The role of hydrogen sulfide in doxorubicin-induced drug resistance in hepatocellular carcinoma cells

by

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Abstract

Doxorubicin is one of the most common chemical agents used in the treatment of cancers. Doxorubicin-treated cancer cells often develop drug resistance due to alterations in the trafficking and metabolism of the drug, which severely limits the drug's effectiveness. This is seen at especially high rates in human hepatocellular carcinoma (HCC), the most common form of liver cancer. H_2S is an important gasotransmitter and is involved in a variety of cellular functions and pathophysiologic processes. The role of H_2S in drug resistance in cancer cells is still unclear. In this study, by using a human hepatocellular carcinoma cell line (HepG2), we found that NaHS (an H_2S donor) was able to reduce cancer cell viability and colony formation in a doxorubicin dose dependent manner, while H_2S alone did not show any effect. The expression of H_2S-generating enzyme cystathionine gamma-lyase (CSE) but not cystathionine beta-synthase (CBS) was reduced by doxorubicin treatment. In addition, H_2S promoted cellular retention of doxorubicin in HepG2 cells, possibly by suppressing the expression of ABCA1 and ABCG8, two drug efflux proteins. LXRα acts as a transcription factor for ABCA1 and ABCG8, however our findings showed that H_2S had no effect on the protein expression and S-sulfhydration of LXRα, suggesting LXRα is not involved in H_2S-regulated expressions of ABCA1 and ABCG8. In comparison with the parental cells, CSE expression was also reduced in doxorubicin-resistant cells. Exogenously applied NaHS reversed the drug resistance in doxorubicin-resistant cells. In conclusion, our study provides a novel solution for reversing drug resistance by targeting H_2S signaling.

Key words: H_2S, Cystathionine gamma-lyase, human hepatocellular carcinoma, doxorubicin, drug resistance

Abbreviations:

3MST: 3-mercaptopyruvate sulfurtransferase

ABC: ATP-binding cassette

CAT: Cysteine aminotransferase

CBS: Cystathionine β-synthase

CSE: Cystathionine γ-lyase

D.R: Doxorubicin drug resistant

EDRF: Endothelial derived growth factor

GST: glutathione-S-transferase

HAIC: Hepatic artery-infusion chemotherapy

HCC: Primary Hepatocellular Carcinoma

LPS: Lipopolysaccharide

LXR: Liver X receptors

MDR: Multi-drug resistance

PAG: DL-propargylglycine

ROS: Reactive oxygen species

SLC: solute carrier

VEGF: vascular endothelial growth factor

WT: Wild-type

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1. Introduction

1.1 Gasotransmitters

In 1980, Drs.Furchgott and Zawadzki described the ability of an unknown factor, released by endothelial cells, to induce the relaxation of smooth muscle cells. They termed this molecule an endothelial derived relaxing factor (EDRF), and in 1987, it was discovered to be the gas molecule nitrous oxide. (1). Their research was awarded the Nobel Prize in 1998 alongside Drs. Ignarro and Murad, sparking the interest in the potential for other endogenously produced gaseous molecules to have similar signalling properties. Since that time, both carbon monoxide (CO) and most recently, hydrogen sulfide (H_2S) have been implicated as physiologically relevant molecules in mammalian physiology. In recognition of these gaseous signalling molecules, Dr. Wang coined and defined the term “gasotranmsmitters”. In order to be recognized as a member of the gasotransmitter group, a molecule needs to meet the 6 criteria outlined below (adapted from (2));

1. They are small molecules of gas.

2. They are freely permeable to the cell membrane. As such, their intracellular and intercellular movements do not exclusively rely on cognate membrane receptors or transport proteins

3. They are endogenously generated in mammalian cells with specific substrates and enzymes; more than the products of metabolism, their production is regulated to fulfill signaling messenger functions.

4. They have well-defined, specific functions at physiologically relevant concentrations.

5. Functions of endogenous gases can be mimicked by their exogenously applied counterparts.

6. They are involved in signal transduction and have specific cellular and molecular targets.

1.2. H_2S in mammalian physiology

H_2S has a molecular weight of 34.1 g/mol and is a flammable gas with no colour. It is most commonly associated with its distinct "rotten egg" smell. Initial H_2S research focused mostly on it's cytotoxic effects, as well as it's potential as a major workplace safety concern in the oil industry (3). At the time of it's discovery, endogenous H_2S production in brain tissues was widely considered to be nothing more than metabolic waste (4). It wasn't until the discovery of nitric oxide as an important signalling molecule researchers began to look at other endogenously produced gaseous molecules. Since then, the endogenous production of H_2S has been well described, and is facilitated by three major enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) in conjugation with cysteine aminotransferase (CAT). These enzymes catalyze the conversion of L-cysteine to H_2S with pyridoxal 5' phosphate (vitamin B6) as a cofactor (Fig 1.). Given the importance of L-cysteine in the production of H_2S, the regulation of appropriate circulating cysteine is critical for maintaining H_2S homeostasis. The production of cysteine in mammalian tissues is performed through the reverse transsulfuration pathway and uses the H_2S generating enzymes CSE and CBS to convert homocysteine to cystathionine, and then finally to cysteine (Fig 1.).

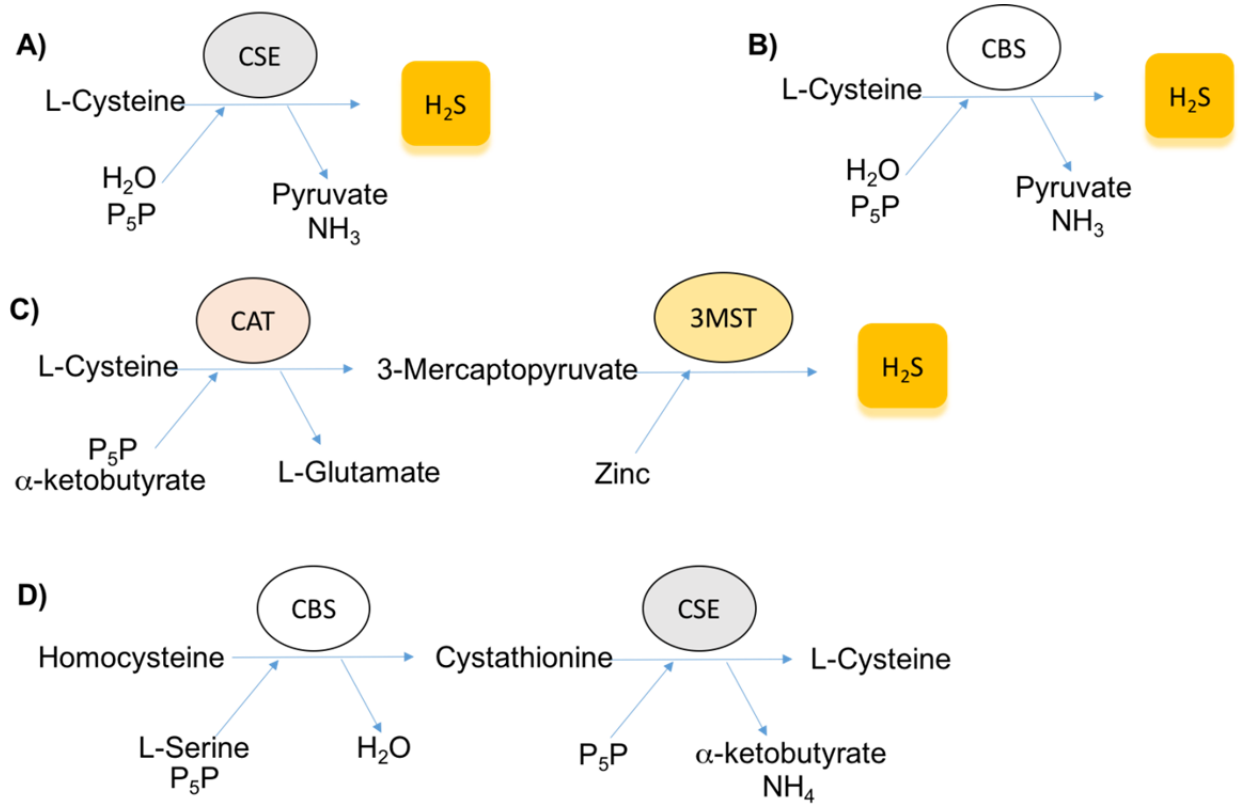

Figure 1. Mammalian endogenous generation of H_2S through reverse transsulfuration

H_2S is capable of inducing cellular changes through a process known as protein S-sulfhydration. This involves the conversion of thiol –SH groups in the cysteine residues of target proteins, to hydropersulfide –SSH groups, changing both the structure and functionality of these proteins (5). In doing so, H_2S is capable of carrying out a huge number of signalling roles in most of the major organ systems. H_2S-mediated S-sulfhydration of the NF-κB p65 subunit has been shown to increase NF-κB transcriptional activity, thus providing increased protection from cellular apoptosis (6). In human endothelial and fibroblast cells, S-sulfhydration of the mitogen-activated protein kinase (MEK1) results in activation of poly (ADP-ribose) polymerase 1 (PARP1), an important enzyme associated with DNA repair mechanisms (7). In response to

injury in a mouse model, increased levels of S-sulfhydration of the ATP synthase alpha subunit was associated with increased ATP synthase activity. This suggests a role S-sulfhydration in regulating and maintaining necessary energy production during times of cellular stress (8).

The first described role of H_2S as a gasotransmitter was it's signalling properties within the nervous system. In the central nervous system, H_2S is capable of enhancing the post-synaptic potentials of excitatory neurotransmission through increasing the sensitivity of NMDA receptors to glutamate signalling (9). H_2S has also been shown to enhance the production of GABA receptors, leading to inhibitory neurotransmission in the central nervous system (10). In the cardiovascular system, exogenous H_2S has been shown to reduce both heart rate and contractility through the opening of K_{ATP} channels, causing subsequent potassium efflux and hyperpolarization of myocytes (11, 12). The effect of H_2S on K_{ATP} channels has also been implicated in the regulation of vascular smooth muscles, inducing the relaxation of blood vessels and thus regulating systemic blood flow (13). Of all the organ systems, H_2S production in the liver occurs at exceptionally high levels, ranging from 30-90% higher than in vascular tissues. Because of this, some researchers have suggested that the liver is one of the major sites of H_2S production in the body, and thus makes it a key target organ for studying the signalling roles of H_2S (14). Currently, the main role of H_2S in the liver is associated with the regulation of hepatic circulation through targeting of K_{ATP} channels. In doing so, H_2S has been shown to induce vasodilation of the hepatic artery, thus ensuring adequate hepatic clearance (15).

1.3 H_2S in pathophysiology

Given the importance of H_2S production in mammalian physiology, researchers have become interested in describing the role of H_2S in new and unique cellular signaling

mechanisms, specifically in the onset and regulation of disease. Today, our understanding of H_2S in pathophysiology continues to open up new possibilities for H_2S as a clinically relevant tool.

Within the immune system, evidence has suggested both a pro and anti-inflammatory role of H_2S. Given the importance of inflammation in the onset of many pathological conditions, as well as the danger that unregulated inflammation itself can pose, the role of H_2S in inflammatory signalling should not be understated. The pro-inflammatory effect of H_2S has been shown in lipopolysaccharide (LPS)-induced inflammation in mouse models, where plasma H_2S levels were significantly increased. Additionally, administration of the H_2S donor sodium hydrosulfide (NaHS) increased histological signs of inflammatory damage in lung and liver tissues (16). Elevated H_2S levels were also shown to be associated with induced systemic inflammation, while the use of the CSE inhibitor DL-propargylglycine (PAG) significantly reduced the inflammatory response, and NaHS treatment further aggravated it (17). Conversely, as an anti-inflammatory molecule, one study showed the ability for inhaled H_2S to restrict LPS induced systemic inflammation, again highlighting the sometimes conflicting regulatory activity of H_2S (18). H_2S donor treatment was shown to restrict the ability for leukocyte attachment to mesenteric endothelium cells, a key step in the initiation of inflammation. Similarly, H_2S treatment was shown to limit induced paw edema in animal models, while inhibition of H_2S production had the opposite effect (19).

Another important function of this gasotransmitter is its ability to regulate apoptosis. In a manner similar to inflammation, the specific role of H_2S in apoptosis is conflicted. H_2S has been shown to induce apoptosis of human aortic smooth muscle cells (HASMC) (20). Overexpression of the gene encoding CSE, a major H_2S-generating enzyme in the vascular system, enhanced endogenous H_2S production and induced significant levels of HASMC apoptosis. Furthermore,

CSE inhibition through the use of CSE-specific short interfering (si)-RNA exaggerated these effects, suggesting the importance of endogenously produced H_2S in sensitizing these cells to pro-apoptotic signaling events. It was shown that exogenous H_2S increased protein expression of p21Cip and decreased protein expression of cyclin D1, further pointing to its role as a pro-apoptotic signaling molecule (20). Building upon these initial findings, researchers next looked at the role of H_2S in regulating apoptotic events in cancerous cell lines. Lee *et. al*. investigated the effects of H_2S exposure on both breast (MCF7) and liver (HepG2) cancer cell lines (21). Through the use of the H_2S donor NaHS, continuous H_2S treatment over a 5-day period resulted in a significant decrease in cell viability. This was also shown to be specific to only cancer cell line variants, with the same treatment having no effect on corresponding wild-type cells. The researchers proposed that this apparent anti-cancer effect was caused by H_2S-induced uncoupling of mitochondrial electron transport chain. As a result, these cells become more heavily reliant on glycolytic ATP production, with lactic acid produced as a by-product. Because of the high metabolic demands of cancerous cells relative to wild-type cells, these effects became exaggerated in the cancerous lines. The increased lactic acid production and therefore decreased intracellular pH led to damaging cytosolic conditions and ultimately, cancer cell death (21). Similar inhibitory effects were also seen in cancerous gastric cell lines. Following 24-hour H_2S treatments at concentrations ranging from 200-800 μM, gastric cancer (SGC7901) cells exhibited significant levels of apoptosis in a dose-dependent manner (22). In the same study, H_2S was also shown to effectively inhibit both gastric cancer invasion and migration, reducing the ability of the cancerous cells to metastasise. It was proposed that the H_2S-induced the up regulation of Bax, Cytochrome C and Caspase 3, important factors in the intrinsic apoptotic pathway, was responsible for these effects (22).

However, equally convincing data has been collected suggesting that instead of stimulating cell apoptosis, H_2S actually increases cell proliferation and metabolic activity. Szabo *et al*. presented these findings, using a colon cancer cell line, HCT116 (23). Szabo and his colleagues found that CBS knockdown resulted in a significant decrease in H_2S production, implicating CBS as the major H_2S-generating enzyme in these cells. CBS-knockdown also resulted in an approximately 50% decrease in the levels of HCT 116 cell proliferation, while also resulting in down regulation of cellular respiration and ATP synthesis. Szabo et al. also treated both a non-cancerous colon cell line (NCM356) and the HCT 116 cells with aminooxyacetic acid (AOAA), a CBS inhibitor, and found that the resultant CBS inhibition had anti-proliferative effects in the HCT116, but not the NCM356 cells. Conversely, CBS overexpression in the NCM356 cells actually increased cell proliferation, highlighting how, even within the same tissue type, pathological cellular conditions can be affected by the signalling role of H_2S. To explain these effects, the researchers pointed to the ability of H_2S to promote cellular bioenergetic processes such as glycolytic and electron transport chain activity, as well as its stimulatory effects on cell survival mechanisms, such as the Akt-pathway (23). Other studies have pointed to the ability of H_2S to scavenge reactive oxygen species (ROS) and to restrict tumor suppressing gene P53 expression to explain its stimulatory effects (24). A summary of some of the current knowledge on the inhibitory (Fig 2A) and stimulatory (Fig 2B) effects of H_2S on cell growth and activity is provided below.

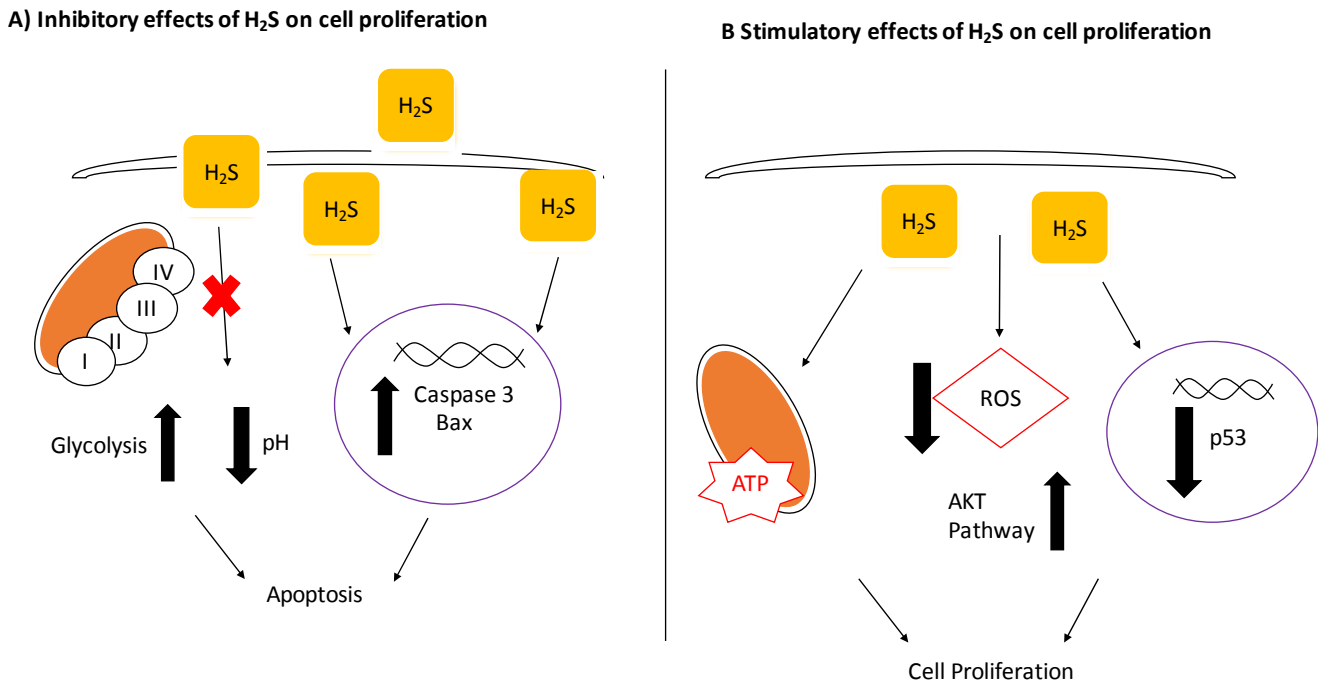

Figure 2. Inhibitory and proliferative signaling properties of H_2S

While it is clear that H_2S plays an important role in the regulation of inflammation and apoptosis, it does so to a wide degree of variability. As a result, it can exhibit sometimes opposing functions depending on factors such as the the type and concentration of H_2S donor used, the modification of either endogenous or application of exogenous H_2S sources, as well as the tissue type being studied. What is clear however, is the ability for H_2S to play a key role in major disease processes. This offers the potential for new discoveries, especially in tissues where H_2S production is significant, but its exact role is still poorly understood, the liver being a prime example of such conditions.

1.4 H_2S post-translational modifications of protein by S-sulfhydration [1]

S-sulfhydration, or S-persulfidation, is a newly discovered protein post-translational modification by yielding a hydropersulfide moiety (–SSH) in the active cysteine residues. By changing local conformation and the final activity of target proteins, *S*-sulfhydration is believed to mediate most of cellular responses initiated by H_2S. Since the first finding of *S*-sulfhydration on proteins was described in 2009 (5), many proteins have been reported to be *S*-sulfhydrated and involved in the physiological and pathological functions of H_2S. H_2S acts as an endothelium-deriving relaxing factor (EDRF) through *S*-sulfhydration of potassium channel proteins (25). *S*-sulfhydration of Keap1 provides protection against cellular senescence via the regulation of Nrf2

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[1] (This section has been published as part of a review paper titled "H_2S-mediated protein *S*-sulfhydration: a prediction for its formation and regulation in *Molecules*. Vol. 2017, doi 10.3390/molecules22081334).

activity (26). Recently, it was found that *S*-sulfhydration of MEK1 is associated with repairing damaged DNA inside the cell (7). eNOS *S*-sulfhydration regulates eNOS activity through the regulation of eNOS dimerization (27). In addition, abnormal protein *S*-sulfhydration have been found to be involved in multiple sclerosis (28), antioxidants (29), neuroprotection (30), and endoplasmic reticulum stress response (31) by altering enzymatic activity, protein localization, protein–protein interactions, and protein stability.

Despite the importance of protein *S*-sulfhydration in diverse cellular functions and pathophysiological responses, the regulatory mechanism of protein *S*-sulfhydration are largely unclear. The interaction or competition between cysteine *S*-sulfhydration and other protein post-translational modifications (Sulfenylation, S-nitrosylation, and glutathionylation, etc) in the same protein need to be determined. Due to the instability and transient nature of s-sulfhydration moieties, the development of better detection technology and methodologies for protein *S*-sulfhydration is required for an improved understanding of its formation and wide biological implications.

1.5 Primary hepatocellular carcinoma (HCC)

Primary HCC is the most common form of primary liver cancer, making up 70-85% of all liver cancer diagnoses. HCC has the second highest mortality rate of all cancers in men, and the sixth highest in women, making it one of the most significant cancer forms worldwide (32). While HCC variants exist, all of them are associated with deregulated hepatocyte cell growth, and in some cases, metastasis (33). HCC etiology in developing countries is most commonly associated with viral hepatitis B/C infection, while in western countries has been linked to alcoholism and obesity (32).

The prognosis associated with HCC is quite poor and the overall survival rate of patients with HCC remains very low. The most successful treatment option available for individuals is complete liver transplantation, with a 5-year survival rate of 61%. Similarly, surgical removal of cancerous tissues has also shown to be an effective treatment strategy, with 5-year survival rates upwards of 50%. Despite their promise, these treatments are severely restricted by long wait periods, and the numerous co-morbidities associated with HCC complicate the procedures, including diabetes, kidney, and cardiovascular diseases (34). Other treatment forms include chemotherapeutic and radiotherapy, as well as selective blockage of blood supply to the tumor. These treatments are, again, limited in their usefulness based on the size and location of the tumor (35).

Chemotherapeutics are some of the most widely used treatment tools for most forms of cancer, including HCC. These chemicals are applied both systemically in a non tumor-specific manner, or locally targeted to the cancerous tissues. While the hepatic artery supplies the liver with 25% of total blood flow, the remainder coming from the portal vein, studies have shown that HCC tumors receive close to 90% of its blood supply from the hepatic artery (36). As such, treatments can be facilitated to supply chemotherapeutic agents directly to target HCC tissues through the hepatic artery, in a method known as hepatic artery-infusion chemotherapy (HAIC) (36, 37). Modern chemotherapeutics can be organized into the following classes as described in table 1.

Table 1. Classes of chemotherapeutic agents based on mode of activity (adapted from (38)**).**

Class	Example	Applications
Antimetabolites	5-fluorouracil	Hepatic artery infusion chemotherapy, HCC treatment
Biological Agents	Bevacizumab	Colorectal metastases associated with HCC
Multi-kinase Inhibitors	Sorafenib	Systemic treatment of HCC and renal cell carcinoma (RCC)
Platinum salts	Cisplatin	HCC and associated metastases treatment
Antineoplastic Antibiotics	Doxorubicin	HCC and associated metastases treatment

Antimetabolites such as 5-fluorouracil work to inhibit the production of essential biomolecules such as nucleotides. Biological agents instead use the patients own immune system to directly target tumors or modulate hormone levels necessary for tumor establishment and growth, and are specifically useful in the treatment of breast and prostate cancers. Agents such as sorafenib, which fall under the multi-kinase inhibitor class, target the signal transduction pathways associated with protein kinases. Sorafenib specifically blocks the protein kinase receptor for vascular endothelial growth factor (VEGF), which in turn prevents the production of new tumor vasculature, depriving the tumor of oxygen and inducing cell death. Platinum salts work through inducing DNA strand cross linkages which interrupts the DNA replication/ repair machinery, leading to apoptosis in the rapidly dividing cancer cells. One of the most well studied and widely used group of chemotherapeutics are the anti-neoplastic antibiotics. First discovered in the *Streptomyces* bacteria as a tool used to inhibit the growth of competitive bacteria, anti-neoplastic antibiotics have clinical usefulness as well (38). Of the antineoplastic antibiotics available, doxorubicin is one of the most well studied and widely used agents, with clinical applications across a wide range of cancers, including HCC (39).

1.6 Doxorubicin as a model chemotherapeutic agent

Doxorubicin, trade name as adriamycin, was first employed as an anticancer agent in 1970. Since that time, doxorubicin has become one of the most commonly used treatment tools for the management of cancers of the lung, breast, and liver to name a few (40–42). Doxorubicin carries out it's cytotoxic effects as outline in figure 3.

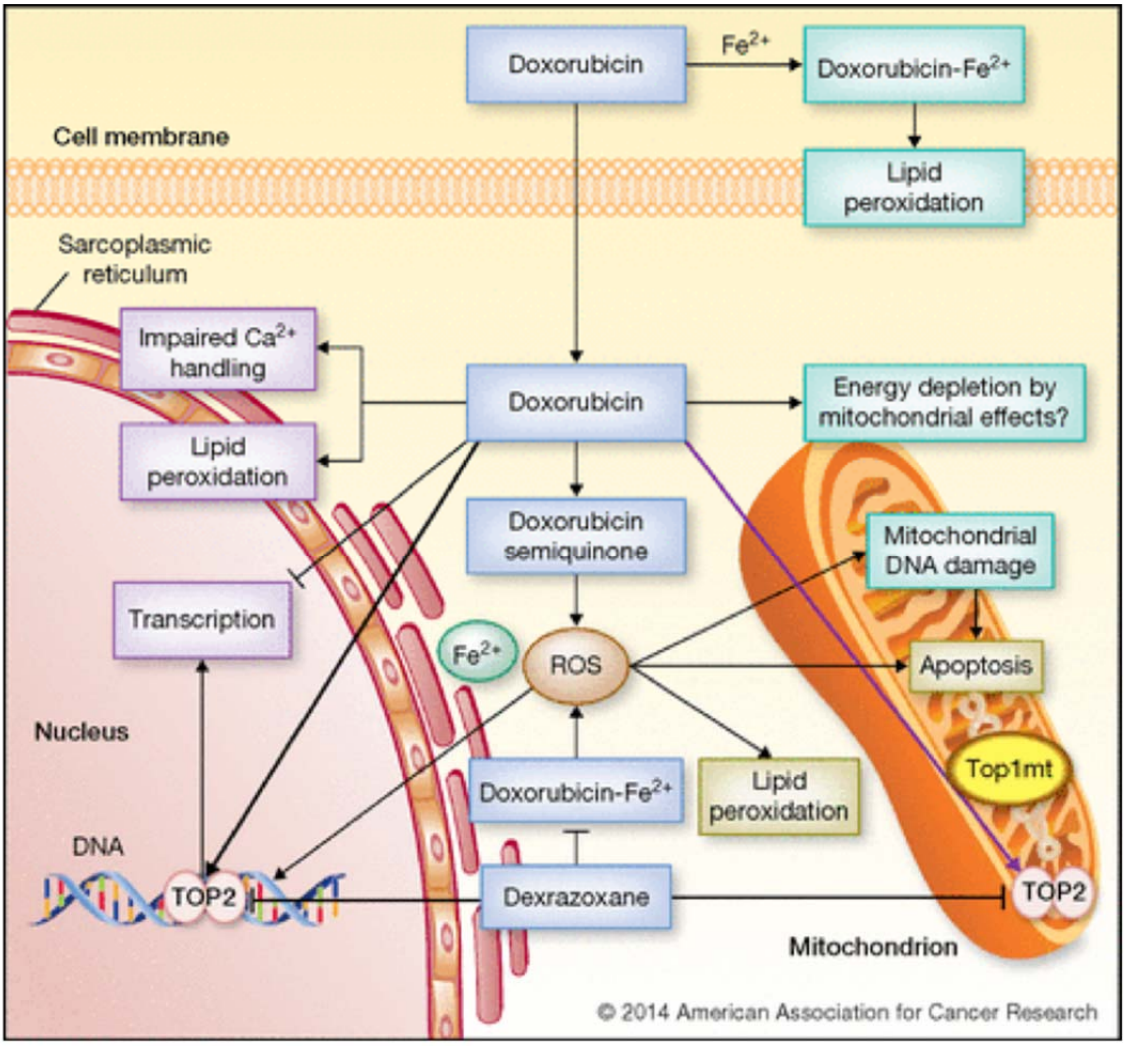

Figure 3. Cytotoxic signaling properties of doxorubicin (adapted from (43))

Doxorubicin is capable of directly inducing lipid peroxidation, thus damaging elements of the cell membrane and nucleus. While these effects have been shown to be especially damaging in cardiomyocytes, resulting in cardiotoxicity, it is not the primary mechanism in which doxorubicin induces cell death (44). The majority of doxorubicin's effects come from interruption of DNA replication machinery and generation of ROS. Once inside the cell, doxorubicin is capable of interacting with mitochondrial reductases to produce a doxorubicin semiquinone, which then reduces free oxygen to a superoxide anion. The superoxide anion is then capable of further reacting with DNA, lipids, and proteins, damaging their functionality and inducing cell death (45). Doxorubicin itself can directly enter the nucleus and intercalate with DNA, inducing strand breaks. It can then bind topoisomerase II during the DNA repair process, sequestering it and reducing topoisomerase availability, thus leading to extensive, irreparable DNA damage, and thus cell death (46). While these effects will occur in both the patients healthy and cancerous cells, the direct targeting of DNA replication and repair machinery makes doxorubicin's cytotoxicity more specific to cancer cells. While it's clear that the anti-cancer effects of doxorubicin are significant and varied, like the other treatment methods mentioned above, it suffers from several limitations as well. One of the most significant and commonly seen in the treatment of HCC, is a reduced anti-cancer response over extended treatment periods, a condition known as multi-drug resistance (MDR).

1.7 Multi-drug resistance (MDR)

Cellular resistance to normally toxic compounds was first studied in bacteria and by looking at the ability to adapt to certain antibiotic treatments (47). Building upon these findings, researchers discovered similar processes occurring in certain forms of human pathology, specifically in the treatment of cancers. Given the many difficulties associated with the curative

treatment of HCC, patients often only have chemotherapeutic related treatments available to them (48). These treatments can last several months, following a reduction in the responsiveness to the chemotherapeutic agents, a phenomenon termed as multi-drug resistance (MDR) (49).

MDR is a multifactorial issue, and is characterized by changes in the metabolism, intracellular targeting, and transport of the chemotherapeutic agents (50). To study the onset and regulation of these mechanisms, researchers study protein expression levels associated with the cellular management of chemotherapeutics. The majority of research on MDR focuses on drug efflux, specifically through a group of membrane bound proteins called the ATP-binding cassette (ABC) transport proteins. There are 48 ABC transport proteins, which are divided into 7 subgroups (A to G). These proteins are responsible for the transport of a wide variety of hydrophobic compounds such as metal ions, lipids, and sterol based metabolites, etc. (51). These ABC transporters facilitate unidirectional movement based on concentration gradients. The structure of most ABC transporters consists of 6 transmembrane α-helices, in which the transmembrane pore is only accessible from one side of the lipid bilayer (52). The transmembrane domains act as the site of intracellular substrate binding, followed by the binding of ATP to the nucleotide binding domains. ATP hydrolysis catalyzes change of the transporter from the inward to outward facing conformation, followed by extracellular substrate release and ADP dissociation to return the protein back to the inward facing conformation, allowing for another round of transport activity (53). Abnormalities in ABC transporter protein expression and function can lead to a host of different disorders. Polymorphisms of ABCA2, expressed in brain tissues, has been associated with the changes in brain cholesterol transport associated with the onset of Alzheimer's Disease (54), while ABCB4 mutations can result in a condition known as Progressive familial intrahepatic cholestasis (PFIC), characterized by bile accumulation in

hepatocytes (55). In cancerous cells, overexpression or increased activity levels of these proteins can result in decreased intracellular accumulation of chemotherapeutic agents, which has been implicated as one of the major causes MDR. The role of ABCB1/MDR1 in chemotherapeutic drug resistance has been extensively studied across several cell types ranging from myelogenous leukemia cells to breast cancer cells (56, 57). Other members of the ABC family have also been implicated in poor chemotherapeutic treatment prognosis. In human lung adenocarcinoma (A549) cells, ABCA1 down-regulation increased the cytotoxic activity of the chemotherapeutic agent nitidine (58). ABCG2/BCRP was shown to be highly expressed in a doxorubicin resistant human breast cancer (MCF-7/AdrVp) cell line, implicating it, and possibly other ABCG subgroup members, as major regulators of MDR. The regulation of these proteins is a tightly controlled process which includes a host of different transcription factors, some of which are shared between different members of the ABC group. Liver X receptors (LXRs) are a group of nuclear bound receptors that, through the binding of their ligands, promote the expression of target genes through the formation of a co-factor protein complex. The two subtypes of LXR's, alpha and beta, are believed to be activated by the same ligands, and differ only in their tissue distribution, with alpha expression occurring mostly in the liver, and beta expression distributed evenly throughout most tissues (59). LXR's are activated through the presence of sterol based metabolites and have been shown to regulate the expression of certain ABC proteins in order to control cholesterol transport. ABCA1 and ABCG8 are two ABC transport proteins that are induced by LXR alpha/beta to promote excess cholesterol efflux in gut lumen tissue (60). While the role of ABCA1 and ABCG8 in cholesterol homeostasis has been well described, their role in the efflux of chemotherapeutic agents has yet to be studied and could offer new insight into the development of MDR.

The ability for cancerous cells to detoxify chemotherapeutic agents has also been shown to have importance in the onset of MDR (61) . The glutathione-S-transferase (GST) group is a large family of enzymes that are capable of catalyzing the conjugation of glutathione to various xenobiotic substrates for the purpose of detoxification (62). Once conjugated to glutathione, the xenobiotic substrates are then transported through the cytoplasm and processed through the mercapturic pathway in the cytosol. Once the resultant mercapturic acids are formed, they are released into circulation and then escape into the urine (63). GST is capable catalyzing the conjugation of glutathione to several different chemotherapeutic agents, removing them from circulation and the intended site of treatment, which is a proposed mechanism for MDR (62). Elevated GST levels have been studied as a prognostic tool for MDR in human ovarian cancer, with one study identifying 90% of drug resistant tumors as having increased GST levels (64). In Mouse embryo fibroblast cells, GST overexpression resulted in a protective effect against doxorubicin, but not other cytotoxic agents such as cisplatin, highlighting the selective role of the GST family in chemotherapeutic metabolism (65). It's also important to recognize the ability for GST group members to regulate key cell survival pathways as well. One GST isoform, GSTP1-1, is capable of inhibiting the activity of c-Jun N-terminal kinase (JNK), thus disrupting the mitogen-activated protein kinase (MAPK) pathway. In doing so GSTP1-1 prevents cellular apoptosis, and improves tumor cell survival (66). It's possible that through both its enzymatic and regulatory signaling activity, the GST family provides a dual mechanism for initiating MDR.

As discussed, one of the most studied and well described mechanisms associated with MDR is changes in the transport of chemotherapeutics. If these agents are not readily transported into the cytoplasm, or they are quickly removed, their ability to carry out their cytotoxic effects are severely restricted. While drug efflux has been shown to have the most significant effect on

MDR development, researchers are now looking at the role chemotherapeutic drug efflux might play in MDR. Most chemotherapeutic agents are transported into the cell through a group of influx transport proteins called the solute carriers (SLC) transport proteins. These proteins function through facilitated diffusion as both co-transporter and counter transporter proteins to allow for the cellular uptake of not only chemotherapeutic drugs, but many other important metabolites (67). SLC transporter dysfunction has been associated with certain disease conditions including inflammatory bowel disease and hyperbilirubinaemia (68). One specific member, SLC22A1, has been associated with doxorubicin influx across tissue types (69). Despite this, our understanding of their regulatory role in MDR requires further investigation.

1.8 H_2S as a putative regulator of MDR

Given the high incidence rate and serious impact that MDR in HCC has on patient survival, the need to study new signaling mechanisms associated with the onset of MDR in these tissues is critical (14). The potential for MDR-related H_2S signaling can be highlighted by one study that found an increase in the response of ovarian cancer cells (A2780) to the chemotherapeutic agent cisplatin, through the silencing of the H_2S-generating gene CBS (24). These findings were shown to extend to in vivo mouse models of ovarian cancer, where CBS-directed siRNA coupled with cisplatin treatment had the most significant reduction in tumor size. In a similar manner, CSE and CBS inhibition increased the sensitivity of lung adenocarcinoma cells (A549) to another chemotherapeutic drug oxaliplatin (70). Other studies have noted increases in the levels of CSE and CBS in chemotherapeutic-resistance colon cancer cells (HCT116) compared to non-resistant cells (71). While these studies highlight the ability for H_2S to induce MDR, the sometimes conflicting nature of H_2S signaling presents the possibility for H_2S to alternatively restrict MDR development, however this required further investigation. The

exceptionally high levels of H_2S production shown in the liver suggests a key importance within these tissues. Given the high levels of liver H_2S production, as well as the ability for H_2S to regulate the response of cancer cells to various chemotherapeutics, the role of H_2S in the regulation of MDR in HCC requires further investigation. Building upon these studies, this project looks to determine the possible involvement of H_2S signaling in MDR in HCC and the underlying mechanisms. Through the use of human HCC (HepG2) cell lines and doxorubicin as a model chemotherapeutic agent, this study aims to investigate: 1), the interaction of doxorubicin and H_2S on HepG2 cell viability and clonogenic formation; 2), the effect of doxorubicin on endogenous H_2S signaling; 3), the regulation of ABC transporters, GST, and/or SLC in H_2S-altered MDR and the possible underlying mechanisms. In doing so, we hope to discover the potential role for H_2S as a clinically relevant tool for the management of chemotherapeutic drug resistance in liver cancer.

2. Methods

2.1. Cell culture

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured with Dulbecco's modified Eagle's medium (Sigma-Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells with 70-80% confluence were used for various treatments.

2.2. Establishment of a doxorubicin-resistant HepG2 cell line

A doxorubicin-resistant HepG2 cell line was established by continuous exposure to low dose doxorubicin for a period of over 2 months. HepG2 cells were treated with an initial dose of 50 nM doxorubicin for one month, followed by another month of 75 nM doxorubicin treatment. The medium was changed every 3 days. Doxorubicin-resistant HepG2 cells were determined based on morphological changes and a decreased sensitivity to doxorubicin treatment. Doxorubicin-resistant HepG2 cells were cultured in normal medium without doxorubicin for 1 week prior to various treatments.

2.3. Cell viability assay

The effect of doxorubicin and NaHS treatment on the cell viability was measured with MTT assay as previously described (72). Briefly, 20,000-40,000 cell/well were seeded into a 96 well plate in 200 μl of medium for 24 hours at 37^{o}C. Cells were then washed with 100 μl PBS. After that, 200 μl of medium containing either doxorubicin and/or NaHS were added for an additional 24-hour incubation. Following the treatment, cells were again washed with 100 μl PBS, and 100 μl of MTT (1 mg/ml) was added to each well. Following a 4-hour incubation at 37^{o}C, MTT was removed and 100 μl of DMSO was added for 10 minutes. Absorbance at 570 nM was read with a FLUOstar OPTIMA microplate spectrophotometer (BMG LABtech, Germany). Cell viability was expressed as a percentage of untreated control

2.4. Clonogenic assay

To determine the effect of doxorubicin and H_2S treatments on the colony forming abilities of HepG2 cells, a clonogenic assay was performed as previously described with some minor changes (73). HepG2 cells were first treated with/without doxorubicin (1 μM) in the presence or absence of NaHS (30 μM) for 24 hours. After that, the cells were washed with 2 mL PBS and trypsinized for cell counting. A total of 15,000 cells were plated into 35 mm dishes containing 2 mL regular medium. The cells were cultured at 37 ^{o}C for 2 weeks with medium changed every 3 days. Following fixation with acetic acid/methanol (1:7) and staining with crystal violet (0.5%), the pictures of the plates were taken and colony numbers were analyzed using Image J 1.43 software.

2.5. Western blotting

After different treatments, the cells were collected and lysed in the presence of protease inhibitors. The resultant extracts were centrifuged at 15,000 rpm for 15 minutes at 4^{o}C for protein separation. Equal amounts of proteins (60 μg) were mixed with loading buffer and placed at 95^{o}C for 5 minutes, followed by separation with a 10% SDS-PAGE gel and transfer onto to nitrocellulose membranes (Pall Corporation, Pensacola, FL). The membranes were probed with appropriate primary antibodies and detected using peroxidase-conjugated secondary antibodies (Sigma-Aldrich, 1:5000) and visualized by ECL (GE Healthcare, Amersham, UK) (55). The following antibodies were diluted as follows with 3% milk in PBST: CSE 1/1000 (Protech, Rosemont, IL), CBS 1/100 (Santa Cruz, Dallas, TX), 3MST 1/1000 (Abnova, Walnut, CA), ABCA1 1/1000 (Abcam Toronto, ON), ABCB1 1/100 (Santa Cruz, Dallas, TX), ABCG8 1/100

(Santa Cruz, Dallas, TX), LXRα 1/1000 (Abcam, Toronto, ON), LXRβ 1/1000 (Cell Signaling Tech., Danvers, MA), and β-actin 1/5000 (Sigma-Aldrich).□□

2.6. Real-time PCR

Total RNA from the cells was collected using TriReagent (Sigma-Aldrich) and then reverse-transcribed into cDNA using random hexamer primers according to manufacturer's protocol (Thermo Fisher Scientific, Ottawa, ON). Controls processed in the absence of reverse transcriptase were used to monitor contamination. The quantification of mRNA expression was performed with an iCycler iQ^5 apparatus (Bio-Rad, Mississauga, ON) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad). The sequences of primers were: *SLC22A16* (5'-CCTGGCGCCGTTCTCTGTGG-3' and 5'-CTGGGGGTAATCGTTCCGTTTTT-3'), *GST* (5'-AGGTTTTTGCCAATCCAGAAGAC-3' and 5'-GTAGATCCGTGCTCCGACAAATA-3'), and *GAPDH* (5'-GCGGGGCTCTCCAGAACATCAT-3' and 5'-CCAGCCCCAGCGTCAAAGGTG-3'). Relative mRNA quantification was calculated by using the arithmetic formula "$2^{-\Delta\Delta CT}$", where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference of GAPDH gene.

2.7. Measurement of H_2S production rate

H_2S production rate, reflecting CSE activity, was measured with a methyl blue method as commonly described (74). Briefly, 10% w/v mouse liver tissue homogenates were mixed with 100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxial 5'-phosphate, with or without 1 μM doxorubicin in the reaction flask. A center well containing 0.5 ml 1% zinc acetate

with a piece of filter paper (2 cm × 2.5 cm) was also put into the reaction flask. After being flushed with N_2, the flask was kept at 37^{o}C for 90 minutes. To stop the reaction, 0.5 ml of 50% trichloroacetic acid was added to the flasks. The flasks were then incubated at 37^{o}C for 60 minutes. After incubation, the contents of the center wells were then transferred to test tubes containing 3.5 ml H_2O, and then 0.5 ml of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 0.5 ml of 30 mM $FeCl_3$ in 1.2 M HCl were added into the test tubes for 20 minutes. The methylene blue generated from the reaction was quantified at 670 nm using a FLUOstar OPTIMA microplate spectrophotometer. The H_2S produced from each reaction was determined with a standard curve of NaHS and expressed in nmole/g/min.

2.8. H_2S direct interaction

The direct interaction of doxorubicin with H_2S was analyzed with a lead sulfur method to determine the ability for doxorubicin to sequester free H_2S in solution (75). Briefly, 100 μl of solution containing 30 μM NaHS with or without 1 μM doxorubicin was placed in a 96-well plate. Lead acetate paper was placed above the liquid phase contained in the 96-well plate with a cover. The reaction was incubated for 1 hour at 37^{o}C in the dark. The well only containing NaHS acted as control. The lead acetate paper was then scanned and analyzed with Image J software.

2.9. Doxorubicin efflux and influx assay

After HepG2 cells were treated with doxorubicin and/or NaHS for 24 hours, the cells were washed twice with PBS, and incubated with 2 mL PBS for additional 2 hours. Afterward, 1 mL sample of PBS was first taken from each plate for measurement of doxorubicin efflux. The cells were then collected for analysis of doxorubicin influx. The fluorescence of doxorubicin was read

at an excitation wavelength of 485 nm and an emission wavelength of 590 nm using a FLUOstar OPTIMA reader as previously described (76). The images of doxorubicin fluorescence were also taken using an inverted Olympus IX70 fluorescence microscope.

2.10. S-sulfhydration assay

LXRα *S*-sulfhydration was performed as described previously (26, 77, 78). Briefly, cells were sonicated in buffer containing 250 mM Hepes buffer (pH 7.7), 1 mM EDTA, 0.1 mM neocuproine and 100 μM deferoxamine following centrifuge at 13,000 × g for 30 min at 4°C. Cell lysates were added to blocking buffer (sonication buffer with 2.5% SDS and 20 mM MMTS) at 50°C for 20 min with frequent mixing using vortex. The proteins were precipitated with acetone at -20°C for 20 min, then the proteins were resuspended in blocking buffer (with 1% SDS) following addition of 4 mM biotin-HPDP. After incubation for 3 hours at 25°C, biotinylated proteins were precipitated by streptavidin-agarose beads, which were then washed with blocking buffer. The biotinylated proteins were eluted by SDS-PAGE gel and subjected to Western blotting analysis using anti- LXRα antibody.

2.11. Statistical Analysis

The data were presented as means ± SEM, representing at least 3 independent experiments. Statistical comparisons were made using two-tailed Student's *t*-tests or one-way ANOVA followed by a post-hoc analysis (Tukey test) where applicable. Values of $p<0.05$ were considered to be statistically significant.

3. Results

3.1 The effects of doxorubicin and H_2S treatment on HepG2 cell survival

First, the effect of doxorubicin on HepG2 cell viability was evaluated with an MTT assay. We observed that cell viability was decreased in a dose-dependent manner when the cells were exposed to increasing concentrations of doxorubicin for 24 hours (Fig. 4A). Doxorubicin at 1 μM decreased cell viability by 35.2%, while cell viability was only 28.2% in 10 μM doxorubicin-incubated cells in comparison with the control cells (100%). We next incubated HepG2 cells with NaHS, a well-known H_2S-releasing donor (79). The result demonstrated that cell viability was not affected by exogenously applied NaHS (1-100 μM) (Fig. 4B). To further study the interaction of doxorubicin and H_2S on cell viability, HepG2 cells were co-cultured with varying concentration of doxorubicin (0.1-10 μM) and NaHS (30 μM) for 24 hours. The growth inhibitory effects of doxorubicin/NaHS combination were significantly higher than those caused by doxorubicin alone (Fig. 4C). The cell viability in the treatment with both doxorubicin (1 μM) and NaHS (30 μM) was only 38.5% when compared with that (62.8%, $p<0.05$) in the treatment with doxorubicin (1 μM) alone. As shown in Fig. 4D, doxorubicin at 1 μM treatment significantly reduced HepG2 cell confluence, which was exaggerated by co-treatment with 30 μM NaHS over 24 hours.

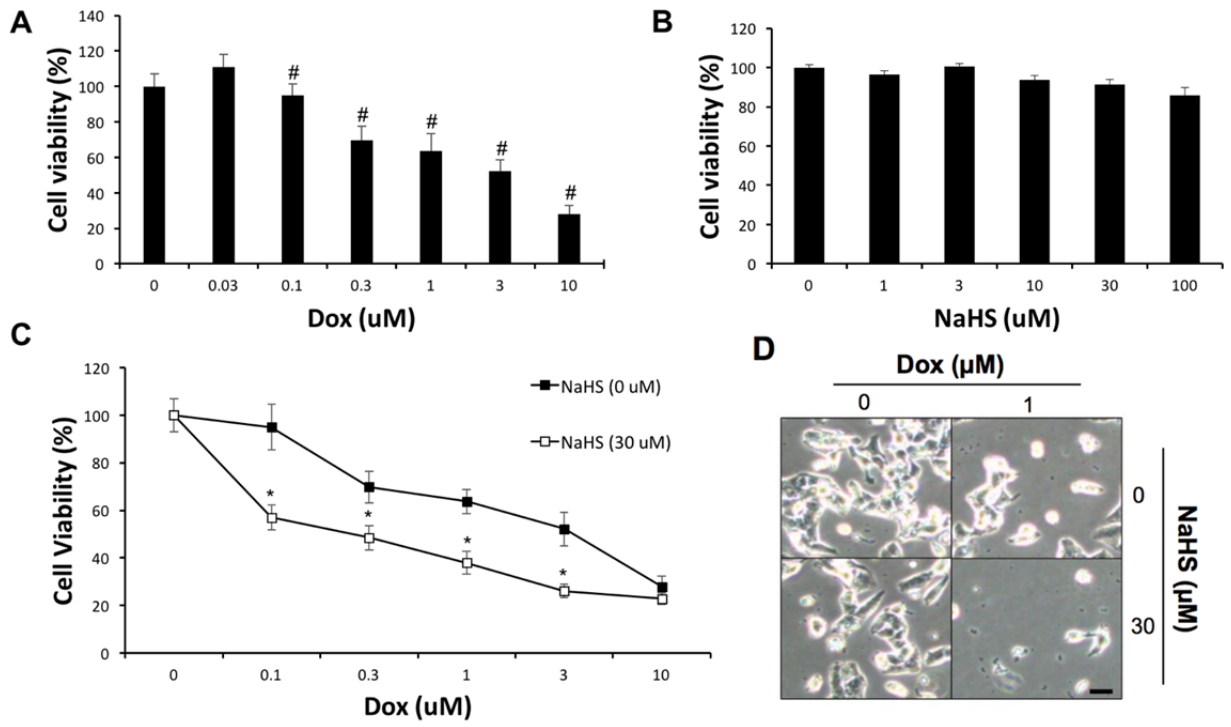

Figure 4. H_2S sensitizes HepG2 cells to doxorubicin cytotoxicity. (***A***) Doxorubicin dose-dependently decreased cell viability. HepG2 cells were incubated with doxorubicin at the indicated concentration (0.03-10 μM) for 24 hours. After that, cell viability was measured by MTT assay. #, $p<0.05$ versus control. (***B***) NaHS had no effect on cell viability. HepG2 cells were incubated with NaHS (1-100 μM) for 24 hours. After that, cell viability was measured by MTT assay. (***C***) NaHS enhanced the sensitivity of HepG2 cells to doxorubicin cytotoxicity. HepG2 cells were co-cultured with varying concentration of doxorubicin (0.1-10 μM) and NaHS (30 μM) for 24 hours. After that, cell viability was detected by MTT assay. *, $p<0.05$ versus the group with doxorubicin alone. (***D***) Morphology and confluence changes in HepG2 cells after treated with doxorubicin (1 μM) and/or NaHS (30 μM) for 24 hours. Scale bar: 20 μm. The data were from at least 3 independent experiments.

3.2 H_2S and doxorubicin-induced changes in the clonogenic formation of HepG2 cells

An important hallmark of cancerous cells is the ability to quickly form large colonies, ultimately resulting in a tumorous mass. A clonogenic assay was then performed to quantify the effects of H_2S and doxorubicin on the colony forming ability of HepG2 cells (73). HepG2 cells were treated 1 μM doxorubicin and/or 30 μM NaHS for 24 hours. The treated cells were then split to new plates at equal cell numbers and cultured for 2 weeks. The resultant colonies were visualized using crystal violet and quantified using image J software (Fig 5A). Co-treatment with both doxorubicin and H_2S significantly inhibited colony formation to a greater degree than doxorubicin treatment alone, while NaHS treatment did not have a statistically significant effect (Fig 5B).

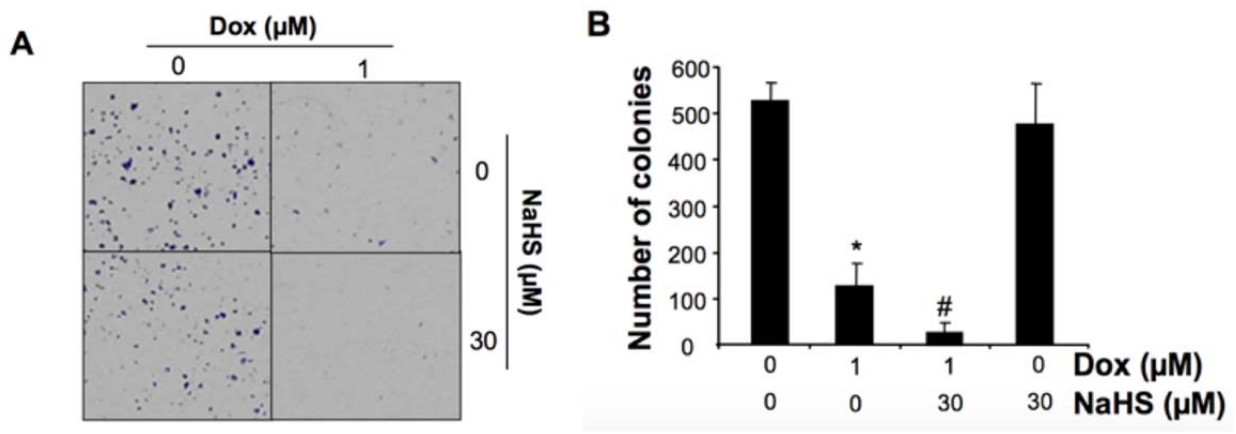

Figure 5. H_2S sensitizes HepG2 cells to colony disruption by doxorubicin treatment.

Following 24-hour treatments with 1 μM doxorubicin and/or 30 μM NaHS for 24 hours, the cells were split into fresh plates and re-cultured for 2 weeks. Colony formation was visualized using crystal violet staining (***A***) and number of colonies were quantified using image J software analysis (***B***). *, $p<0.05$ versus control; #, $p<0.05$ versus doxorubicin alone. The data were from 3 independent experiments.

3.3 The effects of doxorubicin on H_2S-generating enzymes in HepG2 cells

We next determined the effect of doxorubicin on the protein expressions of 3 H_2S-generating enzymes. As observed in Fig. 6A, CSE protein expression was significantly decreased when HepG2 cells were treated with 1 μM doxorubicin for 24 hours, while CBS expression was not affected by doxorubicin. The protein expression of 3MST was not detectable in HepG2 cells. CSE is a major H_2S-generating enzyme in liver tissues (14). We then measured the effect of doxorubicin on endogenous H_2S generation from mouse liver tissues with a methyl blue method. Fig. 6B showed that doxorubicin significantly decreased H_2S production by 80%, indicating that doxorubicin may also directly block CSE enzymatic activity. To further investigate the possibility of direct interaction between doxorubicin and H_2S, doxorubicin at 1 μM was mixed with 30 uM NaHS, which was then exposed to lead acetate paper at 37°C for 1 hour. As shown in Fig. 6C, it seems that there was no direct chemical reaction between doxorubicin and H_2S.

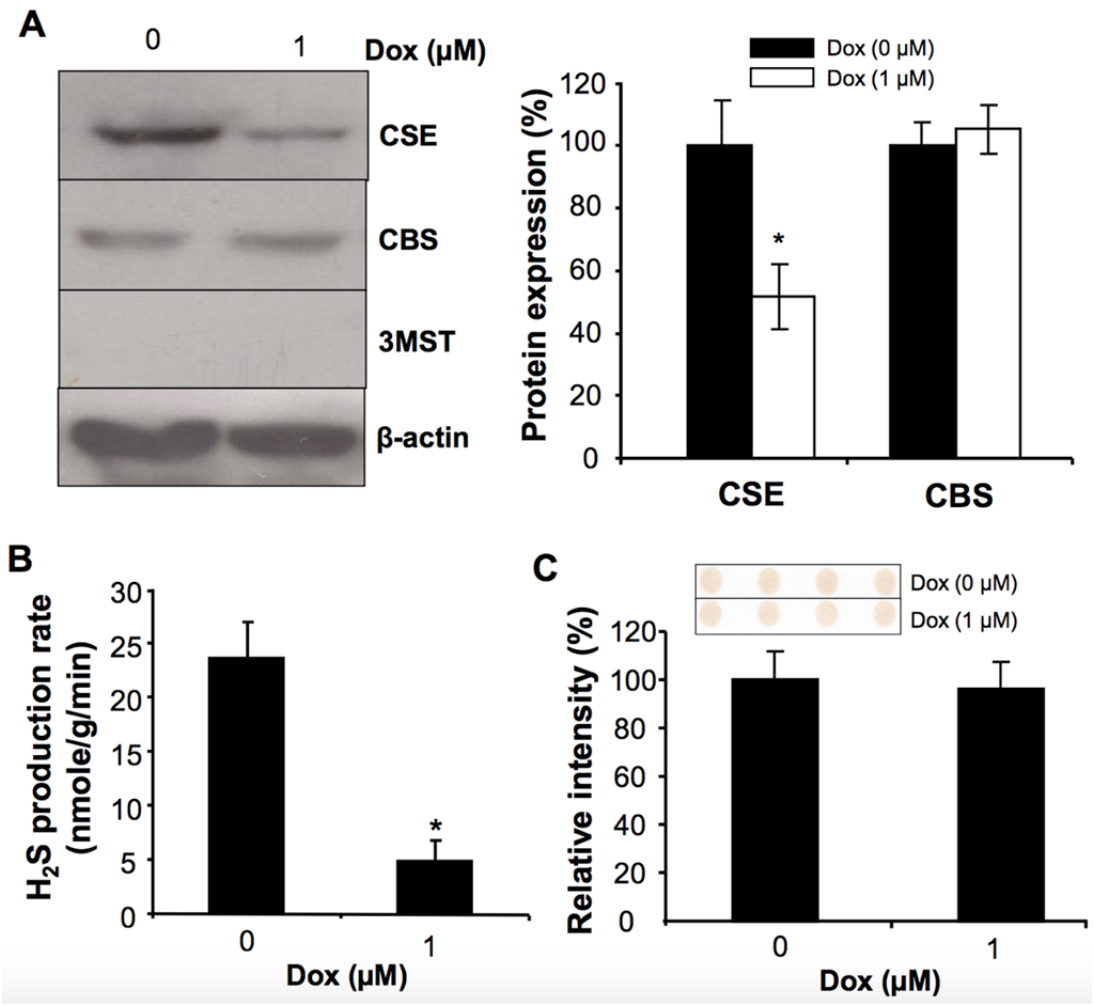

Figure 6. Doxorubicin attenuates H_2S production in HepG2 cells. (***A***) Doxorubicin inhibited the protein expression of CSE but not CBS. After HepG2 cells were treated with 1 μM doxorubicin for 24 hours, the protein expressions were detected by western blotting. *, $p<0.05$ versus control. (***B***) Doxorubicin reduced endogenous H_2S production. Mouse liver tissues were processed for H_2S measurement by methyl blue method in the presence of 1 μM doxorubicin. *, $p<0.05$. (***C***) Doxorubicin did not react with H_2S. Doxorubicin (1 μM) and NaHS (30 μM) were mixed together followed by detection of H_2S release by acetate lead paper at 37°C for 1 hour. *, $p<0.05$. The data were from at least four independent experiments.

3.4. H_2S modulates doxorubicin trafficking in HepG2 cells

To understand the mechanism underlying the synergistic effect of doxorubicin and H_2S combination treatment, the efflux and influx of doxorubicin in HepG2 cells were measured with a fluorescence plate reader. We found that exogenously applied H_2S inhibited the efflux of doxorubicin by 80% in comparison with doxorubicin treatment alone (Fig. 7A). As expected, intracellular doxorubicin concentration was increased significantly upon doxorubicin and NaHS combination treatment compared to that observed with doxorubicin alone (Fig. 7B). The differences of fluorescence intensity between doxorubicin alone and doxorubicin/H_2S co-treatment were further validated with a fluorescent microscopy (Fig 7C)

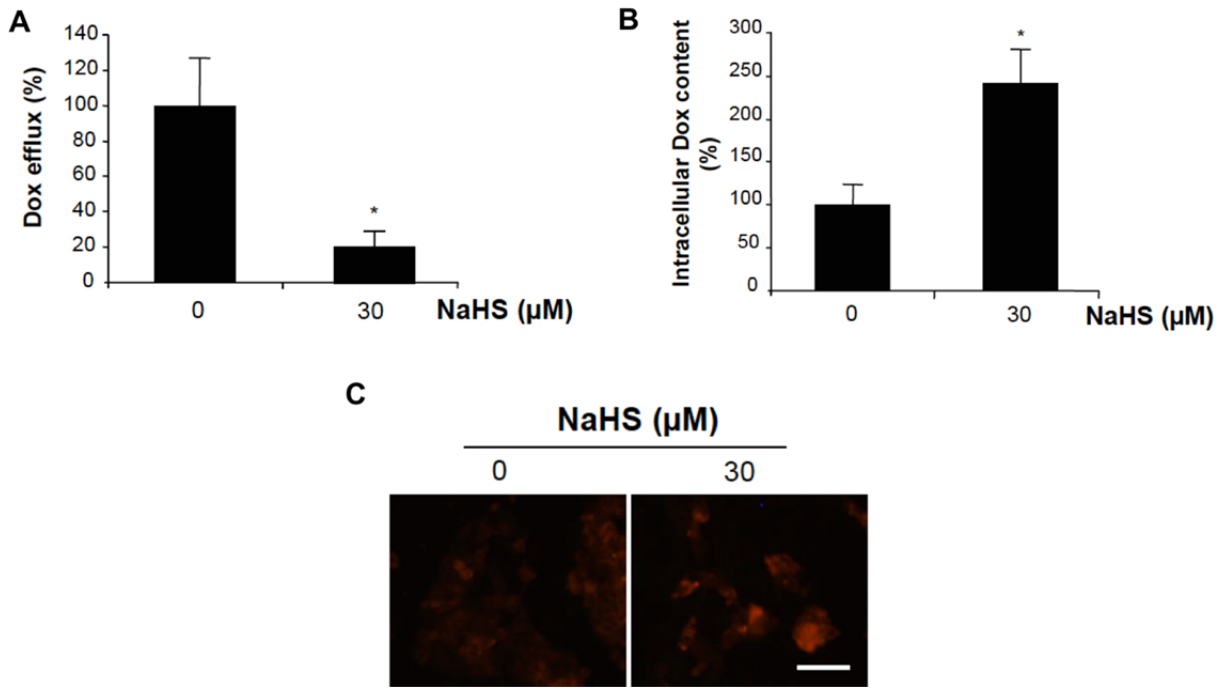

Figure 7. H_2S promotes cellular retention of doxorubicin in HepG2 cells. H_2S inhibited efflux of doxorubicin (***A***) but enhanced cellular retention of doxorubicin (***B***). Twenty-four hours after HepG2 cells were treated with doxorubicin and/or NaHS, the cells were washed and incubated with 2 mL PBS for additional 2 hours. Afterward, 1 mL sample of PBS was first taken for measurement of doxorubicin efflux (***A***). The cells were then collected for analysis of doxorubicin influx (***B***). Fluorescence intensity was normalized to equal amount of proteins loaded, and the intensity in doxorubicin treated group was expressed as 100%. *, $p<0.05$. The images of cellular doxorubicin internalization were captured using fluorescence inverted microscopy (***C***). Scale bar: 20 μm. The data were from at least three independent experiments.

3.5 Effects of doxorubicin and H_2S on the expression of ABC transport proteins in HepG2 cells.

ABC transport proteins are responsible for the efflux of doxorubicin in many cell types (59). We next assessed the effect of both doxorubicin and H_2S on the protein expression of several ABC transporters, including ABCA1, ABCB1 and ABCG8, which have been demonstrated to be highly expressed in HCC. In comparison with the control cells, incubation of HepG2 cells with 1 μM doxorubicin for 24 hours increased the protein expressions of ABCA1 and ABCG8 by 2.1 fold and 1.8 fold respectively (Fig. 8). The stimulatory effect of doxorubicin on ABCA1 and ABCG8 expressions was markedly reversed by exogenously applied H_2S. In contrast, the protein expression of ABCB1 was not affected by either doxorubicin or H_2S. These results suggest that the inhibition of ABCA1 and ABCG8 by H_2S resulting in the increased accumulation of intracellular doxorubicin, is a potential mechanism underlying the synergistic effects of doxorubicin and H_2S on cancer cell death.

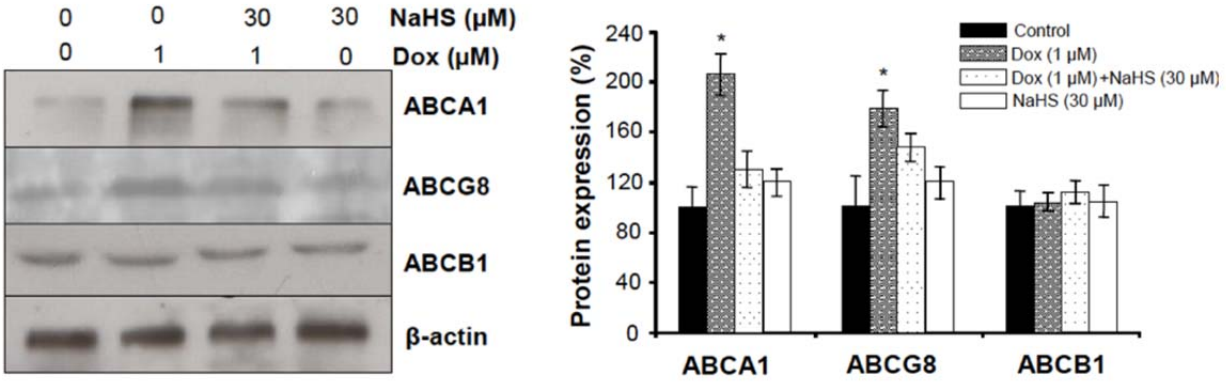

Figure 8. H_2S reverses doxorubicin-induced expression of ABCA1 and ABCG8 but not ABCB1. HepG2 cells were treated with doxorubicin alone (1 μM) and/or NaHS (30 μM) for 24 hours. The protein expressions of ABCA1, ABCG8, and ABCB1 were analyzed with western blotting. *, $p<0.05$ versus all other groups. The data were from four independent experiments.

3.6 The effect of doxorubicin and H_2S on LXRα expression and S-sulfhydration

We further investigated the signalling pathways associated with altered expression of ABCA1 and ABCG8 by doxorubicin and H_2S. The LXR group is a family of transcriptional regulatory proteins that have been associated with the regulation of ABC gene expression (60). Western blotting was further conducted to analyze the expressions of both LXRα and LXRβ in HepG2 cells. No change in the expression of LXRα was seen with doxorubicin and/or H_2S treatment (Fig. 9), while LXRβ was not detectable in this cell type. We then asked whether H_2S can post-translationally modify LXRα via *S*-sulfhydration leading to altered trans-activation activity. With a biotin switch assay, we did not detect LXRα *S*-sulfhydration after the cells were treated with doxorubicin (1 μM) and/or H_2S (30 μM) for 24 hours (data not shown). These data exclude the possibility of LXRα/β in mediating the altered expressions of ABCA1 and ABCG8 by doxorubicin and H_2S.

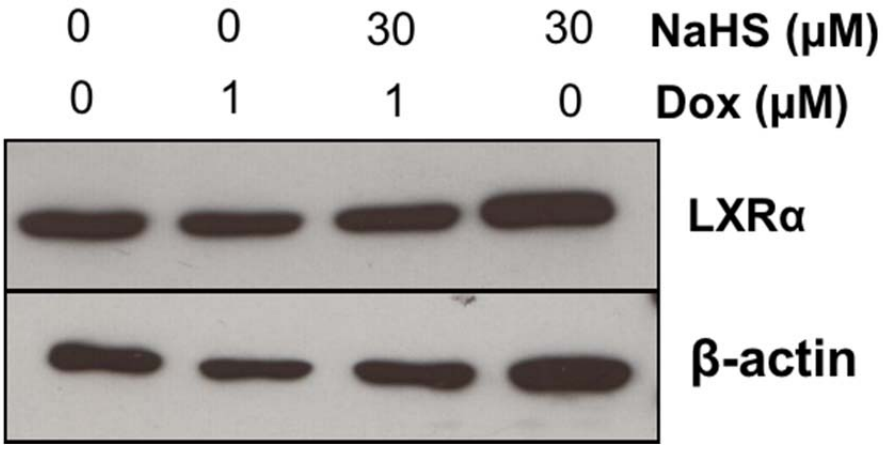

Figure 9. LXRα is not altered by doxorubicin and/or H_2S. HepG2 cells were treated for 24 hours with doxorubicin (1 μM) and/or NaHS (30 μM). The protein expressions of LXRα and LXRβ were then analyzed by western blotting. The experiments were repeated for 3 times.

3.7 Doxorubicin and H_2S regulate GST and SLC22A1 expression in HepG2 cells

To further explore other mechanisms for the increased retention of doxorubicin by H_2S, the mRNA expression level of GST and SLC22A1 were determined by real-time PCR. GST is capable of detoxifying doxorubicin through catalyzing it's conjugation with glutathione (62), while SLC22A1 has been proposed as a potential doxorubicin influx transport protein (67). Doxorubicin slightly induced the mRNA expression of GST, but it did not reach significance. In addition, NaHS alone or doxorubicin and NaHS combination significantly induced GST mRNA expression (Fig. 10). More interestingly, doxorubicin and NaHS combination treatment stimulated the mRNA expression of SLC22A1 by 160% when compared with the control cells. Either doxorubicin or NaHS alone had no significant effect on SLC22A1 expression.

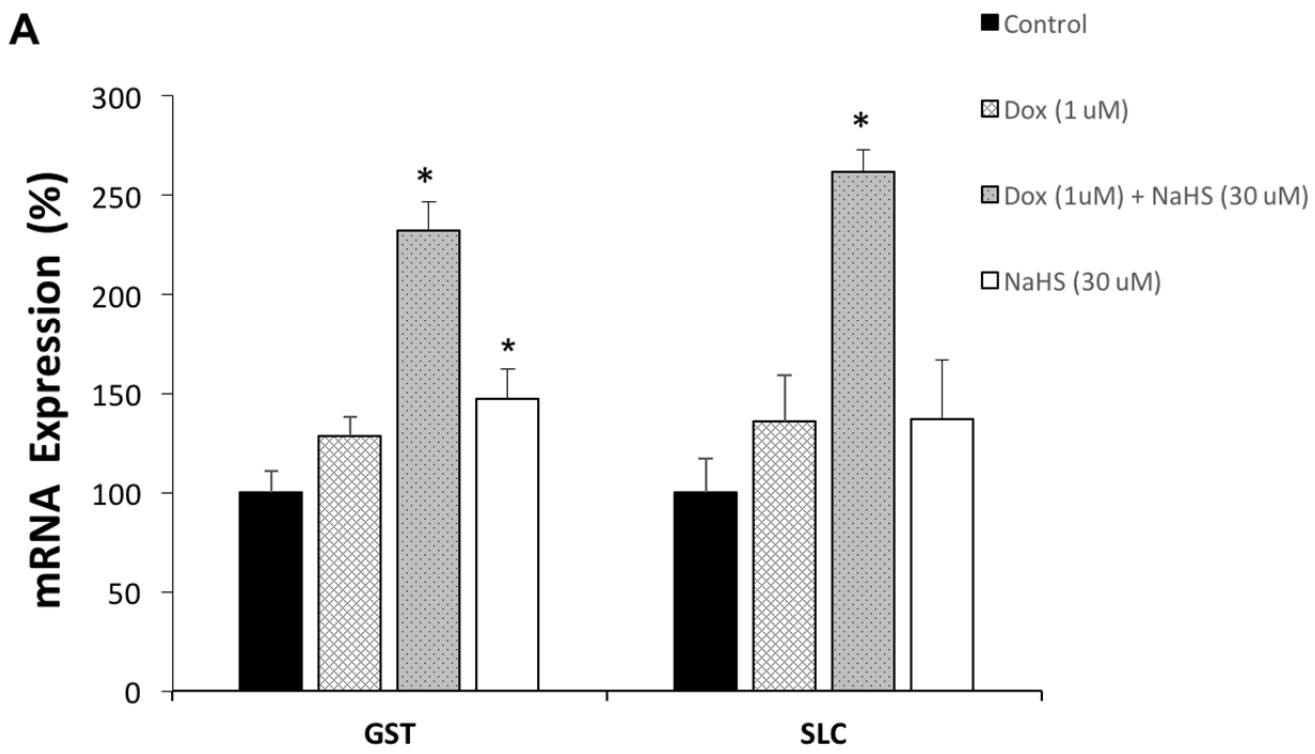

Figure 10. Effect of doxorubicin and H_2S on the mRNA expression of GST and SLC22A1.

HepG2 cells were treated for 24 hours with doxorubicin (1 μM) and/or NaHS (30 μM). The cells were then collected for real-time PCR analysis of GST and SLC22A1 mRNA expressions. *, $p<0.05$ versus control. The data were from four independent experiments.

3.8. Characterization of doxorubicin drug resistance in HepG2 cells

To further elucidate the functional significance of H_2S in drug resistance in HCC, we established an *in vitro* model of doxorubicin-resistant HepG2 cells (D.R. HepG2) by long term treatment of HepG2 cells with low dose of doxorubicin (80). In comparison with the parental (W.T.) HepG2 cells, D.R.-HepG2 cells were flatter with an enlarged cytoplasm (Fig. 11A). We further observed that CSE expression was quite lower in D.R. HepG2 in comparison with the W.T. HepG2 cells (Fig. 11B). Next, we compared the sensitivity of both cell types to doxorubicin and H_2S. After seeding equal numbers of both cell types in new plates, D.R. HepG2 grew faster (122%) than the parental W.T. HepG2 cells (100%) in the normal culture medium. Doxorubicin at 1 μM decreased HepG2 cell viability by 61.5% but only 12.3% in D.R. HepG2 cells, suggesting that D.R. HepG2 cells are resistant to doxorubicin-arrested cell growth. NaHS at 30 μM had no effect on the cell viability of parental W.T. HepG2 cells but significantly decreased the viability of D.R. HepG2 cells. The addition of NaHS restored the sensitivity of D.R. HepG2 cells to doxorubicin-induced cell growth inhibition (Fig. 11C).

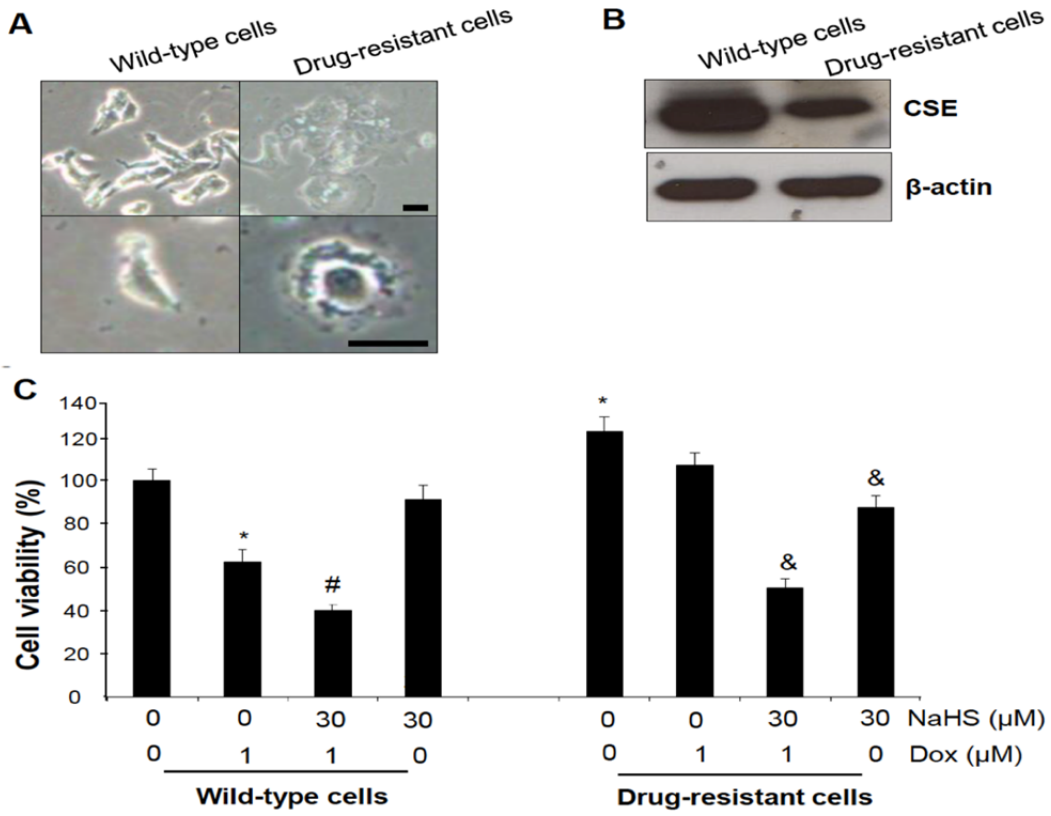

Figure 11. H_2S sensitizes drug resistant D.R. HepG2 cells to doxorubicin treatment. (A) D.R. HepG2 cells were flatter with an enlarged cytoplasm. D.R. HepG2 cells were obtained by continuous exposure to low dose doxorubicin (50-75 nM) for a period of over 2 months. (B) CSE expression was lower in D.R. HepG2 cells. Both parental W.T. HepG2 cells and W.T. HepG2 cells were cultured in normal medium without doxorubicin for 1 week prior to detection of CSE expression by western blotting. (C) H_2S sensitized D.R. HepG2 cells to doxorubicin treatment. Equal number of parental W.T. HepG2 cells and D.R. HepG2 cells were seeded in 96-well plate for 1 days followed by treatment with doxorubicin (1 μM) and/or NaHS (30 μM) for 24 hours. After that, cell viability was analyzed by MTT assay. *, $p<0.05$ versus parental W.T. control; #, $p<0.05$ versus parental W.T. cells with doxorubicin treatment; &, $p<0.05$ versus D.R. HepG2 cells with no treatment or doxorubicin treatment. The experiments were repeated for at least 3 times.

4. Discussion

One of the major limitations in the chemotherapeutic treatment of cancer is the development of MDR. In HCC, MDR is characterized by changes in drug transport, metabolism, and its ability to alter intracellular targets. Given the unique and critical role of H_2S in various cellular functions, the ability of H_2S to regulate the development of MDR in HCC deserves further investigation (81).

The chemotherapeutic agent doxorubicin has been well described as having strong anti-proliferative effects in cancer lines including HepG2. Doxorubicin induces cell death through the direct intercalation with DNA and disruption of topoisomerase II, as well as through the production of ROS. ROS themselves can induce oxidative damage to cellular membranes, DNA, and proteins, due to the reactivity of the oxygen free radicals (82). Our studies here validated that doxorubicin reduces HepG2 cell viability in a dose-dependent manner, and H_2S supplementation (30 μM) sensitizes the cells to doxorubicin cytotoxicity, while H_2S treatment alone has no effect on cell viability. These results were further confirmed by the studies with colony formation. Consistent with the cell viability data, exogenously applied H_2S strengthens the ability of doxorubicin to disrupt HepG2 colony formation, while H_2S alone has no effect on colony formation. A 30 μM NaHS treatment was selected because, upon dissociation, this chemical would supply an H_2S dosage in the low μM range, most closely reflecting endogenous H_2S production levels (14). Also, the rapid release of H_2S from NaHS treatments allowed for effective co-treatment timing with doxorubicin (79).

To further investigate the mechanism associated with these changes in doxorubicin cytotoxicity, the expression of a group of membrane-bound efflux transport proteins known as the ABC transport proteins was studied. Of the members of the ABC group, ABCB1 is the most well described in MDR development for its ability to facilitate the efflux of chemotherapeutic agents including doxorubicin, and thus reduce their cytotoxicity (83). ABCA1 and ABCG8 were also selected for analysis because of their high levels of expression in the liver, their role in hepatic efflux transport, as well as the lack of research investigating their role in MDR development (51, 84). Current understanding on the role of ABCA1 focuses on it's importance as a cholesterol efflux transporter expressed highly in the liver (84), however some evidence has shown an ability for it to regulate chemotherapeutic cytotoxicity in lung cancer cells (58). Likewise, there has been little insight on the role of ABCG8 in MDR development, however ABCG2/BCRP, a member of the same sub-group, has been well described as an MDR protein in breast cancer cells (57). We have shown that in doxorubicin (1 μM) treated HepG2 cells, the expression of both ABCA1 and ABCG8 is increased, and that H_2S supplementation (30 μM) returns the expression level of these proteins back to a control state, while the expression of ABCB1 was shown to not be affected by any of the treatments. H_2S treatment (30 μM) alone did not affect the expression of any of the proteins. Overexpression of ABC transport proteins is one of the most well described mechanisms for the onset of MDR, as the increased number of efflux transporters reduces the ability for chemotherapeutics to accumulate in the cancerous cells and carry out their cytotoxic functions (85). The ability for H_2S treatments to restrict the expression of these proteins could explain the increase in doxorubicin/H_2S co-treatment cytotoxicity seen in the cell viability studies. To determine if doxorubicin/H_2S-induced changes in ABCA1 and ABCG8 levels correspond to changes in doxorubicin transport, a doxorubicin fluorescence assay

was performed in HepG2 cells. Using doxorubicin's intrinsic fluorescent qualities, the intra/extracellular localization of doxorubicin could be tracked (76) . These results show that co-treatment of doxorubicin (1 μM) with H_2S (30 μM) increases the intracellular fluorescence, and therefore doxorubicin content, of HepG2 cells. Some studies have suggested that intracellular doxorubicin fluorescence may not be reflective of intracellular toxicity, as inactivated doxorubicin can still fluoresce (86). However, the doxorubicin/H_2S cell viability studies can confirm that an H_2S-induced increase in doxorubicin accumulation causes increased cytotoxicity and thus HepG2 cell death. Our findings therefore suggest an expanded role for ABCA1 and ABCG8 in HCC cells, as efflux transporters of chemotherapeutic agents, specifically doxorubicin. The importance of ABCB1 in MDR has been well established in several different cancer lines including HepG2, however, our results suggested otherwise (87, 88). It is important to note that previous studies have conducted their experiments with established doxorubicin resistant cell lines, without studying the effects of initial doxorubicin treatment in wild-type cells. As such, their results may reflect the exaggerated changes in ABC transport protein expression seen in already resistant cell lines. Our findings may instead reflect more sensitive changes seen in the initial establishment of doxorubicin resistance in wild-type cells, offering novel insight into the importance of other ABC transporters previously not studied.

The Liver X Receptor (LXR) group is a family of nuclear receptors that are important regulators of cholesterol metabolism. The two subgroups, LXRα and LXRβ, differ only in tissue distribution, with LXRα predominately in the liver, and LXRβ found in most tissues. The importance of both LXR groups in the regulation of cholesterol metabolism makes them key regulators of certain ABC-transport protein expression (89). In one study, it was shown that the

hepatic expression of ABCG8 and ABCA1 is greatly reduced by the inhibition of LXRα and β (60). In a separate study, the use of LXR agonists had no effect on the regulation of ABCB1 in HepG2 cells (90). Taken together, these findings suggest that H_2S is capable of regulating specifically ABCA1 and ABCG8, but not ABCB1, through the targeting of LXR's in HepG2 cells. In order to study this, HepG2 cells were treated with doxorubicin (1 μM) with or without NaHS (30 μM) and NaHS (30 μM alone). We found no effect of any treatment group on the expression of LXRα, while LXRβ was not expressed at measurable levels. Given these results, it is unlikely that H_2S modulates the expression of LXRα to exert its regulatory effects over ABCA1 and ABCG8. We next wanted to investigate the ability of H_2S to instead regulate the activity of LXRα through protein S-sulfhydration. Through the addition of sulfur groups to the cysteine residues of target proteins, H_2S is capable of inducing changes in the structure and thus the function of proteins (5). Again, it was shown that H_2S treatment had no measurable effect on the level of LXRα sulfhydration, allowing us to conclude that the LXR group is unlikely the intermediate signaling complex through which H_2S exerts its effects on ABCA1 and ABCG8. Our future studies will look to investigate other possible regulatory elements through which H_2S regulates ABC protein expression. One possible candidate for this role is the retinoid X receptor (RXR) group, a family of nuclear receptors expressed highly in the liver and associated with regulation of cholesterol metabolism through the formation of heterodimers with LXRα/β (91). Studies have shown the ability for RXR activation to induce the expression of ABCA and ABCG subgroup members, pointing to the possibility of RXR as the intermediate signaling complex between H_2S and ABCA1/G8 (92).

While efflux transport is the best described mechanism associated with MDR, the pathways associated with influx transport and metabolism may offer new research avenues for the prevention of this condition in HCC. The role of the SLC transporter group of proteins is just now becoming recognized for its potential role in the influx transport of various chemotherapeutics, including doxorubicin (93). The SLC group is responsible for regulating metabolite and ion influx but has also shown the ability to regulate chemotherapeutic influx as well, specifically in the SLC22 subfamily. SLC22A1-transfected colon cancer cells showed a significant increase in the accumulation of oxaliplatin, a derivative of cisplatin, improving the cytotoxicity of the drug. Further studies showed differential mRNA expression of SLC22A1in various colon cancer tumors, suggesting tissue specific variations in the degree of chemotherapeutic effectiveness (94). In order to describe the role of SLC22A1 in the regulation of doxorubicin management in HepG2 cells, we treated the cells with doxorubicin (1 μM) with or without NaHS (30 μM) and NaHS (30 μM alone) (Fig 10). The findings showed that doxorubicin treatment alone had no effect on the levels of SLC22A1 mRNA in Hep-G2 cells, and thus is unlikely to be associated with the onset of MDR in this cell line. Co-treatment with both doxorubicin and H_2S showed an increase in SLC22A1 mRNA expression, however H_2S treatment alone had no significant effect. Both doxorubicin alone and H_2S alone treatments showed an increasing trend, and despite not being enough to qualify for statistical significance, could describe the synergistic effect that combined treatment had. As such, it is plausible that both doxorubicin and H_2S treatment function to improve doxorubicin intracellular accumulation, however in a pathway distinct from that regulating ABC transport proteins. The likelihood for distinct regulatory mechanisms controlling both chemotherapeutic influx and efflux offers both daunting and exciting research possibilities.

The metabolism of chemotherapeutic agents is another mechanism in which drug resistance can be established. The GST family is a large group of enzymes capable of processing toxic chemical agents for removal from the body. Elevated levels of GST across various cell lines has been associated with the development of MDR, and thus may be associated with similar regulation in HepG2 cells (95, 96). In the present study, doxorubicin treatment alone had no effect on the levels of GST expression, however we saw an increasing trend, suggesting that longer, or higher dose treatments perhaps could exaggerate these effects. Combined treatment of doxorubicin and NaHS showed a significant increase in GST mRNA, indicating a synergistic effect similar to that seen in the regulation of SLC22A1. H_2S alone also significantly increased GST expression in these cells. These findings taken alone may indicate that H_2S actually perpetuates MDR through the up regulation of GST, but given our cell viability findings and the cellular level effects of H_2S on doxorubicin toxicity, this is unlikely to be the case. While studies have shown that H_2S up regulates GST levels in various cell lines (97, 98), our cell viability and clonogenic studies suggest that the sum of the effects of H_2S on doxorubicin cytotoxicity in HepG2 cells is an increased sensitivity to the drug.

We also tested the effect of doxorubicin on endogenous H_2S signaling in HepG2 cells. The protein levels of all 3 of the H_2S-generatng enzymes, including CSE, CBS and 3-MST, were compared following doxorubicin treatment (1 μM). Our findings demonstrated that only CSE but not CBS was down-regulated by doxorubicin. The protein expression of 3-MST was not detected. It has been observed that CSE is highly expressed in liver tissues, and knockout of CSE diminishes most H_2S production in mouse liver tissues, supporting our findings (14). We also

measured the effect of doxorubicin on H_2S production rate in mouse liver tissues and found that doxorubicin significantly reduced the production rate of H_2S in these tissues. These same effects were also reported in rat cardiac (H9C2) cells (99) following 24 hour doxorubicin (5 μM) treatment. Together, these findings strongly suggest that doxorubicin is capable of inhibiting CSE expression, and therefore endogenous H_2S production in HepG2 cells, possibly through its strong DNA intercalation properties, however this requires further investigation (100). When considered with the data of cell viability, doxorubicin transport, and ABC protein expression data, it seems that this loss of H_2S production ultimately leads to pro-MDR related signaling events in HepG2 cells.

We have also established a doxorubicin resistant (75 nM) HepG2 cell line in order to study changes in H_2S signaling and doxorubicin sensitivity, and to compare these characteristics with wild-type HepG2 cells. Morphological features of the D.R. cell line included an irregular shape and enlarged cytoplasm, characteristics similar to those of doxorubicin resistant breast cancer cells (101). Some studies have focused on the importance of epithelial-mesenchymal transition (EMT) in the morphological changes associated with MDR cells. EMT is a process in which cancerous cells undergo cytoskeletal rearrangement to improve their migratory ability (102). In various cancer cell lines, increased activity of the EMT pathway has been associated with not only metastasis, but also the onset of MDR. Resistant cells often exhibit EMT phenotype changes including increases in cell size and irregular cell structure (103). The role of EMT in HepG2 MDR development has yet to be studied but could present an important avenue of future research. Upon establishment of a D.R. HepG2 cell line, experiments were performed to determine changes in doxorubicin sensitivity and H_2S production between W.T. and D.R. cell

lines. Non-treated D.R. HepG2 cells exhibited a significant decrease in CSE expression compared to W.T. cells, similar to that seen in doxorubicin treated W.T. cells. The ability for D.R. alone to induce CSE down-regulation without direct doxorubicin treatment suggests that it may not be the doxorubicin itself effecting CSE expression, but instead doing so through an indirect signaling mechanism, however this requires further investigation. Decreased H_2S producing enzyme levels in D.R. but not W.T. cells, once again implicates the importance of endogenous H_2S in regulating doxorubicin transport. In order to study the effects of D.R. resistance on doxorubicin/H_2S induced cell death, cell viability analysis was performed on both W.T. and D.R. Hep-G2 cells. The statistically significant decrease seen in the viability of doxorubicin treated W.T. cells, but not in the doxorubicin D.R. cells, confirmed doxorubicin treatment was no longer an effective inhibitor of cell growth, and thus further confirmed this group as a doxorubicin resistant cell line. When exogenous H_2S was combined with doxorubicin treatment, both the W.T. and D.R. groups saw a very similar decrease in cell viability, greater than with doxorubicin alone. This further confirmed that H_2S is capable of sensitizing HepG2 cells to doxorubicin treatment, and in D.R. cells, actually returns their sensitivity back to a WT state. Applied in a clinical context, H_2S therefore could be used in conjugation with doxorubicin to not only improve initial doxorubicin treatment, but also to restrict the onset of MDR in HCC tissues. Recent studies have investigated doxorubicin/H_2S co-treatment in prostate and bone cancers and have even synthesized H_2S-releasing doxorubicin compounds. These compounds have shown an increased ability both in-vitro and in-vivo to reduce tumor size and induce cellular apoptosis through supplementation of doxorubicin with simultaneous H_2S release (104, 105).

5. Conclusions and future studies

These findings have shown that doxorubicin treatment is capable of decreasing CSE expression in HepG2 cells, and that exogenous H_2S is capable of sensitizing HepG2 cells to doxorubicin treatment, possibly by suppressing doxorubicin transporters, ABCA1 and ABCG8. These effects are not mediated by the interaction between H_2S and LXR family members, but perhaps through other related signaling complexes such as the RXR group, since RXR's regulate the transcription of ABC transporters by interaction with LXR. In addition, we discovered that H_2S is capable of returning the sensitivity of doxorubicin resistant Hep-G2 cells to a non-resistant level. The ability for H_2S to sensitize HepG2 cells to doxorubicin treatment offers the possibility for improving the management of HCC through the use of chemotherapeutic agents. H_2S supplementation with doxorubicin may improve both the initial response of HCC treatment, as well as offer the ability to return D.R. HCC cells to a non-resistant state. The discovery of ABCA1 and ABCG8 as important transporters of doxorubicin in HepG2 cells, and the ability for H_2S to regulate their expression improves not only our understanding of the mechanisms associated with MDR development, but also the role of H_2S in liver physiology as whole. We hope that these novel regulatory functions of H_2S in the liver will be the basis for improving current HCC treatment methods, and will contribute to the development of H_2S as a clinically relevant tool. Building upon these findings, future studies will look to determine; A) how doxorubicin alters CSE expression in both W.T. and D.R. HepG2 cells; B) the mechanism in which H_2S affects ABCA1 and ABCG8 expression if not through LXRα/β; C) exploring the role of doxorubicin/H_2S on tumor growth in-vivo, through the use of xenograft animal models with W.T. vs D.R. induced HCC tumors; D) synthesizing novel H_2S-releasing doxorubicin

compounds capable of effectively targeting HCC tissues to improve current HCC treatment tools and strategies.

6. References

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