Interaction of the carbon monoxide-releasing molecule Ru(CO)_3Cl(glycinate) (CORM-3) with Salmonella enterica serovar Typhimurium: in situ measurements of carbon monoxide binding by integrating cavity dual-beam spectrophotometry

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Carbon monoxide (CO) is a toxic gas that binds to haems, but also plays critical signalling and cytoprotective roles in mammalian systems; despite problems associated with systemic delivery by inhalation of the gas, it may be employed therapeutically. CO delivered to cells and tissues by CO-releasing molecules (CO-RMs) has beneficial and toxic effects not mimicked by CO gas; CO-RMs are also attractive candidates as novel antimicrobial agents. Salmonella enterica serovar Typhimurium is an enteropathogen causing gastroenteritis in humans. Recent studies have implicated haem oxygenase-1 (HO-1), the protein that catalyses the degradation of haem into biliverdin, free iron and CO, in the host immune response to Salmonella infection. In several studies, CO administration via CO-RMs elicited many of the protective roles of HO-1 induction and so we investigated the effects of a well-characterized water-soluble CO-RM, Ru(CO)_3Cl(glycinate) (CORM-3), on Salmonella. CORM-3 exhibits toxic effects at concentrations significantly lower than those reported to cause toxicity to RAW 264.7 macrophages. We demonstrated here, through oxyhaemoglobin assays, that CORM-3 did not release CO spontaneously in phosphate buffer, buffered minimal medium or very rich medium. CORM-3 was, however, accumulated to high levels intracellularly (as shown by inductively coupled plasma MS) and released CO inside cells. Using growing Salmonella cultures without prior concentration, we showed for the first time that sensitive dual-beam integrating cavity absorption spectrophotometry can detect directly the CO released from CORM-3 binding in real-time to haems of the bacterial electron transport chain. The toxic effects of CO-RMs suggested potential applications as adjuvants to antibiotics in antimicrobial therapy.

INTRODUCTION

Carbon monoxide (CO) is a colourless, odourless gas, able to exert toxic effects by binding to ferrous haems in cytochromes and globins, and inhibiting respiration (Keilin, 1966). However, at lower concentrations, CO has more recently been recognized for its beneficial roles in mammalian systems (Foresti & Motterlini, 2010; Motterlini & Otterbein, 2010), including neural messaging, vasodilation, anti-inflammatory and anti-apoptotic properties (Boczkowski et al., 2006). In these respects, CO shares some similarities with other ‘gasotransmitters’, i.e. nitric oxide (NO) and hydrogen sulfide (H2S) (Jesse et al., 2013; Tinajero-Trejo et al., 2013). CO is currently being explored for its potential therapeutic benefits, as in the management of ischaemia reperfusion injury (Patel et al., 2012) and its anti-inflammatory effects in patients with chronic obstructive pulmonary disease (Bathoorn et al., 2007; Motterlini & Otterbein, 2010).

However, due to the danger of toxicity when delivering CO systemically, CO-releasing molecules (CO-RMs) have been
developed to safely deliver CO to target areas without significantly increasing carboxyhaemoglobin levels in the blood beyond the recommended safe limit (10%). There is now a wide variety of CO-RMs, mostly metal carbonyl compounds, with differing rates and mechanisms of CO release, including dissociation in biological buffers, photo-dissociation and via enzyme activity (Desmard et al., 2012; Mann, 2010; Romanski et al., 2011; Schatzschneider, 2011). Amongst the best studied is the ruthenium-containing water-soluble compound Ru(CO)3Cl(glycinate) (CORM-3) (Clark et al., 2003). CORM-3 is relatively stable in water or buffer alone (Desmard et al., 2012), but releases CO with a half-life of ~2 min in the standard myoglobin assay, although the sodium dithionite used for globin reduction has been shown to be essential for rapid CO release (McLean et al., 2012).

CORM-3 is bactericidal against Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa (David et al., 2009; Desmard et al., 2009, 2012; Nobre et al., 2007; Wilson et al., 2013), demonstrating its versatility as an antimicrobial compound. The antimicrobial potential of other CO-RMs has been demonstrated by the injection of (Ru(CO)3Cl)2 (CORM-2) into WT and haem oxygenase-1 (HO-1)-deficient mice after the onset of polymicrobial sepsis, which increased phagocytosis and rescued HO-1-deficient mice from sepsis-induced lethality (Chung et al., 2008). Although CO-RMs release CO inside bacteria (David et al., 2009; Nobre et al., 2007), we do not understand the biochemical basis of CO-RM action. In the case of CORM-2 and ALF062, a contributing factor appears to be the generation of oxidative stress (Tavares et al., 2011).

Prior to use in antimicrobial therapy, the direct interaction of CO-RMs with bacterial targets must be fully understood. In this work, we characterized the direct effects of CORM-3 on Salmonella enterica serovar Typhimurium and demonstrated for the first time how CO released from CORM-3 binds to the terminal oxidases of the bacterium in situ in growing bacterial cultures under physiological conditions utilizing a commercial integrating cavity absorption meter (ICAM). We characterized the antimicrobial activity of CORM-3 against S. Typhimurium in a defined minimal medium, and showed that it was toxic to the bacterium at low concentrations (<100 μM) and that the ruthenium was accumulated intracellularly over time.

**METHODS**

**Salmonella strains and growth conditions.** The strain used for this study was WT S. Typhimurium ATCC 14028s. Cells were grown in batch culture conditions in Luria broth (LB), Circlegrow (MP Biomedicals) or defined minimal medium based on that of Evans et al. (1970) with the following exceptions: 2 mM nitrotriacetic acid was used as the metal ion chelator replacing citrate, sodium selenite (30 μg l⁻¹) was added, and the medium was buffered with 17 mM monosodium phosphate and 33 mM disodium phosphate. Glucose (20 mM) was used as the sole carbon source. Cultures were grown at 37 °C with shaking at 200 r.p.m. in conical flasks. For measurement of growth, 20 ml cultures were grown in 250 ml conical flasks fitted with side arms for measurements of optical density with a Klett–Summerson photoelectric colorimeter (Klett Manufacturing) fitted with a no. 66 red filter. For whole-cell spectroscopy, cultures were grown in 600 ml LB in 2 l flasks. Where indicated, cells were grown in 40 ml Circlegrow super-rich medium in 250 ml conical flasks to provide sustained slow release of the carbon source and attainment of high growth yields.

**Preparation of CORM-3 solution.** CORM-3 was prepared as described previously (Clark et al., 2003); a 100 mM stock solution was made fresh daily and kept on ice in the dark.

**Viability assays.** Viability was measured using the surface viable count (Miles–Misra) technique after serial dilutions of samples in PBS, plating 10 μl aliquots on LB agar and incubation overnight at 37 °C.

**Ruthenium uptake.** Cultures were grown to mid-exponential phase of growth (OD₅₇₈ ~0.4) and 20 ml samples were taken both prior to, and at regular intervals after, the addition of 40 μM CORM-3. Samples were treated and analysed for ruthenium content as described previously using inductively coupled plasma MS (David et al., 2009; Graham et al., 2012; McLean et al., 2013).

**Whole-cell spectrophotometric determination.** Difference spectra (CO reduced minus reduced) of S. Typhimurium whole cells suspended in PBS were recorded in two different dual-wavelength spectrophotometers. Scans were taken using an SDB dual-wavelength spectrophotometer (Johnson Foundation) using 10 mm path length (Kalnes et al., 1998). Scans were also taken using an Olis RSM41000 dual-beam rapid scanning monochromator in CLARITY mode (Online Instrument Systems). Data for CO binding to haems of the bacterial electron transport chain were also plotted against time and curves were fitted using Solver (Frontline Systems).

**RESULTS**

**CORM-3 inhibits growth and reduces viability of S. Typhimurium in a concentration-dependent manner**

The sensitivity of S. Typhimurium to CORM-3 was studied using cultures grown to mid-exponential phase (40 Klett units) in a defined minimal medium with glucose as the sole carbon source, before a bolus addition of CORM-3 was made (0, 25, 50, 75 or 100 μM); turbidity was monitored for a further 14 h to assess the effects on growth. Fig. 1(a) shows that growth inhibition was more severe when the concentration of CORM-3 was increased, with as little as 25 μM causing a transient inhibition and 100 μM causing a severe perturbation of growth. To assess further the effects on Salmonella, viability assays were performed of cells exposed to between 75 and 200 μM CORM-3 (Fig. 1b). Viability decreased with increasing CORM-3 concentration; viability dropped to ~3% within 5 min of exposure to 200 μM CORM-3 and was reduced to ~0.01% by 180 min post-treatment. The molecular basis of the toxicity exerted by CORM-3 remains uncertain (see Discussion).

**Growing S. Typhimurium cultures rapidly take up CORM-3**

One explanation of this toxicity is that the metal carbonyl compound released CO extracellularly, perhaps promoted
by sulfites or other ligands in medium (McLean et al., 2012), and that the liberated CO freely diffused to intracellular sites. Interestingly, several CO-RMs are less effective in rich medium than defined medium. Alternatively, as for E. coli (Davidge et al., 2009), CORM-3 may have entered cells to release CO. Indeed, CORM-3 from which CO has been pre-released does not readily permeate E. coli cells (McLean et al., 2013). We therefore tested whether CORM-3 released CO spontaneously in the absence of dithionite or sulfites by avoiding myoglobin, which requires dithionite for reduction, and using oxyhaemoglobin as introduced by McLean et al. (2012). CORM-3 failed to release CO to oxyhaemoglobin, thus forming the spectrally distinct carboxyhaemoglobin in buffer, Evans defined medium or Circlegrow rich medium (Fig. S1, available in the online Supplementary Material). Surprisingly, the addition of CORM-3 to oxyhaemoglobin in LB medium caused oxidation of oxyhaemoglobin to the ferric form; the explanation for this is unclear, but we avoided adding CORM-3 to cells growing in LB.

To test whether CORM-3 penetrated S. Typhimurium cells, ruthenium analyses were performed. Ruthenium is not present naturally inside bacterial cells so that intracellular levels unambiguously report the presence of CORM-3 that has entered the cell. Cultures were grown to ~40 Klett units before CORM-3 addition (40 μM). Samples were removed at regular intervals to monitor the rate of uptake over 80 min. Concentrations of ruthenium inside the cells increased from 0 to ~300 μM within the first 2.5 min, giving an initial uptake rate of ≥120 μM min⁻¹. Subsequently levels rose at a much lower rate to a final concentration of ~550 μM, an intracellular accumulation of ~14 times the level added to the medium (Fig. 2). The mechanisms of accumulation of CORM-3 within cells to concentrations that exceed those outside in the medium is discussed later.

**CO released from CORM-3 binds to the terminal oxidases of S. Typhimurium**

To ascertain whether CO released from the accumulated CORM-3 bound to haem proteins in S. Typhimurium, the

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**Fig. 1.** Growth and viability of S. Typhimurium and effects of increasing concentrations of CORM-3. S. Typhimurium cultures were grown in 20 ml Evans medium in 250 ml side-arm flasks to an optical density of 40 Klett units where varying amounts of CORM-3 were added (0, 25, 50, 75,100, 150 or 200 μM). (a) Growth was monitored for a further 14 h by measuring Klett absorbance units. (b) Viability of the cultures. Data represent the mean ± SEM (n=3).

**Fig. 2.** CORM-3 was rapidly taken into S. Typhimurium cells. Cultures were grown in Evans medium to OD₆₀₀ ~0.4 before addition of CORM-3 (final concentration 40 μM). Cell samples were taken prior to and at regular intervals after CORM-3 addition for quantification of their ruthenium content. Data represent the mean ± SEM (n=3). Dotted line represents extracellular final concentration of CORM-3 added (40 μM).
method of choice is visible spectrophotometry. However, turbid light-scattering suspensions confound spectral assays as light is greatly scattered by the sample, allowing only a small fraction to reach the detector (Fig. 3a). For >60 years, split-beam or dual-wavelength spectrophotometers have nevertheless served invaluable roles in studies of cells, membranes, mitochondria, tissues and frozen samples with unfavourable turbidity (Poole & Kalnenieks, 2000). In the present work, cells were grown to mid-exponential phase, and then harvested, washed and resuspended in PBS to give a highly turbid suspension (OD₆₀₀ ~50). Difference spectra were taken of cells reduced with dithionite and exposed to 100 μM CORM-3 for 20 min against a reference sample of reduced cells (Fig. 3). Using a conventional dual-beam scanning spectrophotometer with a 1 cm path length cuvette, a focused beam of light was directed at the highly turbid samples. Such an instrument has repeatedly been shown to be sufficiently sensitive to scan highly turbid bacterial cultures and yield spectral data on CO binding to haems of the terminal oxidases of the bacterial electron transport chain (Fig. 3a) (Jesse et al., 2013; Jones & Poole, 1985; Poole & Kalnenieks, 2000).

The second method of spectrophotometry used to detect signals from highly turbid solutions utilized a spherical chamber or ICAM, in which scattered light is reflected within a spherical sample chamber containing the turbid suspension until the light reaches the detector (Elterman, 1970; Fry et al., 1992; Hodgkinson et al., 2009; Jávorfi et al., 2006). We used a new commercial system that had been developed employing two matching highly reflective flasks (Fig. 3), allowing dual-beam measurement of turbid samples, and which greatly increased sensitivity and spectral resolution. Here, scatter minimally affected the signal and the enhanced path length (up to 30-fold) due to reflectance greatly increased the sensitivity of the spectrophotometer. When the *Salmonella* samples were scanned using this apparatus, we were able to visualize the characteristic peaks of CO binding to the terminal oxidases along with the associated troughs caused by loss of reduced cytochrome with much greater resolution, especially in the α/β region of the spectrum (Fig. 3b). The kinetics of appearance of the CO-bound form (Fig. 4a; the sum of the rate of CORM-3 entry, CO release and fast CO binding) to haems of the bacterial terminal electron transport chain was apparently first order, with \( k = 2.12 \text{ min}^{-1} \) (Fig. 4b).

**Fig. 3.** The Olis dual-beam spectrophotometer in CLARITY mode showed greater sensitivity to changes in the terminal oxidases of *S. Typhimurium* than a conventional dual-beam scanning instrument. (a) In a standard dual-beam spectrophotometer, a focused beam of light is highly scattered by turbid samples causing only a small percentage to pass through to the detector. This gives lower spectral resolution than if the sample is scanned using (b) a highly reflective flask as in the CLARITY setup. Here, the light beam is reflected until recognized by the detector, which is positioned directly adjacent to the flask. In this case, scatter will minimally affect the signal and the enhanced path length (up to 30-fold) increases the sensitivity of the spectrophotometer. Cells grown to mid-exponential phase were harvested, washed and resuspended in PBS at OD₆₀₀ ~50. Difference spectra (CO reduced minus reduced) were taken in each instrument from the same cell sample.
Olis RSM1000 dual-beam rapid scanning monochromator in CLARiTY mode detects changes in the terminal oxidases of growing cultures

Although improved sensitivity when scanning highly turbid samples is of great benefit to these and many other studies, we wanted to test the boundaries of detection further. In the conventional dual-beam configuration (Fig. 3a), the turbidity of the sample is critical, deviations in which can increase noise and reduce sensitivity. We tested the sensitivity of the dual ICAM system by serially diluting a sample of reduced cells from OD$_{600}$ ~50 down to ~5. We observed a reduction in the amplitude of peaks and troughs caused by the binding of CO to haem; however, even at OD$_{600}$ ~5, all spectral characteristics could be seen (Fig. 5, inset). As cultures of *Salmonella* can be grown in rich media to turbidities much greater than OD$_{600}$ ~5, this indicated that we may be able to observe binding of CO to the terminal oxidases in highly turbid, growing cultures without prior concentration. We tested this hypothesis by growing cultures in a rich Circlegrow medium; when they reached OD$_{600}$ 5–6 (5.175 $\pm$ 10$^9$ viable cells ml$^{-1}$), cultures were transferred directly into the reflective flasks without harvesting. Samples were reduced with grains of sodium dithionite, and 100 $\mu$M CORM-3 was added to one of the flasks and incubated for 15 min. CORM-3 (100 $\mu$M) had no effect on the growth rate of *S.* Typhimurium at OD$_{600}$ 5–6 in Circlegrow medium, but ruthenium was still able to enter cells under these conditions (data not shown).

Fig. 6(a) shows CO binding to the terminal oxidases of the bacterial electron transport chain of a growing *S.* Typhimurium culture. Immediately upon addition of CORM-3 to growing cells, the formation of a ‘W’ shape in the Soret region with a peak at 421 nm, a trough at 433 nm and a further dip at ~445 nm in the CO difference spectra using the CLARiTY mode corresponded well with previously published spectra. This intricate line shape of the Soret region induced by CO binding has complex interpretations, even for purified cytochrome *bd* (Borisov *et al.*, 2001). A blue shift of the ferrous haem *d*-band gives rise to a band near 400 nm (due to the CO-ligated haem *d*, not well resolved here) and a broad minimum centred at 432 nm (decrease in free haem *d*) with an inflection at 420 nm between. Overlapping is a sharp ‘first derivative-shaped’ feature with a maximum at 436 nm and a minima at 420 nm.
minimum at 444 nm dominated by perturbation of the haem b595 band at 440 nm induced by CO binding to haem d (Borisov et al., 2001). Such a feature arises from the formation of a CO-ligated species (seen as a peak in the difference spectrum) with concomitant bleaching of the signal due to the unligated species (seen as a trough in the difference spectrum). In addition, there is a contribution from the Soret absorbance of the CO-ligated cytochrome o (at ~415 nm) and the loss of free haem o at ~432 nm (Poole et al., 1979). Collectively these signals cause the broad bands in Fig. 6(a) observed at 421 and 433 nm. In Fig. 6(a), the 647 nm peak corresponded to the absorbance maximum of the CO-ligated cytochrome d, whilst the 628 nm trough reflected reaction with CO (and therefore removal from the difference spectrum) of the ferrous cytochrome d. These spectral characteristics resembled those seen when CO reacted with cytochrome bd in harvested E. coli whole cells and membranes (Davidge et al., 2009; Jesse et al., 2013). This can be compared with the same sample measured using a standard dual-wavelength scanning spectrophotometer (Fig. 6b). Here, extensive signal smoothing and averaging were needed to resolve the signals in the Soret region, whilst the αβ regions provided almost no useful information without spectral manipulation (Fig. 6b, inset).

Note that, in the spectra discussed above, sodium dithionite was added to reduce quickly the intracellular haems of E. coli cells. We have shown previously that, even in the presence of dithionite, extracellular myoglobin can be used as a monitor of the passage of CO (as CORM-3) into intact bacteria (Davidge et al., 2009). However, a potential complication arises, as McLean et al. (2012) showed that dithionite promotes release from CORM-3 in the absence of cells. Therefore, to demonstrate that dithionite was not implicated directly in the release of CO and the formation of the CO adducts visualized in Fig. 6(a, b), we used glucose in lieu of dithionite in a separate experiment (Fig. 6a, inset). It was clear that uptake of the CORM and the reaction of CORM-derived CO with intracellular cytochromes was not dependent on dithionite.

**DISCUSSION**

S. Typhimurium is a well-known enteropathogen and leading cause of gastroenteritis in humans, and survives and proliferates within innate immune cells. HO-1 and CO play an important role in the host response to salmonellosis, possibly in combination with nitrosative mechanisms. Zaki et al. (2009) found evidence that HO-1, induced by NO-mediated 8-nitro-cGMP formation, contributes to host defence during murine Salmonella infection via its potent cytoprotective function. A more recent study investigating the effects of HO-1 and CO on the clearance of enteric infection found that either HO-1 induction or CO administration prevented colonic inflammation and enhanced bacterial killing/clearance in mice challenged with conventional microbiota or S. Typhimurium infection models (Onyiah et al., 2013). The authors used the CORM fac-[Mo(CO)3(histidinate)]Na (ALF186) to administer CO to macrophages and suggested that its antimicrobial activity was due to increasing phagolysosomal formation/acidification in macrophages, which was associated with enhanced killing of intracellular E. coli, Enterococcus facialis and S. Typhimurium. Surprisingly, it was also reported that, under *in vitro* experimental conditions, there was no bacterial growth inhibition after exposure of cultures to 100 μM ALF186 grown in LB, suggesting that the compound itself was not toxic to these bacterial species. However, the...
bactericidal activity of CO-RMs is reduced by the presence of a number of molecules, including N-acetylcyesteine, cysteine and reduced glutathione (Desmard et al., 2009; Jesse et al., 2013; McLean et al., 2013), and so is likely to be diminished in a complex medium such as LB. The reasons are unclear.

In contrast, the growth rate and viability of S. Typhimurium is decreased by exposure to CORM-3 in a concentration-dependent manner (Fig. 1), in agreement with other studies that found CORM-3 to be toxic against a variety of bacterial species (Davidge et al., 2009; Desmard et al., 2009, 2012; Nobre et al., 2007; Smith et al., 2011). However, S. Typhimurium seems to be more sensitive than E. coli to this CO-RM (McLean et al., 2013).

Concentrations of CORM-3 used in this study that are highly bactericidal against S. Typhimurium (>150 μM CORM-3, viability ≥0.01 % after 180 min, Fig. 1) were shown previously to cause no significant drop in viability of RAW 264.7 macrophages after 24 h incubation (Desmard et al., 2009). Furthermore, there was significant toxicity to the bacterium at concentrations as low as 75 μM CORM-3. Together, these data show that CORM-3 is bactericidal against Salmonella at concentrations >6.5 times lower than those required to cause toxicity in RAW 264.7 macrophages. The basis of this toxicity is unclear. However, CO gas is not a very effective inhibitor of growth or respiration (Nobre et al., 2007; Reeder et al., 2011; Wilson et al., 2013) of bacteria and CO-depleted (inactive) CORMs are also non-toxic. It has been suggested that CORMs exert their toxicity by effectively delivering CO direct to intracellular targets, such as haem proteins, more effectively than the free gas (the Trojan Horse hypothesis; Wilson et al., 2013) or that the high concentrations of CORMs accumulated by cells (see below) provide high local concentrations of the gas. The precise basis of toxicity is so far unresolved for any CORM and further study is needed. Whatever the mechanism, it should be noted that CO competes effectively with oxygen for binding to haems. In the case of human haemoglobin A, the ratio of CO to oxygen bound is 217 times greater than the ratio of these gas tensions at equilibrium (Engel et al., 1969).

Upon exposure to CORM-3, there is a rapid influx of the ruthenium complex into Salmonella cells. By 80 min post-addition, the levels of ruthenium found inside the cells far exceeded the final concentration added to the extracellular medium (~14 times, Fig. 2). This indicates that either CORM-3 can actively enter the cell against the concentration gradient, driven by an unidentified transporter, or that CORM-3 diffuses in and is altered once inside the cell so that influx can continue passively.

Any attempt to study CO binding or other absorbance changes of pigments in cell or membrane suspensions is confounded by the scattering of light from the particles. The theory of this has been well studied and documented. For example, Koch (1961, 1968, 1970) made a detailed study of these problems as they apply to the use of spectrophotometry for measuring bacterial population density. For the most sensitive measurement of culture density (by far the most common application in microbiology), the propensity for scattering should be maximized so that a small number of cells causes maximum deviation of the incident light away from the optical axis and the photomultiplier or other detector. This can be achieved most easily by reducing the wavelength of the measuring light or increasing the distance between sample and detector (Fig. 1a). However, for in situ measurements of absorbance, as here of haemproteins within cells, optimal detection is achieved when as much of the light scattered by the sample is captured by the detector by positioning it as close as possible to the sample (Poole & Kalnienieks, 2000; Yang & Legallais, 1954). The development of a dual-beam spectrophotometer utilizing an ICAM has allowed the visualization of CO binding to cytochromes of actively growing S. Typhimurium cultures, representing the first measurements of CO binding in situ in growing bacterial cells. Previously, Blake & Griff (2012) used a similar apparatus to record spectra of intact Leptospirillum ferrooxidans cells, after bacteria had been harvested from ferrous ion-oxidizing cultures, and concentrated to ~5 × 10^8 cells ml^{-1} and ‘roughly as turbid as non-fat milk’.

As highly turbid cultures of growing Salmonella can be achieved using rich media such as Circlegrow, we found no requirement for centrifugal concentration of growing cell suspensions of S. Typhimurium in order to measure the optical changes associated with CO binding. Another feature of this spectrophotometer, not exploited in the present work, is that it permits the rapid acquisition of up to 100 absorbance scans s^{-1} over a wavelength range of 300 nm, giving extremely fast time-resolved spectra. Alternatively, the integrating cavity facility allows the generation of mean scans from the 100 taken over 1 s, greatly increasing the signal-to-noise ratio.

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