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H_2S-Donating Doxorubicins May Overcome Cardiotoxicity and Multidrug Resistance

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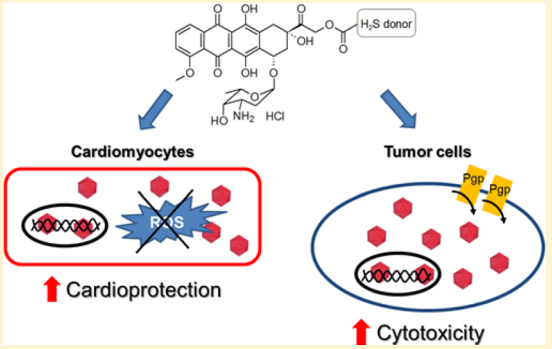
Supporting Information

ABSTRACT: Doxorubicin (DOXO) is one of the most effective antineoplastic agents in clinical practice. Its use is limited by acute and chronic side effects, in particular by its cardiotoxicity and by the rapid development of resistance to it. As part of a program aimed at developing new DOXO derivatives endowed with reduced cardiotoxicity, and active against DOXO-resistant tumor cells, a series of H_2S-releasing DOXOs (H_2S-DOXOs) were obtained by combining DOXO with appropriate H_2S donor substructures. The resulting compounds were studied on H9c2 cardiomyocytes and in DOXO-sensitive U-2OS osteosarcoma cells, as well as in related cell variants with increasing degrees of DOXO-resistance. Differently from DOXO, most of the products were not toxic at 5 μM concentration on H9c2 cells. A few of them triggered high activity on the cancer cells. H_2S-DOXOs **10** and **11** emerged as the most interesting members of the series. The capacity of **10** to impair Pgp transporter is also discussed.

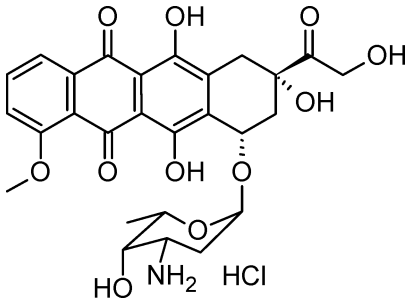


INTRODUCTION

Doxorubicin (DOXO) **1** (Chart 1), known also as Adriamicin, is a potent broad-spectrum antineoplastic antibiotic, isolated from *Streptomyces* species, widely used as single agent or in combination with other anticancer drugs in treating of hematological cancers and solid tumors, lymphomas, and sarcomas.[1] Its use is accompanied by a number of clinical toxicities, of which cardiomyopathy is the most important. Clinically, there are two kinds of cardiomyopathy: an acute form, with rapid onset after the administration of a single dose of antibiotic, and a chronic, cumulative, dose-related form.[2,3] The former is characterized by abnormal electrocardiographic changes, and is rarely a serious problem; the latter can lead to congestive heart failure that is unresponsive to digitalis. The mortality rate in patients with congestive heart failure is close to 50%. The production of high levels of reactive oxygen species (ROS), including peroxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), by induction of the antibiotic's redox cycle at complex 1 of the mitochondrial electron transport chain, is the most likely of the mechanisms proposed to explain this cardiotoxicity.[4,5] The heart is very sensitive to oxidative stress because of its poor antioxidant defenses compared to other organs.[6,7] The cardiomyocyte damage appears to be principally due to impairment of mitochondrial functioning.[4,7,8]

Several synthetic DOXOs characterized by lower cardiotoxicity have been described. For instance, the DOXO's analogs N-trifluoroacetyladriamycin-14-valerate (AD32), N-trifluoroacetyladriamycin-14-O-hemiadipate (AD143) and N-benzyladriamycin-14-valerate (AD198) induce lower toxicity in rat hearts thanks to their different effects on mitochondrial energy

Chart 1. Doxorubicin (DOXO)



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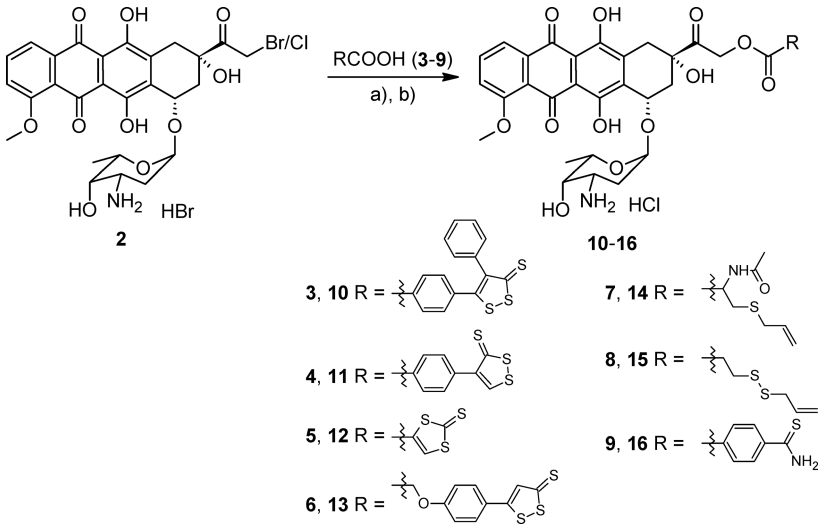
Scheme 1[a]



[a]Reagents and conditions: (a) KF, DMF, rt; (b) dry THF, HCl in dry dioxane.

metabolism.[9] Semisynthetic DOXOs conjugated with the antioxidant ferulic or caffeic acids were less cardiotoxic but still retain their antitumor efficacy.[10] Similarly, a synthetic doxorubicin targeting mitochondria, which is effective against ovarian cancer cells, induces at the same time a lower mitochondria-dependent cardiac damage.[11] A critical issue in the production of DOXO analogs with lower cardiotoxicity is to prevent the loss of their cytotoxicity against tumors with low sensitivity to the drug. Indeed, DOXO's efficacy in cancer therapy is also hampered by the ease with which resistance to it develops. This occurs through a number of mechanisms, principally the increased capacity of the cancer cells to efflux the drug, thus limiting its cellular accumulation and reducing its toxicity.[12] Interestingly, DOXO resistance is decreased by nitric oxide (NO) donors, namely compounds able to increase the intracellular NO concentration.[13−17]

Hydrogen sulfide (H_2S) is a colorless gas with a characteristic smell of rotten eggs that, as a biochemical agent, has long only been considered as a poisonous and toxic pollutant. It is a weak acid ($pK_{a1} \approx 6.9$, $pK_{a2} > 12$ at 25 °C), and at physiological pH (7.4) the two species present in equilibrium are HS^- and H_2S, in a ratio of about 3:1. At 37 °C its water solubility is about 80 mM.[18] It is able to cross lipid membranes by simple diffusion, and does not require facilitation by membrane channels.[19] A huge amount of experimental evidence has accumulated in the past 15 years to show that hydrogen sulfide is an endogenous gasotransmitter produced from L-cysteine by the action of two pyridoxal-5′-phosphate (PLP)-dependent enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase, also called cystathionase (CSE).[20]

Like the other two gasotransmitters, NO and carbon monoxide (CO), H_2S performs a variety of homeostatic functions, especially in the nervous and cardiovascular systems.[20−25] Several reports indicate that there is cross-talk between H_2S and NO. This relation is complex, and it requires further investigation. It has been suggested that these two species may interact, producing an unidentified nitrosothiol, which triggers a weak vasorelaxing effect, if any.[26,27] In addition, H_2S plays roles in modulating the cellular S-nitrosothiol profile, probably through the formation of thionitrous acid (HSNO), which is a source of nitrosonium ion (NO^+), NO, and nitroxyl (HNO).[28,29] H_2S exhibits strong cytoprotective effects through a combination of antioxidant and antiapoptotic signals.[30−33] It is able to scavenge ROS and reactive nitrogen species (RNS).[33] In particular, it is able to suppress peroxinitrite (HOONO) induced cell damage, owing to its ability to react with HOONO, giving rise to sulfinyl nitrite ($HSNO_2$), a new NO donor.[34] A number of recent reports show that sodium hydrosulfide (NaSH), which at physiological pH is in equilibrium with H_2S, attenuates DOXO-induced cardiotoxicity in H9c2 embryonic rat cardiac cells, by inhibiting endoplasmatic reticulum (ER) stress and oxidative stress.[35] Inhibition of the p38 MAPK pathway, activated by DOXO, seems to be an important mechanism underlying this protection.[36] Exogenous H_2S has also recently been shown to attenuate DOXO-induced cardiotoxicity in H9c2 cells, by inhibiting calreticulin (CRT) expression.[37] On the basis of this rationale, DOXO linked to H_2S donors (H_2S-DOXO), namely compounds capable of releasing H_2S under physiological conditions,[38−40] might be expected to give rise to chimeras endowed with improved biochemical profiles compared to the antibiotic lead.

This paper describes a number of such compounds (Scheme 1) obtained by combining DOXO with H_2S donors through an ester linkage at C-14. They are shown to be less cardiotoxic than the lead, and they maintain high levels of activity against DOXO-resistant cancer cell lines.

RESULTS AND DISCUSSION

Synthesis. The H_2S-DOXOs **10−16** were obtained by the reaction of 14-bromo/chloro daunomicin hydrobromide (**2**) with the appropriate H_2S donor acids **3−9** (Scheme 1). The reaction was performed in dry DMF in the presence of KF at room temperature. The resulting products were purified by flash chromatography, successively suspended/dissolved in dry THF, and treated with 1 equiv of HCl in dry dioxane to obtain the corresponding hydrochlorides. The purity of the compounds was evaluated by RP-HPLC techniques.

Stability of the H_2S-DOXOs. The stability of all the new DOXO derivatives was evaluated by high-performance liquid chromatography (HPLC) in Dulbecco's Modified Eagle Medium (DMEM), in Iscove's Modified Dulbecco's Medium (IMDM), as well as in human serum. The products were

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hydrolyzed following pseudo-first-order kinetics. The half-lives ($t_{1/2}$), determined by fitting the data to a one-phase exponential decay equation, are reported in Table 1.

Table 1. Stability ($t_{1/2}$) of H_2S-DOXOs in Cell Culture Medium (DMEM) and in Human Serum[a]

Compound	DMEM $t_{1/2}$	Human serum $t_{1/2}$
10	>24 h	2.8 h
11	21.5 h	4.6 h
12	17 min	17 min
13	16 min	3 min
14	<1 min	11 min
15	5.6 h	3.8 h
16	3.4 h	38 min

[a]Data derived from 3−5 independent experiments.

In DMEM, the compounds may be separated into four clusters: compounds possessing high (**10**, **11**, $t_{1/2}$ > 20 h), medium (**15**, **16**, $t_{1/2}$ = 3−6 h), low (**12**, **13**, $t_{1/2}$ = 16−17 min), and very low (**14**, $t_{1/2}$ < 1 min) stability (Table 1).

Similar results (data not shown) were obtained in IMDM. In human serum, the products **12**−**14** and **16** showed $t_{1/2}$ < 38 min, while the more stable **10**, **11**, and **15** showed $t_{1/2}$ values in the range 2.8−4.6 h. The $t_{1/2}$ values measured in the different media principally reflect the presence of two vulnerable moieties in the H_2S-DOXOs: the ester group, and the H_2S donor substructure. HPLC analysis (see Supporting Information) showed that both these moieties contribute to determining this parameter, in different degrees depending on the specific product.

Hydrogen sulfide release from the H_2S-DOXOs. H_2S release was assessed using a fluorometric assay based upon the reaction of H_2S with dansyl azide (DNS-Az) to give the fluorescent related amide, which was detected with a HPLC system equipped with a fluorimetric detector.[41] The results of H_2S release in DMEM are reported in Figure 1. The most potent H_2S donor was **11**, a derivative of benzoic acid bearing, in *para*-position, the 3-thioxo-3*H*-1,2-dithiol-5-yl group. Introduction into **11** of a phenyl substituent at the 4-position of the thione ring (**10**) reduced this capacity. Substitution of the thione ring with a thiocarbamoyl group (**16**), and in particular

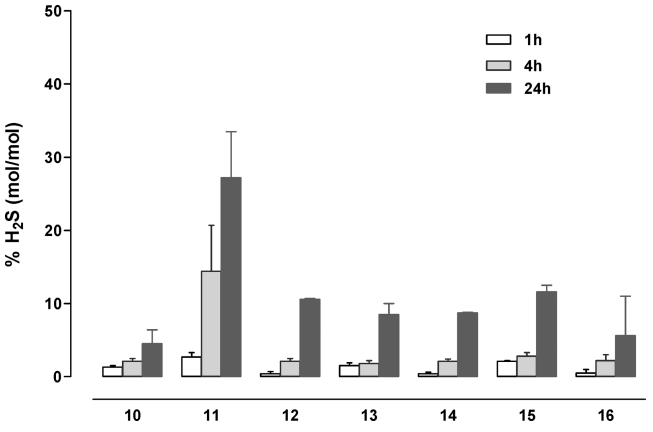

Figure 1. H_2S release assessment from the H_2S-DOXOs at 1, 4, and 24 h incubation time in DMEM. Data are presented as mean ± SEM (SEM ≤ 3; number of determinations = 3−5).

manipulation of the ester bridge (**13**), but also transformation of 2-thioxo-1,3-dithiole-4-carboxylic acid into the ester (**12**), decreased H_2S release. Compounds **14** and **15**, two aliphatic esters, were markedly less potent H_2S donors than **11**. Very similar results were obtained when the experiments were performed in IMDM (data not shown). In human serum, H_2S release was increased, except in the case of compound **14** (Supporting Information Figure 1).

BIOLOGICAL ASSAYS

Accumulation of H_2S-DOXOs and their cytotoxic effects in H9c2 cardiomyocytes. We compared the effect of DOXO and H_2S-DOXOs at 5 μM concentration, that we previously found to be cytotoxic in H9c2 cardiomyocytes[10,16,17] and in DOXO-sensitive cancer cells, but not in DOXO-resistant ones.[13,16,42,43] The accumulation of H_2S-DOXOs within H9c2-cardiomyocytes was measured by a fluorimetric assay, and their cytotoxic effects were assayed by detecting the activity of lactic dehydrogenase (LDH) in the extracellular medium, considered as an index of doxorubicin-induced damage.[13,15] As shown in Figure 2A, H_2S DOXOs were retained within H9c2 cells at

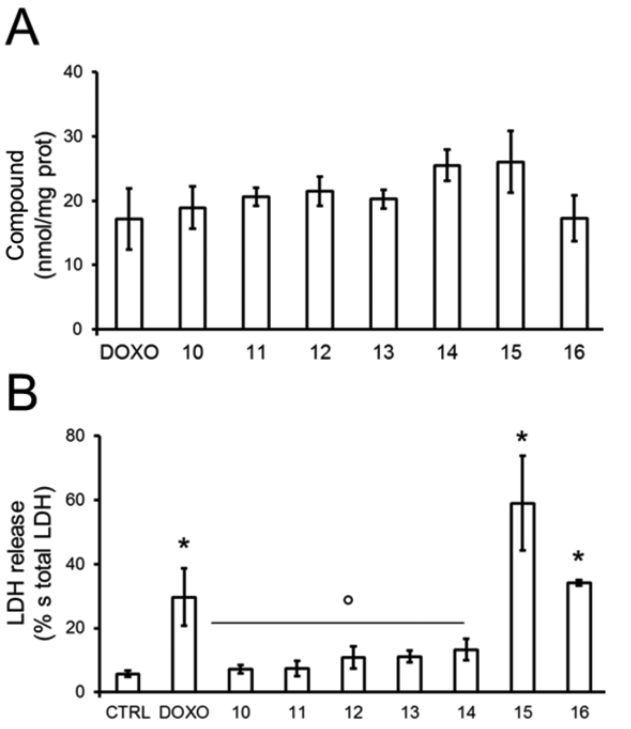

Figure 2. Intracellular accumulation and cytotoxicity of H_2S-DOXOs in cardiomyocytes. H9c2 cardiomyocytes were incubated for 24 h in fresh medium (CTRL) or in medium containing 5 μM DOXO or H_2S-DOXOs (**10**−**16**). (A) The amount of intracellular DOXOs was measured fluorimetrically in cell lysates in duplicate (n = 3). Data are presented as means ± SD. Vs DOXO: * p < 0.002. (B) The release of extracellular LDH in the culture medium was measured spectrophotometrically in duplicate (n = 3). Vs CTRL: * p < 0.001; vs DOXO: ° p < 0.05.

similar concentrations as DOXO was. However, while DOXO induced significant cytotoxicity, the hybrid antibiotics **10**−**14** were significantly less toxic than the lead, and did not produce significantly higher toxicity compared to the untreated cells (Figure 2B).

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Compound **3**, the H_2S-releasing moiety present in **10**, did not modify the intracellular retention of DOXO within H9c2 cells when co-incubated at an equimolar concentration (Supporting Information Figure 2A), and it was not toxic when used alone. Conversely, co-incubation significantly reduced DOXO's toxicity, suggesting that the H_2S-DOXOs' lack of cardiotoxicity is due to the presence of the H_2S-releasing moieties (Supporting Information Figure 2B). The case is different with compounds **15** and **16**, which are significantly more cardiotoxic than the lead (Figure 2B). To shed light on this difference, the ethyl ester of acid **8** and the methyl ester of acid **9** (**17** and **18** respectively; see Supporting Information), the H_2S donor substructures present in **15** and **16**, were tested for their toxicity on H9c2 cells. The results clearly indicate that these two products are more toxic than the lead (Supporting Information Figure 3). It may thus reasonably be supposed that the cardiotoxicity of H_2S-DOXOs **15** and **16** is due to the presence in their structure of these two moieties.

Effect of H_2S-DOXOs on ROS intracellular levels. The intracellular levels of ROS, which are critical mediators of DOXO-induced cardiotoxicity,[7] were measured by a fluorimetric assay. DOXO significantly increased the ROS levels in H9c2 cardiomyocytes; by contrast, the intracellular ROS levels measured in the same cells treated with H_2S-DOXO were significantly lower than those produced by the parent antibiotic and did not differ from untreated cells (Figure 3A). To determine whether the reduced ROS levels were caused by the release of H_2S, ROS levels were again measured in the presence of hydroxocobalamin (OHCbl), a well-known H_2S scavenger.[44] The product did not affect the ROS levels in cells treated with DOXO, but markedly increased them in those exposed to H_2S-DOXOs, producing the same range of ROS measured in DOXO-treated cells (Figure 3A). In line with this data, OHCbl was not toxic in untreated cardiomyocytes, and did not modify the cytotoxicity of DOXO; conversely, it significantly increased the cytotoxic effects induced by all H_2S-DOXOs except for **15** and **16**, which showed the opposing trend (Figure 3B). Compound **3** alone was sufficient to reduce basal ROS levels in H9c2 cells, an event that was reversed by OHCbl (Supporting Information Figure 4A). Similarly, the presence of OHCbl increased the cytotoxicity of **3**, alone or co-incubated with DOXO (Supporting Information Figure 4B). Similarly, the antioxidant N-acetyl-L-cysteine (NAC), used at a concentration that significantly reduced the amount of ROS produced by DOXO (Supporting Information Figure 5A), strongly reduced the release of LDH (Supporting Information Figure 5B), as did compounds **10**–**14**. By contrast, the co-incubation of DOXO with dexrazoxane, which achieves significant cardioprotection acting through an ROS-independent mechanism,[45] failed to reduce DOXO's cytotoxicity in H9c2 cells (Supporting Information Figure 6).

As demonstrated by the experiments with OHCbl and NAC, the presence of appropriate H_2S-releasing moieties linked to DOXO or the presence of an excess of an antioxidant molecule plays a pivotal role in lowering ROS levels and in preventing toxicity in cardiomyocytes. These results strongly support the hypothesis that the reduced cardiotoxicity of compounds **10**–**14** was due to the decreased intracellular level of ROS.

H_2S-DOXOs as effective anticancer agents. Since oxidative stress is one of the main mechanisms involved in DOXO's antitumor activity,[7] the next point to clarify was whether the H_2S-DOXOs still retained their activity on cancer cells. The attention was focused on human osteosarcoma cells,

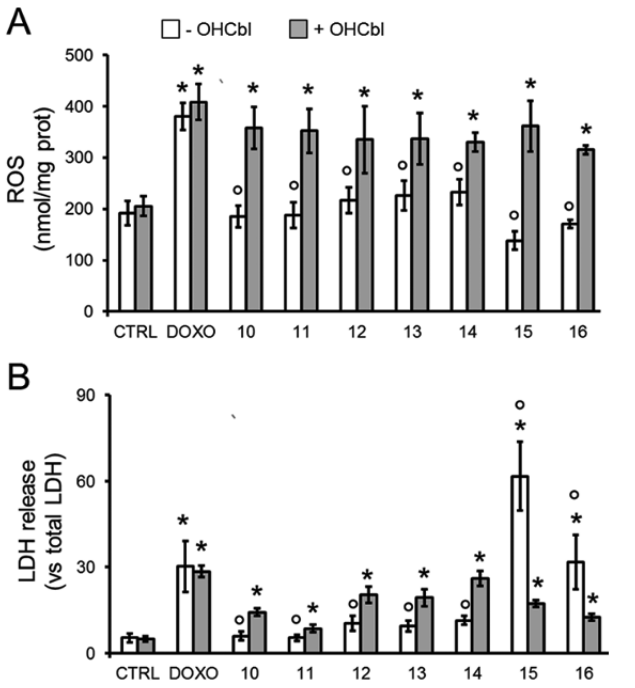

Figure 3. Intracellular ROS and cytotoxicity of H_2S-DOXOs in cardiomyocytes treated with the H_2S scavenger hydroxocobalamin. H9c2 cardiomyocytes were incubated for 24 h in fresh medium (CTRL) or in medium containing 5 μM DOXO or H_2S-DOXOs (**10**–**16**), in the absence (−) or in the presence (+) of 100 μM OHCbl, chosen as H_2S scavenger. (A) The amount of intracellular ROS was measured fluorimetrically in cell lysates in duplicate (n = 3). Data are presented as means ± SD. Vs respective CTRL: * $p < 0.01$; vs DOXO: ° $p < 0.002$; − OHCbl vs + OHCbl: $p < 0.01$ for all compounds (not shown). (B) The release of extracellular LDH in the culture medium was measured spectrophotometrically in duplicate (n = 3). Vs respective CTRL: * $p < 0.05$; vs DOXO: ° $p < 0.05$; − OHCbl vs + OHCbl: $p < 0.05$ for all compounds (not shown).

since DOXO is the first-line drug in this kind of tumor;[46] however, its efficacy is limited in osteosarcoma expressing P-glycoprotein (Pgp/ABCB1),[47] a membrane transporter that effluxes DOXO and limits its anticancer efficacy.[12] The effects of H_2S-DOXOs in DOXO-sensitive U-2OS osteosarcoma cells, and in the related cell variants with increasing degrees of DOXO-resistance, namely U-2OS/DX30, U-2OS/DX100, and U-2OS/DX580, were investigated. The DOXO IC_{50} values of these variants show a progressive increase (Supporting Information Table 1), according to the increasing expression of Pgp.[48] As expected, DOXO was accumulated in progressively smaller amounts in the resistant cells (Figure 4A), where it progressively lost its toxicity (Figure 4B). By screening the intracellular retention of the H_2S-DOXOs, **10**, **11**, **15**, and **16** were identified as being accumulated to a significantly greater extent than DOXO itself, in both sensitive and resistant cells (Figure 4A).

In line with their higher intracellular retention, these compounds were significantly more cytotoxic than DOXO in both drug-sensitive and drug-resistant cells (Figure 4B). Co-incubation of **3** with DOXO increased drug retention and cytotoxicity in U-2OS/DX30 and U-2OS/DX100 cells; however, this combination did not show superior efficacy than DOXO in U-2OS/DX580 cells (Supporting Information Figure 7A, B). These data suggest that the sole presence of a H_2S-releasing group may be sufficient to improve DOXO's

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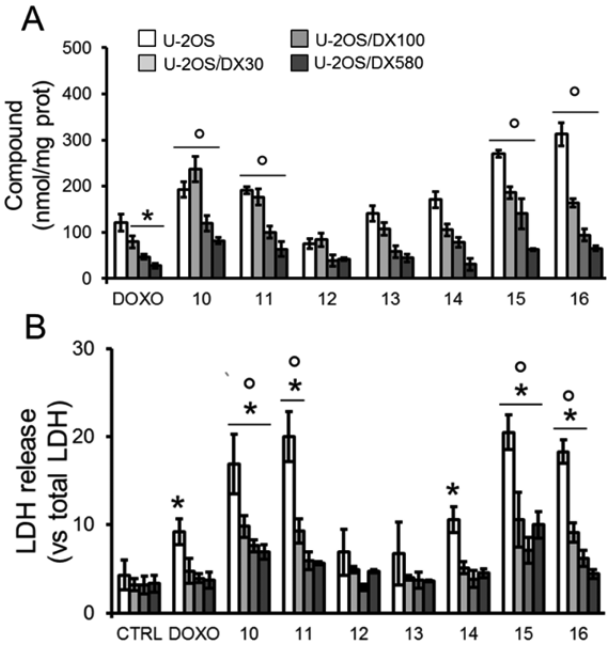

Figure 4. Intracellular accumulation and cytotoxicity of H_2S-DOXOs in osteosarcoma cells. DOXO-sensitive U-2OS cells and the DOXO-resistant variants (U-2OS/DX30, U-2OS/DX100, U-2OS/DX580) were incubated for 24 h in fresh medium (CTRL) or in medium containing 5 μM DOXO or H_2S-DOXOs (**10**–**16**). (A) The amount of intracellular DOXOs was measured fluorimetrically in cell lysates in duplicate (n = 3). Data are presented as means ± SD. For DOXO, resistant cells vs U-2OS cells: * $p < 0.05$; for all compounds, vs DOXO: * $p < 0.05$. (B) Release of extracellular LDH into the culture medium was measured spectrophotometrically in duplicate (n = 3). Data are presented as means ± SD. Vs respective CTRL: * $p < 0.01$; vs DOXO: ° $p < 0.05$.

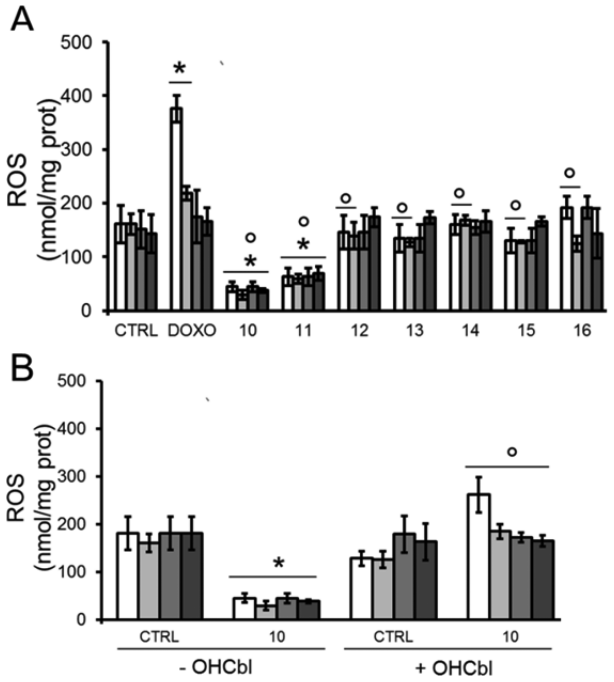

Figure 5. Intracellular ROS and cytotoxicity of H_2S-DOXOs in osteosarcoma cells treated with OHCbl. DOXO-sensitive U-2OS cells and the DOXO-resistant variants (U-2OS/DX30, U-2OS/DX100, U-2OS/DX580) were incubated for 24 h in fresh medium (CTRL) or in medium containing 5 μM DOXO or H_2S-DOXOs (**10**–**16**), in the absence (−) or in the presence (+) of 100 μM OHCbl. (A and B) The amount of intracellular ROS was measured fluorimetrically in cell lysates in duplicate (n = 3). Data are presented as means ± SD. For panel A: vs respective CTRL: * $p < 0.001$; vs DOXO: ° $p < 0.05$. For panel B: vs respective CTRL: * $p < 0.001$; − OHCbl vs + OHCbl: $p < 0.001$.

efficacy in mildly chemoresistant cells, but not in strongly resistant ones. Interestingly, while DOXO increased the ROS levels in U-2OS cells but not in resistant cells, in sensitive cells the H_2S-DOXOs produced significantly less ROS than DOXO did (Figure 5A). Of note, **10** and **11**, which exerted high cytotoxicity, also significantly reduced the ROS amount compared with untreated osteosarcoma cells (Figure 5A). This reduction of ROS levels is likely due to the presence of the H_2S-releasing groups, since ROS levels were decreased in osteosarcoma cells exposed to DOXO plus **3** to a significantly greater extent than they were in cells exposed to DOXO alone (Supporting Information Figure 7C). The observed increase in ROS production in cells treated with **10** in the presence of OHCbl is in line with this result (Figure 5B).

Similar data were obtained with **11** (not shown). These results indicate that **10** and **11** are cytotoxic in both drug-sensitive and drug-resistant cells, notwithstanding the lower ROS production, leading to the hypothesis that they may exert their anticancer effects through an oxidative stress-independent mechanism. Several works highlight that part of the anticancer efficacy of anthracyclines is due to the inhibition of topoisomerase II.[49] By contrast, all H_2S-DOXOs were unable to inhibit this enzyme (Supporting Information Figure 8), leading to exclusion of the possibility that their anticancer effects were mediated by this mechanism.

H_2S-DOXOs are less effluxed by Pgp in osteosarcoma-resistant cells. To shed light on how the presence of H_2S-releasing groups improves the efficacy of doxorubicin in resistant cells, the efflux kinetics of parental DOXO, DOXO co-incubated with **3**, and **10** were measured in the Pgp overexpressing U-2OS/DX580 cells. As shown in Figure 6, the presence of **3** reduced the V_{max} of DOXO efflux, without affecting the K_m; by contrast, **10** had a lower V_{max} and a higher K_m than DOXO.

These results suggest that the release of H_2S by an appropriate H_2S donor impairs the catalytic efficacy of Pgp, thus explaining the increased intracellular accumulation of DOXO in the presence of **3**. In the case of **10**, the presence of an H_2S donor linked to the anthracycline moiety not only impairs the catalytic activity Pgp, but also reduces the compound's affinity for the protein, producing maximal benefit in terms of drug accumulation and toxicity.

CONCLUSION

A series of H_2S-releasing moieties were linked through an ester bridge to C-14 of DOXO to improve its pharmaceutical profile, in view of their ability to release H_2S. All products were tested on cardiac H9c2 cells, and on U-2OS osteosarcoma cells and related cell variants with increasing degrees of DOXO resistance. The specific H_2S-releasing moiety strongly influenced the products' biological behavior. All H_2S-DOXOs were able to reduce the oxidative stress induced by the antibiotic on the cardiomyocytes, and most of them were significantly less toxic on the cardiomyocytes than the lead. Compounds **15** and **16** reduced the oxidative stress but not the cardiotoxicity, paralleling the intrinsic toxicity of the two H_2S-releasing

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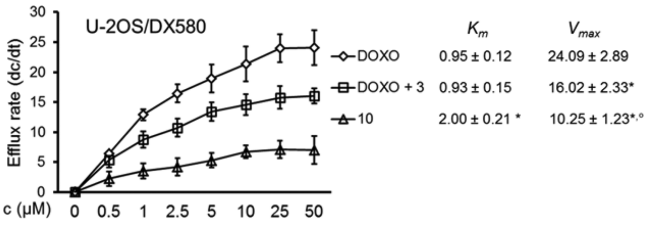

Figure 6. Efflux kinetics of DOXO, DOXO + **3**, and **10**, from drug-resistant osteosarcoma cells. DOXO-resistant U-2OS/DX580 cells were incubated for 20 min with increasing concentrations (0−50 μM) of DOXO, DOXO with the H_2S-releasing compound **3**, or H_2S-DOXO (**10**). Cells were washed and tested fluorimetrically for their intracellular drug content. The procedure was repeated on a second series of dishes, incubated under the same experimental conditions and analyzed after 10 min. Data are presented as means ± SD (n = 3). The rate of DOXOs efflux (dc/dt) was plotted versus the initial concentration of the drug. V_{max} (nmol/min/mg proteins), and K_m (nmol/mg proteins) values were calculated with the Enzfitter software. DOXO + **3** vs DOXO: $p \leq 0.001$, for concentrations 1−50 μM; **10** vs DOXO: $p \leq 0.001$, for concentrations 0.5−50 μM; **10** vs DOXO + **3**: $p \leq 0.01$, for concentrations 0.5−50 μM (not shown). For K_m, vs DOXO: * p 0.01; for V_{max}, vs DOXO: * $p < 0.001$; vs DOXO + **3**: ° $p < 0.05$.

moieties **7** and **8** present in their structures. Compounds **10** and **11** emerged as the most interesting members of the series. When tested on H9c2 cells, they did not produce a significantly higher toxicity than the control. In addition, when tested on sarcoma cell lines, they displayed significantly more potent cytotoxic effects than those of the lead. Preliminary studies carried out on **10**, taken as a representative member of the class, suggest that the increased cytotoxicity is likely due to a reduced efflux of the product by Pgp. Dedicated investigations to shed light on the action mechanisms of this new class of modified doxorubicins, as well as in *vivo* studies of **10** and **11**, are now in progress.

EXPERIMENTAL SECTION

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvance 300, at 300 and 75 MHz, respectively, using $SiMe_4$ as internal standard. The following abbreviations indicate peak multiplicity: *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet, *bs* = broad singlet. ESI MS spectra were recorded on a Micromass Quattro API micro (Waters Corporation, Milford, MA, USA) mass spectrometer. Data were processed using a MassLynxSystem (Waters). High resolution MS spectra were recorded on a Bruker Bio Apex Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Apollo I ESI source, a 4.7 T superconducting magnet, and a cylindrical infinity cell (Bruker Daltonics, Billerica, MA, USA). Melting points were determined with a capillary apparatus (Büchi 540) in open capillary. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM). The progress of the reactions was followed by thin-layer chromatography (TLC) on 5 × 20 cm plates Merck Kieselgel 60 F254, with a layer thickness of 0.20 mm. Anhydrous sodium sulfate (Na_2SO_4) was used as drying agent for the organic phases. Organic solvents were removed under reduced pressure at 30 °C. Synthetic-purity solvents dichloromethane (DCM), acetonitrile (CH_3CN), methanol (MeOH), diethyl ether (Et_2O), diisopropyl ether (i-Pr_2O), dimethylformamide (DMF) and 40−60 petroleum ether (PE) were used. Dry tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone under positive N_2 pressure. Dry DMF was obtained through storage on 4 Å molecular sieves. Commercial starting materials were purchased from Sigma-Aldrich, Alfa Aesar, and TCI Europe. For the synthesis of **2**−**9**, see the Supporting Information.

The purity determination of compounds. RP-HPLC analyses were run with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), and a diode-array detector (DAD) (model G1315B) integrated into the HP1100 system. Data were processed using a HP ChemStation system (Agilent Technologies). The analytical column was a Tracer Excel 120 ODS-B (250 × 4.6 mm, 5 μm; Teknokroma, Barcelona). Compounds were dissolved in the mobile phase and injected through a 20 μL loop (Rheodyne, Cotati, CA). The mobile phase consisted of 0.1% aqueous HCOOH (solvent A) and CH_3CN (solvent B) and elution was in gradient mode: initially 35% of solvent B until 5 min, from 35 to 80% of solvent B between 5 and 10 min, 80% of solvent B until 20 min, and from 80 to 35% of solvent B between 20 and 25 min. HPLC retention times (t_R) were obtained at flow rates of 1.0 mL·min^{-1}, and the column effluent was monitored at 234 and 480 nm referenced against a 700 nm wavelength. All products displayed purity ≥95% with the exception of **10** and **12** (93% purity).

*General procedure for the synthesis of H_2S donor doxorubicins **10**−**16**.* To a solution of the appropriate acid (1.35 mmol) in dry DMF, KF (155 mg, 2.70 mmol) was added in one portion, and the reaction was vigorously stirred for 15 min. 14-Bromo/chloro daunorubicin hydrobromide (**2**) (300 mg, 0.45 mmol) was added and the reaction was stirred at r.t. until completed (LC control). Solvent was removed under reduced pressure at 30 °C and the resulting mixture was separated by flash chromatography (eluent: gradient from 98/2 to 80/20 CH_2Cl_2/MeOH) to give a red solid. The resulting compound was dissolved/suspended in dry THF, and 2 equiv of HCl solution in dry dioxane were added. The resulting mixture was stirred for 2 h at r.t., then diluted with Et_2O and the precipitate was filtered, washed with Et_2O and dried in desiccators to give a title compound as a red powder.

*Doxorubicin 14-[4-(4-phenyl-5-thioxo-5H-[1,2]dithiol-3-yl)]-benzoate (**10**).* Yield: 52%; pHPLC 93% (t_R = 12.2 min); mp 166−170 °C (dec.); ^{1}H NMR (CD_3OD + $CDCl_3$) δ ppm: 1.36 (d, J^3_{HH} = 6.22 Hz, 3H, $^{6'}CH_3$); 1.91 (m, 1H), 2.06 (m, 1H) ($^{2'}CH_2$); 2.19 (m, 1H), 2.51 (m, 1H) ($^8CH_2$); 3.05 (m, 1H), 3.28 (m, 1H) ($^{10}CH_2$); 3.35 (m, 1H, $^{3'}CH$); 3.56 (m, 1H, $^{4'}CHOH$); 3.79 (br. s, 1H, $^{4'}CHOH$); 4.08 (s, 3H, OCH_3); 4.28 (m, 1H, $^{5'}CH$); 5.23 (m, 1H, ^7CH); 5.42 (m, 1H), 5.57 (m, 2H, $^{1'}CH$ and $^{14}CH_2$); 7.15 (m, 1H, ^2CH); 7.36 (m, 2H), 7.51 (m, 5H), 7.83 (m, 2H), (m, 1H.), 8.01 (m, 2H) (C*H* Ar.); MS (ESI$^+$) m/z 856 (M+H)$^+$. HRMS (ESI$^+$) m/z calcd for $C_{43}H_{37}NO_{12}S_3$ (M+H)$^+$ 856.1551, found 856.1551.

*Doxorubicin 14-[4-(3-thioxo-3H-1,2-dithiol-4-yl)]benzoate (**11**).* Yield: 20%; pHPLC 97% (t_R = 11.9 min); mp 159−163 °C (dec.); ^{1}H NMR (DMSO-d_6) δ ppm: 1.21 (d, J^3_{HH} = 6.22 Hz, 3H, $^{6'}CH_3$); 1.71 (m, 1H), 1.92 (m, 1H) ($^{2'}CH_2$); 2.12 (m, 1H), 2.38 (m, 1H) ($^8CH_2$); 2.89 (d, J^2_{HH} = 18.3 Hz, 1H), 3.12 (d, J^2_{HH} = 18.3 Hz, 1H) ($^{10}CH_2$); 3.62 (m, 1H, $^{4'}CHOH$); 3.98 (s, 3H, OCH_3); 4.29 (m, 1H, $^{5'}CH$); 4.97 (m, 1H, ^7CH); 5.32 (br. s, 1H, $^{4'}CHOH$); 5.54 (m, 3H, $^{1'}CH$ and $^{14}CH_2$); 5.77 (s, 1H, ^9COH); 7.64 (m, 1H, ^2CH); 7.69 (d, 2H), 7.90 (m, 2H), 8.08 (d, 2H) (C*H* Ar.); 9.30 (s, 1H, SC*H*), 13.24 (s, 1H), 14.02 (s, 1H) (2ArO*H*); MS (ESI$^+$) m/z 780 (M+H)$^+$. HRMS (ESI$^+$) m/z calcd for $C_{37}H_{33}NO_{12}S_3$ (M+H)$^+$ 780.1238, found 780.1237.

*Doxorubicin 14-[2-thioxo-1,3-dithiole-4]carboxylate (**12**).* Yield: 43%; pHPLC 93% (t_R = 13.0 min); mp 190−195 °C (dec.); ^{1}H NMR (DMSO-d_6) δ ppm: 1.17 (d, J^3_{HH} = 6.31 Hz, 3H, $^{6'}CH_3$); 1.70 (m, 1H), 1.90 (m, 1H) ($^{2'}CH_2$); 2.09 (m, 1H), 2.30 (m, 1H) ($^8CH_2$); 2.88 (d, J^2_{HH} = 18.0 Hz, 1H), 3.12 (d, J^2_{HH} = 18.4 Hz, 1H) ($^{10}CH_2$); 3.60 (m, 1H, $^{4'}CHOH$); 3.99 (s, 3H, OCH_3); 4.24 (m, 1H, $^{5'}CH$); 4.95 (m, 1H, ^7CH); 5.30 (br. s, 1H, $^{4'}CHOH$); 5.50 (m, 3H, $^{1'}CH$ and $^{14}CH_2$); 5.88 (br. s, 1H, ^9COH); 7.67 (m, 1H, ^2CH); 7.93 (m, 2H, 2C*H* Ar.); 8.60 (s, 1H, C*H*S); 13.25 (s, 1H, ArO*H*); MS (ESI$^+$) m/z 704 (M+H)$^+$; HRMS (ESI$^+$) m/z calcd for $C_{31}H_{29}NO_{12}S_3$ (M+H)$^+$ 704.0925, found 704.0929.

*Doxorubicin 14-[4-(5-Thioxo-5H-[1,2]dithiol-3-yl)phenoxy]-acetate (**13**).* Yield: 45%; pHPLC 95% (t_R = 14.2 min); mp 159−162 °C (dec.); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16 (d, J^3_{HH} = 6.59 Hz, 3H, $^{6'}CH_3$); 1.71 (m, 1H), 1.89 (m, 1H) ($^{2'}CH_2$); 2.08 (m, 1H), 2.29 (m, 1H) ($^8CH_2$); 2.87 (d, J^2_{HH} = 17.9 Hz, 1H), 3.09 (d, J^2_{HH} = 18.0

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Hz, 1H) ($^{10}CH_2$); 3.60 (m, 1H, $^{4'}CHOH$); 3.98 (s, 3H, OCH_3); 4.23 (m, 1H, $^{5'}CH$); 4.95 (m, 1H, ^{7}CH); 5.09 (s, 2H, CH_2COO); 5.29 (br. s, 1H, $^{4'}CHOH$); 5.44 (m, 3H, $^{1'}CH$ and $^{14}CH_2$); 5.76 (s, 1H, ^{9}COH); 7.13 (d, 2H, CH Ar.); 7.65 (m, 1H, ^{2}CH); 7.78 (s, 1H, SCH); 7.90 (m, 4H, CH Ar.); 13.23 (s, 1H), 14.02 (s, 1H) (2ArOH); MS (ESI^+) m/z 810 $(M+H)^+$. HRMS (ESI^+) m/z calcd for $C_{38}H_{35}NO_{13}S_3$ $(M+H)^+$ 810.1343, found 810.1342.

Doxorubicin 14-[2(S)-2-acetylamino-3-allylsulfanyl]propionate ***(14)***. Yield: 44%; pHPLC 97% (t_R = 10.3 min); mp 143−146 °C (dec.); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16 (d, J^3_{HH} = 6.59 Hz, 3H, $^{6'}CH_3$); 1.71 (m, 1H), 1.89 (m, 4H) ($^{2'}CH_2$ and CH_3CONH); 2.08 (m, 1H), 2.27 (m, 1H) ($^{8}CH_2$); 2.71 (dd, 1H), 2.83 (d, J^2_{HH} = 18.0 Hz, 1H), 2.94 (dd, 1H), 3.07 (d, J^2_{HH} = 18.3 Hz, 1H) ($^{10}CH_2$ and $SCH_2CH(NHAc)COO$); 3.19 (d, 2H, SCH_2CHCH_2); 3.60 (m, 1H, $^{4'}CHOH$); 3.98 (s, 3H, OCH_3); 4.24 (m, 1H, $^{5'}CH$); 4.57 (m, 1H, CHCOO); 4.94 (m, 1H, ^{7}CH); 5.13 (m, 2H, $CH{=}CH_2$); 5.29 (br. s, 3H, $^{4'}CHOH$ and $^{14}CH_2$); 5.47 (m, 1H, $^{1'}CH$); 5.74 (m, 2H, $CH{=}CH_2$ and ^{9}COH); 7.65 (m, 1H, ^{2}CH); 7.91 (m, 2H, CH Ar.); 8.48 (d, 1H, NH); 13.22 (s, 1H, ArOH); MS (ESI^+) m/z 729 $(M+H)^+$. HRMS (ESI^+) m/z calcd for $C_{35}H_{40}N_2O_{13}S$ $(M+H)^+$ 729.2324, found 729.2326.

Doxorubicin 14-(3-allyldisulfanyl)propionate ***(15)***. Yield: 42%; pHPLC 98% (t_R = 12.4 min); mp 135−138 °C (dec.); ^{1}H NMR (DMSO-d_6) δ ppm: 1.15 (d, J^3_{HH} = 6.31 Hz, 3H, $^{6'}CH_3$); 1.56 (m, 1H), 1.74 (m, 1H) ($^{2'}CH_2$); 2.05 (m, 1H), 2.29 (m, 1H) ($^{8}CH_2$); 2.71 (d, J^2_{HH} = 12.4 Hz, 1H) (^{10}CHH), 2.88 (m, 4H, CH_2CH_2), 3.06 (m, 1H) (^{10}CHH); 3.40 (d, 2H, SCH_2CH); 3.57 (m, 1H, $^{4'}CHOH$); 3.98 (s, 3H, OCH_3); 4.16 (m, 1H, $^{5'}CH$); 4.94 (br. s, 1H, ^{7}CH); 5.21 (m, 4H, $^{14}CH_2$, $CH{=}CH_2$); 5.60 (br. s, 1H, $^{1'}CH$); 5.81 (m, 1H, $CH{=}CH_2$) 7.64 (m, 1H, ^{2}CH); 7.92 (m, 2H, CH Ar.); MS (ESI^+) m/z 704 $(M+H)^+$. HRMS (ESI^+) m/z calcd for $C_{33}H_{37}NO_{12}S_2$ $(M+H)^+$ 704.1830, found 704.1829.

Doxorubicin 14-(4-thiocarbamoyl)benzoate ***(16)***. Yield: 51%; pHPLC 99% (t_R = 10.5 min); mp 179−183 °C (dec.); ^{1}H NMR (DMSO-d_6) δ ppm: 1.20 (d, J^3_{HH} = 6.31 Hz, 3H, $^{6'}CH_3$); 1.72 (m, 1H), 1.91 (m, 1H) ($^{2'}CH_2$); 2.12 (m, 1H), 2.37 (m, 1H) ($^{8}CH_2$); 2.89 (d, J^2_{HH} = 18.9 Hz, 1H), 3.13 (d, J^2_{HH} = 18.9 Hz, 1H) ($^{10}CH_2$); 3.62 (m, 1H, $^{4'}CHOH$); 3.98 (s, 3H, OCH_3); 4.28 (m, 1H, $^{5'}CH$); 4.96 (m, 1H, ^{7}CH); 5.31 (br. s, 1H, $^{4'}CHOH$); 5.50 (m, 3H, $^{1'}CH$ and $^{14}CH_2$); 5.82 (s, 1H, ^{9}COH); 7.65 (m, 1H, ^{2}CH); 7.96 (m, 6H, CH Ar.); 9.75 (s, 1H), 10.12 (s, 1H) (NH_2); MS (ESI^+) m/z 707 $(M+H)^+$. HRMS (ESI^+) m/z calcd for $C_{35}H_{34}N_2O_{12}S$ $(M+H)^+$ 707.1905, found 707.1906.

Chemicals. Fetal bovine serum (FBS) and culture medium were supplied by Invitrogen Life Technologies (Carlsbad, CA); human serum (sterile filtered from human male AB plasma) was supplied by Sigma-Aldrich; plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA); the protein content of cell monolayers and lysates was assessed with the BCA kit from Sigma Chemical Co (St. Louis, MO). Doxorubicin and reagents not specified were from Sigma Chemical Co.

Stability in culture medium and in human serum. Before addition of the compound, culture medium (DMEM or IMDM) was preheated at 37 °C. Then solution of each H_2S-DOXO in DMSO was added to culture medium to have a final concentration of compound 200 μM. The obtained solution was incubated at 37 ± 0.5 °C, and at appropriate time intervals, an aliquot of 200 μL of sample were collected and diluted with the same amount of acetonitrile containing 0.1% HCOOH to final concentration of 100 μM. The obtained mixture was vortexed, filtrated (PTFE filters, 0.45 μM) and immediately analyzed by RP-HPLC. All experiments were performed at least in triplicate.

The stability of the H_2S-DOXOs to the esterase was evaluated by incubating the compounds in human serum; the solution of compound (10 mM in DMSO) was added to human serum preheated at 37 °C; the final concentration of the compound was 100 μM. The solution was incubated at 37 ± 0.5 °C, and at appropriate time intervals, an aliquot of 300 μL of reaction mixture was withdrawn and added to 300 μL of acetonitrile containing 0.1% HCOOH, in order to deproteinize the serum. The sample was sonicated, vortexed, and centrifuged for 10 min at 2150 g. The clear supernatant was filtered through 0.45 μm PTFE filters and analyzed by RP-HPLC. All experiments were performed at least in triplicate.

In both the assays the HPLC analyses were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) in the experimental conditions previously described. The reverse-phase HPLC procedure allowed separation and quantitation of H_2S-DOXOs and of degradation products (DOXO, H_2S donor substructure); quantitation of H_2S-DOXOs and of DOXO was done using calibration curve obtained with standards solutions chromatographed in the same conditions in a concentration range of 1−200 μM (r^2 > 0.99).

The half-lives ($t_{1/2}$) of all the H_2S-DOXOs were determined by fitting the data with one phase exponential decay equation using Prism software vers. Five (Graph Pad, San Diego, CA, USA).

H_2S release in culture medium and human serum. To 1900 μL of culture medium (DMEM or IMDM) or human serum preheated at 37 °C, 60 μL of dansylazide solution (10 mM in Ethanol) and 40 μL of H_2S-DOXO stock solution (10 mM in DMSO) were added to have an initial H_2S-DOXO concentration of 200 μM. Compounds **10** and **11** were incubated at half concentration due to their low solubility. The solution was incubated at 37 ± 0.5 °C, and at fixed time (1, 4, and 24 h) 200 μL of reaction mixture was withdrawn and diluted with 200 μL of CH_3CN to have a final concentration of compound 100 μM. The mixture was vortexed, centrifuged for 10 min at 2150 × g. The clear supernatant was filtered through 0.45 μm PTFE filters and analyzed by RP-HPLC. All experiments were performed at least in triplicate. HPLC analyses were performed with a HP 1200 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1322A), a UV detector, MWD (model G1365D) and a fluorescence detector (model G1321A) integrated in the HP1200 system. Data analysis was done using a HP ChemStation system (Agilent Technologies). The sample was eluted on Tracer Excel 120 ODSB C18 (250 × 4.6 mm, 5 μm; Teknokroma); injection volume was 20 μL. The mobile phase consisted of 0.1% aqueous HCOOH and CH_3CN 20/80 v/v; elution was in isocratic mode at a flow rate of 0.8 mL/min. The signals were obtained on fluorescence using an excitation and emission wavelength of 340 and 535 nm, respectively (gain factor = 10). Data manipulation was performed by Agilent ChemStation. The values obtained from integration of the peak of dansyl amide were interpolated in a calibration line, prepared using NaHS as a standard, so the concentration of dansyl amide in each sample is an index of H_2S amount.

Cell lines. Rat H9c2 cardiomyocytes and human DOXO-sensitive U-2OS cells were purchased from ATCC (Manassas, VA) and cultured in DMEM medium. The corresponding variants with increasing resistance to DOXO (U-2OS/DX30, U-2OS/DX100, U-2OS/DX580), selected by culturing parental cells in a medium with 30, 100, 580 ng/mL DOXO, were generated as described elsewhere.[50] Culture media were supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. Cell cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Intracellular DOXOs accumulation. The amount of DOXOs in cell lysates was measured spectrofluorimetrically, as described elsewhere,[13] using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Winooski, VT). Excitation and emission wavelengths were 475 and 553 nm, respectively. A cell-free blank was prepared for each set of experiments, and its fluorescence was subtracted from that measured in the presence of cells. Fluorescence was converted to nmol DOXOs/mg cell proteins using a previously prepared calibration curve.

Cytotoxicity assays. To verify the cytotoxic effect of DOXOs, the extracellular medium was centrifuged at 12,000 × g for 5 min to pellet cellular debris, while cells were washed with fresh medium, detached with trypsin/EDTA, resuspended in 0.2 mL of 82.3 mM triethanolamine phosphate-HCl (pH 7.6) and sonicated on ice with two 10 s bursts. Lactic dehydrogenase (LDH) activity was measured in extracellular medium and cell lysate, as reported elsewhere.[16] The

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reaction was monitored for 6 min, measuring absorbance at 340 nm with a Synergy HT Multi-Detection Microplate Reader, and was linear throughout the measurement time. Both intracellular and extracellular enzyme activities were expressed in μmol NADH oxidized/min/dish. Extracellular LDH activity was calculated as the percentage of the total LDH activity occurring in the dish.

To determine the IC_{50} of DOXO, the cell viability was measured by the neutral red staining method, as reported elsewhere.[42] The absorbance of untreated cells was considered as 100% viability; the results are expressed as percentages of viable cells versus untreated cells. IC_{50} was considered as the concentration of each drug that reduces to 50% the cell viability versus untreated cells.

ROS measurement. The amount of intracellular ROS was measured fluorimetrically as described elsewhere.[43] 1×10^6 cells were resuspended in a final volume of 0.5 mL PBS, incubated for 30 min at 37 °C with 5 μM of the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester (DCFDA-AM), centrifuged at 13,000 × g at 37 °C and resuspended 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 ROS/mg cell proteins.

Doxorubicin efflux. DOXOs efflux kinetics was measured as described elsewhere.[51] Cells were incubated for 20 min with increasing (0−50 μM) concentrations of parent or synthetic DOXOs, then washed and analyzed for intracellular DOXO concentration. A second series of dishes, incubated in the same experimental conditions, were left for a further 10 min at 37 °C, then washed and tested for intracellular drug content. The difference of DOXO concentration between the two series, expressed as nmol DOXOs extruded/min/mg cell proteins, was plotted versus initial drug concentration. Values were fitted to the Michaelis−Menten equation to calculate V_{max} and K_m, using the Enzfitter software (Biosoft Corporation, Cambridge, United Kingdom).

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

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00184.

Synthesis of intermediate compounds, supplementary biological tests, HPLC chromatograms of final compounds, ^1H and ^{13}C NMR spectra, and hydrolytic profile of H_2S-DOXOs (PDF)

Molecular formula strings for compounds **3**−**23** (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DOXO, doxorubicin; Pgp, P-glycoprotein; ROS, reactive oxygen species; PLP, pyridoxal-5′-phosphate; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; RNS, reactive nitrogen species; ER, endoplasmatic reticulum; MAPK, mitogen-activated protein kinase; CRT, calreticulin; DMEM, Dulbecco's Modified Eagle Medium; IMDM, Iscove's Modified Dulbecco's Medium; DNS-Az, dansyl azide; LDH, lactic dehydrogenase; OHCbl, hydroxocobalamin; ABCB1, ATP binding cassette B1; FBS, fetal bovine serum; BCA, bicinchoninic acid; DCFDA-AM, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester; BSA, bovine serum albumin

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