W.; Schultz, G. *Circ. Res.* **1991**, *69*, 1476.
- Cells were lysed and the whole cell lysate was subjected to Western blotting. Expression of actin, as product of a housekeeping gene, was used as control for equal protein loading. The figure is representative of three experiments with similar results.
- Cells were incubated for 20 min with increasing concentrations (0-100 $\mu\text{mol/L}$) of DOX or **7**, then washed and tested for intracellular drug content. The procedure was repeated on a second series of dishes, incubated in the same experimental conditions and analysed after a further 10 minutes. Measurements ($n = 3$) were in duplicate and data (means $\pm$ SD) are presented as rate of drug efflux (dc/dt) plotted versus initial drug concentration. $V_{\max}$ (nmol/min/mg cell proteins) and K_m (nmol/mg cell proteins) were calculated using the Enzfitter software package.
- Cells were incubated for 24 h in fresh medium (ctrl), or in 5 $\mu\text{mol/L}$ DOX or **7**. **A**: ROS levels. Cells were incubated with the ROS-sensitive probe DCFDA-AM, then analysed in duplicate. Data are presented as means $\pm$ SD ($n = 3$). Vs. ctrl: * $p < 0.001$; **7** vs. DOX: ° $p < 0.005$. **B**: Lipoperoxidation assessment. The amount of MDA was measured in triplicate in the cell lysates. Data are presented as means $\pm$ SD ($n = 3$). Vs. ctrl: * $p < 0.05$; **7** vs. DOX: ° $p < 0.01$.
- Serafim, T. L.; Carvalho, F. S.; Marques, M. P.; Calheiros, R.; Silva, T.; Garrido, J.; Milhazes, N.; Borges, F.; Roleira, F.; Silva, E. T.; Holy, J.; Oliveira, P. J. *Chem. Res. Toxicol.* **2011**, *24*, 763.
- Kancheva, V. D.; Saso, L.; Angelova, S. E.; Foti, M. C.; Slavova-Kasakova, A.; Daquino, C.; Enchev, V.; Firuzi, O.; Nechev, J. *Biochimie* **2012**, *94*, 403.
- Gottesman, M. M.; Fojo, T.; Bates, S. E. *Nat. Rev. Cancer* **2002**, *2*, 48.
- Leonard, R. C. F.; Williams, S.; Tulpule, A.; Levine, A. M.; Oliveros, S. *Breast* **2009**, *18*, 218.