Sdox is a hydrogen sulfide (H$_2$S)-releasing doxorubicin effective in P-glycoprotein-overexpressing/doxorubicin-resistant tumor models and not cytotoxic, as the parental drug, in H9c2 cardiomyocytes. The aim of this study was the assessment of Sdox drug-like features and its absorption, distribution, metabolism, and excretion (ADME)/toxicity properties, by a multi- and transdisciplinary in silico, in vitro, and in vivo approach. Doxorubicin was used as the reference compound. The in silico profiling suggested that Sdox possesses higher lipophilicity and lower solubility compared to doxorubicin, and the off-targets prediction revealed relevant differences between Dox and Sdox towards several cancer targets, suggesting different toxicological profiles. In vitro data showed that Sdox is a substrate with lower affinity for P-glycoprotein, less hepatotoxic, and causes less oxidative damage than doxorubicin. Both anthracyclines inhibited CYP3A4, but not hERG currents. Unlike doxorubicin, the percentage of zebrafish live embryos at 72 hpf was not affected by Sdox treatment. In conclusion, these findings demonstrate that Sdox displays a more favorable drug-like ADME/toxicity profile than doxorubicin, different selectivity towards cancer targets, along with a greater preclinical efficacy in resistant tumors. Therefore, Sdox represents a prototype of innovative anthracyclines, worthy of further investigations in clinical settings.

Keywords: cytochrome P450, doxorubicin, hepatotoxicity, hERG, in silico profiling, off-targets, P-glycoprotein, zebrafish
INTRODUCTION

A library of hydrogen sulfide (H$_2$S)-releasing doxorubicin analogues was synthesized to overcome the well-known cardiotoxicity (Sawicki et al., 2021) and the drug resistance (Assaraf et al., 2019) characterizing doxorubicin (Dox) treatment and limiting its use in cancer therapy (Chegaev et al., 2016). A series of derivatives, devoid of toxicity in H9c2 cardiomyocytes, but still retaining their efficacy in U-2OS osteosarcoma cells, characterized by increasing levels of P-glycoprotein (P-gp) and resistance to Dox, have been synthesized (Chegaev et al., 2016).

Sdox, for example, displayed cytotoxic activity in vitro in an androgen-independent and Dox-resistant DU-145 prostate cancer cell line and reduced tumor mass volume in a castration-resistant prostate cancer xenograft model (Bigagli et al., 2018). In U-2OS and Saos-2 chemoresistant osteosarcoma cells, Sdox, unlike Dox, accumulated within the endoplasmic reticulum (ER), where it releases H$_2$S that sulfhydrated nascent proteins, including P-gp, increasing their misfolding and triggering ER-dependent apoptosis (Buondonno et al., 2019). This process enhanced both retention and toxicity of Sdox in resistant cells.

Sdox and, to a greater extent, Sdox in a liposomal formulation decorated with hyaluronic acid (HA-Lsdox) reduced the growth of osteosarcoma refractory to both Dox and Caelyx®, the pegylated liposomal Dox, currently used in clinical setting (Gazzano et al., 2019).

Additionally, Sdox was not cytotoxic as Dox in H9C2 cardiomyocytes (Chegaev et al., 2016; Buondonno et al., 2019) and displayed the same cardiotoxicity profile of Caelyx® in osteosarcoma xenograft (Gazzano et al., 2019).

Elevated liver enzymes and acute liver injury with jaundice, which can be serious and even fatal, have been reported during Dox treatment (LiverTox, 2018; Prasanna et al., 2020). After all, Dox is eliminated in large part by the liver, and several mechanisms of Dox-induced hepatotoxicity have been proposed, including free radical formation and mitochondrial dysfunction (Kalender et al., 2005; Prasanna et al., 2020).

Abnormal liver blood test result is a rare side effect reported also in Caelyx® summary of product characteristics (European Medicines Agency, 2021).

Therefore, Dox and Caelyx® dosage should be adjusted in patients with hepatic impairment (Food and Drug Administration, 2013).

Several strategies have been attempted to overcome MDR and either prevent or alleviate these Dox unwanted effects.

Although Sdox may represent a lead compound for the treatment of chemoresistant tumors, its clinical development might be limited by severe adverse reactions that characterize the parent compound (Food and Drug Administration, 2013). Moreover, nonclinical safety-related attrition represents a major issue for research and development of novel drugs, particularly during lead identification and optimization stages (Blomme and Will, 2016).

The principal side effects of a drug originate from (1) chemical-based effects, related to its physicochemical features; (2) on-target, mechanism-based effects; and (3) off-target effects, due to binding to unintended targets (e.g., proteins or nucleic acids) (Rudmann, 2013).

Therefore, the aim of this study was the in silico as well as in vitro and in vivo evaluation of Sdox drug-like characteristics, and anti-cancer efficacy, absorption, distribution, metabolism, and excretion/toxicity (ADME/Tox) properties, using Dox as a reference compound.

This multidisciplinary work was performed by a group of researchers from COST Action STRATAGEM “New diagnostic and therapeutic tools against multidrug resistant tumors.”

MATERIALS AND METHODS

In Silico Drug Profiling

The ACD/Percepta software (version 2020.2, Advanced Chemistry Development, Inc., Toronto, ON, Canada) was initially used for Dox and Sdox profiling.

The individual predictions were categorized according to predefined thresholds for the following groups of parameters: (1) PhysChem (logP, solubility, H-bonding, molecular size and flexibility, drug and lead-likeness rules); (2) ADME [human intestinal absorption (HIA) and Caco-2 permeability, plasma protein binding (PBI), blood–brain barrier penetration (BBB)]; (3) drug safety [P-gp substrate, cytochrome P450 (CYP450) inhibitor, and hERG inhibitor specificity].

Categorization was based on either continuous properties (numerical thresholds set on the scale of the corresponding property values) or probabilistic predictors (for drug safety). Each prediction was assigned a score according to the equation: Score = (p—0.5) * RI + 0.5, where p is the obtained probability, and RI is the reliability index value. The score relies on the assumption that predicted probability close to 0.5 and low RI are both indicators of an inconclusive prediction. The term (p—0.5) * RI considers predictions where low RI values would ultimately yield scores in the intermediate range even if the original p-value is quite high or low. The final category assignment was based on the default classification score ranges.

The partition coefficient logP was calculated using two different predictive algorithms: ACD/LogP Classic (fragment-based approach) and ACD/LogP GALAS (similarity-based approach). The consensus logP was calculated as a weighted average of both predictions by assigning dynamic adaptive coefficients: each model obtained larger weight in those regions of chemical space where it performed most reliably. The models were as follows: for Dox (0.12 Classic+ 0.88 GALAS) and for Sdox (0.4 Classic+0.6 GA-LAS). In addition, the logP values of the compounds were estimated by Molecular Operating Environment (MOE) software (version 2019.01, Chemical Computing Group, ULC, Montreal, QC, Canada) and by Marvin (version 14.8.25, ChemAxon, Budapest, Hungary). In MOE, the “h_logP” descriptor was calculated by a multiparameter model built on 1,836 molecules [r$^2$ = 0.84, root mean square error (RMSE) = 0.59]. In Marvin, the logP values were calculated by an atom-based approach (Hansch et al., 1995).
The distribution coefficients (logD) of Dox and Sdox at pH = 7.0 were also calculated using ACD/Labs.

The Derek Nexus expert system (version 6.0.1, Lhasa Ltd., Leeds, United Kingdom) was used for toxicity prediction. The algorithm relies on the concept of "structural alerts" (toxicophores) defined as a set of structural features that makes a user suspect that the molecule may exert a particular effect. It compares the structural features of the query compound to the structural alerts in the knowledge database of the program. Prediction is based on a reasoning scheme, which considers the presence of a set of toxicophores in the query structure (Sutter et al., 2013). Seven likelihood levels were used as follows (in highest to lowest order of likelihood): certain—there is proof that the proposition is true; probable—there is at least one strong argument that the proposition is true and there are no arguments against it; plausible (baseline)—the level of likelihood indicating the weight of evidence supports the proposition; equivocal—there is an equal weight of evidence for and against the proposition; doubted—the weight of evidence opposes the proposition; improbable—there is at least one strong argument that the proposition is false and there are no arguments that it is true; and impossible—there is proof that the proposition is false (Judson et al., 2013). The following settings were applied: restrict to mammal species; perceive tautomers (yes); reasoning level threshold (plausible); and show negative predictions (yes).

**Metabolism Predictions**

Metabolic transformations were predicted by the knowledge-based expert system Meteor Nexus (version 3.1.0, Meteor KB 2018 1.0.0, Lhasa Ltd., Leeds, United Kingdom).

Since reactive metabolites are generally produced by Phase I reactions (Njuguna et al., 2012), only those were considered in this study. The system allows for analysis of the assigned likelihood levels by either considering the relevance of each metabolite (absolute likelihood) or taking into account the likelihood of occurrence of a preceding metabolite as a factor affecting the appearance of metabolites resulting in the following steps (on path likelihood). The absolute reasoning (AR) method was used with the following default parameters: maximal depth (number of metabolic steps) = 3 and maximal number of metabolites = 1,000. The AR method relies on a similar reasoning scheme as toxicity predictions by Derek (see above) operating with five likelihood levels (probable, plausible, equivocal, doubted, and improbable) for a biotransformation to occur (Judson et al., 2013). The minimal likelihood level was set to consider only probable and plausible predictions.

**Docking and Binding to Pregnen-X-Receptor and Sulfotransferase**

The protein structures of the pregnane-X-receptor (PXR) and sulfotransferase (SULT) were searched in the Protein DataBank (PDB) (Berman et al., 2000). Crystal structures and optimization protocols were as described previously (Glisic et al., 2016; Viira et al., 2016; Garcia-Sosa et al., 2009). Briefly, the crystal structures 1M13.pdb and 2A3R.pdb were downloaded, hydrogens added, and preprocessed with Maestro (Release 2021-4, Schrödinger LLC, New York, NY, United States). Thresholds for docked compounds were based on previously known binders (Glisic et al., 2016; Viira et al., 2016; Garcia-Sosa et al., 2009) and docking scores color-coded black, gray, or white, depending on closeness to the determined threshold.

Dox, Sdox, and their metabolites were drawn, hydrogens added, and geometry-optimized with Maestro (Schrödinger Release 2021-4, LLC, New York, NY, United States). Ligprep (Release 2021-4, Schrödinger LLC, New York, NY, United States) calculated tautomers, ionization states, and conformers for the metabolite structures using a pH of 7.4 with threshold of 2 units.

Docking was carried out using programs Glide XP (Schrödinger Release 2021-4, LLC, New York, NY, United States) and AutoDock (Morris et al., 2009), in addition to Vina (Trott and Olson, 2010). Each docking program uses a different algorithm for pose prediction and scoring in order to hedge values and increase confidence in the results. The best score was recorded for each different species of the same ligand. AutoDock 4 and Vina calculations were performed with 100 runs per ligand. Settings were default except for those previously described in (Glisic et al., 2016; Viira et al., 2016; Garcia-Sosa et al., 2009).

**Off-Target Prediction**

Interactions of Dox and Sdox with 85 different targets were analyzed by computational docking using MOE software (version 2019.01, Chemical Computing Group, ULC, Montreal, QC, Canada). For β-tubulin, interactions at both the colchicine binding site (CBS) and the Vinca binding site (VBS) were investigated. Crystallographic structure of targets was obtained from PDB (list of PDB target IDs available in Supplementary Information) (Berman et al., 2000). Protein structures were energetically minimized using Amber10 force field with EHT parameters for small molecules, R-field solvation model, and dielectric constant of 1 for the protein interior and 80 for exterior. Dox and Sdox structures were drawn in MOE software (version 2019.01, Chemical Computing Group, ULC, Montreal, QC, Canada), and their energies were minimized according to the above parameters using as stop criterion an RMS gradient lower than 0.01 kcal/mol/Å. For docking calculations, the Triangle Matcher algorithm with the London dG scoring function in the placement stage was used. The receptor was rigid, and the GBVI/WSA dG scoring function was used in the refinement stage.

Results were expressed as docking score (DS) values and difference in DS (ADS) values obtained for Dox and Sdox against the same target in order to provide more relevant information in their mechanism of action (Palmeira et al., 2012).

**In Vitro P-gp Assay**

To study the effect of P-gp overexpression in the antiproliferative activity of Dox and Sdox, a cell-line-based assay was performed by using wild-type cell line (SW1573) and its P-gp-overexpressing variant (SW1573/P-gp) (Baas et al., 1990). Compounds were tested against both cell lines in the presence or absence of 10 μM verapamil (a known P-gp and CYP3A4/5 inhibitor).
The GI<sub>50</sub> (the concentration that causes 50% growth inhibition) (Skehan et al., 1990) values after 48 h of exposure to drugs in absence (w/o) or presence (w) of verapamil were determined in both cell types. The microtubule-interacting drugs paclitaxel (known substrate of P-gp), colchicine, vincristine, and vinblastine were selected as reference compounds for comparison purposes (Baas et al., 1990).

**In Vitro Inhibition of Human CYP3A4**

Dox and Sdox were tested *in vitro* for their ability to inhibit CYP3A4 enzyme recombinant human CYP3A4 (BD Biosciences Discovery Labware, Woburn, MA, United States). 3-(3-Benzylxoxophenyl-7-methoxycoumarin was used as the substrate, the well-known CYP3A4 inhibitor ketoconazole as positive control, while negative controls were performed in the absence of substrate or enzyme. Kinetic assays were carried out in a final volume of 100 µl containing 10 µM substrate, 2.5 nM recombinant CYP3A4, various concentrations of the tested compounds, and 20% NADPH regenerating system in 100 mM Tris–HCl (pH 7.4). Incubations took place in duplicate at 37°C in 96-multiwell plates; fluorescence was measured with a Victor2 plate reader (PerkinElmer Life Sciences, Turku, Finland). The reaction was started by adding NADPH, and fluorescence was measured at 2-min intervals for 40 min using excitation at 405 nm and emission at 460 nm. IC<sub>50</sub> (the concentration causing a 50% reduction of maximal activity) values were calculated using the equation \( \frac{v_i}{v_0} = 1/(1 + [I]/IC_{50}) \), where \( v_i \) is the rate at a fixed concentration of inhibitor, \( v_0 \) is the rate recorded in the absence of the inhibitor (100% rate), and \( I \) is the inhibitor concentration (Juvenon et al., 2020).

**In Vitro Hepatotoxicity Studies**

Human hepatoma (HepG2) cells (European Collection of Authenticated Cell Cultures) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO<sub>2</sub> humidified atmosphere.

Primary hepatocyte cultures were obtained from male Wistar rats (body weight, 220 ± 20 g, 3 months of age, National Breeding Center, Sofia, Bulgaria) sacrificed under sodium pentobarbital anesthesia (60 mg/kg, i.p.). In *situ* liver perfusion and cell isolation were performed as previously described (Kondeva-Burdina et al., 2014). All procedures were approved by the Bulgarian Food Safety Agency (No. 304 valid until June 28, 2026) and by the Animal Ethics Committee of the University of Sofia (No. 220, April 13, 2021). The principles stated in the European Union Guidelines for the Care and the Use of Laboratory Animals, Directive 2010/63/EU were strictly followed throughout the experiment.

HepG2 and rat hepatocytes viability after 24, 48, or 72 h treatment with Dox and Sdox were investigated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) and the trypan blue exclusion assay (Strober, 2001), respectively.

The extracellular release of lactate dehydrogenase (LDH), considered an index of cell damage and necrosis, was measured in both cell models as previously reported (Decker and Lohmann-Matthes, 1988; Fau et al., 1992). Based on its extent, the magnitude of the cytotoxic effect was classified as follows: weak cytotoxicity (20–40% decrease in viability), medium cytotoxicity (40–60% decrease in viability), and strong cytotoxicity (60–80% decrease in viability) (López-García et al., 2014).

Glutathione (GSH) depletion was evaluated after 24, 48, or 72 h treatment with both anthracyclines. Isolated rat hepatocytes were centrifuged at 400 g for 3 min, and the pellet was used for measuring intracellular GSH by means of the spectrophotometric method based on the conversion of the sulphydryl reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) at 412 nm (Fau et al., 1992).

Lipid peroxidation was estimated by evaluating malondialdehyde (MDA) in the culture supernatant treated with 0.67% (w/v) 2-thiobarbituric acid (TBA). The absorbance was measured at 535 nm, and the amount of TBA reactants was calculated using a molar extinction coefficient of MDA 1.56 × 10<sup>5</sup> M<sup>−1</sup> cm<sup>−1</sup> (Fau et al., 1992).

**In Vitro hERG Study**

hERG-HEK293 cells (BPS Bioscience, San Diego, United States), expressing hERG K<sup>+</sup> channel (Kv11.1), were cultured in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% Na pyruvate, 1% penicillin/streptomycin, and 400 µg/ml geneticin (Lazzerini et al., 2021).

The bath solution contained (in mM): 136 NaCl, 5.4 KCl, 10 HEPES, 10 D-glucose · H<sub>2</sub>O, 1 CaCl<sub>2</sub> · 2 H<sub>2</sub>O, 1 MgCl<sub>2</sub> · 6 H<sub>2</sub>O, pH 7.4 with NaOH, whereas the pipette solution consisted of (in mM): 130 KCl, 10 EGTA, 10 HEPES, 5 MgATP, 1 MgCl<sub>2</sub> · 6 H<sub>2</sub>O, pH 7.2 with KOH.

Whole-cell recording was performed as previously described (Lazzerini et al., 2021), and a depolarizing step from a holding potential of −80–20 mV was applied to elicit the Kv11.1 current. Tail current was evoked by repolarizing the cell membrane to −50 mV for 4 s.

Kv11.1 current values were corrected for leakage using 1 µM E4031, which completely blocked hERG channels.

**Zebrafish Embryo Toxicity Studies**

Adult wild-type Tübingen zebrafish (*Danio rerio*) were used in all experiments and maintained at 28 ± 1°C under continuous water aeration and filtering and artificial light with a 12/12 h dark/light cycle, according to The Zebrafish Book (Westferfield, 2000). The fish were regularly fed twice a day with commercial dry-flake food (TetraMin™ flakes; Tetra Melle, Germany) supplemented with *Artemia nauplii* once a day. The day before spawning, males and females were placed in separated breeding tanks at a ratio of 1:2 before the onset of darkness and left undisturbed overnight. At the onset of light, the separators were removed from the breeding tanks. After 30 min, the eggs were collected, rinsed twice from debris using fresh embryo medium (Instant ocean), and transferred into Petri dishes containing the embryo medium.

Fertilized eggs were selected under a binocular stereomicroscope (PXX-VI, Optica) and transferred into 24-well plates. Twelve embryos were placed in each well in a 2-ml of embryo medium, treated with
either Dox or Sdox at 6 h post fertilization (hpf). The embryo medium and 0.1% DMSO were used as controls. Treated embryos and controls were then incubated at 28 ± 0.5°C. Each experiment was repeated three times from three independent breedings. In each experiment, at least 3 wells with 12 embryos per treatment per concentration were used.

The zebrafish embryo toxicity assay was performed when fertilization rate was ≥90%. An assay was considered valid if the overall survival of embryos in negative controls was ≥90% until hatching.

All embryos were inspected for morphological characteristics at different developmental stages (48 and 72 hpf) as described by Kimmel (1989). Lethal and teratogenic effects were recorded according to the Organisation for Economic Co-operation and Development (OECD) (236) guidelines for testing of chemicals. Mortality was determined by counting embryos with or without a visible heart beat at 72 hpf. For visual documentation of the malformations and heartbeat, embryos were imaged and recorded using a camera connected to the microscope (CKX41; Olympus, Tokyo, Japan). Each heartbeat was manually counted for 20 s and multiplied by 3 to calculate heart rate (beat/minute) (Hoage et al., 2012). At least three embryos per group were recorded and their heart beat counted. Teratogenic effects were recorded if the fingerprint end-point was observed in at least 50% of all embryos showing teratogenic effects and if these effects were concentration-related.

Zebrafish experiments were conducted under the standard national and EU regulations.

Materials
The materials used included DMEM, RPMI 1640, FBS, L-glutamine, penicillin, streptomycin, Dox, sodium pyruvate, geneticin, accutase, E-4031, trypsin/EDTA, MTT, SRB, Tris, and verapamil (Sigma-Aldrich, Milan, Italy; Sigma Aldrich, Germany), MEM, non-essential amino acids, FBS (Gibco, Life Technologies, New York, United States), amd LDH cytotoxicity Kit (TaKaRa, Germany).

Sdox (2-((25,4S)-4-((2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotracenc-2-yl)-2-oxoethyl 4-(4-phenyl-3-thioxo-3H-1,2-dithiol-5-yl)benzoate) was synthetized as previously reported (Chegaev et al., 2016).

Statistical Analyses
Statistical analysis of data was performed with GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, CA, United States). Comparisons among groups were performed by Student’s t-test for unpaired samples, one-way ANOVA with Dunnett’s multiple comparisons post hoc test, or multiple t-tests with a Holm–Sidak correction, assuming similar dispersions. Values of p < 0.05 were considered statistically significant.

RESULTS
In Silico Studies
The in silico studies aimed at a preliminary evaluation of the ADME/Tox properties of Sdox to guide further experimental studies. A multistep procedure was used involving the following types of drug profiling: physicochemical and ADME; drug safety; metabolic transformations; docking and binding to PXR and SULT; and off-targets prediction. In all steps, the results obtained for Sdox (Figure 1) were compared to those calculated for Dox to help in assessing the reliability of predictions.

Physicochemical Characterization and ADME
Table 1 summarizes the results on the physicochemical characterization of the studied compounds by ACD/Percepta.

The results point to two main differences in the physicochemical properties between Dox and Sdox: lipophilicity and solubility (shaded rows in Table 1). Considering the importance of the partition coefficient logP, this fundamental molecular descriptor was calculated by several software programs employing different predictive algorithms (Table 2). The calculated logP values of Dox differed depending on the applied algorithms; however, the average value of 1.13 obtained by the different methods was close to the experimental one, corresponding to 1.27 (Viswanadhan et al., 1989), suggesting reliable prediction of this descriptor. In addition, to consider the property of the compound at physiological pH, the distribution coefficient logD was calculated (Table 2). Obviously, the presence of the H,S-donor group has an essential impact on the lipophilicity of Sdox, the compound remaining highly hydrophobic even at pH = 7.0.

The results of ADME profiling showed that both drugs possess poor permeability across Caco-2 monolayers (Pe ≤ 1.10–6 cm/s at pH = 7 and 500 rpm stirring rate) and their capacity for BBB penetration (the model takes into account only the BBB governed by passive diffusion) scored a low value, suggesting inactivity in the CNS.

While Dox was ranked as strongly bound to plasma proteins (80% < PPB ≤ 90%), Sdox was estimated as “undefined” with RI < 0.3. It should be noted that the predictions related to HIA were not considered, taking into account that Dox (and presumably Sdox) is not administrated orally.

Drug Safety
The predictions of the ability of Sdox to interact with the membrane transporter P-gp and the major CYP450 isoforms responsible for drug metabolism (CYP1A2, 2C9, 2C19, 2D6, 3A4) were classified Dox as a P-gp substrate and as a non-inhibitor of all CYP450 isoforms. For Sdox, all predictions fell into the category “undefined” with the exception of CYP1A2 and CYP2D6 for which the compound was estimated as a non-inhibitor.

Cardiac and hepatic drug safety profiles were further predicted using ACD/Percepta and Derek Nexus expert systems (Table 3). The reported values showed that, according to ACD/Percepta estimation, both compounds had low probability to bind to hERG channel; however, these predictions were outside the applicability do-main of the model used for prediction. The results of Derek Nexus gave plausible levels for Sdox cardiotoxicity and
hepatotoxicity and a higher likelihood level for Dox hepatotoxicity (probable).

**Metabolic Transformations**

The prediction of the biotransformations resulted in 37 different metabolites of Dox and 69 of Sdox. Supplementary Tables S1, S2 illustrate the structures of predicted metabolites. Duplicated metabolites appeared more than once at different steps of the biotransformation cascade. Reproduction of the original input structures during the liver enzymatic reactions was also predicted. The comparative analysis of Phase I metabolic profiles of Dox and Sdox underlined 19 shared metabolites (Supplementary Table S5) related to their common molecular scaffold.

By focusing on the “on path likelihood” of the predicted biotransformations, all 37 Dox and 54 Sdox metabolites appeared more than once at different steps of the biotransformation cascade. Reproduction of the original input structures during the liver enzymatic reactions was also predicted. The comparative analysis of Phase I metabolic profiles of Dox and Sdox underlined 19 shared metabolites (Supplementary Table S5) related to their common molecular scaffold.

A comparative analysis of the individual biotransformations frequency was performed for both dox and Sdox (Figure 2). Eleven different biotransformations were predicted to be catalysed by four enzymes. Nearly 80% (47) of Dox metabolites resulted from reactions catalyzed by the alcohol

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**TABLE 1 | Physicochemical profiles of Dox and Sdox as predicted by ACD/Percepta software (differences are shaded).**

| Physicochemical parameter | Value | Category/range | Value | Category/range |
|---------------------------|-------|----------------|-------|----------------|
| logP\(^{a}\)             | 0.92  | Optimal \((-1 < + \leq 4.2)\) | 6.32  | Very lipophilic \(>5\) |
| MW                        | 543.52| Bad \(>500\) | 855.95| Bad \(>500\) |
| No. of H-bond donors      | 7     | Bad \(>5\) | 6     | Bad \(>5\) |
| No. of H-bond acceptors   | 12    | Bad \(>10\) | 13    | Bad \(>10\) |
| No. of rotational bonds   | 5     | Good \(<10\) | 10    | Good \(<10\) |
| No. of rings              | 5     | Bad \(>4\) | 8     | Bad \(>4\) |
| Lipinsky rule of \(S\) (No. of violations) | 3 | Bad (less than 2 rules apply) | 4 | Bad (less than 2 rules apply) |
| Lead-like rule\(^{b}\) (No. of violations) | 3 | Bad (less than 2 rules apply) | 4 | Bad (less than 2 rules apply) |
| Solubility, mg/ml         | 12.7  | Soluble       | 5.10  | Highly insoluble |

\(^{a}\)Consensus algorithm combining classical fragment- and similarity-based approach.

\(^{b}\)Applies to the following thresholds: \(-2 \leq \text{log}P < 4.2\); MW < 460; No. of H-bond donors \(\leq 5\); No. of H-bond acceptors \(\leq 9\). The main differences are shaded.

**TABLE 2 | Calculated logP and logD (at pH = 7.0) of Dox and Sdox using different software.**

| Software     | logP/logD |
|--------------|-----------|
|              | Dox       | Sdox      |
| ACD/Percepta\(^{a}\) | 0.92/0.93 | 6.05/4.20 |
| MOE          | 0.96/0.26 | 6.79/5.57 |
| Marvin       | 1.50/0.10 | 7.14/5.73 |
| Average value | 1.13/0.32 | 6.75/5.30 |
| Experimental value\(^{b}\) | 1.27/n.d.\(^{c}\) | n.d. |

\(^{a}\)Consensus algorithm combining classical fragment-based and similarity-based approach.

\(^{b}\)Viswanadhan et al., 1989.

\(^{c}\)Not determined.
dehydrogenase (ADH), and the remaining 20% (12) resulted from CYP450-catalysed biotransformations. The Sdox metabolic profile revealed 49% (74) of the reactions involving ADH, 33% (50) of the reactions catalyzed by hydrolase, 17% (25) by CYP450, and only one reaction mediated by the glutathione-disulfide reductase (GSR) (Figure 2A). Overall, the ADH-catalyzed oxidation of secondary (acyclic) alcohols and the reduction of aliphatic ketones accounted for twice more metabolites in the Sdox profile than in the metabolic tree of Dox (Figure 2B). A similar result was obtained with the oxidative O-demethylation operated by the CYP450 system (Figure 2C).

All Sdox biotransformations involving hydrolases were related to “hydrolysis of acyclic carboxylic esters” and were responsible for the enzymatic cleavage of the 3-thioxo-3H-1,2-dithiol-5-yl group (or its metabolites) from the Dox scaffold (in its original or metabolized form). These reactions were listed exclusively in the Sdox profile. They were predicted to result in two plausible (M65 and M109) and one probable (M15) metabolites, possibly relevant to the hydrogen sulfide release from Sdox. Therefore, these metabolites were suggested to act as H2S donor substructures (Figure 3A). Several Sdox metabolites either reproduced the scaffold of the known and most relevant Dox metabolite doxorubicinol or included its derivative presenting a carbonyl instead of a carboxyl substituent on the glycosidic moiety (Figure 3A, M13 and M32). Other contained a metabolized form of the H2S donor group attached to a core sub-structure, reproducing doxorubicinol (Figure 3B, M63 and M95).

The above predictions prompted further studies to evaluate Sdox hepatotoxicity, hERG liability, and CYP450 inhibition.

Docking and Binding to Pregnane-X-Receptor and Sulfotransferase
Docking and binding of both anthracyclines (Supplementary Figure S1) and some of their predicted metabolites to PXR and SULT were performed.

Glide (XP) was not able to calculate docking scores for Sdox and some of its metabolites to PXR and SULT, possibly due to inability of the applied algorithm to fit them properly into the respective binding sites because of their large molecular size (Table 4).

According to Vina and AutoDock, Dox, Sdox, and most of the Sdox metabolites displayed comparable docking scores for PXR binding, although lower than the previously determined

### TABLE 3 | Toxicity predictions of Dox and Sdox.

| Toxicity                  | Dox               | Sdox              |
|---------------------------|-------------------|-------------------|
| ACD/Percepta: 0.33 < Score ≤ 0.67 (undefined) | Score = 0.36 p = 0.01 RI = 0.28 | Score = 0.43 p = 0.05 RI = 0.15 |
| hERG inhibition (K<sub>i</sub> < 10 μM, patch-clamp method) | plausibility      | plausible         |
| Derek Nexus: likeliness levels | Cardiotoxicity in mammals plausible | Hepatotoxicity in mammals plausible |

For Score, p, RI, and likelihood levels, see Materials and Methods.
threshold. Instead, Dox (only with AutoDock) and Sdox metabolites M15, M65, and M109 showed better scores for SULT binding compared to Sdox.

**Off-Target Prediction**

Dox forms complexes with DNA by intercalation between base pairs, and it inhibits TOPO II activity by stabilizing the DNA-topoisomerase II complex, while Sdox releases H₂S in the ER. Therefore, any other interaction with proteins must be considered an off-target effect (side effect, non-therapeutic, toxicological, etc.) unless a proof of the pharmacological (therapeutic) effect is reported.

Predictive *in silico* off-target profiling for Sdox was performed by using the reverse docking method, to assess possible, relevant differences between Dox and Sdox towards several cancer targets. Neither a standard protocol nor a list of recommended targets to study is reported in the literature. Based on our prior findings (Silveira-Dorta et al., 2015), we selected for docking purposes our in-house set of 85 common human cancer targets, which are available in PDB ([Supplementary Table S6](#)), whose resolution is valid for docking studies. Although not covering all possible protein–drug interactions, the present approach showed how small chemical differences between Dox and Sdox led to relevant changes in the selectivity towards cancer targets.

Among the top 10 ranked interactions (**Table 5**), only PKC-α was common to both compounds.

**Figure 4** shows the DS for both drugs against the selected targets, plotted in order of increasing ΔDS with the largest ΔDS.
All compounds exhibited CYP3A4 inhibition potency comparable to ketoconazole [IC_{50} 6.8 µM, 95% confidence interval (CI) 3.5–10.1 for Dox; 3.1 µM, 95%CI 1.9–4.3 for Sdox; 13.7 µM, 95% CI 3.8–23.6 for ketoconazole] with 95% CI overlapping for all compounds (p > 0.05). Thus, Dox and Sdox inhibit CYP3A4 to a degree comparable to the reference inhibitor ketoconazole.

**In Vitro Safety Evaluation of Sdox and Dox on HepG2 Cells and Primary Rat Hepatocytes**

To characterize deeper the safety profile of Sdox vs. Dox, a comparative in vitro evaluation of both compounds on two complementary liver-derived cell models, the HepG2 cells and the freshly isolated rat hepatocytes, was performed. HepG2 cells are non-tumorigenic cells with high proliferation rates and an epithelial-like morphology that perform many differentiated hepatic functions (Donato et al., 2015).

The effects of Dox and Sdox on HepG2 cell viability were assessed by using the MTT assay (Table 8). At all scheduled times, the estimated IC_{50} values demonstrated that HepG2 cells were more sensitive to Dox than to Sdox (Table 8).

LDH release from cultured HepG2 cells, an indication of cell membrane injury, was examined. After 24 and 48 h of treatment, Sdox increased LDH release only at the maximum concentration tested (10 µM) and at 72 h also at 4 µM. However, Dox became cytotoxic at concentrations lower than those of Sdox, regardless of timing of exposure (Figures 5A–C).

To further confirm the data obtained in HepG2 cells, we analyzed more in depth a second non-transformed liver model, i.e., primary isolated rat hepatocytes, which, although characterized by short life-span, exhibit higher levels of expression and activity of drug-metabolizing enzymes than HepG2 cells (Soldatow et al., 2013; Poldidoro et al., 2021).

Dox and Sdox reduced the number of viable cells, estimated by the trypan blue exclusion assay, with IC_{50} values of 9.68 ± 0.89 µM and 16.49 ± 1.01 µM, respectively (p < 0.05 vs. Dox, Student’s t-test for unpaired samples) and increased the release of LDH in a concentration-dependent manner, Dox being significantly more potent and toxic than Sdox (Figure 5D).

Moreover, Sdox produced a lower decrease in the reduced GSH and a lower increase in the MDA levels, considered an index of lipid peroxidation (Figures 5E,F), indicating that it causes a lower oxidative stress as compared to Dox.

The data from in vitro hepatic safety evaluation on both HepG2 cells and isolated hepatocytes are consistent with the in silico predicted drug safety profiles of Sdox vs. Dox in terms of hepatic drug safety (shown in Table 3).

**Effect of Dox and Sdox on hERG (KV11.1) Current**

Dox (up to 100 µM) and Sdox (up to 10 µM) did not affect hERG currents recorded in hERG-HEK293 recombinant cells by using the patch-clamp technique (Figure 6).

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**Table 4** Docking scores of Dox and Sdox binding to pregnane-X-receptor (PXR, PDB ID:1m13) and sulphotransferase (SULT, PDB ID: 2a3r) [kcal/mol].

| Compound | PXR | SULT |
|----------|-----|------|
|          | Glide XP | Vina | AutoDock | Glide XP | Vina | AutoDock |
| Threshold | -7.7 | -10.3 | -12.5 | -6.3 | -6.0 | -7.5 |
| Dox      | -11.89 | -8.8 | -8.46 | -3.2 | -8.83 | -3.2 |
| Sdox     | - | -8.5 | -9.28 | +0.7 | -2.05 | +0.7 |
| M5       | - | -6.8 | -9.77 | +7.3 | +0.19 | +7.3 |
| M13      | - | -6.9 | -9.35 | +6.3 | -2.20 | +6.3 |
| M15      | -8.39 | -7.6 | -8.0 | -6.21 | -7.7 | -7.51 |
| M32      | - | -7.8 | -9.58 | +3.7 | -2.84 | +3.7 |
| M63      | - | -6.7 | -8.89 | +5.0 | +1.78 | +5.0 |
| M65      | -8.15 | -7.2 | -7.43 | -6.74 | -8.2 | -8.11 |
| M95      | - | -7.3 | -9.33 | +4.8 | -0.98 | +4.8 |
| M109     | -6.66 | -6.7 | -7.57 | -6.33 | -7.2 | -7.31 |
| M153     | - | -8.2 | -9.81 | +4.4 | -2.13 | +4.4 |

Docking score of Dox, Sdox, and Sdox common metabolites (M) calculated according to three different software (Glide XP, Vina, and AutoDock). Values in bold indicate differences being summarized in Table 6. The results predicted a significant difference (>2 kcal/mol) for the binding interaction with CDK6, MAPK 8, DNA Topo I, and PKC-ζ. A higher ΔDS (>5 kcal/mol) was found for β-tubulin (VBS), tankyrase (TNKS), GSK-3 β, and Cyclin D3 (CCND3). Thus, the proteins CDK6, MAPK 8, DNA Topo I, and PKC-ζ were predicted to represent preferential targets for Sdox, whereas Dox was anticipated to bind preferentially the proteins β-tubulin (VBS), TNKS, GSK-3 β, and CCND3.

On the left side of the graph, we can observe the targets for which hypothetically Sdox binds stronger than Dox. The targets with more affinity for Dox than for Sdox are reported on the right-hand side.

**In Vitro Studies**

To further characterize the differential impact in terms of efficacy, metabolism, and safety between Dox and Sdox, four parameters were analyzed in vitro: the efficacy in cell lines overexpressing P-gp, the inhibition of CYP3A4, involvement in Dox and several drugs metabolism, and the toxic effects on hepatocytes and that on hERG channel.

**P-Glycoprotein Assay**

The effect of P-gp overexpression in the antiproliferative activity of Sdox is shown in Table 7. Gl_{50} values recorded in P-gp overexpressing and wild-type cell lines and their ratio (resistance factor, RF) are given. In the absence of verapamil, Sdox was slightly affected by P-gp overexpression (RF = 3.5) as compared to Dox and, in particular, to paclitaxel (RF = 564). Co-treatment with verapamil caused a decrease in RF for all antimitotic drugs. This result is consistent with the data on Table 5.

**In Vitro Inhibition of Human CYP3A4**

Previous studies have suggested that CYP3A4 enzyme plays a role in the oxidative metabolism of Dox in humans (Kivistö et al., 1995).
Zebrafish Embryo Toxicity Studies
To further deepen the comparison between the toxicity of Sdox and Dox, the lethality induced by the drugs, applied at 6 hpf, was monitored in zebrafish embryos at 72 hpf (Figure 7). As shown in Figure 7A, 10 µM Dox markedly reduced the number of live embryos, whereas Sdox was ineffective with nearly 90% of embryos alive. Similar results were observed at 96 hps (data not shown).

The toxic effects of both anthracyclines, at 10 µM concentration, on embryo development were analyzed at 48 and 72 hpf. In particular, at 48 hpf, neither visible teratogenic effects nor differences in hatching rate were observed (Figure 7B), and at 72 hpf, Dox and Sdox did not display developmental toxicity in zebrafish embryos (Figure 7B). Consistently, neither compound affected the embryo heartbeat rate at 72 hpf when applied at 10 µM concentration (Figure 7C).

As expected, when higher concentrations were applied (up to 100 µM), a Dox-induced concentration-dependent developmental toxicity was evident. Notably, cardiotoxicity was manifested through the presence of pericardial edema and deformity of the heart, and a reduction of zebrafish embryo heartbeat rate (data not shown). Instead, Sdox was tested up to 10 µM because a precipitate was observed at higher concentrations.

To test whether the presence of chorion can interfere with the diffusion of Sdox and Dox, zebrafish embryos were manually dechorionated at 24 and 48 hpf and exposed to either drug. Malformations detected in dechorionated embryos were similar to those observed in embryos with intact chorion.

DISCUSSION
Sdox and its liposomal formulation were recently proposed as an innovative and effective therapeutic tools against P-gp-overexpressing/Dox-resistant tumors (Bigagli et al., 2018; Gazzano et al., 2019).

The present study evaluated Sdox drug-like characteristics and ADME/Tox properties in silico, in vitro, and in vivo.

The in silico physicochemical profiling of Sdox demonstrates that it maintains some of Dox properties but has specific features different from its parental drug that may constitute an advantage in terms of reduced toxicity, increased efficacy against P-gp-overexpressing cells, metabolism, and reduced off-target effects.

For instance, Sdox possesses high lipophilicity and low solubility compared to Dox, suggesting a different pharmacokinetic behavior. The lipophilicity of anthracyclines has been previously investigated using spectrofluorimetric...
TABLE 6 | Top-ranked ΔDS values obtained for Sdox and Dox.

| Preferred drug | PDB ID | DS (kcal/mol) | ΔDS (kcal/mol) | Protein |
|----------------|--------|---------------|----------------|---------|
|                |        | Sdox          | Dox            |         |
| Sdox           | 19        | −11.344       | −8.119         | −3.225  | CDK6   |
| Dox            | 4w68     | −1.82         | −7.729         | 7.957   | β-tubulin (VBS) |
| Vincristine     | 3g33     | −1.705        | −7.340         | 5.635   | CCND3  |

TABLE 7 | Antiproliferative activity (GI50) of Sdox, Dox, and tubulin-interacting drugs in SW1573 and SW1573/P-gp cell lines.

| Drug         | SW1573   | SW1573/P-gp | SW1573   | SW1573/P-gp |
|--------------|----------|-------------|----------|-------------|
|              | w/o verapamil | w Verapamil |          | w Verapamil |
| Sdox         | 873 ± 410 | 3,015 ± 531 | 3.5      | 975 ± 330   |
| Dox          | 70 ± 8.6  | 1,601 ± 169 | 23       | 26 ± 8.2    |
| Vincristine  | 0.53 ± 0.22| 98 ± 113    | 564      | 0.46 ± 0.21 |
| Colchicine   | 67 ± 15   | 2,366 ± 937 | 35       | 0.36 ± 0.21 |
| Paclitaxel   | 3.9 ± 1.5 | 76 ± 15     | 22       | 0.60 ± 0.11 |
| Vinblastine  | 9.4 ± 0.35| 16 ± 4.7    | 17       | 0.55 ± 0.11 |

TABLE 8 | Effects of Dox and Sdox on HepG2 cell viability.

| Compound | IC50 (μM) | Rf |
|----------|----------|----|
|          | 24 h     | 48 h | 72 h |
| Dox      | 6.11 ± 0.66 | 0.46 ± 0.03 | 0.19 ± 0.01 |
| Sdox     | 8.69 ± 0.31**| 3.21 ± 0.16***| 1.39 ± 0.03***|

Potency of compounds is expressed as estimated IC50 values (μM). Data are reported as mean ± SD, of 3 independent experiments. **p < 0.01, ***p < 0.001 vs. Dox (Student’s t-test for unpaired samples).

A method to evaluate cell uptake, and a positive correlation between their influx rates and logP has been reported (Rivory et al., 1996). Thus, it may be expected that the higher lipophilicity of Sdox compared to Dox confers an increased uptake within tumor cells, also in the presence of high levels of P-gp, as demonstrated previously (Buondonno et al., 2019) and suggested by the higher cytotoxicity of Sdox compared to Dox in P-gp-overexpressing cells observed in the present work. On the other hand, the intracellular uptake of Dox from the solution was found to be higher than from liposomal formulation at therapeutically relevant concentrations (Kullenberg et al., 2021), while low solubility warns about the need of administering Sdox-like drugs as liposomal formulations to ensure a higher efficacy (Gazzano et al., 2019).

The in silico studies classified Dox as a non-inhibitor of all CYP450 isoforms included in prediction (CYP1A2, 2C9, 2C19, 2D6, and 3A4); Sdox was assigned as a non-inhibitor of two of them (CYP1A2 and 2D6). On the other hand, in vitro data showed that Dox and Sdox inhibited CYP3A4 comparably to ketoconazole, suggesting that both anthracyclines have relatively high affinity to the human CYP3A4 enzyme, isofrom paramount to drug metabolism. This is an important information to acquire in the perspective of future clinical applications of Sdox-like drugs, since many oncological patients are treated with different drugs, and therefore, they are very susceptible to drug–drug interactions. To limit undesired drug–drug interactions, once again, the liposomal formulations of Sdox that have a higher tumor-to-healthy tissue delivery (Gazzano et al., 2019) are crucial.

Differences were also recorded in the effects of both drugs on the membrane transporters, including P-gp. Dox is a well-known P-gp substrate (Food and Drug Administration, 2013), while according to the in silico prediction, Sdox fell into the category “undefined” in relation to this protein.

In the present study, co-treatment of P-gp-overexpressing cells with the P-gp inhibitor verapamil demonstrated that Sdox is a substrate with low affinity for this transporter, and it is less affected (ca. 6.5 times) by P-gp when compared with its parent compound Dox. This agrees with previous studies demonstrating the efficacy of Sdox towards P-gp-overexpressing cells (Chegaev et al., 2016; Bigagli et al., 2018; Gazzano et al., 2019). It was proposed that Sdox may trigger the P-gp ubiquitination by altering the disulfide bonds in the protein structure and subsequently its conformation and stability (Buondonno et al., 2019; Gazzano et al., 2019). Indeed, results obtained in this study on Sdox metabolic transformations confirm the presence of metabolites that can cause P-gp sulfhydration.
Moreover, as PXR has a key role in the regulation of both drug metabolism and drug efflux by activating the expression of genes encoding CYP450 enzymes and drug efflux transporters (Ekins and Schuetz, 2002; Albermann et al., 2005) and SULT1C4 is involved in Dox disposition, catalyzing its sulfation (Luo et al., 2016), both anthracyclines and some of their predicted metabolites were in silico screened for their interaction with these targets. Docking results indicate that Dox had stronger predicted binding to PXR than all the other compounds, whereas only Dox, M15, M65, and M109 could bind to SULT. This suggests a better profile of Sdox against efflux transporters and SULT, compared to Dox and its metabolites.

Pharmacodynamics is the quantitative study of the relationship between drug exposure and pharmacological (desired) or toxicological (unwanted) responses. In recent years, several efforts were made to ensure extensive identification of relevant cellular targets for a given drug both at the experimental (Bendels et al., 2019) and the computational level (Agamah et al., 2019; Bruno et al., 2019). In silico methods could help to anticipate for any given drug undesired interactions with other cellular targets, the so-called off-target effects (Rao et al., 2019). The study of these unwanted interactions had been proposed as strategy for drug repurposing (Lim et al., 2016). The main limitations are related to the availability of the three-dimensional structure with high resolution of the biological target and to the computational capacity. In the effort to expand the possibilities offered by this in silico approach, a method to profile differences between a parent drug and its derivatives was envisioned. In this work, we explored for Dox and Sdox relevant differences in the interaction with potential off-targets. For our pilot study, we selected a set of 85 common cancer targets that are available in PDB. The objective was not to identify all possible off-targets for the two compounds but to point out relevant dissimilarity that might aid to explain the observed experimental differences in the biological assays.

FIGURE 5 | Effects of Sdox and Dox treatment on HepG2 cells and primary rat hepatocytes. LDH release after (A) 24 h, (B) 48 h, and (C) 72 h treatment with Dox and Sdox in HepG2 cells. (D) LDH release and oxidative stress estimated by measuring (E) reduced GSH and (F) MDA in primary rat hepatocytes. Data are means ± SD of triplicate assays (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001 vs. controls, one-way ANOVA with Dunnett’s post-test. #p < 0.05 vs. Dox, multiple t-test, corrected by the Holm–Sidak method.

FIGURE 6 | Effect of Dox and Sdox on Kv11.1 (hERG) current recorded in hERG-HEK293 cells. Concentration-dependent effect of Dox and Sdox of hERG tail currents. On the ordinate scale, current amplitude is reported as a percentage of the value recorded just before the addition of the first concentration of the drug. Data points are the mean ± SD (n = 6).

Moreover, as PXR has a key role in the regulation of both drug metabolism and drug efflux by activating the expression of genes encoding CYP450 enzymes and drug efflux transporters (Ekins and Schuetz, 2002; Albermann et al., 2005) and SULT1C4 is involved in Dox disposition, catalyzing its sulfation (Luo et al., 2016), both anthracyclines and some of their predicted metabolites were in silico screened for their interaction with these targets. Docking results indicate that Dox had stronger predicted binding to PXR than all the other compounds, whereas only Dox, M15, M65, and M109 could bind to SULT. This suggests a better profile of Sdox against efflux transporters and SULT, compared to Dox and its metabolites.

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Our method predicts that, differently from Dox, Sdox targets preferentially several proteins that are important for cell cycle progression, such as CDK6, mediating the G1/S transition; CDK7, which is required for the G2/M transition; KIF11,
involved in the spindle dynamics during mitosis; ROCK1, necessary for the positioning of centrosome during mitosis; and DNA Topo I, allowing the rejoining of DNA single strands. Moreover, Sdox targets several proteins involved in proliferation and survival pathways, often oncogenically mutated and activated in tumors, such as RET tyrosine kinase, PKC-ζ, PKC-α, and the pro-angiogenic receptor VEGFR2. We are aware that all these predicted interactions and their biological meaning must be further validated experimentally, but the present findings suggest a multitarget profile of Sdox that acts simultaneously on different crucial proteins driving tumor progression.

We found larger ΔDS values among Dox targets. Worth mentioning is the strongest predicted interaction of Dox with the Vinca binding site of β-tubulin (ΔDS = 7.957 kcal/mol). The cardiac toxicity of anthracyclines was reported to be a consequence of the disruption of microtubule organization in cardiac myocytes (Fromes et al., 1996). Thus, it is reasonable to hypothesize that Sdox could exhibit a lower cardiac toxicity. The toxicity studies performed in zebrafish embryos and that obtained by Bigagli et al. (2018) and Gazzano et al. (2019) agree with this hypothesis.

The docking score is only a broad approximation to the predicted binding energy (not attempted to correlate with experimental ligand–receptor affinity) and is only used to give clues to possible interactions and ranks among compounds, prompting the further use of experimentation on these. Further biological tests will be necessary to validate the relevance of the off-targets, here outlined, in the different effects induced by both anthracyclines. Notably, the predicted differences seem to correlate with the experimental results. To the best of our knowledge, this work represents the first attempt to run such approach and the premise to further explore the potency of the predictive off-targets method in a future dedicated work. Overall, the differential target profile suggests that Dox and Sdox are two pharmacodynamically distinct drugs.

Kv11.1 (hERG) K⁺ channels mediate the cardiac IKr current that acts as an important determinant of action potential repolarization in the human ventricle and of pacemaking activity in heart nodes (Vandenbroucke et al., 2012). hERG blockade or dysfunction, therefore, results in prolongation of the electrocardiogram QT interval, leading, in rare cases, to Torsade de Pointes, a polymorphic ventricular tachycardia that can degenerate into ventricular fibrillation and death (Antzelevitch, 2007). Indeed, hERG channel liability, along with hepatotoxicity, is the major reason for drug attrition during preclinical development, clinical trials, and post-marketing drug withdrawal (Valentin, 2010).

In silico results predicted and in vitro data demonstrated that Dox and Sdox did not affect hERG currents recorded in hERG-HEK293 recombinant cells by using the patch-clamp technique. Furthermore, Sdox, differently from Dox, was not cytotoxic in H9C2 cardiomyocytes (Chegaev et al., 2016; Buondonno et al., 2019), since the presence of H₂S prevented the increase in reactive oxygen species (ROS) (Chegaev et al., 2016). Moreover, Sdox did not show any evidence of cardiac toxicity in prostate cancer.
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... of Dox and Sdox, respectively. Previous findings concerning liver toxicity indicated that Dox induces focal necrosis, hepatocytes vacuolation, degeneration of hepatocyte cords, and bile duct hyperplasia, mainly due to ROS generation during its hepatic metabolism, resulting in imbalanced redox potential leading to oxidative stress, reduced levels of antioxidant enzymes, apoptosis, inflammation, and mitochondrial dysfunction (Prasanna et al., 2020). In both hepatic cell-based models used here, Dox decreased cell viability and increased necrosis and oxidative stress, while Sdox was less cytotoxic and caused less oxidative damage.

These experimental findings were in agreement with previous works showing that Sdox did not display signs of liver toxicity, according to the hematochemical parameters (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and creatine phosphokinase) in osteosarcoma xenograft (Gazzano et al., 2019).

Several studies have already assessed the effects of Dox on zebrafish embryo development (Yang et al., 2011; Chang et al., 2014; Han et al., 2015; Cavalcante et al., 2021), and its toxicity was demonstrated at different developmental stages (Chang et al., 2014).

The results obtained in the present work demonstrated that unlike Dox, Sdox did not reduce the number of live embryos at 72 hpf. It is known that the chorion can act as a selective barrier for some compounds to reach the embryo (Nishimura et al., 2016), but our results showed that this was not the case for both compounds.

The zebrafish expresses ABCB4 and ABCB5 transporters that are structurally very similar to mammalian P-gp (ABCB1); in particular, ABCB4 is responsible for embryo resistance to P-gp substrates (Fischer et al., 2013). However, Dox, a P-gp substrate, has no major effect on the zebrafish ABCB4 ATPase activity when applied at concentrations up to 100 μM [67].

At present, the interaction of Sdox with ATP-binding cassette membrane transporters such as ABCB4 and ABCB5, which are constitutively expressed in various tissues during early zebrafish embryo development, is unknown (Fischer et al., 2013).

In vitro, Sdox displayed a much lower Rf in SW1573 cells overexpressing P-gp as compared to Dox, indicating a different interaction pattern with this membrane transporter, either as a weak P-gp substrate or indirectly modifying its function. Therefore, it is conceivable to speculate that Sdox’s direct interaction with zebrafish ABCB4 transporter is also not prominent: further studies, however, are needed to clarify this issue.

The addition of H2S to Dox treatment in rats ameliorates its cardiotoxic effects by inhibiting oxidative stress, reducing inflammation, and suppressing apoptosis (Li et al., 2021). As Sdox treatment is accompanied by a reduced oxidative stress compared to Dox, the lack of toxic effects of the drug in zebrafish is likely due to the same mechanisms.

CONCLUSION

Dox, discovered in the late 1960s, still represents the mainstay for the treatment of numerous solid and hematological malignancies, despite its therapeutic value being hampered by cross-resistance towards different anticancer drugs and severe dose-dependent cardiotoxicity.

Recent studies revealed that Sdox, a novel H2S-releasing Dox, besides being effective in several preclinical Dox-resistant tumor models, is also devoid of cardiotoxic effects.

This study aimed to further characterize Sdox integrating a multi- and trans-disciplinary approach.

In silico profiling suggested that Sdox possesses higher lipophilicity and lower solubility compared to Dox, and the off-targets prediction indicated that the proteins CDK6, MAPK 8, DNA Topo I, and PKC-ζ represent preferential targets for Sdox, whereas Dox was anticipated to bind preferentially the proteins β-tubulin (VBS), TNKS, GSK-3 β, and CCND3.

In vitro studies demonstrate that Sdox is a substrate with lower affinity for P-gp; it is less hepatotoxic and causes less oxidative damage than Dox.

Unlike Dox, it did not affect the percentage of Zebrafish live embryos at 72 hpf. Although we cannot infer any conclusion about the clinical profile of Sdox, taken together, the in silico, in vitro, and in vivo findings demonstrate that it displays a higher efficacy against Pgp-positive cells, different selectivity towards cancer targets, and a more favorable ADME/toxicity profile than Dox, thus representing a significant advancement in the treatment of Dox-resistant/Pgp-overexpressing tumors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Bulgarian Food Safety Agency (No. 304 valid until June 28, 2026) and Animal Ethics Committee of the University of Sofia (No. 220, April 13, 2021).

AUTHOR CONTRIBUTIONS

Conceptualization: JP, IP, and SS. Formal analysis PA, MA, DA, JD, AD, FF, AG-S, MK-B, JP, IP, HR, CR, IT, VT, and YY.
Investigation: PA, MA, DA, KC, JD, AD, MF, FF, AG-S, RJ, MK-B, IP, TP, AP, IT, and YY. Writing—original draft: JD, FF, AG-S, MK-B, JP, IP, HR, VT, and SS. Writing—review and editing: JD, AG-S, FF, JP, MA, PA, IT, TP, CR, and SS. All authors have read and agreed to the published version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.831791/full#supplementary-material

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