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Light-Regulated NO Release as a Novel Strategy to Overcome Doxorubicin MultiDrug Resistance

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Supporting Information

ABSTRACT: Nitric oxide (NO) release from a suitable NO photodonor (**NOP**) can be fine-tuned by visible light stimuli, at doses that are not toxic to cells but that inhibit several efflux pumps; these are mainly responsible for the Multidrug Resistance of the anticancer agent Doxorubicin (**DOX**). The strategy may thus increase **DOX** toxicity against resistant cancer cells. Moreover, a novel molecular hybrid covalently joining **DOX** and **NOP** showed similar increased toxicity towards resistant cancer cells and, in addition, lower cardiotoxicity than **DOX**. This opens new and underexplored approaches to overcoming the main therapeutic drawbacks of this chemotherapeutic based on light-controlled release of NO.

The development of multidrug resistance (MDR) to chemotherapy remains a major challenge in treating cancer. Doxorubicin (**DOX**) (Chart 1) is an antibiotic isolated from the culture broth of bacteria belonging to the genus *Streptomyces* that is widely used in treating a variety of tumors, including solid tumors, soft tissues sarcomas, and many malignancies of the blood.[1] Several molecular mechanisms have been proposed to underlie its antitumoral activity, including DNA intercalation, topoisomerase II inhibition, and free-radical generation.[2] The clinical use of this important antibiotic is hampered by the development of resistance to it and its cardiotoxicity. Several mechanisms have been proposed to explain the onset of resistance in cancer chemotherapy. Of these, one of the most studied is the increased efflux of antineoplastic drugs from tumor cells consequent on overexpression of ATP binding cassette (ABC) transporters. These proteins use the energy deriving from ATP hydrolysis to extrude xenobiotics from the cells.[3,4] P-glycoprotein (P-gp/ABCB1), MDR–Related Protein 1 (MRP1/ABCC1) and Breast Cancer Resistance Protein (BCRP/ABCG2) are the most common transporters responsible for the failure of **DOX** efficacy.

A number of strategies have been proposed to overcome MDR, in particular co-administration of antineoplastic agents with compounds able to interact with ABC transporters and consequently block drug extrusion.[5,6] These compounds have been studied in association with a number of anticancer drugs, included **DOX**.[7] However, this strategy suffers from a number of pharmacokinetic and pharmacodynamic limitations that are only partially overcome by co-incapsulation of antibiotic and pump inhibitor within appropriate matrixes.[8]

Nitric oxide (NO) is a ubiquitous gaseous messenger that plays a variety of roles in human physiology and pathophysiology.[9] NO is involved in a number of biological processes, including vasodilation, platelet aggregation, neurotransmission, and macrophage-mediated immunity.[10] NO is also involved in tumour biology and can display either stimulatory or inhibitory effects on cancer progression and metastasis, depending on several factors including concentration, cellular sensitivity, and duration of exposure.[11] NO-donors are products that can release NO under physiological conditions, and that can consequently be used as NO-prodrugs.[12] Previous research by the present group has shown that classical NO-donors, such as *S*-nitrosopenicillamine (SNAP), sodium nitroprusside (SNP), and *S*-nitrosoglutathione (GSNO), are able to reduce the efflux of **DOX** in human cancer cells. The mechanism responsible for this effect is the nitration of critical tyrosine residues of P-gp, ABCB1, and MRPs/ABCCs transporters.[13,14] Furoxan derivatives, which are known to release NO under the action of thiol co-factors, can also similarly inhibit P-gp and MRP1 pumps in cell lines of Madin-Darby canine kidney cells.[15] On these bases, new **DOX** derivatives have been proposed in which moieties containing NO-releasing groups are covalently linked to **DOX**. Some of these products can overcome resistance by inhibiting the ABC transporters that extrude the drug.[16-18] However, these NO donors have different NO release kinetics, and spatiotemporal control is totally lacking. This makes it necessary to use high concentrations of the NO donors and prolong their incubation time to reach an intracellular concentration sufficient for protein nitration.

Light is a powerful and minimally invasive "microsyringe" for the injection of NO into biological systems, with excellent spatiotemporal accuracy, using suitable NO photodonors (NOPs).[19] These compounds must satisfy several prerequisites for bio-application, including excitation with visible light, and formation of non-toxic and non-visible-light-absorbing side photoproducts.

In this paper we report for the first time that photoregulation of NO release at doses not toxic to cells, but able to inhibit several efflux pumps that are mainly responsible for MDR, may be a suitable approach not only to potentiate the anticancer activity of **DOX**, but also to reduce its toxicity towards healthy cells such as cardiomyocytes and fibroblasts.

As prototype **NOP**, a derivative of 4-nitro-3-(trifluoromethy)aniline (**NOP1** in Chart 1) developed in recent years was chosen.[20] This compound was used as such in combination with **DOX**, or covalently linked to the anticancer drug through a suitable bridge (**DOX-NOP1** in Chart 1).

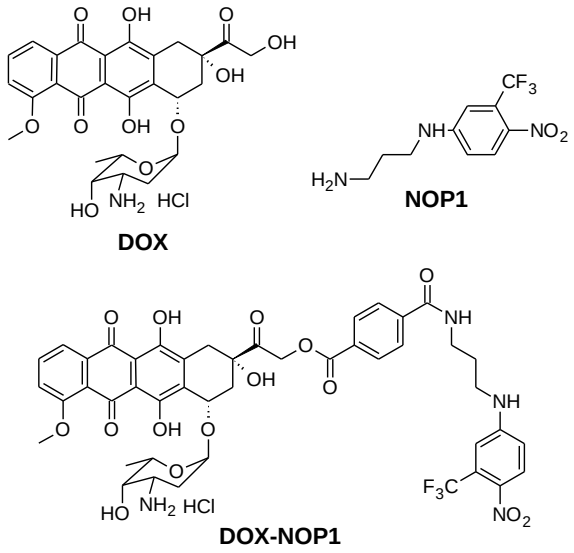

CHART 1. Molecular structure of **DOX**, the NO photodonor **NOP1** and the molecular hybrid **DOX-NOP1**.

NOP1 can be excited with visible light (λ > 400 nm) undergoing a nitro-nitrite rearrangement, followed by release of NO and formation of a phenol derivative as a stable product that does not absorb in the visible region.[20] Further, it can easily be modified structurally, making it a simple matter to produce the hybrid **DOX-NOP1** through simple synthetic procedures (see SI). This compound is soluble and stable in PBS with 3% DMSO and its half-life in DMEM medium exceeds 24 h. A key prerequisite for this conjugate to be active is retention of the two independent components' main properties after their covalent linking. In the case of photoactivable compounds, this is not a trivial requirement, since photoinduced intermolecular processes (*i.e.* energy and/or electron transfer) may preclude correct functioning of the conjugate. The absorption spectrum of **DOX-NOP1** (Figure 1A) matches that of an equimolar mixture of **DOX** and **NOP1** quite closely, exhibiting absorption bands at *ca*. 400 nm and 500 nm, typical respectively for the **NOP1** and **DOX** chromophores, thus ruling out any relevant interaction between the two functional units in the ground state. Further, the static and dynamic emission properties of the **DOX** unit are well preserved in the conjugate (see Figure S1,S2). This provides insights into the DNA intercalating capability of the conjugate by fluorescence spectroscopy. Analogously to what observed for **DOX**, the fluorescence emission of **DOX-NOP1** was significantly quenched upon addition of double-strand calf thymus DNA (ct-DNA) (Figure S3) in good agreement with the typical intercalation process of **DOX**.[21] A binding constant of 6.3×10^5 $M^{-1}s^{-1}$ and a number of **DOX** units bound per DNA base pairs of *ca*. 1 were obtained (see SI). While the latter value is similar to that reported for unfunctionalized **DOX**[21] (*ca*. 0.8), the former is almost one order of magnitude larger, accounting for a significant cooperative effect of the NO photodonor unit in the whole binding process. An increase in the binding constant of **DOX** derivatives has also been observed for **DOX** derivatives containing the trifluoromethyl substituents[21] (like that present in the structure of **NOP1**). The NO photoreleasing properties were also well preserved in the conjugate. NO release was firstly monitored by the typical Griess assay, which detects the content of nitrite, the main stable degradation product of NO oxidation under aerobic conditions. As shown in Figure 1B, the nitrite photogenerated from **NOP1** and from the hybrid **DOX-NOP1** did not differ significantly. NO photogeneration was also monitored by direct amperometric detection. **DOX-NOP1** is stable in the dark but releases NO exclusively under visible light excitation (***a*** in Figure 1C). Note that DNA intercalation does not change the NO photoreleasing capability of **DOX-NOP1**, which takes place with an efficiency very similar to that observed in the absence of DNA (***b*** in Figure 1C). It is also noteworthy that the spectral evolution observed upon light excitation of the **DOX-NOP1** complex with DNA (Figure 1D) showed bleaching only in the **NOP1** band (*ca*. 400 nm), typical for the formation of the non-absorbing product after NO release. In contrast, absorption in the region of **DOX** (*ca*. 500 nm) was almost unchanged. This finding explains the good preservation of the integrity of the **DOX** unit in the conjugate upon light excitation, a fundamental requisite for its anticancer action to be maintained.

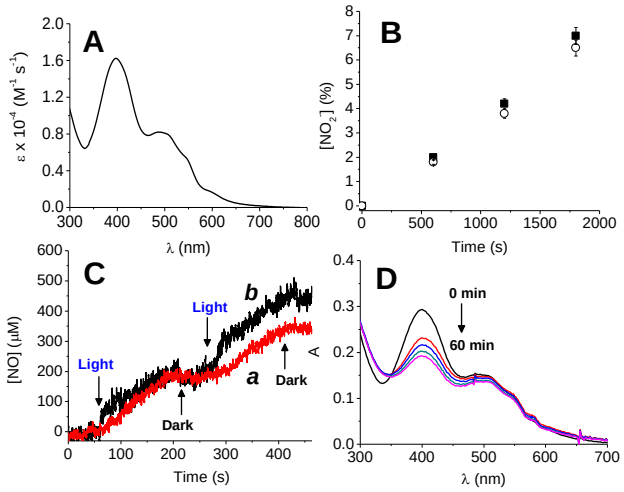

Figure 1. (A) Absorption spectrum of **DOX-NO1**. (**B**) Generation of nitrite observed by Griess assay at different irradiation times of a solution of **DOX-NOP1** (100 μM) (○) and, for comparison, an optically matched solution of **NOP1** (■). (**C**) NO release profile observed upon alternate cycles of light (λ_{exc} = 405 nm) and dark for a solution of **DOX-NOP1** (10 μM) in the absence (***a***) and in the presence (***b***) of ct-DNA (18 μM). (**D**) Absorption spectral changes observed after 0, 30, 40, 50 and 60 min of irradiation (λ_{exc} = 405 nm) of a solution **DOX-NOP1** (10 μM) in the presence of ct-DNA (18 μM). PBS (pH 7.4, 10 mM with 3%DMSO).

NOP1 and **DOX-NOP1** were tested in human melanoma M14 cells, which express several ABC transporters (Figure S4). **DOX** did not elicit any relevant increase of nitrites, independently of cell

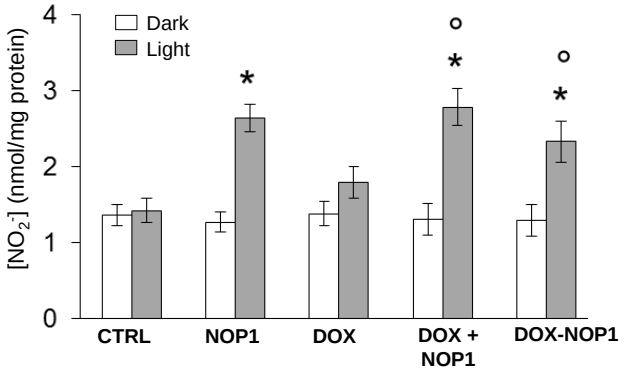

Figure 2. Nitrite observed by Griess assay in M14 melanoma cells maintained for 20 minutes at room temperature in PBS in the dark or irradiatiated (λ_{exc} = 400 nm), in the absence (**CTRL**) or in the presence of 5 μM **NOP1**, **DOX**, **DOXO** + **NOP1** or **DOX-NOP1**. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated cells (**CTRL**): * $p < 0.005$; *vs.* **DOX**-treated cells (D): ° $p < 0.005$.

irradiation (Figure 2). In contrast, and in line with NO release in acellular systems, both **NOP1** and **DOX-NOP1** significantly and similarly increased nitrite levels in irradiated cells. As expected, none of the compounds increased nitrite in non-irradiated cells (Figure 2).

In principle, it could be argued that the NO released in cells treated with **NOP1** and **DOX-NOP1** may also originate from the upregulation of inducible nitric oxide synthase (iNOS) induced by **DOX**.[13,14] However, this is not the case: the ability of **DOX** to induce iNOS is proportional to the drug's intracellular accumulation. This event usually occurs in **DOX**-sensitive cells, but not in **DOX**-resistant cells, from which the drug rapidly effluxes by action of the ABC transporters.[14] Since melanoma cells express at least three transporters involved in **DOX** efflux (Pgp, MRP1, and BCRP), it is unlikely that **DOX** reaches an intracellular concentration sufficient to induce iNOS. The increase of nitrite is more likely due to the release of NO from **NOP1** and **DOX-NOP1**, for two reasons: i) it occurs exclusively upon irradiation; and ii) the amount of nitrite released by compounds **NOP1** and **DOX-NOP1** in acellular systems is compatible with the amount of nitrite measured in the supernatants of cells treated with those compounds.

As mentioned above, NO inhibits the drug efflux activity of ABC transporters by nitrating critical tyrosines.[13-18] In line with these findings, Pgp, MRP1, MRP4, and BCRP were nitrated on tyrosines when incubated with either **NOP1** (alone or co-incubated with **DOX**) or **DOX-NOP1**, upon irradiation (Figure 3A). When nitrated, the pumps displayed reduced catalytic activity (Figure 3B). Since Pgp, MRP1, and BCRP are the main transporters involved in

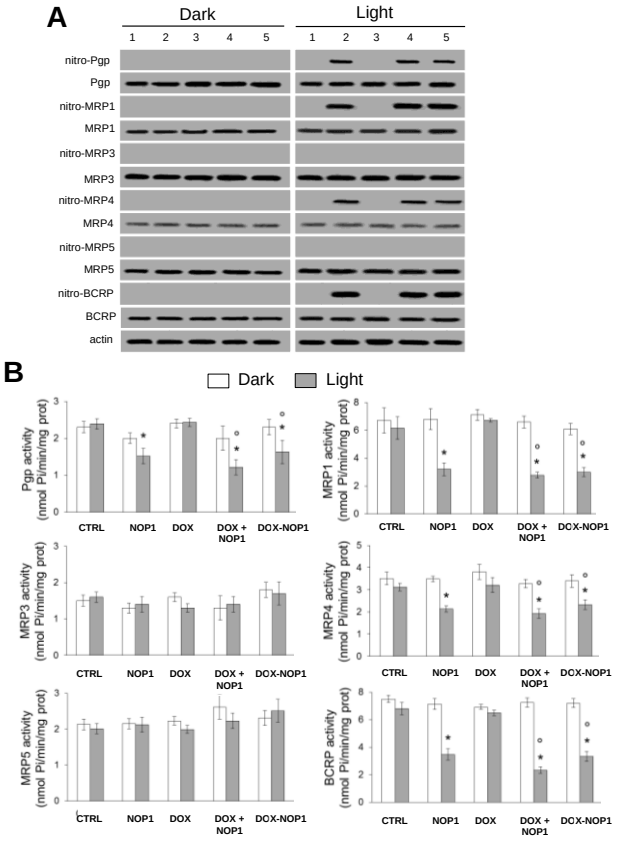

Figure 3. (**A**) Immunoblot detection of nitrated ABC transporters in M14 melanoma cells maintained for 20 minutes at room temperature in PBS in a dark room or irradiated (λ_{exc} = 400 nm), in the absence (**CTRL,** 1) or presence of 5 µM **NOP1** (2), **DOX** (3), **DOXO** + **NOP1** (4) or **DOX-NOP1** (5). After 24 h in complete medium, cells were subjected to the following assays. (**B**) ATPase activity. Data are presented as means ± SD (n = 3); *vs.* untreated cells (*CTRL*): * $p < 0.02$; *vs.* **DOX**-treated cells: ° $p < 0.002$.

DOX efflux, their nitration may increase intracellular retention of **DOX**, as has been reported.[13,14] It should be noted that, in this study, **DOX** and **NOP1** were used at equimolar concentration, *i.e.* 5 µM. This concentration is 20 times lower than that used in classical NO donor studies (e.g. SNAP, SNP, GSNO) to observe effective nitration and inhibition of ABC transporter activity.[13,14] It thus emerges that the use of NOP offers the advantage of fine-tuning the amount of NO released, allowing closely-controlled nitrating concentrations to be achieved, and enabling the amount of NO releaser used to be significantly reduced. Not all the ABC transporters present in M14 cells were nitrated. Nitration is critically influenced by the amount and localization of target proteins, the amount and accessibility of tyrosines, and the type of aminoacids surrounding tyrosines.[22] It cannot be excluded that, changing tumor type and/or incubation conditions, a different spectrum of nitrated ABC transporters might be obtained.

The anti-tumor efficacy of **DOX** and **DOX-NOP1** was evaluated by measuring the extracellular release of lactate dehydrogenase (LDH), an index of cell damage and necrosis.[13] As expected, **DOX** did not induce any cell damage in M14 cells (Figure 4), in line with what has been observed in other resistant cancer cells with low intracellular accumulation of **DOX** and high drug efflux rate via ABC transporters.[13,14,16-18] Interestingly, the mixture of **DOX** and **NOP1**, as well as the hybrid **DOX-NOP1**, significantly induced cytotoxicity upon irradiation, overcoming **DOX** resistance. If note, this enhanced cytotoxicity is not due to the cytotoxic action of NO: as Figure 4 shows, **NOP1** was not toxic either in the dark or under irradiation. The amount of NO released from **NOP1**, and similarly from **DOX-NOP1** (see above) is in the nanomolar range, i.e. below the cytotoxic micromolar range for NO.[11]

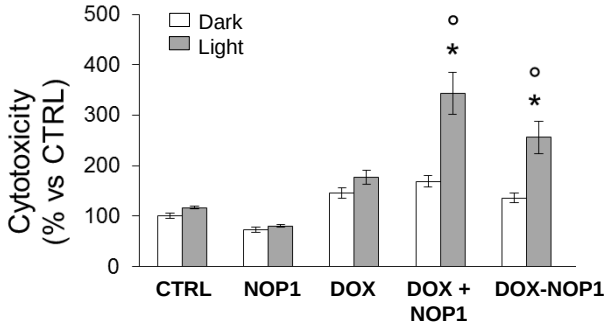

Figure 4. Cytotoxicity observed in melanoma M14 cells maintained for 20 minutes at room temperature in PBS in the dark or irradiated (λ_{exc} = 400 nm), in the absence (**CTRL**) or in the presence of 5 µM **NOP1**, **DOX**, **DOXO** + **NOP1** or **DOX-NOP1**. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated cells (**CTRL**): * $p < 0.001$; *vs.* **DOX**-treated cells (D): ° $p < 0.001$.

The toxicity of co-incubated **DOX** and **NOP1**, the hybrid **DOX-NOP1**, and for comparison that of **DOX**, was also tested in non-transformed cells, namely fibroblasts and cardiomyocytes, well-known targets of **DOX** (Figure 5).[23]

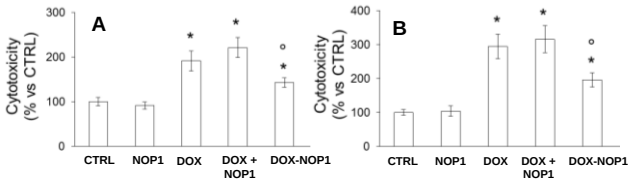

Figure 5. Cytotoxicity observed human fibroblasts (A) and rat H9c2 cardiomyocytes (B) maintained for 20 minutes at room temperature in PBS in the dark or irradiated (λ_{exc} = 400 nm), in the absence (**CTRL**) or in the presence of 5 µM **NOP1**, **DOX**, **DOXO** + **NOP1** or **DOX-NOP1**. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated cells (**CTRL**): * $p < 0.001$; *vs.* **DOX**-treated cells (D): ° $p < 0.001$.

As expected, **DOX** was toxic in both cell populations. Interestingly, co-incubation of **DOX** with **NOP1** did not further increase the cytotoxicity of **DOX**, and the conjugate **DOX-NOP1**, which was cytotoxic against melanoma cells, displayed lower toxicity than the parent **DOX**.

In summary, it has been demonstrated for the first time that the use of **DOX** and **NOP1**, either in combination or under the form of the molecular hybrid **DOX-NOP1**, offers the advantage of precisely regulating the amount of NO released, allowing the concentration of NO precursors required to reverse chemo-resistance to be reduced by 20 times compared to non-activated NO releasers. The **DOX-NOP1** conjugate exhibits similar NO photoreleasing properties than free **NOP1**, combined with enhanced DNA binding capability compared with **DOX**. Another remarkable finding is that **DOX-NOP1** overcomes both main drawbacks of **DOX**: the molecular hybrid leads to a reversion of MDR, and also possesses reduced toxicity towards healthy cells, paving the way for innovative and underexplored approaches to overcome the main therapeutic drawbacks of **DOX**.

Finally, it should be stressed that melanoma is a highly chemo-resistant tumor expressing multiple ABC transporters. It has been demonstrated that inhibiting only one transporter is not sufficient to effectively reverse chemoresistance.[24] The strategy reported here inhibits the activity of different ABC transporters at the same time, reducing the **DOX** extrusion activity of the dedicated transporters (Pgp, MRP1, BCRP). This broad-spectrum inhibition of ABC transporters may lead to increased retention and cytotoxicity of other chemotherapeutic drugs, determining an efficient reversion of chemoresistance that is not limited to **DOX**. These studies are currently underway in our laboratories to investigate this possibility.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, steady-state and time resolved emission spectra, determination of DNA binding constant, ABC transporters expression in melanoma cells **(PDF).**

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Author Contributions

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Notes

The authors declare no competing financial interests.

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