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Pharmacological induction of mesenchymal-epithelial transition via inhibition of H2S biosynthesis and consequent suppression of ACLY activity in colon cancer cells

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ABSTRACT

Hydrogen sulfide (H_2S) is an important endogenous gaseous transmitter mediator, which regulates a variety of cellular functions in autocrine and paracrine manner. The enzymes responsible for the biological generation of H_2S include cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST). Increased expression of these enzymes and overproduction of H_2S has been implicated in essential processes of various cancer cells, including the stimulation of metabolism, maintenance of cell proliferation and cytoprotection. Cancer cell identity is characterized by so-called "transition states". The progression from normal (epithelial) to transformed (mesenchymal) state is termed epithelial-to-mesenchymal transition (EMT) whereby epithelial cells lose their cell-to-cell adhesion capacity and gain mesenchymal characteristics. The transition process can also proceed in the opposite direction, and this process is termed mesenchymal-to-epithelial transition (MET). The current project was designed to determine whether inhibition of endogenous H_2S production in colon cancer cells affects the EMT/MET balance *in vitro*. Inhibition of H_2S biosynthesis in HCT116 human colon cancer cells was achieved either with aminooxyacetic acid (AOAA) or 2-[(4-hydroxy-6-methylpyrimidin-2-yl)sulfanyl]-1-(naphthalen-1-yl)ethan-1-one (HMPSNE). These inhibitors induced an upregulation of E-cadherin and Zonula occludens-1 (ZO-1) expression and downregulation of fibronectin expression, demonstrating that H_2S biosynthesis inhibitors can produce a pharmacological induction of MET in colon cancer cells. These actions were functionally reflected in an inhibition of cell migration, as demonstrated in an *in vitro* "scratch wound" assay. The mechanisms involved in the action of endogenously produced H_2S in cancer cells in promoting (or maintaining) EMT (or tonically inhibiting MET) relate, at least in part, in the induction of ATP citrate lyase (ACLY) protein expression, which occurs via upregulation of ACLY mRNA (via activation of the ACLY promoter). ACLY in turn, regulates the Wnt-β-catenin pathway, an essential regulator of the EMT/MET balance. Taken together, pharmacological inhibition of endogenous H_2S biosynthesis in cancer cells induces

Abbreviations: 3-MP, 3-mercaptopyruvate; 3-MST, 3-mercaptopyruvate sulfurtransferase; ACLY, ATP citrate lyase; ADP, adenosine diphosphate; AKT, (AKT1) protein kinase B; AOAA, aminooxyacetic acid; ATCC, American Type Culture Collection; ATP, adenosine triphosphate; BSA, bovine serum albumin; CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lyase; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; ETHE1, ethylmalonic encephalopathy 1 protein; EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GOT, glutamic-oxaloacetic transaminase / aspartate aminotransferase; GSH, glutathione; H_2S, hydrogen sulfide; HMPSNE, 2-[(4-hydroxy-6-methylpyrimidin-2-yl)sulfanyl]-1-(naphthalen-1-yl)ethan-1-one; HRP, horseradish peroxidase; IgG, immunoglobulin G; LDH, lactate dehydrogenase; MET, mesenchymal-to-epithelial transition; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIH, National Institute of Health; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDVF, polyvinylidene fluoride; PLP, pyridoxal phosphate; RIPA, radioimmunoprecipitation assay; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSOH, sulfenic acid; RT, room temperature; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TBST, mixture of tris-buffered saline (TBS) and polysorbate 20 (Tween 20); TGF-β1, transforming growth factor-β1; TJP1, tight junction protein-1; TRX, thioredoxin; TST, thiosulfate sulfurtransferase; ZO-1, zonula occludens-1 (also known as Tight Junction Protein-1: TJP1).

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MET. We hypothesize that this may contribute to anti-cancer / anti-metastatic effects of H_2S biosynthesis inhibitors.

1. Introduction

Hydrogen sulfide (H_2S) is an endogenous gaseous transmitter mediator, which regulates a variety of cellular functions in autocrine and paracrine manner [1,2]. The enzymes responsible for the biological generation of H_2S in mammalian cells include cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) [1–6]. Simultaneous pharmacological inhibition of CBS and CSE in cultured cells is possible via the application of aminooxyacetic acid (AOAA), which, however, is also known to inhibit other enzymatic targets (several enzymes that possess the PLP prosthetic group) [1,7,8]. Pharmacological inhibition of 3-MST has become possible recently using HMPSNE (2-[(4-hydroxy-6-methylpyrimidin-2-yl)sulfanyl]-1-(naphthalen-1-yl)ethan-1-one) [9–12].

Using a combination of pharmacological and genetic approaches, significant roles of endogenously produced H_2S have been identified over the last decade in health and disease. The field of endogenously produced H_2S and cancer is an emerging area of H_2S biology, with significant translational potential. This field started with a set of observations in colon cancer surgical specimens and colon cancer cell lines *in vitro*, demonstrating the overexpression of CBS and an increased endogenous production of H_2S, and demonstrating the inhibitory effect of CBS inhibitors on colon cancer cell bioenergetics and proliferation [13]. Multiple lines of studies have confirmed and extended these early findings. It is now well established that increased endogenous H_2S production (due to CBS, CSE and/or 3-MST overexpression, depending on the tumor type) has been attributed to the increased proliferation rate of various cancer cells, as well increased cellular bioenergetics (aerobic and anaerobic ATP production), pro-inflammatory and cytoprotective signalling processes, chemotherapeutic resistance, tumor cell stemness, as well as local vasodilatation and peritumor angiogenesis [12–36]. Accordingly, pharmacological inhibitors of CBS, CSE or 3-MST have been found to suppress tumor cell bioenergetics, slow down tumor cell proliferation, migration and invasion and restore chemotherapeutic responsiveness; taken together, these studies identify the various H_2S-producing enzymes as potential novel experimental therapeutic targets [12–37].

Cancer cell identity is significantly characterized by so-called "transition states". The progression from normal (epithelial) to transformed (mesenchymal) state is termed epithelial-to-mesenchymal transition (EMT). EMT represents a complex sequence of cellular events, typically governed by the Wnt/β-catenin signaling pathway, whereby epithelial cells lose their cell to-cell adhesion capacity and gain mesenchymal characteristics. β-catenin (gene entry: CTNNB1) is constitutively bound to a multiprotein "destruction complex", whose fate is to be degraded by the proteasome. In the presence of Wnt ligands such as Wnt3, the formation of the destruction complex is prevented, followed by the β-catenin translocation in the nucleus, where it mediates the expression of genes, such as Snail1 and Twist1, involved in cell proliferation, migration and invasion. The end results of this process are commonly detected by EMT markers, e.g. increased N-cadherin expression and loss of membranous E-cadherin; it is also functionally reflected in the cells' increased migratory capabilities. In the context of colon cancer, EMT is known to occur during the transition of the transformed colon epithelial cells into metastatic type cells [38–40]. The above transition process can also go in the opposite direction, i.e. from transformed (mesenchymal) to normal (epithelial) state, and this process is termed mesenchymal-to-epithelial transition (MET); the result of this process is that mesenchymal cells decrease their motility and establish epithelial characteristics (signified by increased expression of various EMT markers, for instance E-cadherin and increased cellular polarity) [38–40]. Pharmacological induction of MET may be useful to force the transition of cancer cells into a less metastatic (i.e. less aggressive) phenotype.

Recently, ATP-citrate lyase (ACLY), a pivotal enzyme in the lipid metabolism and currently evaluated as a potential target in cancer therapy, has been shown to be involved in promoting epithelial-to-mesenchymal transition in colon cancer cell line through interacting with β-catenin, thus favoring its translocation in the nucleus and the expression of Wnt downstream target genes [41,42]. Pharmacological induction of MET, therefore, may be possible by targeting ACLY or its transcription factors (Sp1 and Sp3 [43]).

The current project was designed to investigate whether endogenous H_2S synthesis is necessary to maintain the cancer cells in the mesenchymal phenotype and to test if pharmacological inhibition of H_2S biosynthesis can induce MET, and if so, to begin to characterize some of the underlying molecular regulatory mechanisms.

2. Materials and methods

2.1. Cell culture

The HCT116 human colorectal carcinoma cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and was cultured in McCoy's 5A (Modified) Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % FBS (Gibco, Thermo Fisher Scientific), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Gibco, Thermo Fisher Scientific).

2.2. Reagents and antibodies

The 3-MST inhibitor HMPSNE was purchased from MolPort (Riga, Latvia), the CBS/CSE inhibitor AOAA was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and the H_2S donor GYY4137 (4-methoxyphenyl(morpholino)phosphinodithioate morpholinium salt) was purchased from AdipoGen (San Diego, CA, USA). The ACLY inhibitor (3R,5S)-rel-5-[6-(2,4-dichlorophenyl)hexyl]tetrahydro-3-hydroxy-2-oxo-3-furanacetic acid (SB 204990) [44] was purchased from MedChemExpress (Monmouth Junction, MJ, USA). Rabbit monoclonal anti-CBS (D8 F2P), anti-E-cadherin (24E10), anti-ZO-1 (D7D12), anti-ACLY (D1 × 6P), anti-β-catenin (D10A8), anti-AKT (pan) (C67E7), anti-p-Akt (Ser473) (D9E), rabbit polyclonal anti-p-ACLY (Ser455) and anti-mouse IgG HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti--β-Actin (AC-15) was purchase from Sigma-Aldrich. Anti-rabbit IgG (H + L) cross-adsorbed secondary antibody-HRP was purchased from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal anti-CSE (ab151769), anti-3-MST (ab224043), anti-TST (ab231248), rabbit monoclonal anti-ETHE1 (ab174302) and goat anti-rabbit IgG H&L (Alexa Fluor® 488) were purchased from Abcam (Cambridge, England).

2.3. Western blotting

The cells were collected and lysed with RIPA lysis buffer (Thermo Fisher Scientific) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and the protein concentrations were determined with Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate) using Infinite 200 Pro reader (Tecan, Männedorf, Switzerland).

Proteins from whole-cell lysate were reduced and denatured by boiling in lithium dodecyl sulfate (LDS) sample buffer (Bolt™ LDS Sample Buffer, Invitrogen, Thermo Fisher Scientific) or sodium dodecyl

sulfate (SDS) sample buffer (Novex™ Tris-Glycine SDS Sample Buffer, Invitrogen, Thermo Fisher Scientific) supplemented with 50 mM dithiothreitol (DTT; Bolt™ Reducing Agent, Invitrogen, Thermo Fisher Scientific) at 95 °C for 5 min. Equivalent protein samples were loaded and separated using Bolt™ 4%–12% Bis-Tris Plus Gels (Invitrogen, Thermo Fisher Scientific) or Novex™ 8 % Tris-Glycine Mini Gels, WedgeWell™ format (Invitrogen, Thermo Fisher Scientific) and run at the constant voltage of 100 V. Proteins were transferred to nitrocellulose or PDVF membranes by dry transfer using the iBlot™ 2 Device and Transfer Stacks nitrocellulose or PVDF (Invitrogen, Thermo Fisher Scientific). Membrane blocking was performed in 5 % Milk-TBS/0.1 % Tween Buffer (5 % Milk-TBST). Primary antibodies were prepared in 5 % BSA-TBST or 5 % Milk-TBST and incubated 1 h at RT or overnight at 4 °C. Afterwards, the blots were rinsed with TBST for 3 times and further incubated with anti-rabbit IgG or anti-mouse IgG, HRP-linked antibody diluted 1:5,000 in 5 % Milk-TBST and incubated 1 h at room temperature. After being washed 3 times with TBST, the blots were developed with Radiance Plus (AC2103, Azure Biosystems, Dublin, CA, USA) chemiluminescence solutions using Azure Imaging System 300 (Azure Biosystems) and quantified using the ImageJ software (NIH, Bethesda, MD, USA). For human 3-MST, which produces double bands related to splice variants of the enzyme [1], the two bands were quantified together. Intensity values of related bands were normalized to values of β-actin housekeeping protein. Representative blots of at least three independent experiments are shown.

2.4. Quantitative real-time PCR (qRT-PCR)

Total cellular RNA was extracted and isolated from the cultured cells using the NucleoSpin® RNA Plus kit according to the manufacturer's instructions (Macherey-Nagel, Dueren, Germany) and quantified with a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). Reverse-transcribed complementary DNA was synthesized from 1 µg total RNA with a M-MLV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific) or PrimeScript RT reagent kit (Takara, Shimogyō-ku, Kyoto, Japan) in the presence of random primers, according to the manufacturer's instructions.

Quantitative real-time PCR was performed using the SensiFAST SYBR Hi-ROX Kit (Meridian Bioscience, Cincinnati, OH, USA) or TEB green (Takara) supplemented with 10 µM of specific primers on the StepOne Plus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The following thermocycling conditions for PCR were used: initial denaturation at 95 °C for 2 min, followed by 40 amplification cycles consisting of denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s. Each assay was performed in duplicates for each sample, and the GAPDH expression was used as an internal control. The relative expression ratio was calculated using the 2-ΔΔCt method. Representative qRT-PCRs of at least three independent experiments are shown. The pairs of primers used are listed in Table 1.

2.5. Immunofluorescence

The HCT116 cells were seeded in an 8-well chamber and treated with 300 µM of HMPSE or AOAA for 24 h. After treatment the cells were fixed in 4 % paraformaldehyde in PBS for 15 min at RT. Specimens were washed 3 times, 5 min in PBS and blocked in blocking buffer (1x PBS/5 % goat serum/0.3 % Triton™ X-100) for 1 h and then incubated with primary antibodies diluted in antibody dilution buffer: (1x PBS/1 % BSA/0.3 % Triton™ X-100) overnight at 4 °C. After 3 washes, 5 min in PBS, the cells were incubated 1−2 h with fluorochrome-conjugated secondary antibodies diluted in antibody dilution buffer at RT in the dark. Then the specimens were washed 3 times again and mounted in ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology). Staining was visualized with a fluorescent microscope (Leica DFC360 FX, 10x objective). Representative immunofluorescence pictures of at least three independent experiments are shown.

Table 1
Primer sequences for real-time qRT-PCRs used in the current study.

Target Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	AGG GCT GCT TTT AAC TCT GGT	CCC CAC TTG ATT TTG GAG GGA
E-Cadherin	AAA GGC CCA TTT CCT AAA AAC CT	TGC GTT CTC TAT CCA GAG GCT
TJP1	ACCAGTAAGTCGTCCTGATCC	TCGGCCAAATCTTCTCACTCC
Fibronectin	AGG ACG GAC ATC TTT GGT GC	TGT GGT TGT TGT ATA GGA AGG G
Snail1	ACT GCA ACA AGG AAT ACC TCA G	GCA CTG GTA CTT CTT GAC ATC TG
Twist1	GGA CAG AGA TTC CCA GAC GG	GGC TGA TTG GCA CGA CCT
Sp1	GTGGAGGCAACATCATTGCTG	GCCACTGGTACATTGGTCACAT
Sp3	CCAGGATGTGGTAAAGTCTA	CTCCATTGTCTCATTTCCAG
CTNNB1	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATACTGTCCAT
ACLY	ATCGGTTCAAGTATGCTCGGG	GACCAAGTTTTCCACGACGTT
Wnt3	AGGGCACCTCCACCATTTG	GACACTAACACGCCGAAGTCA
Akt1	TCCTCCTCAAGAATGATGGCA	GTGCGTTCGATGACAGTGGT
Akt2	AGGCACGGGCTAAAGTGAC	CTGTGTGAGCGACTTCATCCT

2.6. Cell migration assay

Cell migration was assessed as previously described [12]. Briefly, HCT116 cells were seeded in a 96-well plate at 50,000 cells/well in 100 µl of complete culture medium and incubated 24 h at 37 °C and 5 % CO_2 to create a monolayer of cells. The WoundMaker from Essen BioScience was used to create homogeneous wide wounds and the culture medium was carefully replaced with fresh complete medium containing HMPSNE or AOAA serial dilutions. The cells were then incubated in IncuCyte device (10x objective) (Essen Bioscience, Herforthshire, UK) and the confluence was recorded every 2 h by phase/contrast scanning for 48 h at 37°C and 5 % CO_2. Images were analyzed using the Incucyte ZOOM software.

2.7. Cell viability and mitochondrial metabolic activity assay

HCT116 cells were seeded in a 96-well plate at 6700 cells/well (protein and RNA assay control) or 50'000 cells/well (migration assay control) in 200 µl of complete culture medium. After 24 h, fresh completed medium containing several concentrations of HMPSNE or AOAA was added to the cells and the plate was incubated for 48 h at 37 °C and 5 % CO_2. Then 50 µL of the supernatant was used to test the lactate dehydrogenase (LDH) activity and with the remaining cells an MTT assay was performed. To measure the LDH activity, the Cytotoxicity Detection Kit (LDH, Roche, Basel, Switzerland) was used according to manufacturer's instructions and absorbance was measured at 490 nm with 680 nm used as background [12]. To conduct the MTT assay, which quantify the ability of the cells to convert MTT to formazan, a principally mitochondrion-dependent process, 40 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 2 mg/mL in PBS was added to the cells and incubated for 3 h at 37 °C and 5 % CO_2. Formazan produced by cells with active metabolism was solubilized in 150 µl/well of MTT solvent by mixing up and down. The absorbance was measured at 590 nm and 690 nm (background) using Infinite 200 Pro reader (Tecan) [12]. The LDH and MTT assays were performed at least 3 times in triplicates per compound's concentration.

2.8. Vector amplification

The ACLY promoter vector (HPRM42924-LvPM02) and its negative control (NEG-LvPM02) were obtained from GeneCopoeia (Rockville, MD, USA). The vectors were transformed into NEB® Stable Competent E. coli (New England Biolabs, Ipswich, MA, USA) and random ampicillin-resistant colonies were cultured in LB medium with 100 µg/mL ampicillin. The vectors were then purified using a PureLink™ HiPure

Plasmid Midiprep Kit (Invitrogen, Thermo Fisher Scientific) according to manufacturer's protocol. The integrity of the vectors was performed by digestion with HindIII and MluI enzymes.

2.9. Lentiviral production and HCT116 cell transduction

HEK293 T cells (ATCC) were seeded at 70–80 % confluence in a 6-well plate and transiently transfected 4−6 h later with the expression vector, a packaging plasmid pLP1, pLP2, and an envelope plasmid pLP/VSVG in a ratio of 4.2:2:2.8 using JetOptimus (Polyplus, Strasbourg, France) according to the manufacturer's instructions. The transfection mixture and medium were replaced with fresh culture medium after o/n incubation. Lentiviral supernatants were collected after 24 h and filtered via a 0.45 μm filtration unit and subsequently aliquoted and stored in −80 °C until use. Human HCT116 cells were seeded in 12-well plates to reach approximately 50 % confluence on the day of transduction. The cells were transduced with lentiviral supernatant in the presence of 6 μg/mL protamine sulfate. Fresh complete McCoy's medium was added 24 h after transduction. Seventy-two hours following transduction, puromycin (1 μg/mL) was added to the culture to select transduced cells for 3 days.

2.10. ACLY promoter assay

HCT116 cells were seeded in 96-well plates at 6700 cells/well in 200 μl of complete culture medium and incubated 24 h at 37 °C and 5 % CO_2. The day after the culture medium was carefully replaced with fresh complete medium containing GYY4137 serial dilutions and incubated for 48 h at 37 °C and 5 % CO_2. After incubation with various concentrations of the H_2S donor GYY4137 the cells were detached and incubated in an optimal dilution of a LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fisher Scientific) for 15 min. The cells were analyzed using a BD LSRFortessa™ Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Emission signals for mCherry (610/20) and Live/dead IR (780/60) were detected. Data were analyzed with FlowJo (v10.7.1) software. Data shown is representative of FACS from at least 3 independent experiments in triplicates.

2.11. ACLY activity assay

The ACLY activity assay was adapted from Wei and colleagues [41]. Sulfide and trisulfide stock solutions were prepared by dissolving Na_2S or Na_2S_3 powder in degassed ultra-pure (Milli-Q®, Merck Millipore, Burlington, MA, USA) water under a N_2 atmosphere. The enzymatic activity of the recombinant human ACLY (Sigma-Aldrich, St Louis, MO, USA) was measured using the ADP-Glo kit from Promega (Madison, WI, USA). Enzymatic activity assay was carried out in a 384-white well plate (Fisherbrand, Fisher Scientific AG, Reinach, Switzerland) in a total assay volume of 5 μL. In each well was dispensed 50 mM Hepes buffer (pH 8.0), 300 μM ATP, 300 μM Coenzyme A, 1 mM Citrate and 10 mM $MgCl_2$ previously degassed in N_2 atmosphere. Reduced or oxidized form of the enzyme were obtained by adding 4 mM DTT or 100 μM H_2O_2, respectively. For kinetic studies, the reaction mixture was supplemented with increasing concentration of Na_2S_3 or Na_2S (0–3 mM). Eventually, the reaction was triggered by adding ACLY (0.05 μg/well). Baseline condition was obtained in the absence of ACLY, under otherwise identical assay conditions. The plate was sealed with an adhesive film and incubated at room temperature in the dark for 90 min. The reaction was stopped by adding 5 μL ADP-Glo™ Reagent (which depletes the excess of ATP) and incubated for 40 min. Eventually, the reaction mixture was supplemented with 10 μL of Kinase Detection Reagent and further incubated for 60 min, as recommended by the manufacturer. The luminescent signal, which is proportional to the ADP concentration, was acquired with an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland).

2.12. Statistical analysis

Data are shown as mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was used to detect differences between groups. Statistically significant differences between these two groups are indicated by $*p < 0.05$ or $**p < 0.01$. Statistical calculations were performed using Graphpad Prism analysis software.

3. Results

3.1. Expression of H_2S-producing enzymes in HCT116 cells

All three H_2S-producing enzymes (3-MST, CBS, CSE) and key enzymes involved in H_2S degradation (ETHE1 and rhodanese [also known as TST]) were markedly expressed in HCT116 cells, as determined by Western blotting (Fig. 1**A–F**). We have determined the potential effect of 3-MST inhibitor (HMPSNE) and CBS/CSE inhibitor (AOAA) on the expression of these enzymes. Unexpectedly, HMSPNE at 300 μM markedly suppressed the expression of CBS (Fig. 1**A,B**). HMPSNE at 100 and 300 μM also decreased at the expression of its own enzymatic target, 3-MST (Fig. 1**A,C**). Moreover, HMPSNE also suppressed the expression of both H_2S-degrading enzymes ETHE1 and TST (Fig. 1**A,D,E**). CSE expression was slightly downregulated at the intermediate concentration (100 μM) of HMPSNE only (Fig. 1**A,F**). No significant effect of AOAA on the expression of H_2S-producing enzymes was observed (Fig. 1**A–C,F)**. However, a slight increase of ETHE1 protein expression was noted at 100 μM AOAA (Fig. 1**A,D**). These results suggest that in HCT116 cells, 3-MST-derived (but not CBS/CSE-derived) H_2S exerts various transcriptional regulatory actions; the underlying mechanisms remain to be further characterized.

The practical implications of these findings for the current study are multiple. **(a)** The functional effects of AOAA can be attributed to the dual inhibitory effect of CBS/CSE, according to its well-characterized pharmacological action [7,8] without major secondary effects on any of the other H_2S-producing or H_2S-degrading systems, although – as previously discussed [8] – pharmacological effects on other PLP-dependent enzymes cannot be excluded. **(b)** The cellular action of 100 μM HMPSNE in HCT116 cells may be considered selective to 3-MST, although it is likely result from a combination of the inhibition of 3-MST catalytic activity [9], as well as the downregulation of 3-MST protein. **(c)** The cellular action of 300 μM HMPSNE in HCT116 cells must be interpreted as the result of a "dual 3-MST/CBS inhibitory action" due to the combined action of direct inhibition of 3-MST catalytic activity [9], as well as the downregulation of CBS as well as 3-MST enzymes.

3.2. Pharmacological inhibitors of H_2S biosynthesis promote mesenchymal to epithelial transition (MET) in HCT116 cells

To evaluate the influence of H_2S enzymes inhibitors on the mesenchymal/epithelial status of human colon cancer cells, we incubated HCT116 cells with various concentrations of HMPSNE or AOAA for 24 h and investigated the expression of several epithelial markers (E-cadherin, tight junction protein ZO-1 [TJP1]) and a mesenchymal marker (fibronectin). HMPSNE significantly increased the expression of E-cadherin and ZO-1 at RNA and protein level and decreased the mRNA expression of fibronectin (Figs. 2**A–E,** 3 **A–D**). Although AOAA increased the level of ZO-1 mRNA (Fig. 2B), this effect was not significantly reflected at the protein level, where a trend of a 25 % increase was noted (Fig. 3D). AOAA (similar to HMPSNE) decreased the mRNA expression of fibronectin (Fig. 2C). Our findings suggest that pharmacological inhibitors of H_2S biosynthesis promote MET in HCT116 cells. HMPSNE exerts a more prominent effect on the regulation of these markers than AOAA, most possibly due to its "dual 3-MST/CBS inhibitory action" [as discussed in the previous section].

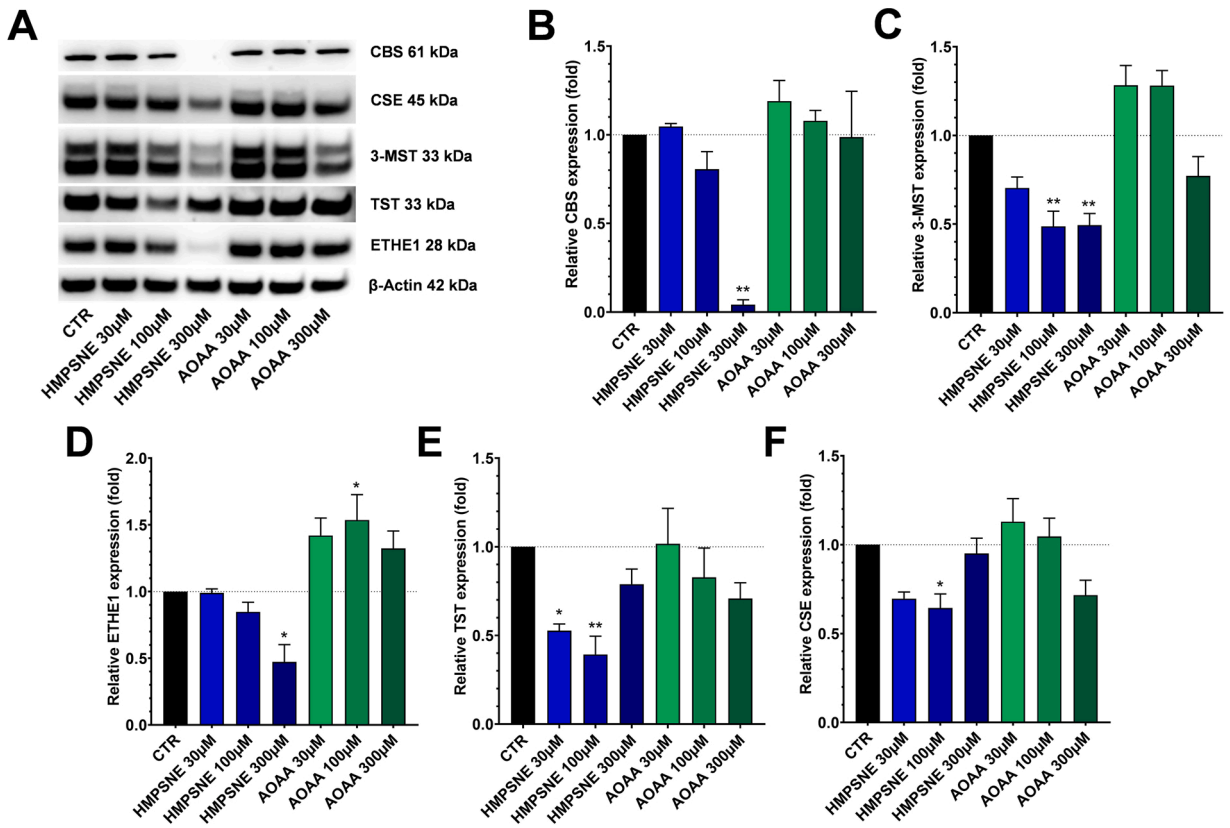

Fig. 1. Effect of pharmacological inhibitors of H_2S biosynthesis on the expression of various H_2S producing and H_2S metabolizing enzymes in HCT116 cells. (A): Representative Western blot of CBS, 3-MST, CSE, ETHE1 and TST expression in HCT116 cells in presence of various concentrations of HMPSNE or AOAA for 48 h. (B-F): Numerical quantification of the expression data. Data are presented as mean ± SEM of at least 3 independent experiments. *$p < 0.05$, **$p < 0.01$ compared to control.

3.3. Pharmacological inhibitors of H_2S biosynthesis induce MET via the modulation of the Sp3-ACLY-Wnt-β-Catenin pathway in HCT116 cells

To investigate the potential signaling pathways that may regulate MET and may be affected by pharmacological inhibitors of H_2S biosynthesis, we have evaluated the expression of some markers related to Wnt signaling pathway, as it is considered an important signaling pathway regulating EMT/MET [37–40] (Fig. 4**A–J**). The mRNA levels of Wnt3 and Ctnnb1 as well as β-catenin protein expression were significantly downregulated after treatment of the cells with 100 or 300 μM HMPSNE (Fig. 4**A,B,H,I**). AOAA also decreased the mRNA and protein levels of β-catenin at the highest concentration tested (300 μM) (Fig. 4**B, H,I**).

The HMPSNE- or AOAA-induced decrease of β-catenin was also associated with significant decreases in the mRNA levels of the transcription factors Snail1 and Twist1 (Fig. 4**C,D**).

We have also investigated ACLY, a key enzyme in acetyl-CoA synthesis, that is recently associated to Wnt signaling pathway and implicated in epithelial-mesenchymal transition in colon cancer cells lines [42]. First, we confirmed the role of ACLY in the regulation of MET/EMT in our current experimental system. Indeed, and in line with prior reports [42] pharmacological inhibition of ACLY using SB 204990 induced significant changes in the mRNA expression patterns of the HCT116 cells that are consistent with the induction of MET (Fig. 5**A–H**). Next, we tested the effect of inhibition of H_2S biosynthesis on ACLY protein and mRNA expression. We have observed that HMPSNE and AOAA (at the highest concentration used) induced significant decreases of ACLY mRNA and protein levels (Fig. 4**E,H,J**).

These findings led us to further investigate the potential regulation by H_2S of ACLY gene transcription and/or ACLY activity. The ACLY promoter is known to be regulated by Sp1 and, more significantly, by Sp3 [43]. Accordingly, we have quantified mRNA levels of Sp1 and Sp3 and found that there are both decreased in response to HMPSNE (Fig. 4**F,G**). The effects were more significant with Sp3. These results point to a potential crosstalk between H_2S, Sp3, ACLY and the Wnt signaling pathway.

3.4. H_2S does not stimulate ACLY enzymatic activity

ACLY is a cytosolic enzyme and as such, it functions in a reducing environment. The discovery that ACLY has a disulfide bond between Cys_{293} and Cys_{748} [45] was intriguing in the context of our current project, since it opens to the possibility to be regulated by redox changes (i.e. possibly those regulated by changes in intracellular H_2S levels). In air equilibrated buffer, the reduced (active) form and oxidized (inactive) form coexist, and typically ACLY activity assay are carried out in the presence of artificial reducing agents, such as dithiothreitol (DTT). In order to minimize the effect of oxygen on ACLY's activity, herein all the solutions used in the assay were degassed in N_2 atmosphere. As expected, treatment with DTT induced an increase of ~ 30 % of the enzymatic activity, suggestive that the thiolic form of the enzyme is the active form (Fig. 6A), as already reported by Wells and colleagues [46]. The role of reactive oxygen species (ROS) in cancer survival and progression is widely known [47]. Thus, we have evaluated whether exposing ACLY to a source of ROS may interfere with its catalytic activity. As shown in Fig. 6A, H_2O_2 induced a loss of 20 % of the enzymatic activity, which is possibly associated to the oxidation of protein thiols exposed to the solvent to sulfenic acid (RSOH) [48]. As expected, incubation of ACLY with H_2O_2 followed by treatment with equimolar concentration of H_2S partially reverted the ROS-induced inactivation of ACLY. This is consistent with the supposed role of biological reductants, such as H_2S ($E^{\circ\prime} = -0.23$ V), which has been postulated to have a

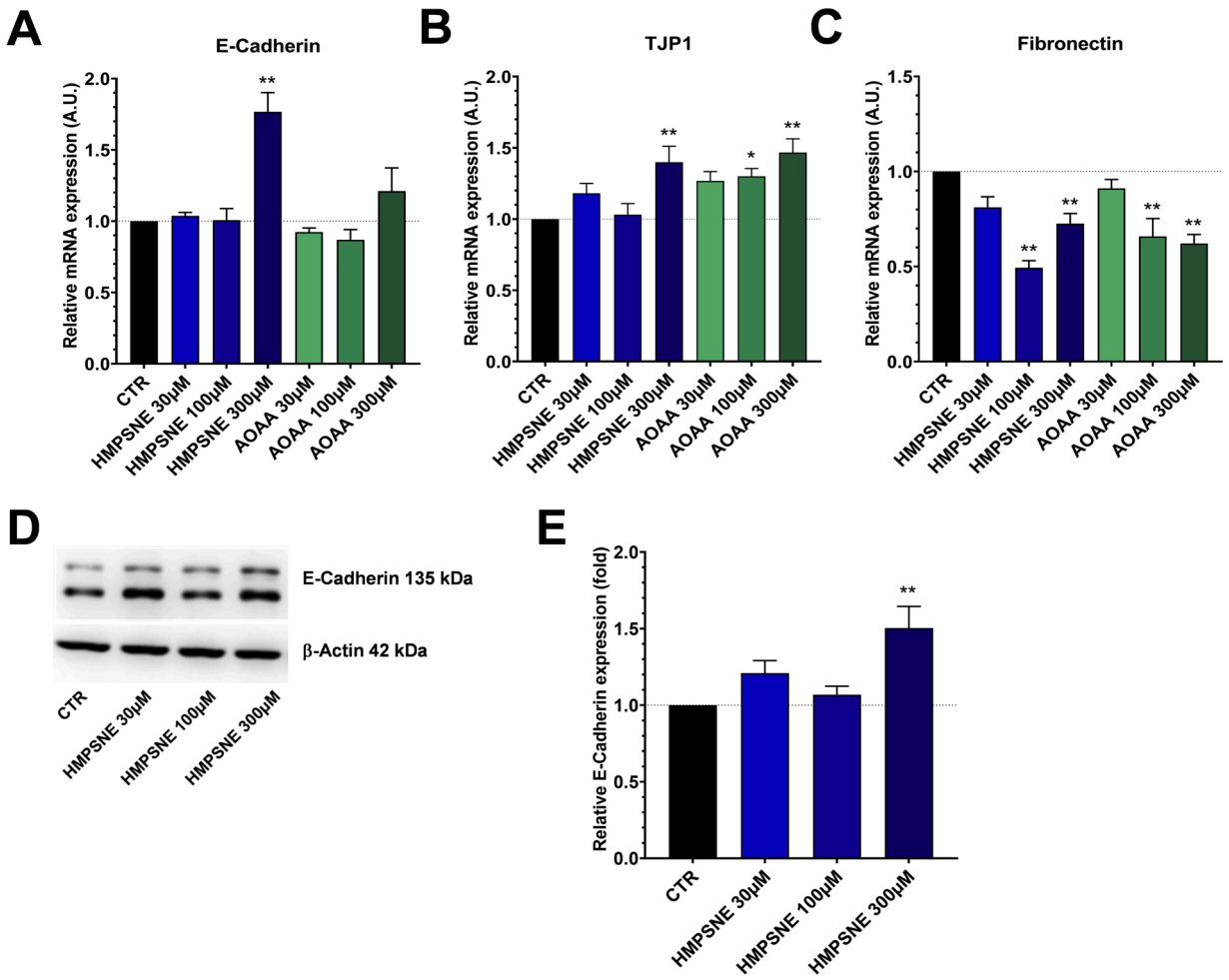

Fig. 2. Effect of pharmacological inhibitors of H_2S biosynthesis on the expression of various H_2S mesenchymal and epithelial markers in HCT116 cells. (A-C): Quantitative real-time PCR (qPCR) analysis of endogenous mRNA levels of E-cadherin, TJP1 (Tight Junction Protein-1 / ZO-1) and fibronectin after incubation of HCT116 cells with various concentrations of HMPSNE or AOAA for 24 h. Data are shown as mean ± SEM, n = 4, *p < 0.05, **p < 0.01 compared to control. (D, E): Western blot analysis of E-cadherin protein in presence of HMPSNE for 24 h. Data are shown as mean ± SEM, n = 5, **p < 0.01 compared to control.

protective role against ROS-induced oxidation of protein cysteine thiols [49].

However, unlike the stronger reductant DTT ($E^{\circ\prime} = -0.33$ V), H_2S alone failed to increase ACLY's activity from basal levels (Fig. 6B), suggesting that H_2S is not able to reduce the protein disulfide to its thiolic form. In physiological conditions the reducing species possibly involved in maintaining ACLY in its reduced (active) state are glutathione (GSH) or thioredoxin (TRX) [46,50]. In the cellular milieu, the ROS-scavenging action of H_2S conceivably leads to formation of reactive sulfur species such as sulfane sulfurs and polysulfides. Hence, we wondered whether these species were able to exert any effect on ACLY activity. To explore this possibility, ACLY was exposed to increasing concentration of sodium trisulfide (Na_2S_3) and it was observed a concentration-dependent inhibition of the enzymatic activity with an $IC_{50} \sim 11$ μM (Fig. 6C). As summarized in Fig. 6D, the partial inactivation resulting from exposure to ROS may be reverted by H_2S. Conversely, sulfane sulfur species may be responsible for the full inactivation of ACLY. It is possible that the mechanism behind the sulfane sulfur-mediated inactivation of the enzyme relies on the persulfidation of the cysteine residues Cys_{293} and Cys_{748}, followed by formation of a disulfide bridge. This interpretation is supported by a previous study addressing the nature of the interaction between ACLY and oxidized glutathione [45].

3.5. H_2S activates the ACLY promoter

To further investigate the relationship between H_2S and ACLY, we have evaluated the potential effect of H_2S on the ACLY promoter. To this end, we introduced in HCT116 cell line a viral vector containing the ACLY promoter that, when activated, induces the expression of mCherry protein. Pharmacological H_2S generation via GYY4137 induced an increase of mCherry expression in a concentration-dependent fashion (Fig. 7**A–C**). It is possible that this increase in ACLY promoter activation is mainly due to the induction of transcription factor Sp3 (since H_2S biosynthesis inhibitors were noted to downregulate this factor; see above) (Fig. 4G). The highest GYY4137 concentrations began to exert a slight cytotoxic effect in HCT116 cells (Fig. 7D), but the actions of the donor on the ACLY promoter were already pronounced at lower concentrations of GYY6137 (1–3 μM) where the donor did not affect cell viability (Fig. 7**A–C**).

Taken together, the results presented above and in the previous section suggest that H_2S overproduction in colon cancer cells which overexpress the various H_2S-producing enzymes leads to an increase of Sp3 transcription factor and a consequent rise in ACLY expression. ACLY then can interact with β-catenin to block its ubiquitination. This, in turn, would lead to an accumulation of β-catenin in the cytoplasm which, in turn, will translocate to the nucleus [42]. In the nucleus β-catenin binds to LEF and activates Twist1 and Snail1 transcription factors, thereby stimulating fibronectin expression and repressing E-cadherin promoting

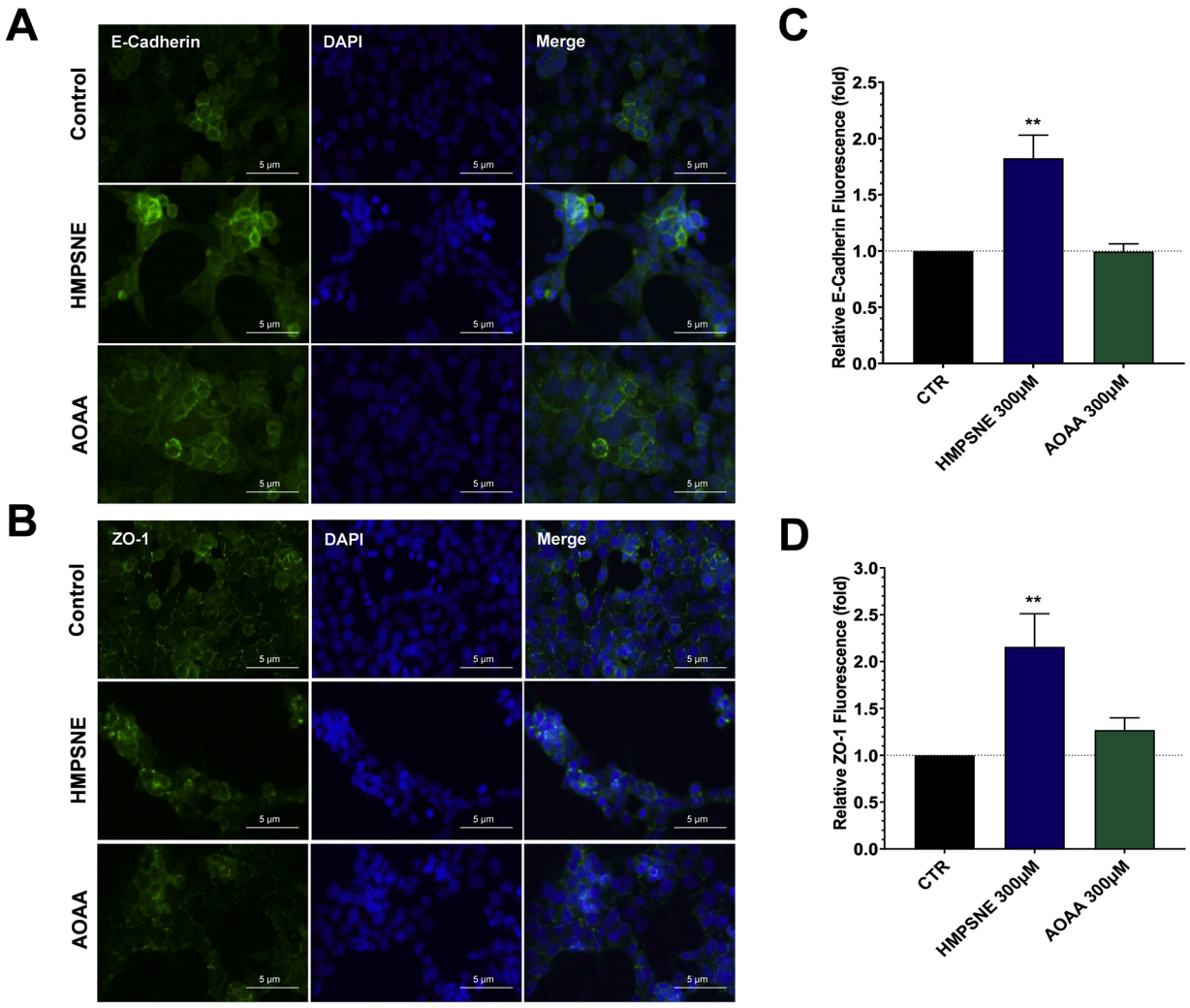

Fig. 3. Effect of pharmacological inhibitors of H_2S biosynthesis on the expression of various H_2S mesenchymal and epithelial markers in HCT116 cells. (A, B): Representative Immunofluorescence images (objective 10x). E-Cadherin and ZO-1 (Tight Junction Protein ZO-1, TJP1) protein levels in presence of HMPSNE or AOAA for 24 h. (C, D): Numerical quantification of the data. Data are shown as mean ± SEM, n = 4, **p < 0.01 compared to control.

EMT, and, potentially, facilitating the metastasis of tumor cells. After inhibition of the H_2S-producing enzymes, these processes are blocked or reversed, thereby the process of MET will be pharmacologically facilitated.

3.6. *Effects of pharmacological inhibitors of H2S biosynthesis on Akt activation*

Since our data indicated that inhibition of H_2S biosynthesis exerts an inhibitory effect on ACLY expression, we also investigated if inhibition of H_2S biosynthesis may have additional, potential effects on ACLY, on the level of enzymatic activity. It is known that H_2S has an effect on Akt expression and phosphorylation [51–54], and it is also known that Akt is able to phosphorylate ACLY, thereby inducing its activation [55]. We anticipated that pharmacological inhibition of H_2S biosynthesis would decrease Akt expression and that, in turn, ACLY phosphorylation may become suppressed.

Indeed, our data demonstrate that the mRNA levels of Akt1 and Akt2 and total Akt protein expression are significantly reduced with 300 µM of HMPSNE (Fig. 8**A–D**). However, this reduction did not coincide with a decrease in Akt phosphorylation; in fact, HMPSNE increased Akt phosphorylation (Fig. 8E). In contrast to HMPSNE, AOAA did not induce significant changes in mRNA levels of Akt1 and Akt2; but, at the concentration of 300 µM it decreased the protein levels of total Akt (Fig. 8D). Moreover, similar to prior findings [26] AOAA increased Akt phosphorylation. AOAA – in contrast to HMPSNE – did not significantly inhibit the phosphorylation of ACLY, although p-ACLY levels at 300 µM AOAA tended to be lower than at 30 or 100 µM AOAA (Fig. 8F). These data suggest that endogenously produced H_2S may regulate Akt expression and phosphorylation, but are not fully consistent with the hypothesis that inhibition of H_2S biosynthesis in cancer cells regulates ACLY and MET via an inhibition of the activation of the Akt pathway, because **(a)** HMPSNE exerted opposite effect on Akt expression and Akt phosphorylation (i.e. activation) – suppression of the former and induction of the latter; and **(b)** AOAA (in contrast to HMPSNE) did not produce any effects that would be consistent with inhibition of the Akt pathway (it only exerted a relatively modest inhibitory effect on total Akt expression and it increased Akt phosphorylation).

3.7. *Effect of H_2S donation in HCT116 cells*

Next, we examined how the pharmacological, exogenous donation of H_2S could affect the expression patterns of genes and proteins orchestrating EMT/MET and whether H_2S donation can abrogate the ability of H_2S biosynthesis inhibitors to promote MET (Fig. 9**A–J**). The slow-releasing donor GYY4137 did not induce any significant increase in the gene expression of Snail1 or Sp3, nor did the H_2S donor affect the protein levels of total Akt (Fig. 9**A,B,J**). Similarly, it did not change the

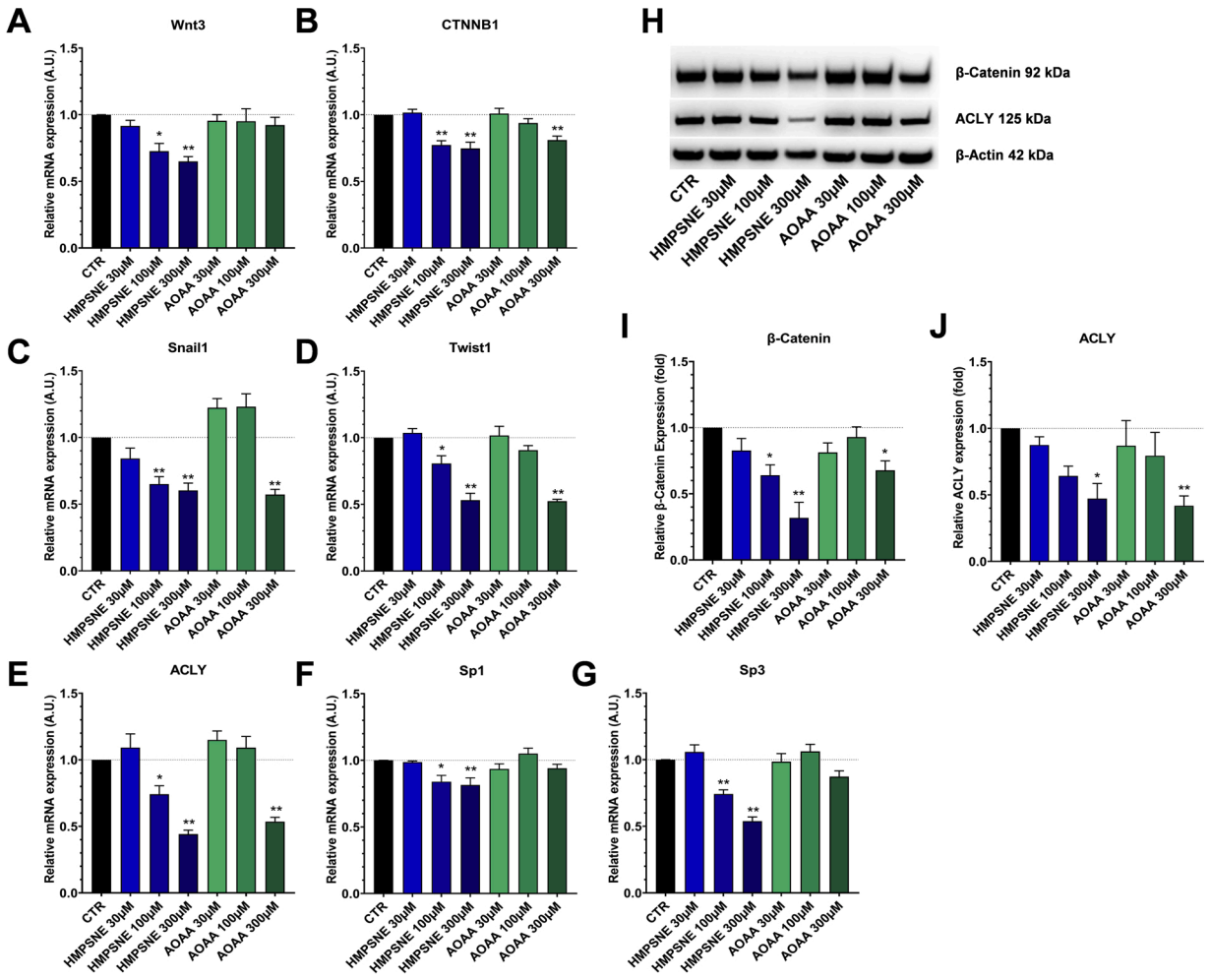

Fig. 4. Effect of pharmacological inhibitors of H_2S biosynthesis on various markers of the Sp3-ACLY-Wnt-β-catenin pathway in HCT116 cells. (A-G): Quantitative real-time PCR (qPCR) analysis of endogenous mRNA levels of various EMT-associated markers after incubation of HCT116 cells with SB204990 for 24 h. Data are shown as mean ± SEM of at least 4 independent experiments, *$p < 0.05$, **$p < 0.01$ compared to control. (H-J): Western blot analysis of β-catenin and ACLY proteins in presence of HMPSNE or AOAA for 48 h. Data are shown as mean ± SEM, $n = 4$, *$p < 0.05$, **$p < 0.01$ compared to control.

mRNA and protein levels of CTNNB1 and E-cadherin. Nevertheless, it significantly increased the mRNA levels of ACLY (Fig. 9C). Taken together, our findings indicate that the exogenous H_2S donation (at least in the concentration range used here) does not exacerbate the mesenchymal phenotype of HCT116 colon cancer cells, which may account for a pre-existing saturation of these systems for the mesenchymal transition by the ambiently high endogenous H_2S levels.

Importantly, when dual administration of either AOAA + GYY4137 or HMPSNE + GYY4137 was evaluated, H_2S "replacement" was able to partially reverse the effect of the H_2S inhibitors on selected EMT/ME markers and effectors (Fig. 9), indicating, that the effects of AOAA and HMPSNE on MET are, indeed, related to the regulation of cellular H_2S levels (as opposed to upstream pharmacological actions, i.e. modulation of substrate levels of these enzymes, or other non-specific actions of these agents that are unrelated to H_2S biosynthesis).

3.8. Pharmacological inhibitors of H_2S biosynthesis inhibit the migratory function of HCT116 cells

Cancer cells with a pharmacologically induced shift into a more epithelial-type phenotype (pharmacological induction of MET) would be expected to be more stationary and less migratory. To assess the impact of HMPSNE and AOAA in HCT116 cell migration a wound healing assay was performed. As shown in Fig. 10, HMPSNE and AOAA both suppressed the migration of HCT116 cells in a concentration-dependent manner. This reduction was stronger with HMPSNE; at 300 μM the cells reached a complete immobility.

Finally, in order to evaluate the effect of HMPSNE and AOAA on HCT116 cell viability and mitochondrial activity, we performed an LDH assay with the supernatant from cells after 48 h incubation with various concentrations of these inhibitors. As the ratio cells number/well surface can have an impact on the viability of cells, two different ratios were used (one corresponding to the same ratio cells number/well surface than the protein and RNA assays and another corresponding to the same ratio cells number/well surface than the migration assays). As shown in Fig. 11**A–D**, both H_2S biosynthesis inhibitors produced a concentration-dependent inhibition of mitochondrial activity of MTT conversion, in line with the previously demonstrated role of H_2S in maintaining cellular bioenergetics and ATP generation in cancer cells; in some cases (especially with the highest concentration of HMPSNE, evidence of overt necrotic cell death (increased LDH levels in the supernatant) was also noted, which may be either due to the fact that the combined inhibition of the activity and expression of 3-MST and the suppression of CBS expression, together, may suppress cellular H_2S levels below a critical level, at which an impairment of the viability of the cells may develop. (Nevertheless, as with all pharmacological inhibitors, non-specific toxicity of the compound, via mechanisms unrelated to the H_2S pathway, cannot be excluded.).

These data indicate that after inhibition of H_2S biosynthesis, HCT116 cells experience a suppressed mitochondrial (or, more generally, cellular

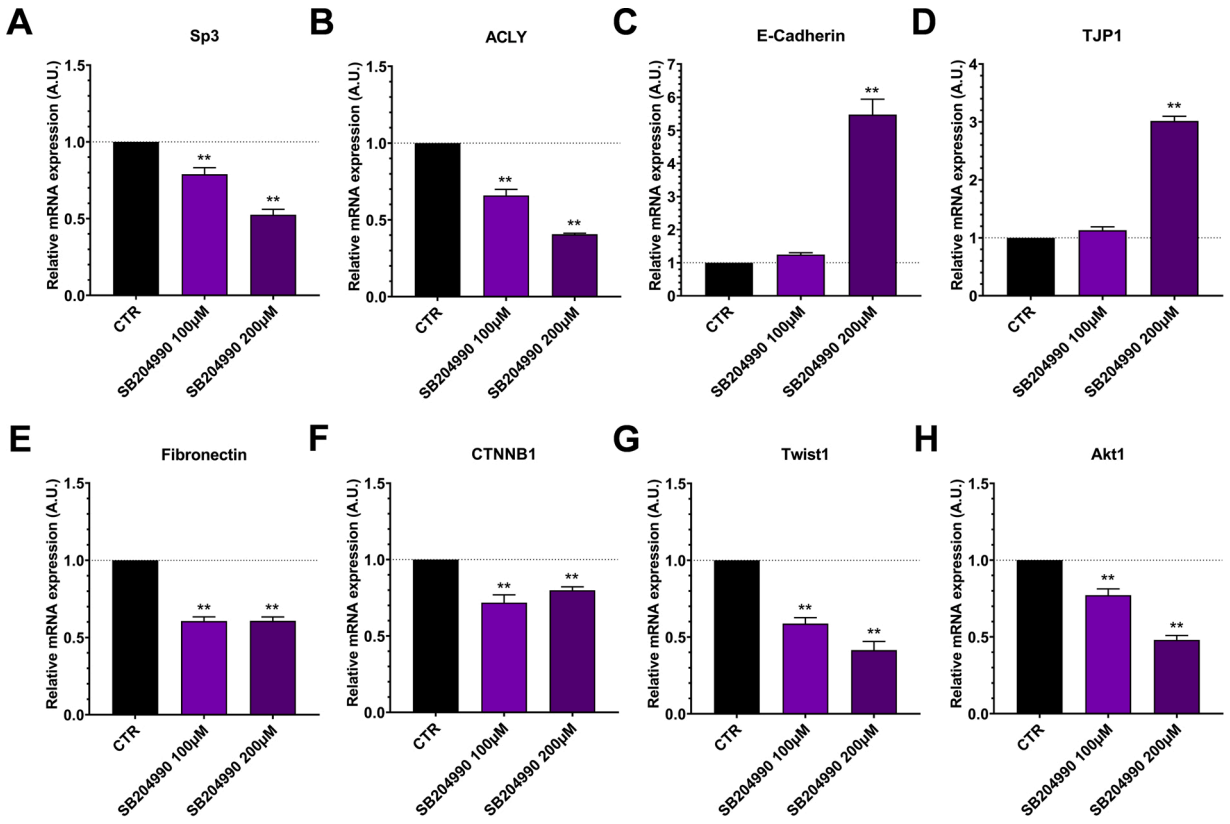

Fig. 5. Effect of a pharmacological inhibitor of ACLY on the expression of various H_2S mesenchymal and epithelial markers in HCT116 cells. (A-H): Quantitative real-time PCR (qPCR) analysis of endogenous mRNA levels of various EMT-associated markers after incubation of HCT116 cells with HMPSNE or AOAA for 24 h. Data are shown as mean ± SEM, n = 4, **p < 0.01 compared to control.

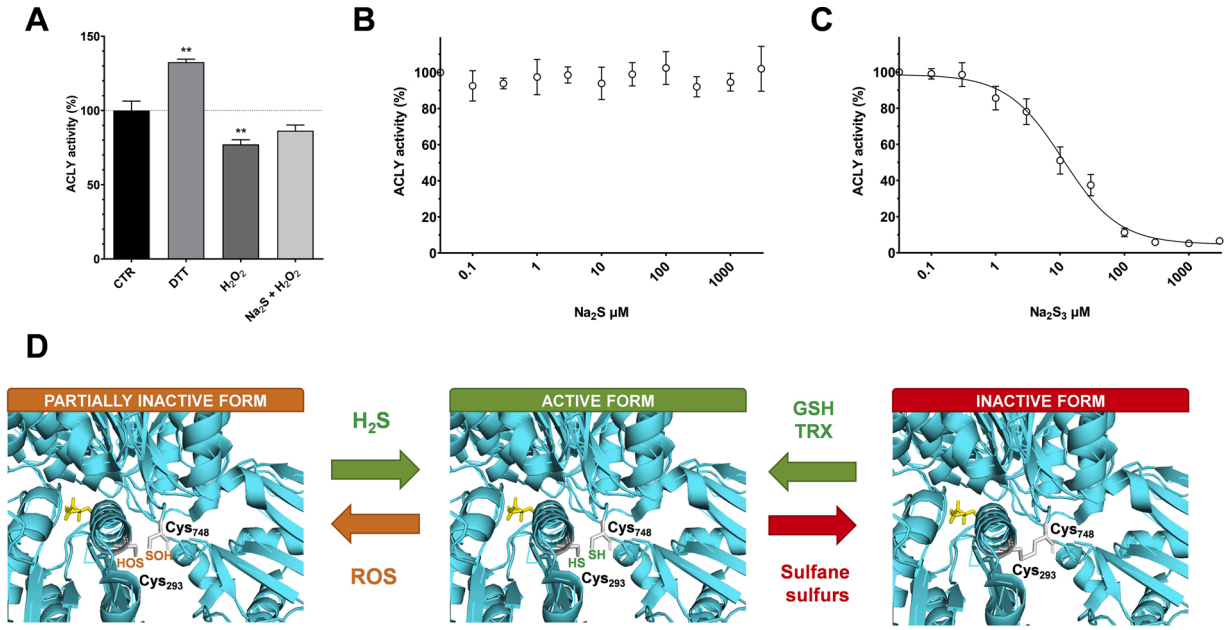

Fig. 6. ACLY is subject to redox regulation, but is not directly activated by H_2S. (A): Effect of 4 mM dithiothreitol (DTT), 100 μM hydrogen peroxide (H_2O_2), 100 μM H_2O_2 + 100 μM Na_2S and (B, C): sodium sulfide (Na_2S) and trisulfide (Na_2S_3) on recombinant human ACLY's activity. Data represent values of at least n = 3 independent experiments (mean ± SEM). **p < 0.01 indicate significant differences as compared to control. (D) Representation of the thiolic (active), disulfide (inactive) and cysteine sulfenic (partially inactive) form of ACLY. Citrate complexed with the protein active site is shown as stick model colored yellow, the Cys_{293} – Cys_{748} disulfide bond is colored in grey, the thiolic and sulfenic cysteines in green and orange, respectively. The figure was drawn using PyMOL and protein three-dimensional structure was generated from PDB entry 3MWD.

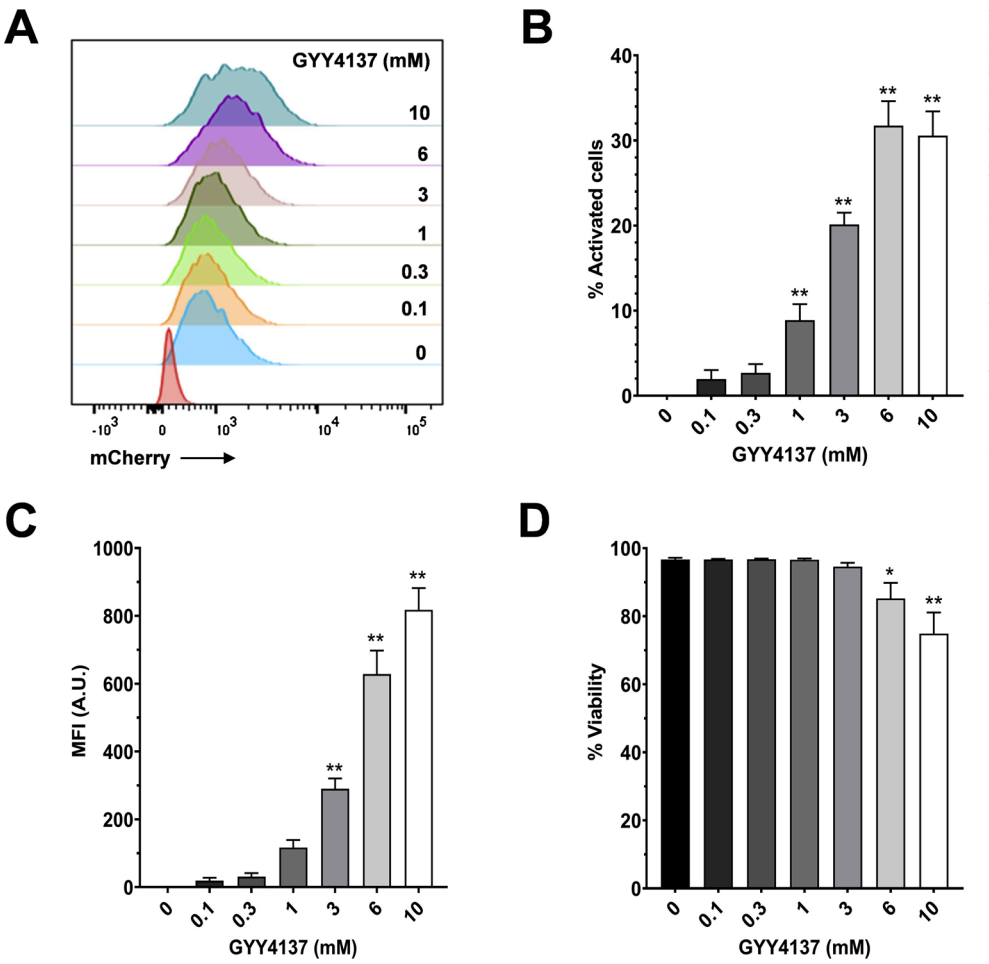

Fig. 7. H_2S activates the ACLY promoter in HCT116 cells. Flow-cytometric analysis of mCherry protein in presence of the H_2S donor GYY4137 for 48 h. The mCherry protein is produced when the ACLY promoter is activated. (A): Histogram showing the increase of the mCherry positive population in presence of increasing concentration of GYY4137. (B): Percentage of activated cells compared to control. Data are shown as mean ± SEM, n = 4, **p < 0.01 compared to control. (C): mCherry mean fluorescence intensity (MFI) compared to control. Data are shown as mean ± SEM, n = 4, **p < 0.01 compared to control. (D): HCT116 viability in presence of increasing concentrations of GYY4137. Data are shown as mean ± SEM, n = 4, *p < 0.05, **p < 0.01 compared to control.

bioenergetic) function, in line with previous findings in various colon cancer cell lines [12,13]. However, the data also show that inhibition of H_2S biosynthesis does not drive the cells into overt necrosis. Cell migration is a highly energy-dependent process. Based on these findings, we hypothesize that a suppressed cellular energetic function (in addition to induction of MET and a consequent re-establishment of cell-to-cell adhesion properties) may also have contributed to the HMPNSNE- or AOAA-induced inhibition of cell migration.

4. Discussion

The main observations and conclusions of the current report can be summarized as follows: **(1)** In HCT116 human colon cancer cells, pharmacological inhibition of H_2S-producing enzymes induces significant changes in gene and protein expression that are consistent with a shift of the cells from the mesenchymal to the epithelial phenotype (i.e. MET). Consequently **(2)** endogenous H_2S production in colon cancer cells plays a significant role in promoting EMT or maintaining the cells in a dedifferentiated (mesenchymal) phenotype. **(3)** The mechanism(s) involved in the action of H_2S in maintaining EMT relate, at least in part, in the activation of ACLY and the consequent modulation of the Wnt-β-catenin pathway, a well-known regulator of MET/EMT balance; these actions may be (at least in part) related to the maintenance of the expression Sp3, a key regulator of this pathway. **(4)** The functional consequence of the above processes is that the cancer cells, after the functional induction of MET, are less mobile and more stationary, as evidenced by a reduction in their migratory capacity. Pharmacological induction of MET, in an *in vivo* situation, would be expected to be therapeutically relevant, because it may reduce the invasive and metastatic capacity of the colon cancer cells. Direct testing of this possibility, however, remains to be performed, for instance in an *in vivo* model of metastatic colon cancer, which is beyond the scope of the current project. Nevertheless, it is interesting to note that several studies have already demonstrated that pharmacological inhibition or genetic deletion of various H_2S-producing enzymes in various forms of cancer has the capacity to reduce metastasis to the liver (in a colon cancer model) or to lymph nodes (in a prostate cancer model) [20,56,57].

What, then, is the mechanism by which endogenously (over)produced H_2S induces and/or maintains ACLY activation in HCT116 cells? In the current study we have examined three distinct mechanisms. The first one relates to a transcriptional activation of ACLY via promoter activation. This mechanism is supported by two independent sets of data. First, pharmacological inhibition of H_2S producing enzymes in HCT116 cells reduced ACLY mRNA (as well as protein) expression (Fig. 4**E, J**) and second, H_2S donation with GYY4137 showed a direct, concentration-dependent promoter activation in a promoter-reporter construct assay (Fig. 7). The concentrations of the H_2S donor used in this assay may be confusing to investigators not familiar with the H_2S pharmacology literature; although the H_2S donor was used in high micromolar or millimolar concentration range, this molecule is a slow releaser of H_2S (which, when placed in solution, releases its H_2S 'load' over several days to up to a week or longer) and therefore the actual flux and the actual local steady-state H_2S concentration is several orders of magnitude lower [58–61]. Based on direct measurements of H_2S generation from GYY4137 in various solutions *in vitro* [61,64] we estimate that GYY4137 in our experiments generated steady-state H_2S concentrations in the range of 100 nM – 10 μM. (Please also note, that the GYY4137 concentrations used in the current project are identical to

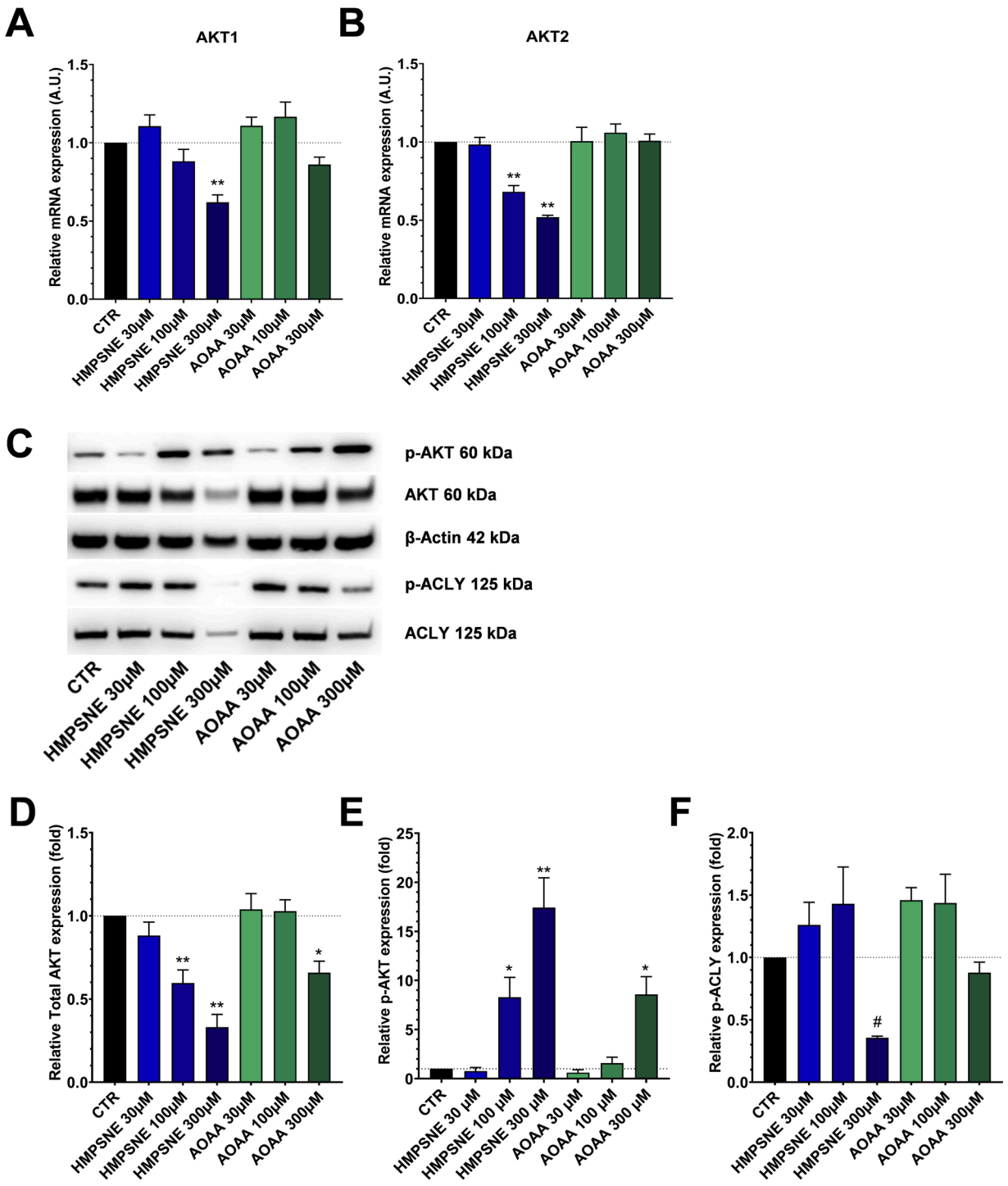

Fig. 8. Effect of pharmacological inhibitors of H_2S biosynthesis on Akt activity and ACLY phosphorylation in HCT116 cells. (A, B): Quantitative real-time PCR (qPCR) analysis of endogenous mRNA levels of Akt1 and Akt2 after incubation of HCT116 cells with HMPSNE or AOAA for 24 h. Data are shown as mean ± SEM, n = 5, **p < 0.01 compared to control. (C-F): Western blot analysis of total AKT, p-Akt and p-ACLY proteins in presence of HMPSNE or AOAA for 48 h. Data are shown as mean ± SEM of at least 3 independent experiments, *p < 0.05, **p < 0.01, #p = 0.058 compared to control.

what is typically used in cell culture experiments to generate biologically relevant levels of H_2S [62–66].).

The second potential mechanism by which H_2S could potentially increase ACLY activity is via its phosphorylation. Phosphorylation is known to increase the catalytic activity of ACLY [67–70]; there are several enzymes that have the ability to phosphorylate ACLY including Akt, the src-family kinase (SFK) Lyn and IKKβ [67–70]. According to our results, ACLY in HCT116 cells is constitutively phosphorylated on Ser455, but unexpectedly – in contrast to prior reports demonstrating that exogenous administration of pharmacological H_2S donors can increase Akt phosphorylation [51–53] – was not suppressed by pharmacological inhibition of H_2S biosynthesis; in fact, both AOAA and HMPSNE *increased* Akt phosphorylation (Fig. 8E). Nevertheless, H_2S biosynthesis inhibition (at least with the highest concentration of HMPSNE used, but not with any of the AOAA concentrations employed in our study), decreased the phosphorylation of ACLY. Since both AOAA

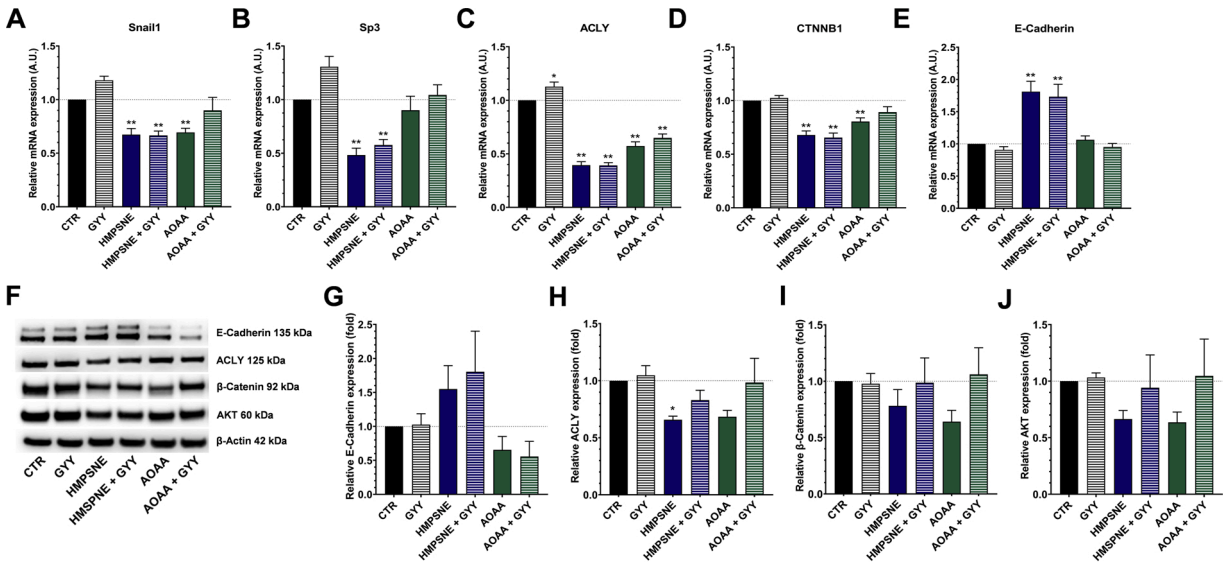

Fig. 9. Effect of the H_2S donor GYY4137 alone, or in combination with inhibitors of H_2S biosynthesis, on the expression of various H_2S mesenchymal and epithelial markers in HCT116 cells. (A-E): Quantitative real-time PCR (qPCR) analysis of endogenous mRNA levels of EMT-associated markers after incubation with HMPSNE 300 μM, AOAA 300 μM, GYY4137 300 μM alone or in combination, for 24 h. Data are shown as mean ± SEM of at least 4 independent experiments, *$p < 0.05$, **$p < 0.01$ compared to control. (F): Representative western blot of various EMT-associated markers after 24 h incubation with HMPSNE, AOAA, GYY4137, HMPSNE + GYY4137 or AOAA + GYY4137. (G-J): Numerical quantification of the expression data. Data are shown as mean ± SEM of at least 3 independent experiments, *$p < 0.05$ compared to control.

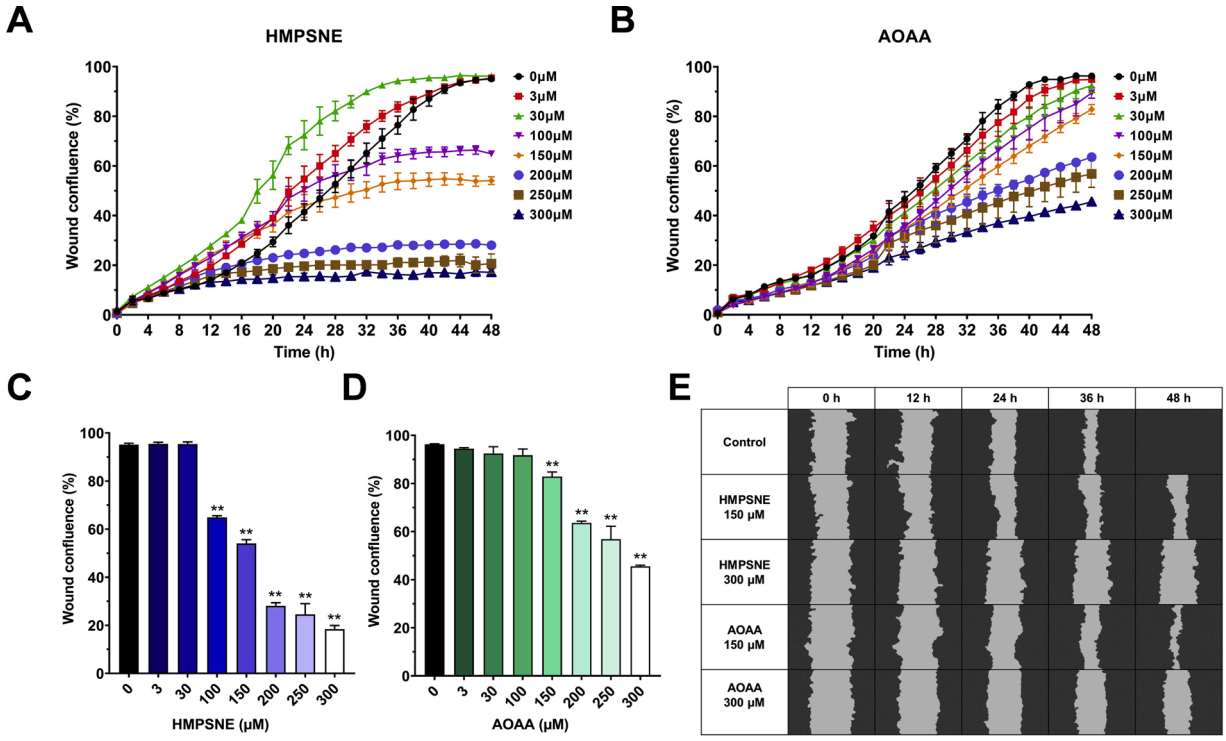

Fig. 10. Effect of pharmacological inhibitors of H_2S biosynthesis on HCT116 cell migration. (A, B) Representatives curves of wound confluence in percentage after incubation with HMPSNE or AOAA for 48 h. (C, D): Quantitative analysis of wound healing rates in presence of HMPSNE or AOAA for 48 h. Data are shown as mean ± SEM, n = 3, **$p < 0.01$ compared to control. (E): Representative tracings of migration of HCT116 cells in the presence of HMPSNE or AOAA for 48 h in the wound healing assay.

and HMPSNE were found to induce MET, but only HMPSNE was found to decrease ACLY phosphorylation, we conclude that the transcriptional regulation of ACLY by endogenous H_2S in colon cancer (as discussed in the previous paragraph) is probably more relevant than a phosphorylation-mediated activation of ACLY in the context of the regulation of the EMT/MET balance. Nevertheless, the downregulation of AKT1 and AKT2 protein expression by HMPSNE may have functional effects; in this context it is, however, interesting to note that in some cancer cells (e.g. breast cancer cells) AKT1 and AKT2 have been shown to exert differential modulatory effects on cell migration, ERK activation and MET [71]. The potential complex role of AKT isoforms in the regulation of the EMT/MET processes in colon cancer; the mechanisms

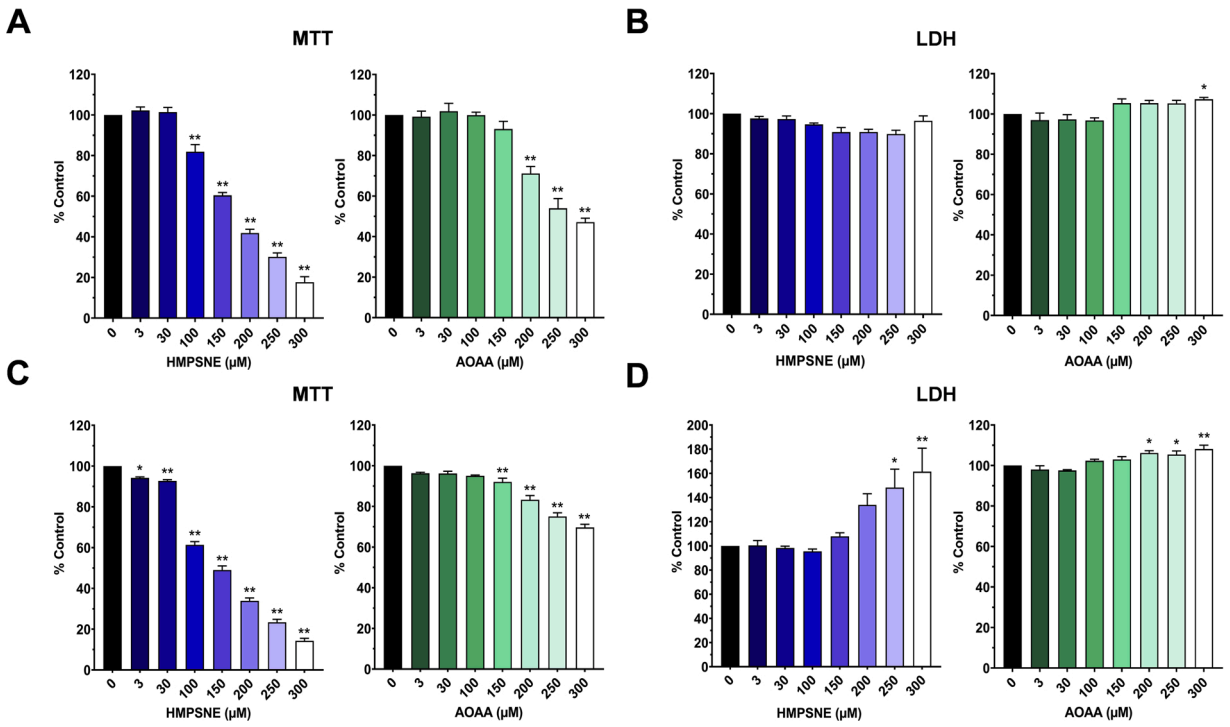

Fig. 11. Effect of pharmacological inhibitors of H_2S biosynthesis on HCT116 cell's mitochondrial activity and viability. (A): MTT assay of HCT116 cells seeded at the same ratio cells number/well surface as for the protein and RNA assays shown in Figs. 1–4 and 8. (B): Lactate dehydrogenase (LDH) activity in medium of HCT116 culture at same ratio cells number/well surface than protein and RNA assays shown in Figs. 1–4 and 8. (C): MTT assay of HCT116 cells at same ratio cells number/well surface than migration assays shown in Fig. 10. (D): Lactate dehydrogenase (LDH) activity in medium of HCT116 culture at same ratio cells number/well surface than migration assays shown in Fig. 10. Data are shown as mean ± SEM of at least 3 independent experiments, *$p < 0.05$, **$p < 0.01$ compared to control.

by which HMPSNE (and, thus, 3-MST and H_2S) modulates AKT expression, and the potential role of AKT in modulating ACLY phosphorylation, remain to be further clarified in follow-up studies.

A third, potential (indirect) mechanism by which H_2S may regulate ACLY activity is through antioxidant effects. Multiple lines of data indicate that H_2S exerts antioxidant effects in various cells. Some of these effects may be direct (reactions of H_2S with various oxidant species), although the rate constants of these reactions are fairly slow and indirect mechanisms, e.g. via upregulation of antioxidant systems is more (patho)physiologically relevant [1,72]. Irrespective of the mechanism(s) involved, the following mechanism is possible: **(a)** in cancer cells, there is an upregulation of ROS and RNS production [47,73], due to a combination of cellular events (induction of the enzymes responsible for the generation of these species, dysregulation of endogenous antioxidant systems, mitochondrial dysfunction and leakage of electrons off the respiratory chain etc.). **(b)** These events produce an increased intracellular ROS 'load', which, in turn, may lead to an inhibition of ACLY activity (the ROS-mediated inhibition of ACLY is demonstrated in our experiments when recombinant ACLY was subjected to H_2O_2 challenge *in vitro*; Fig. 6). **(c)** In a cellular environment, H_2S may protect against this inhibition, thereby maintaining the activity of ACLY.

Although H_2S failed to increase ACLY's basal activity in our cell-free assay, a reversion of the H_2O_2-mediated enzymatic inactivation was observed. Hence, in a cellular environment, H_2S may protect against this inhibition, thereby maintaining the activity of ACLY in the colon cancer cells. Conversely, the treatment with sulfane sulfurs induced a fully inhibition of this enzyme, conceivably due to formation of an internal disulfide bridge. This observation is in line with the complex and multifaceted nature of reactive sulfur compounds. Indeed, a consistent body of literature, in contrast to the supposed pro-tumoral role attributed to H_2S, report anti-cancer action of sulfane sulfur and polysulfide species, as reviewed in [37]. In this scenario, ACLY may represent an interesting model of study of the opposite effects of H_2S vs. polysulfides in cancer biology.

ACLY is an essential player in cancer cell biology: it is generally viewed as an emerging potential experimental anticancer therapeutic target [74–79]. ACLY is recognized as an essential supporter of cancer cell metabolism through the potential deprivation of cytosolic citrate, a process, which promotes glycolysis through the enhancement of the activities of PFK 1 and 2. These processes may also lead to the activation of oncogenic drivers such as PI3K/AKT which can create a positive feedforward cycle through phosphorylation (and further activation) of ACLY and the stimulation of the Warburg effect in cancer cells [74]. The role of ACLY in inducing and maintaining lipid biogenesis has also been demonstrated to play a significant role in cancer cell proliferation and progression [75]. Importantly, ACLY has been recently implicated in the regulation of EMT/MET balance; pharmacological inhibitors of ACLY have been shown to induce MET in various cancer cell types [42,67,77,75–79], a finding that is recapitulated by our current results (Fig. 5). In this context, ACLY was found to stabilize β-catenin 1 protein, and the complex, in turn, was suggested to promote β-catenin 1 translocation through cytoplasm to nucleus, subsequently promoting its transcriptional activity [77], thereby stimulating migration and invasion abilities of colon cancer cells via the mechanisms depicted in Fig. 12.

The finding that ACLY mRNA expression and activity is stimulated by H_2S, to our knowledge, has not previously been observed in the literature. Given the pathophysiological role of this enzyme in a variety of conditions, ranging from neurodegenerative and cardiovascular diseases to diabetes and obesity [79,80] the current observations may be useful to stimulate further work in these fields. The actual molecular mechanism of this action remains to be further characterized; the promoter of ACLY is well characterized [81–83] and contains many regulatory elements that may be affected by H_2S in a direct or indirect fashion.

H_2S has been previously linked to the regulation of various EMT/MET-related processes in several studies in the context of cancer, lung fibrosis or kidney disease, although many of these prior studies have

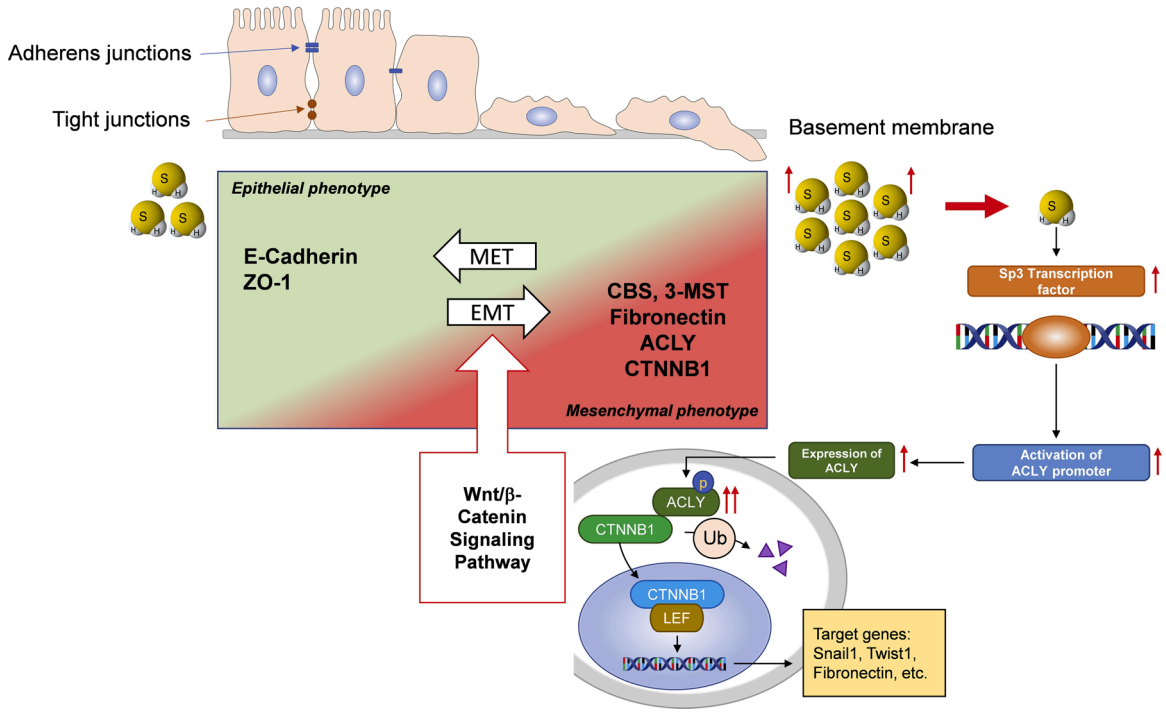

Fig. 12. Endogenous H_2S biosynthesis is involved in the maintenance of mesenchymal phenotype in HCT116 cells via the upregulation of ACLY. Accordingly, pharmacological inhibition of H_2S biosynthesis suppresses ACLY activity and induces MET. The increased H_2S production in colon cancer overexpressing H_2S enzymes leads to an increase of Sp3 transcription factor, in turn, induces ACLY mRNA transcription and ACLY protein expression. ACLY interacts with β-catenin and may block β-catenin ubiquitination leading to its accumulation in the cytoplasm which, in turn, also translocates into the nucleus. In the nucleus, β-catenin binds to LEF and activates Twist1 and Snail1 transcription factors and fibronectin expression and represses E-cadherin, promoting EMT, potentially facilitating the migration, invasion and metastasis of tumor cells.

focused on the effect of exogenous H_2S donation, as opposed to the modulation of endogenous H_2S production [24,57,84–92]. Moreover, the underlying molecular sequence of events and the potential interrelationship between these various effectors have not yet been fully characterized. Mechanistically, the findings reported in the current study have the closest relation to the work of Guo and colleagues who studied the modulatory effect of H_2S donation on the transition process of quiescent renal fibroblasts to myofibroblasts [86]. These investigators noted that exogenous H_2S administration (using the fast-acting H_2S donor NaHS) decreased the expression of α-SMA and fibronectin, and increased the expression of E-cadherin in a model of transforming growth factor-β1 (TGF-β1)-induced EMT, through the modulation of ERK-dependent and Wnt/catenin-dependent pathways, suggesting the ability of exogenously supplied H_2S to suppress the process of EMT; these effects were also reflected in the ability of exogenously administered H_2S to suppress the TGF-β1 induced migratory response in these cells [86]. The findings reported in the current study, however, are directionally opposing; in colon cancer cells, our data show that it is the inhibition of endogenous H_2S producing enzymes (i.e. the reduction of cellular H_2S levels) which can induce MET (i.e. suppress EMT), and this is reflected by an inhibitory effect of HMPSNE or AOAA to inhibit cancer cell migration. The difference between the results of the two studies may be due to the well-known bell-shaped concentration-response of H_2S [1,23], where different concentrations of H_2S can exert opposing cellular responses on proliferation, bioenergetics, viability and many additional parameters.

Directionally, the current results are most consistent with the recently published report of Wang and colleagues, which – similar to our study – evaluated the effect of inhibition of endogenous H_2S production on various parameters (including MET/EMT parameters) in non-small-cell lung cancer lines, and reported that inhibition of CBS and CSE suppressed E-cadherin and vimentin expression, and suppressed cell proliferation, all of which is consistent with the concept that endogenous H_2S production promotes EMT, while inhibition of H_2S production can reverse this process and can induce MET [91]. Moreover, forced expression of CBS into NCM356 cells (a slowly proliferating non-tumorigenic human colonic epithelial cell line) upregulated multiple genes related to increased extracellular matrix, cell adhesion, and epithelial-to-mesenchymal transition [57].

Throughout the current study, we preferred to refer to HMPSNE as an "inhibitor of endogenous H_2S biosynthesis" rather than a "3-MST inhibitor", because the results shown in Fig. 1 indicate that this compound can induce the downregulation of 3-MST as well as CBS. The mechanism of this action remains to be evaluated; it may be related to a potential positive feedforward cycle between H_2S production and the transcriptional or post-translational regulation of various H_2S-producing enzymes. The underlying molecular mechanisms remain to be further investigated. Nevertheless, the practical implication of these findings is that when applying HMPSNE to HCT116 cells (at 300 μM), H_2S generation from both 3-MST and CBS is inhibited. In contrast, the lower concentration of HMPSNE (30 and 100 μM) will primarily affect 3-MST (although through a combination of inhibition of its expression and activity). In contrast, AOAA does not inhibit the expression of any of the H_2S-producing enzymes, but it is well known that it inhibits the catalytic activity of both CBS and CSE, as well as the activity of GOT/cysteine aminotransferase (which feeds into the 3-MST pathway through the synthesis of its substrate 3-MP) [1,8]. Thus, both HMPSNE and AOAA have the potential to inhibit both CBS- and 3-MST-associated H_2S biosynthesis in a cellular system and AOAA has the additional potential to inhibit CSE-associated H_2S biosynthesis as well. Moreover, AOAA inhibits many other PLP-dependent enzymes that are not related to H_2S pathways (for example, GABA-T or GOT) [8]. It is unclear if any of these enzymes regulate the MET/EMT balance, although some of these enzymes (e.g. GOT1) have been implicated in cancer cell bioenergetic processes. The above data illustrate the complexities of working with pharmacological modulators of the H_2S-producing pathways. In some

cases, the selectivity of the pharmacological tools used can be tested by evaluating if the cellular action of a H_2S biosynthesis inhibitor can be reversed by H_2S donation (i.e. probing for a functional antagonism); and, in the current study, this approach was also employed, using the H_2S donor GYY4137, which, indeed, attenuated some of the MET-inducing capacity of the H_2S biosynthesis inhibitors (Fig. 9). However, these experiments are also somewhat difficult to interpret, because H_2S donors have their own effects on the MET/EMT processes, and H_2S exerts a bell-shaped concentration-response in most biological systems [1,6].

In addition to modulating MET/EMT-related processes, there are multiple mechanisms by which endogenous H_2S production modulates the biology of cancer cells. H_2S does not have a single unique traditional "receptor", but rather affects multiple targets, through the modulation of redox processes, post-translational modifications (e.g. cysteine modification, via a process termed sulfhydration) [1–8]. Undoubtedly, endogenously produced H_2S in cancer cells, above and beyond actions on Akt and ACLY (as shown in the current report) can (and will) affect a wide range of signalling processes (many of which have the potential to modulate MET/EMT balance), a variety of aerobic and anaerobic processes (influencing cellular bioenergetics, which, on its own, also has the potential to influence MET/EMT processes). Clearly, the complex interaction of the various signalling and bioenergetic systems regulating MET/EMT and the various roles of H_2S in modulating these processes remain to be investigated in future studies.

As with all pharmacological agents, non-specific and off-target effects are always possible. In fact, as one uses and characterizes novel agents in various contexts, additional effects and targets are commonly discovered. This phenomenon is poignantly stated in the old pharmacologists' proverb "The specificity of drugs decreases over time" [93]. For instance, we have employed the ACLY inhibitor SB204990 in some of the current work. The selectivity and specificity of this compound has not yet been fully characterized. Nevertheless, there are dozens of reports in the literature that utilize this compound and rely on it for various conclusions. Some of these reports are relevant for the current study. For instance, there are multiple reports, using this compound, that connect ACLY to the regulation of Sp1 and Sp3 [43,81,82] and ACLY to the regulation of differentiation and/or EMT/MET [76,78,94–97]. These reports indirectly support the conclusion of the current report.

This non-specificity issue should also be discussed with regard to with HMPSNE, a recently discovered (and therefore, only relatively incompletely characterized) inhibitor that the current study relies on. While HMPSNE does not directly inhibit the two other major H_2S-producing enzymes [9,32], the current report shows that it can, in fact, interfere with the expression of some of these enzymes. There are, also, transcriptional (most likely compensatory) effects that may occur when 3-MST is experimentally inhibited or downregulated. For example, 3-MST silencing was reported to induce the upregulation of rhodanese in the mouse liver [98]. Moreover, off-target actions on other enzymes and/or transcription factors cannot be excluded. At higher concentrations, the decrease in cell viability noted in some of the current experiments, may also be, in part, related to off-target effects and/or non-specific cytotoxicity. Non-H_2S-pathway-related actions may, in fact, explain why the H_2S donor GYY4137 was only partially effective in reversing the actions of HMPSNE on MET/EMT-related parameters in the current study. Additional or non-specific actions of HMPSNE remain to be further characterized.

5. Conclusions

In conclusion, pharmacological inhibition of H_2S-producing enzymes induces significant changes in gene and protein expression that are consistent with a shift of the cells from the mesenchymal to the epithelial phenotype; the underlying mechanism, at least in part, relates to the activation of ACLY and the modulation of the Wnt-β-catenin pathway. Based on the current results, inhibition of endogenous H_2S biosynthesis in cancer cells has the potential to pharmacologically induce MET, which would be expected to contribute to the anti-cancer (e.g. anti-metastatic) potential of these inhibitors. Further studies (including *in vivo* studies evaluating the effect of H_2S biosynthesis inhibitors on the MET/EMT balance and on colon cancer metastasis) should be conducted to expand on the results of the current work. Moreover, the modulatory effect of H_2S on ACLY reported in the current study should stimulate further work in areas above and beyond cancer where ACLY has been implicated in a pathophysiological context; these areas include, for instance, various forms of cardiovascular and metabolic disease.

Ethics approval and consent to participate

The current report does not contain human studies.

Consent for publication

The current report does not contain human studies or studies that would require consenting.

Availability of data and material

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Authors' contributions

All authors contributed to various parts of the study design, method development, experimentation, data interpretation and writing of the manuscript. C.S. and N.D. were responsible for the planning and coordination of the experiments. K.A. and C.S. drafted the first version of the manuscript and C.S. finalized the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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