Article

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Inhibition of Mitochondrial Bioenergetics by Esterase-Triggered COS/H_2S Donors

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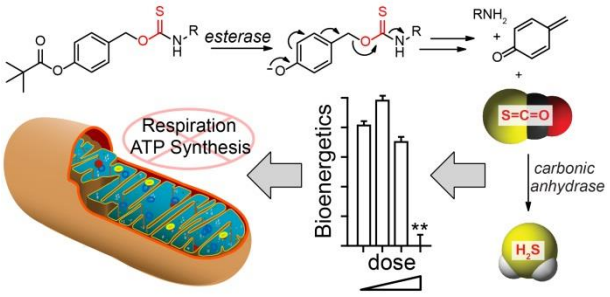
Abstract

Hydrogen sulfide (H_2S) is an important biological mediator, and synthetic H_2S donating molecules provide an important class of investigative tools for H_2S research. Here we report esterase-activated H_2S donors that function by first releasing carbonyl sulfide (COS), which is rapidly converted to H_2S by the ubiquitous enzyme carbonic anhydrase (CA). We report the synthesis, self-immolative decomposition, and H_2S release profiles of the developed scaffolds. In addition, the developed esterase-triggered COS/H_2S donors exhibit higher levels of cytotoxicity than equivalent levels of Na_2S or the common H_2S donors GYY4137 and AP39. Using cellular bioenergetics measurements, we establish that the developed donors reduce cellular respiration and ATP synthesis in BEAS 2B human lung epithelial cells, which is consistent with COS/H_2S inhibition of cytochrome c oxidase in the mitochondrial respiratory chain although not observed with common H_2S donors at the same concentrations. Taken together, these results may suggest that COS functions differently than H_2S in certain biological contexts or that the developed donors are more efficient at delivering H_2S than other common H_2S-releasing motifs.

Table of Contents Image



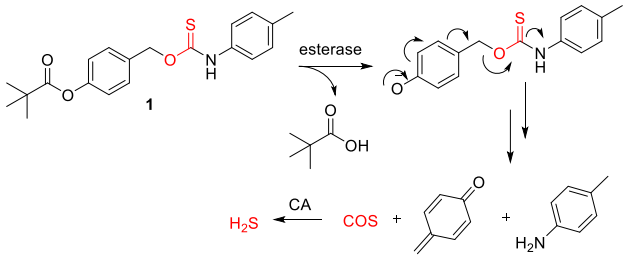
Introduction

Hydrogen sulfide (H_2S) is an endogenously-produced signaling molecule that plays critical roles in mammalian biology. Physiological sulfide levels are tightly regulated, and enzymatic production derives primarily from cysteine and homocysteine metabolism by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate transferase (3-MST).[1] Continually broadening in scope, H_2S plays important roles in cardioprotection,[2] inflammation,[3] vasodilation,[4] as well as other processes. Because of this diversity, there is significant interest in developing both research and therapeutic strategies for regulating sulfide levels in different biological contexts.[5] At the biological level, inhibition, knockout, and/or overexpression of H_2S-producing enzymes can be used to modulate endogenous H_2S levels. Alternatively, chemical approaches using exogenous H_2S donation often provide a more convenient and broader approach. For example, inorganic sulfide salts, such as NaSH and Na_2S provide a convenient source of sulfide, however, the large and instantaneous bolus of sulfide released from such salts is often rapidly oxidized and fails to mimic the continuous H_2S release associated with enzymatic synthesis, thus limiting the utility of these exogenous H_2S sources.[6] Because of these limitations, developing small molecules that undergo specific reactions to release H_2S in a controlled manner to more closely mimic well-regulated enzymatic production remains an important goal.[7, 8] Aligned with these needs, naturally-occurring polysulfides such as diallyl trisulfide (DATS), which releases sulfide upon reaction with thiols,[9, 10] hydrolysis-based H_2S donors such as GYY4137 and ADT-OH,[11, 12] which slowly produce H_2S in water, and a palette of thiol-activated donors, have all been developed.[7, 13] Although such donors have been used in applications ranging from probe development to *in vivo* studies, key challenges include low H_2S donation efficiencies from hydrolysis-based donors and thiol consumption and redox perturbation from polysulfides and thiol-activated donors.

As a step toward addressing these challenges, our group recently developed a new H_2S donation strategy based on the intermediate release of carbonyl sulfide (COS), which is rapidly hydrolyzed to H_2S by the ubiquitous enzyme carbonic anhydrase (CA).[14] By leveraging well-established work on the triggered decomposition of benzylic carbamates commonly used in pro-drug and fluorophore release strategies,[15-17] we demonstrated that self-immolative thiocarbamates can be readily engineered to respond to different stimuli and release COS/H_2S. Specifically, cleavage of an analyte-specific protecting group unmasks a phenol, which then undergoes a self-immolative decomposition to release COS. Importantly, this strategy enables significant control over H_2S donation depending on the trigger and provides access to important carbamate control compounds, which release CO_2/H_2O rather than COS/H_2S. Furthermore, although COS hydrolysis by CA is rapid, some evidence suggests that COS itself may have unique roles in chemical biology, as evidenced by COS detection in the headspace above *ex vivo* porcine coronary arteries and by increased COS levels in exhaled breath from patients

with cystic fibrosis, organ rejection, or liver disease.[18-21] Therefore, although self-immolative thiocarbamates have recently been shown to be useful as responsive H_2S donors, they may also provide a platform for future studies of COS chemical biology.[22] Following our initial report of caged COS/H_2S release, we have expanded this approach to include COS/H_2S donors activated by reactive oxygen species (ROS),[23] bio-orthogonal "click-and-release" donors based on *trans*-cyclooctene / tetrazine click chemistry,[24] and light-activated COS/H_2S donors.[25] Related COS-based donors based on nucleophilic addition to small molecule and polymeric cyclic N-thiocarboxyanhydrides resulting in the release of COS have also emerged recently.[26] Although the nucleophile-based donors provide slow H_2S release akin to enzymatic synthesis, the required consumption of cellular nucleophiles, such as thiols, to release COS/H_2S is similar to other thiol-activated donors.

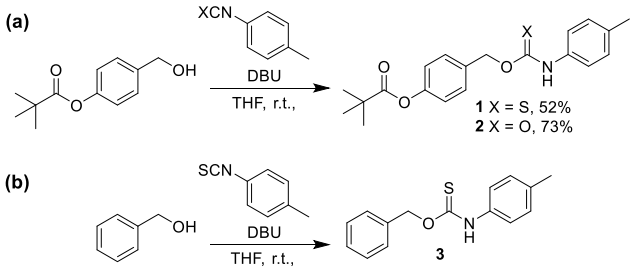
To address these limitations, we viewed that installation of an ester as the triggering group to thiocarbamate-based platforms would provide access to slow-release COS/H_2S donors upon ester cleavage by intracellular esterases that do not require consumption of cellular nucleophiles for activation (Scheme 1). The strategy of using intracellular esterases to cleave esterified moieties on small molecules is a well-established method used extensively to impart cellular trappability, improve membrane permeability, and in the activation of caged pro-drugs and other biological payloads.[27-30] Additionally, activation by intracellular esterases eliminates the consumption of cellular nucleophiles for activation. Consistent with this design strategy, the Wang group recently reported esterase-triggered H_2S donors utilizing a trimethyl lock unmasking of caged thioacids and demonstrated their anti-inflammatory effects.[31] Similarly, during the preparation of this manuscript an esterase-activated *S*-alkyl thiocarbamate COS/H_2S donor was reported, but detailed biological applications were not investigated.[32] Here, we report the design, evaluation, and application of esterase-activated COS/H_2S donors and provide the first insights into the influence of COS donors on cellular toxicology and mitochondrial bioenergetics.



Scheme 1. Design of esterase-triggered self-immolative COS/H_2S donors and proposed COS/H_2S release mechanism.

Results and Discussion

Triggered COS/H_2S donors benefit from a high degree of modularity and facile introduction of different triggering functionalities. To access an esterase-functionalized thiocarbamate, we first prepared 4-pivaloyl benzyl alcohol in one step,[33] which was then treated with *p*-tolylisothiocyanate in the presence of DBU to afford donor **1** in moderate yield (Scheme 2). The choice of pivalic acid to construct the donor was due to the increased hydrolytic stability of pivalic ester over other ester protecting groups, and 4-methyl aniline was used as the payload of the donor to provide a simple spectroscopic handle. The analogous carbamate control **2** was prepared in good yield by treating 4-pivaloyl benzyl alcohol with *p*-tolylisocyanate and DBU. Carbamate **2** provides an important control compound that undergoes the same self-immolative decomposition as **1**, but releases CO_2 instead of COS, thus enabling separation of the action of COS/H_2S from that of the organic byproducts formed after donor activation. Additionally, we also prepared triggerless thiocarbamate **3** as a control compound to test the thiocarbamate stability toward esterases.



Scheme 2. (a) Synthesis of H_2S donor **1**, CO_2-releasing analogue **2**, and triggerless thiocarbamate **3**.

The mechanism of the cascade decomposition of similar benzyl carbamates has established previously to release CO_2, the amine payload, and a quinone methide intermediate.[15-17, 34] The final fate of the electrophilic quinone methide is dependent on which nucleophiles are present in the system. For example, it is often scavenged by water to form 4-hydroxy benzylalcohol, or can be scavenged by biological nucleophiles to afford thiol or amine-derived conjugates.[35, 36] To confirm similar reactivity in the present system, we first demonstrated that the addition of porcine liver esterase (PLE) initiated a self-immolative decomposition reaction as anticipated. After stirring **1** (14 mM) with PLE (28 U/mL) in PBS (pH 7.4) with 10% DMSO for 48 hours, the organic layer was extracted and analyzed by NMR spectroscopy and mass spectrometry (Figure 1). Loss of the benzylic and thiocarbamate N-H protons at ~5.5 ppm and 11.1 ppm, respectively, in the ^{1}H NMR spectrum of the reaction mixture confirmed self-immolation (Figure 1a). As further evidence of the triggered cascade decomposition, the broad NMR resonances characteristic of O-alkyl thiocarbamates, which is due to the slow rotation around the thiocarbamate moiety on the NMR time scale, sharpen

significantly upon ester cleavage with PLE. Additionally, the $^{13}C\{^{1}H\}$ NMR spectrum after treatment with PLE (Figure 1b), clearly showed the loss of the C=S and benzylic carbon resonances at 185 and 70 ppm, respectively. Both the ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra also show the formation of new aromatic species corresponding to several products, which were further characterized using mass spectrometry. Because the generated *p*-quinone methide is electrophilic, we expect that it would be scavenged by biological nucleophiles, such as thiols, amines, or water, under physiological conditions. Given that similar scaffolds have been reported previously for drug and fluorophore delivery, no significant biological interference is anticipated from these side-reactions of the byproducts alone. The mass spectrum acquired of the reaction mixture after treatment with PLE clearly showed formation of *p*-toluidine (m/z: 107.0) and the product corresponding to *p*-toluidine trapping of the *o*-quinone methide intermediate (m/z: 214.1) as expected (See Figure S1 for MS data and formation details). Taken together, the NMR spectroscopy and mass spectrometry studies confirm self-immolation of the scaffold as described in Scheme 1.

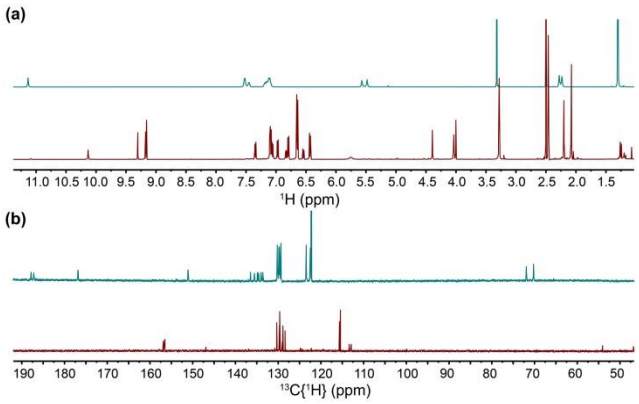


Figure 1. (a) ^{1}H NMR spectrum of **1** before (top) and after (bottom) treatment with PLE. (b) $^{13}C\{^{1}H\}$ NMR spectrum of **1** before (top) and after (bottom) stirring with PLE.

With esterase-triggered donors in hand, we first confirmed that **1** was stable in aqueous solution in the absence of esterase. We next verified that addition of **1** to pH 7.4 PBS buffer containing physiologically relevant levels of CA (25 µg/mL) did not result in H_2S generation when monitored using an H_2S-selective electrode, confirming that the esterase does not cleave thiocarbamates directly (Figure 2a). Further control experiments using the parent benzyl thiocarbamate **3**, which lacks the ester trigger, confirmed that the benzyl thiocarbamate moiety is not cleaved directly by PLE. Having confirmed the stability of the donor platform prior to activation, we next treated **1** with 1 U/mL PLE in the presence of CA and observed immediate H_2S release (Figure 2a). As expected, increasing the PLE concentration to 20 U/mL under otherwise identical conditions resulted in significantly faster H_2S release. Additionally, treatment of **1** with acetazolamide (AAA), a known CA inhibitor, significantly reduced the rate of H_2S production, confirming that CA is necessary for COS conversion to H_2S under

the reaction conditions. Supplementing the H_2S electrode measurements, we also confirmed H_2S release from **1** using an H_2S-responsive fluorescent probe (Figure 2b). Consistent with the electrode data, incubation of 50 μM **1** with 5 μM MeRho-Az[37] in the presence of CA and 1 U/mL PLE resulted in a fluorescence turn-on consistent with H_2S release. We also attempted to obtain fluorescent live cell images by incubating **1** in BEAS 2B cells with MeRho-Az, but the high cytotoxicity of **1** and limited permeability of MeRho-Az in BEAS 2B cells limited our ability to obtain high quality images. As a whole, these data demonstrate that the thiocarbamate donors are stable until activated by esterases and release H_2S in a COS-dependent manner.

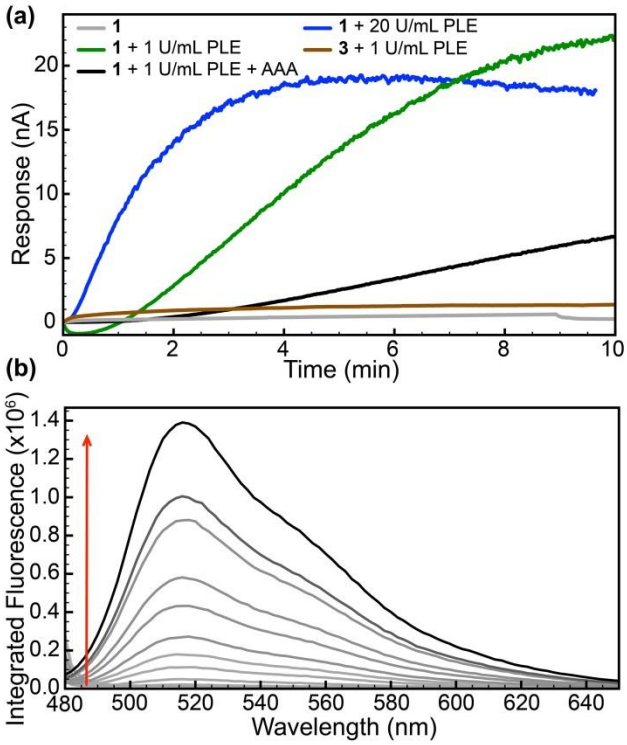


Figure 2. (a) H_2S release from **1** in PBS (pH 7.4) in the presence of CA (25 μg/mL) with 1 U/mL (green trace) or 20 U/mL (blue trace) PLE. Addition of CA inhibitor AAA (2.5 μM) significantly reduces H_2S release (black trace). No H_2S release was observed from **1** in the absence of esterase (grey trace) or from thiocarbamate **3** lacking an ester trigger (brown trace). (b) Detection of H_2S released from **1** with the H_2S-responsive probe MeRho-Az. Conditions: 50 μM 1, 5 μM MeRho-Az, 25 μg/mL CA, 1 unit/mL PLE, 3 mL PBS (pH 7.4). 37 °C, λ_{ex}= 476 nm, λ_{em}= 480-650 nm.

We next investigated the cytotoxicity of **1**-**3** on BEAS 2B, human lung epithelial cells, by measuring the reduction of a tetrazolium compound (MTT) to formazan by metabolically active cells, and by measuring the release of lactate dehydrogenase (LDH) due to permeability of the plasma membrane, which is a sign of necrotic cell death. BEAS 2B cells exhibit low expression of all three canonical H_2S-producing enzymes, CBS, CSE, and 3-MST.[38] To provide suitable comparisons with commonly-used synthetic donors, we first obtained comparable cytotoxicity data for known H_2S

donors GYY4137, which does not localize in the mitochondria, and also with mitochondrially-targeted AP39 (Figure 3a,b).[39] When compared to the DMSO vehicle, GYY4137 showed no significant cytotoxicity up to 10 μM using either the MTT or LDH assay. Similarly, AP39 did not exhibit cytotoxicity at 10 μM nor at lower concentrations, indicating that neither of these H_2S donors are significantly cytotoxic. Additionally, no cytotoxicity was observed when BEAS 2B cells were treated with up to 10 μM of Na_2S, a direct source of sulfide (See Figure S2 for MTT and LDH assays with Na_2S). By contrast, 10 μM of **1** resulted a significant decrease cell viability and increase in LDH levels, which was not observed for control compounds **2** or **3** (Figure 3c,d). The lack of cytotoxicity of control compound **2** suggests that the mechanism of cytotoxicity does not result from the formation of the electrophilic *p*-quinone methide intermediate[40] because this species is formed upon activation of both donor **1** and control compound **2**. Similarly, compound **3** does not reduce cell viability, confirming that the observed cytotoxicity of **1** relies on triggering by cellular esterases and is not a result of the thiocarbamate scaffold itself. We note that although the extensive cytotoxicity data reported is only in BEAS 2B cells, preliminary cytotoxicity evaluations in HeLa cells additionally showed increased cytotoxicity of donor **1** below 100 μM with no cytotoxicity observed for control compound **2** (See Figure S3 for viability evaluation in HeLa cells). Importantly, the esterase-triggered COS/H_2S donor **1** provides a significantly different toxicological profile from other commonly-used H_2S donors.

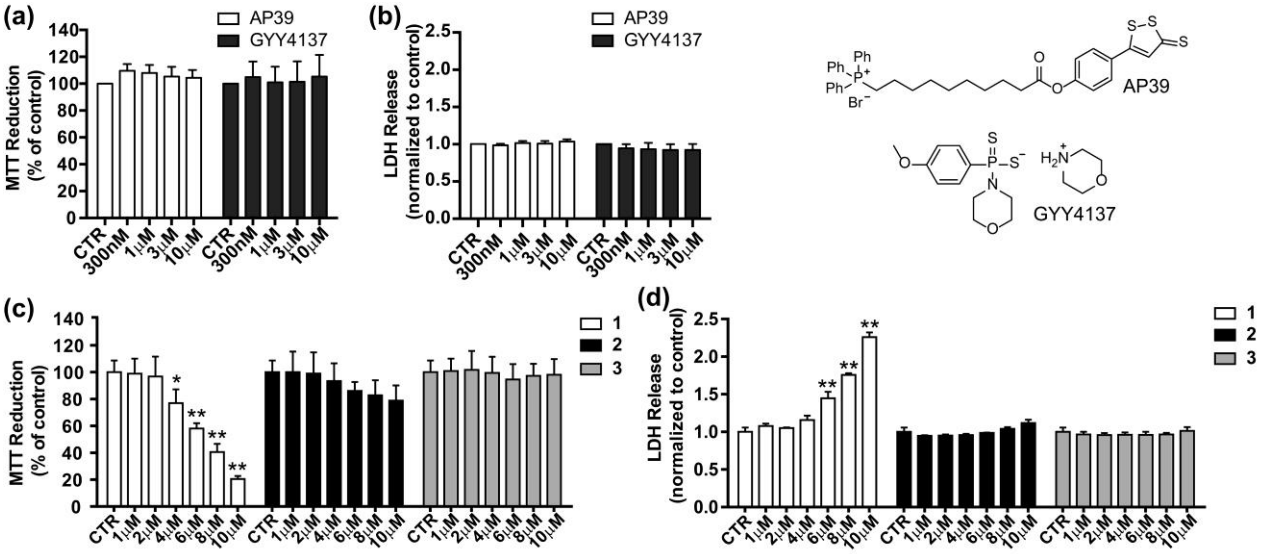


Figure 3. Cell viability studies of AP39, GYY4137, and **1**-**3** in BEAS 2B using the (a,c) MTT and (b,d) LDH cell viability assays. The chemical structures of AP39 and GYY4137 are shown in the upper right panel.

To investigate the underpinnings of the increased cytotoxicity of **1**, and because H_2S is a well-known inhibitor of mitochondrial cytochrome c oxidase,[41] we analyzed the mitochondrial respiration of BEAS 2B cells treated with **1**-**3** using an Extracellular Flux Analyzer.[42] We chose this assay because H_2S is well known to impact mitochondrial respiration, even if the H_2S is not delivered directly to the

mitochondria, and because COS has been suggested to inhibit the mitochondrial respiratory chain. An Extracellular Flux Analyzer measures the cellular oxygen consumption rate (OCR) as an indicator of mitochondrial respiration in real time. Using this technique, we were able to probe three key parameters of mitochondrial function upon exposure to compounds **1-3** as well as AP39 and GYY4137 through sequential addition of oligomycin (an ATP synthase inhibitor), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (a mitochondrial uncoupler), and a combination of rotenone and antimycin A (complex I and complex III inhibitors, respectively).[43] As expected, incubation of BEAS 2B cells with increasing concentrations of **1** for 24 hours negatively affected all major bioenergetics parameters, namely oxygen consumption linked with basal respiration, maximal respiration, and ATP synthesis (Figure 4a-f). These reductions are consistent with known inhibitory effects of H_2S on cellular bioenergetics, primarily through inhibition of mitochondrial cytochrome c oxidase (Complex IV). By contrast, control compound **2** did not negatively impact the measured bioenergetics parameters, but rather resulted in increases in basal respiration and ATP synthesis. Compound **3** had no effect on basal respiration or ATP synthesis, and only slightly decreased maximal respiration at 10 μM. To better compare these results with known H_2S donors, we also analyzed the mitochondrial respiration of BEAS 2B cells incubated with AP39 and GYY4137 under the same conditions (Figure 4g-i). We failed to observe significant inhibition of cellular bioenergetics at the observed concentrations, raising the possibility that the inhibitory effects of **1** may not be from H_2S release alone, but could also be due to direct COS inhibition of cytochrome c oxidase,[44, 45] which has been reported previously but received less scrutiny than direct H_2S inhibition. Our previous investigations with ROS-activated[23] and "click-and-release"[24] COS/H_2S donors did not reveal donor cytotoxicity below 100 μM, suggesting that the observed cytotoxicity from **1** may be either specific to lung epithelial cells or may be amplified by preferential release in certain cellular locales that enable efficient interaction with the mitochondrial respiratory chain.

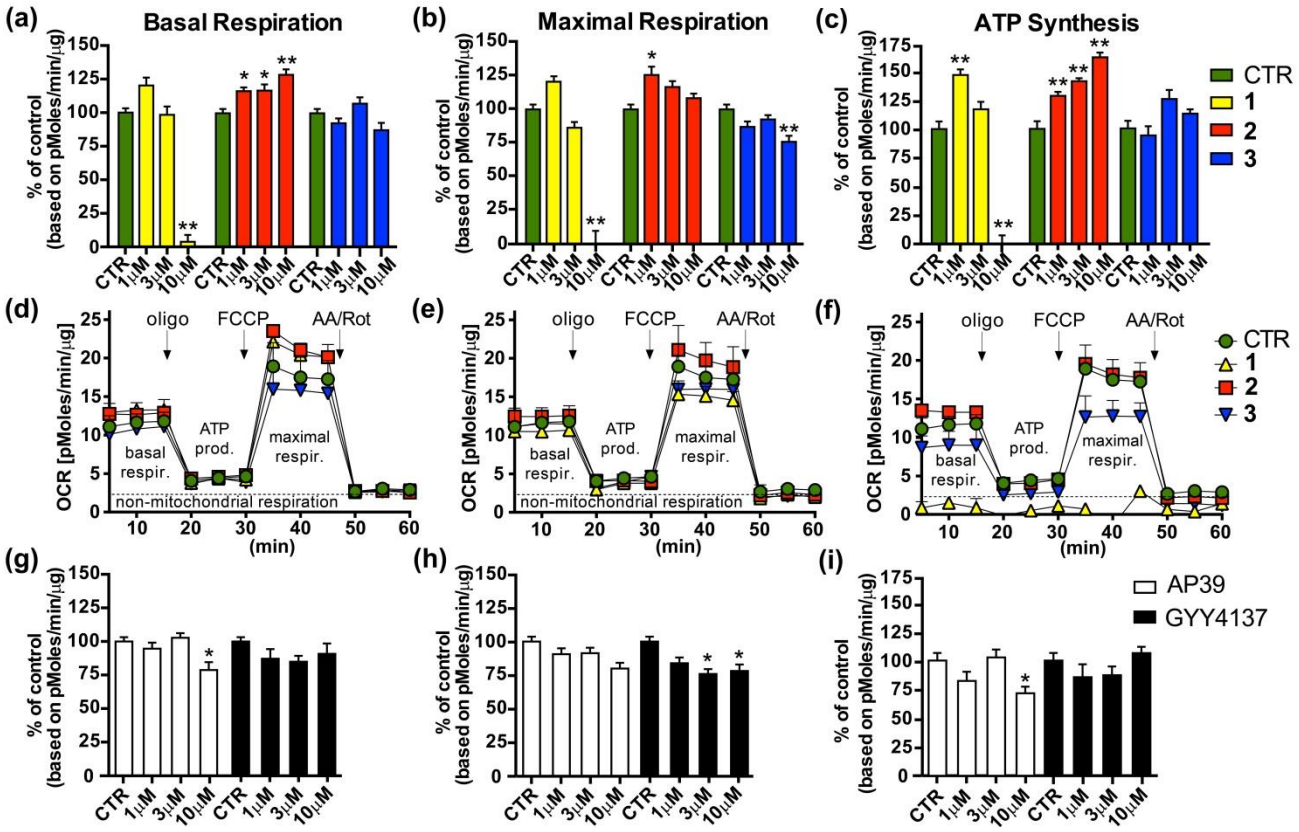


Figure 4. Cellular bioenergetics analysis including (a) basal respiration, (b) maximal respiration, and (c) ATP synthesis in BEAS 2B cells upon addition of 1-10 μM of **1**-**3** as well as full bioenergetics data at (d) 1 μM, (e) 3 μM, and (f) 10 μM. For comparison, data was additionally collected for (g) basal respiration, (h) maximal respiration, and (i) ATP synthesis of BEAS 2B cells incubated with AP39 and GYY4137. Abbreviations: oligo: oligomycin, FCCP: carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, AA/Rot: antimycin A and rotenone. Values represent the means ± SEMs from three independent biological experiments each with five technical replicates. The values that are significantly different by Student's t test are indicated by asterisks as follows: **, $P < 0.01$; *, $P < 0.05$

In conclusion, we have designed an easy-to-access, esterase-triggered COS/H_2S donor and shown that it is rapidly activated by esterases to generate H_2S *in vitro* using isolated PLE. Using toxicity assays and bioenergetics measurements, we demonstrated that the increased cytotoxicity of **1** is due inhibition of mitochondrial respiration, whereas carbamate control compound **2** and triggerless thiocarbamate **3** failed to negatively perturb normal bioenergetics. Using mitochondrial-targeted AP39 and the non-targeted donor GYY4137, we confirmed that the disruption of cellular bioenergetics observed from **1** is significantly different than is seen with other H_2S donors, potentially suggesting that either the efficiency of H_2S release, specific localization of the esterase-triggered scaffold, or direct inhibition of mitochondrial respiration by COS itself is responsible for the observed cytotoxicity. As a whole, the reported esterase-cleaved donor provides a slow-release method of COS/H_2S release in cellular environments. Further work investigating the impacts of H_2S/COS donors on cellular

bioenergetics and applications toward harnessing the selective cytotoxicity of these donor motifs is ongoing in our labs and will be reported in due course.

Methods

Materials and Methods. Reagents were purchased from Sigma-Aldrich or Tokyo Chemical Industry (TCI) and used as received. 4-Pivaloyl benzyl alcohol was synthesized as previously reported.[33] Spectroscopic grade, inhibitor-free THF was deoxygenated by sparging with argon followed by passage through a Pure Process Technologies solvent purification system to remove water. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used as received. Silica gel (SiliaFlash F60, Silicycle, 230-400 mesh) was used for column chromatography. Column chromatography was performed using a Biotage Isolera One automatic flash chromatography purification system. ^{1}H and $^{13}C\{^{1}H\}$ spectra were recorded on a Bruker 600 MHz instrument. Chemical shifts are reported in ppm relative to residual protic solvent resonances. H_2S electrode data were acquired with a World Precision Instruments (WPI) ISO-H2S-2 sensor connected to a TBR4100 Free Radical Analyzer. All air-free manipulations were performed under an inert atmosphere using standard Schlenk techniques or an Innovative Atmospheres N_2-filled glove box.

H_2S Electrode Materials and Methods. Phosphate buffered saline (PBS) tablets (1X, CalBioChem) was used to make buffered solutions (PBS, 140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) in Millipore water. Buffer solutions were sparged with N_2 to remove dissolved oxygen and stored in an N_2-filled glovebox. Carbonic anhydrase (CA) from bovine erythrocytes (≥3,500 W/A units/mg) was obtained from Sigma Aldrich and a 10 mg/ mL CA stock solution was prepared in deoxygenated buffer (PBS, 140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) in a glovebox. The stock solution was stored under nitrogen at 4 °C and warmed to room temperature immediately before use. Stock solutions of porcine liver esterase (PLE) and acetazolamide (AAA) were prepared under N_2 with degassed buffer (PBS, pH 7.4) immediately prior to use. Thiocarbamate and carbamate stock solutions were prepared in an N_2-filled glovebox in DMSO and stored at -25 °C until immediately before use.

General Procedure for H_2S Electrode Experiments. Scintillation vials containing 20.00 mL of phosphate buffer (140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) were prepared in an N_2-filled glovebox. A split-top septum cap was placed on the vial after probe insertion. The WPI electrode was then inserted into the vial and the measured current was allowed to equilibrate before starting the experiment. With moderate stirring, the CA stock solution (50 µL, 25 µg/mL) was injected, followed by subsequent injections of thiocarbamate donor, carbamate control, PLE, or AAA.

MTT assay. The MTT assay was performed as reported.[1] Briefly, BEAS 2B cells were incubated with vehicle (DMSO), **1-3** at the desired concentration for 24 hours, then 3-(4,5-dimethyl-2-thiazolyl)-

2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/mL and cells were cultured at 37 ºC for 1 hour. The cells were washed with PBS and the formazan dye was dissolved in isopropyl alcohol. The amount of converted formazan dye was measured at 570 nm with background measurement at 690 nm on a Powerwave reader (Biotek).

LDH assay. Lactate dehydrogenase (LDH) release was determined as a cytotoxicity assay, a measurement of necrotic cell death, as described previously.[46] Briefly, 30 µL of supernatant was saved before addition of MTT and mixed with 100 µL of freshly prepared LDH assay reagent to reach final concentrations of 85 mM lactic acid, 1040 mM nicotinamide adenine dinucleotide (NAD+), 224 mM *N*-methylphenazonium methyl sulfate (PMS), 528 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and 200 mM Tris (pH 8.2). The changes in absorbance were read kinetically at 492 nm for 15 min (kinetic LDH assay) on a monochromator-based reader (Powerwave HT, Biotek) at 37 ºC. LDH activity values are shown as V_{max} for the kinetic assays in mOD/min.

Bioenergetic analysis in cultured cells. The XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure bioenergetic function as described previously.[46] Briefly, BEAS 2B cells were incubated with vehicle (DMSO), **1-3** (1, 3, 10 µM) for 24h. Oxygen consumption rate (OCR) after oligomycin (1.5 µg/mL) was used to assess ATP production rate and OCR after FCCP (0.5 µM) to assess maximal mitochondrial respiratory capacity. Antimycin A (2 µg/mL) and rotenone (2 µM) were used to inhibit the flux of electrons through complex III and I, to detect residual non-mitochondrial OCR, which is considered to be due to cytosolic oxidase enzymes.

Synthesis

O-(4-pivaloylbenzyl)-*N*-(*p*-tolyl)thiocarbamate (**1**). 4-Pivaloylbenzyl alcohol (131 mg, 0.668 mmol) and *p*-tolylisothiocyanate (107 mg, 0.717 mmol) were combined in anhydrous THF (3 mL) and DBU (125 µL, 0.835 mmol) was added dropwise. The reaction mixture was stirred under nitrogen at room temperature for 4 hours. The solvent was removed under reduced pressure and the crude mixture was purified using column chromatography (hexanes:EtOAc gradient) to yield the pure product as a white solid (123 mg, 52%). Note: The resonances of thiocarbamates are doubled as a result of slow rotation and the peaks associated with these rotamers are denoted with “major” and “minor” when delineation is possible. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 11.13 (s, 1H), 7.54-7.42 (m, 3H), 7.21-7.07 (m, 5H), 5.57 (minor); 5.48 (major) (s, 2H), 2.28 (major); 2.23 (minor) (s, 3H), 1.30 (s, 9H). ^{13}C{^{1}H} NMR (150 MHz, DMSO-d_6) δ (ppm): 187.8, 187.2, 176.9, 151.1, 136.5, 135.5, 134.9, 134.6, 134.0, 133.6, 130.2, 129.9, 129.7, 129.4, 123.4, 122.5, 122.3, 71.9, 70.3, 39.0, 27.2, 20.9. FTIR (ATR, cm^{-1}): 3207, 2972, 1750, 1704, 1541, 1423, 1107, 1043, 810. HRMS (*m/z*): [M + Na]$^{+}$ calcd for [$C_{20}H_{23}NO_3SNa$]$^{+}$ 380.1296, found 380.1299.

O-(4-pivaloylbenzyl)-N-(p-tolyl)carbamate (**2**). 4-Pivaloyl benzyl alcohol (100 mg, 0.480 mmol) was dissolved in anhydrous THF (4 mL) and DBU (89.6 µL, 0.600 mmol) was added. *p*-Tolylisocyanate (64.3 mg, 0.480 mmol) was added dropwise in anhydrous THF (1 mL). The reaction mixture was stirred under nitrogen at room temperature for 18 hours. The solvent was removed under reduced pressure and the crude mixture was purified using column chromatography (hexanes:EtOAc gradient) to yield the pure product as a clear oil (119 mg, 73%). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.65 (s, 1H), 7.47 (d, *J*=8.2 Hz, 2H), 7.32 (d, *J*=8.8 Hz, 2H), 7.07 (d, *J*=8.1 Hz, 2H), 6.85 (d, *J*=8.7 Hz, 2H), 5.05 (s, 2H), 2.23 (s, 3H), 1.30 (s, 9H). ^{13}C{^{1}H} NMR (150 MHz, DMSO-d_6) δ (ppm): 176.9, 153.8, 150.9, 137.1, 134.8, 131.9, 129.9, 129.6, 122.2, 118.6, 65.5, 39.0, 27.3, 20.8. FTIR (ATR, cm^{-1}): 3364, 2974, 1725, 1595, 1524, 1508, 1192, 1111, 1015, 812, 785, 766. HRMS (*m/z*): [M + Na]$^{+}$ calcd for [$C_{20}H_{23}NO_4Na$]$^{+}$ 364.1525, found 364.1525.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. NMR spectra, mass spectrometry data, additional cytotoxicity data.

Acknowledgements

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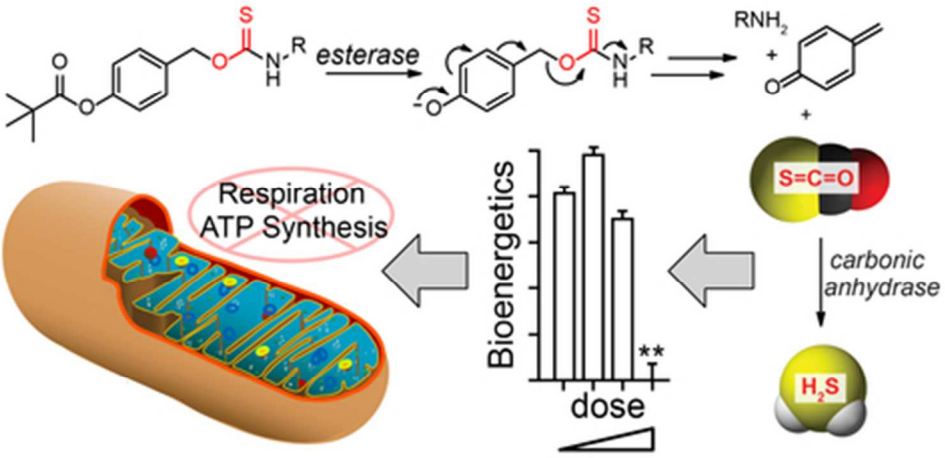

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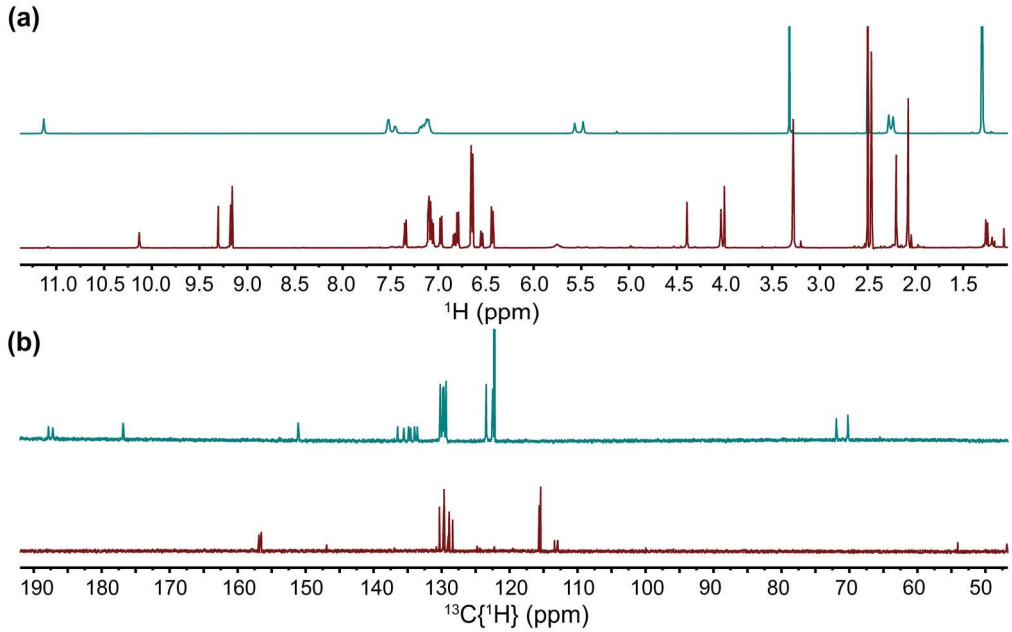

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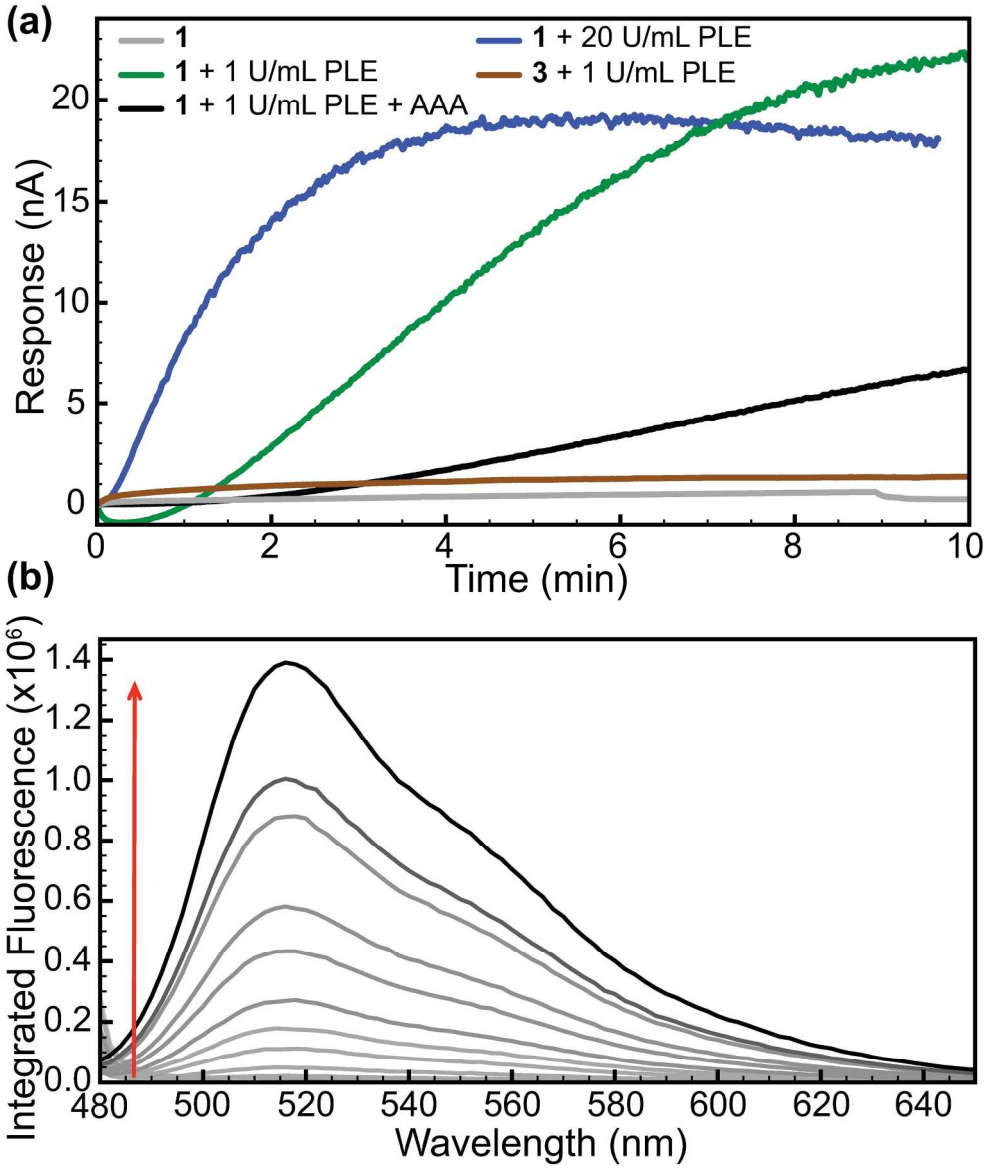

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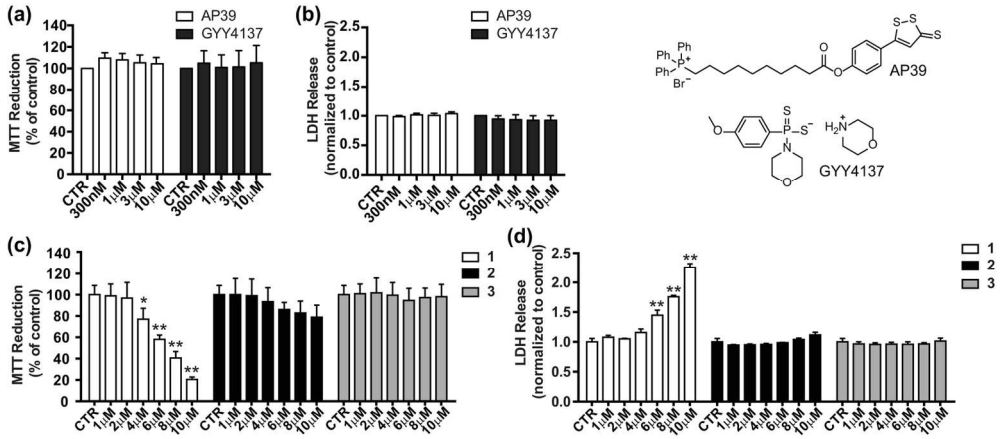

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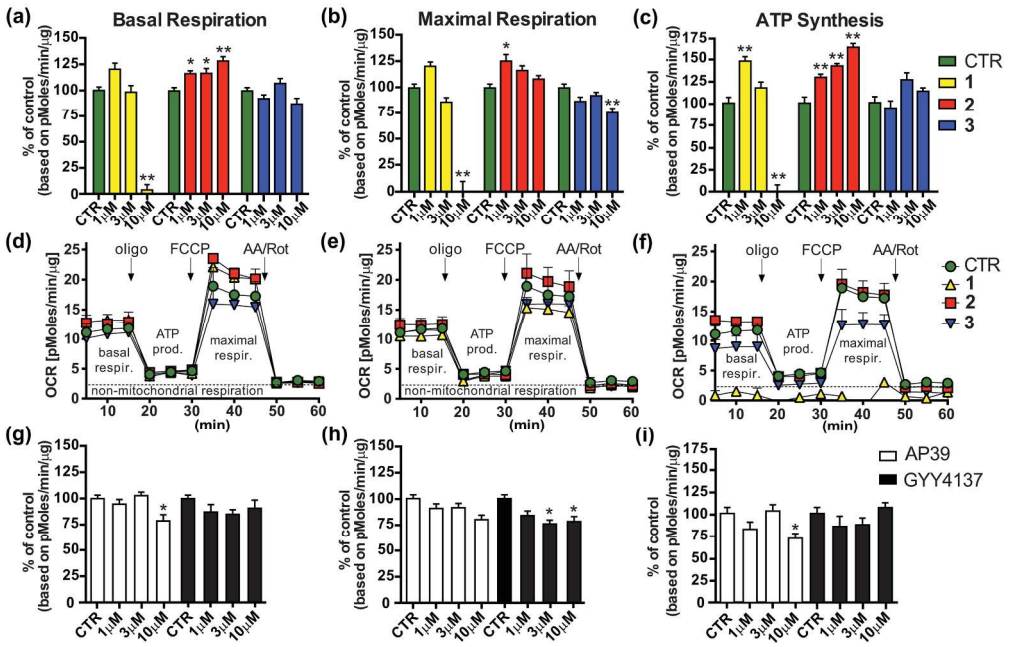

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