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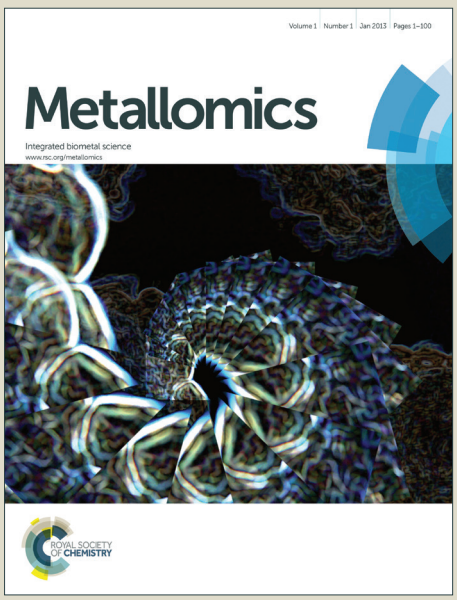

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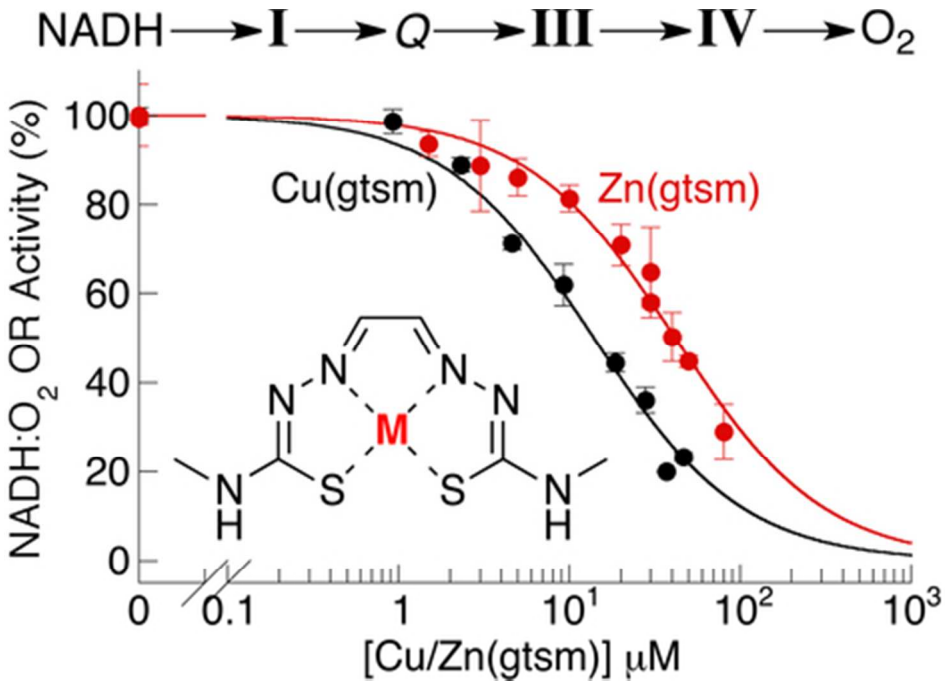

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Inhibition of respiratory Complex I by copper(II)-*bis*(thiosemicarbazonato) complexes

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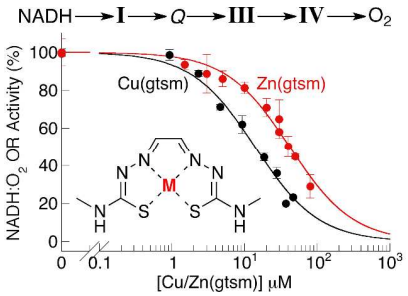

The family of Cu^{II}-*bis*(thiosemicarbazone) complexes inhibits Complex I of the respiratory chain without apparent release of bioavailable Cu ions.

ABSTRACT

Several copper(II) complexes of *bis*(thiosemicarbazones) [(Cu(btsc)s] show promise as therapeutics for the treatment of certain neurological diseases, cancers and bacterial infections. These complexes are thought to act primarily as "copper boosting" agents, whereby the Cu^{II} centre is reduced by cytosolic reductants and Cu^{I} is released as "free" or "bioavailable" ions. It is then assumed that the dissociated Cu^{I} ion is the species responsible for many of the observed biological effects of Cu(btsc)s. We recently showed that Cu(btsc) complexes inhibited NADH dehydrogenases in the bacterial respiratory chain. In this work, we demonstrate that Cu(btsc) complexes also inhibit mitochondrial respiration and that Complex I in the mitochondrial electron transport chain is a specific target of inhibition. However, bioavailable Cu ions do not appear to contribute to the action of Cu(btsc) as a respiratory inhibitor. Instead, the intact Cu(btsc) molecule may bind reversibly and competitively to the site of ubiquinone binding in Complex I. Our results add to the growing body of evidence that the intact complex may be important in the overall cellular activity of Cu(btsc) complexes and further the understanding of their biological effects as a potential therapeutic.

INTRODUCTION

The family of neutral, planar and lipophilic *bis*(thiosemicarbazonato)Cu^{II} complexes (Cu(btsc), Figure 1A) shows potential as therapeutics in the treatment of cancer and neurodegenerative diseases.[1] We and others have also shown recently that these complexes exert potent antibacterial activities against medically significant bacterial pathogens.[2-4] The present model for the biological action of Cu(btsc) complexes as developed from studies in mammalian cells has highlighted their ability to increase intracellular Cu pools.[5-8] This "copper-boosting" action depends on reduction of the Cu^{II} centre and release of this metal ion as "bioavailable" Cu^{I}.

H_2btsc ligands doubly deprotonate upon binding Cu^{II} to generate neutral complexes such as Cu(gtsm) (glyoxal-*bis*[*N*4-methylthiosemicarbazonato]Cu^{II}) and Cu(atsm) (diacetyl-*bis*[*N*4-methylthiosemicarbazonato]Cu^{II}) (Figure 1A). The complexed Cu^{II} ion is stable with respect to dissociation ($K_D \leq 10^{-17}$ M^{-1})[8] but it may accept an electron from cytosolic reductants (Figure 1B). The reduced $[Cu^{I}(btsc)]^-$ complex may be re-oxidised, presumably by O_2, or the Cu^{I} ion may be released as bioavailable ions. Despite a high affinity to Cu^{I} ($K_D \sim 10^{-13}$ M^{-1})[8], the btsc ligand cannot compete with cytosolic thiols, which are often present in millimolar levels and act as a thermodynamic sink for Cu^{I} ($K_D \sim 10^{-39}$ M^{-1}).[9] Thus, the Cu^{I} ion becomes bioavailable as a consequence of its dissociation from the btsc ligand and subsequent transfer to available thiols (Figure 1B).

Generation of bioavailable Cu^{I} ions from Cu(btsc) depends primarily on the Cu^{II}/Cu^{I} reduction potential. This property is dictated largely by alkylation at the diimine carbons (R^1 and R^2 in Figure 1A).[5,7] In the case of Cu(gtsm), the reduction potential is physiologically accessible (E_m -440 mV *vs*. Ag/AgCl or -210 mV *vs*. SHE). Thus, reduction of the Cu^{II} centre and subsequent release of bioavailable Cu^{I} from Cu(gtsm) occur efficiently. By comparison,

Cu(atsm) is harder to reduce due to a lower reduction potential (by ca. 160 mV, E_m -600 mV *vs.* Ag/AgCl). This difference in reduction potentials results in Cu(gtsm) and Cu(atsm) having different biological activities.[2,10,11]

Alternative models for mechanisms of action that do not rely on the release of bioavailable Cu ions have also been proposed. Cu(btsc) complexes interact strongly with lipid bilayers, where they would not encounter cytosolic reductants.[2,12,13] Furthermore, Cu(btsc) complexes are able to persist in their intact and unreduced forms and accumulate as hydrophobic aggregates inside the reducing cytosolic environment.[12,14] These intact forms of Cu(btsc) may exert a cellular effect. For instance, Cu(gtsm) derivatives inhibit DNA synthesis inside the nucleus presumably by intercalation and/or binding to DNA topoisomerases.[14,15] Cu(atsm), which is not thought to release bioavailable Cu ions, was neuroprotective in animal models of Parkinson's disease due to, at least in part, modulation of peroxynitrite-mediated toxicity.[16]

We have recently explored the antimicrobial activities of Cu(btsc) complexes against the bacterial pathogen *Neisseria gonorrhoeae*.[2] Cu(gtsm) suppressed aerobic respiration in this bacterium and NADH dehydrogenases in the electron transport chain were the primary sites of inhibition.[2] One of the NADH dehydrogenases in *N. gonorrhoeae* (Nuo) is closely related to Complex I in mammalian mitochondria. Bacterial and mitochondrial Complex I homologues display key variations in structural organisation, molecular details of catalysis and susceptibility to various inhibitors, but they nonetheless share common defining features. They are usually membrane-bound, multi-subunit, multi-cofactor enzyme complexes that provide primary entry points for electrons into the electron transport chain to O_2 (Figure 2A).[17] Complex I homologues catalyse electron transfer from NADH to ubiquinone (Coenzyme *Q*) and this process is coupled with the translocation of protons across the lipid bilayer. Ubiquinol (QH_2) subsequently provides reducing equivalents for downstream

enzymes that transfer electrons to molecular O_2 as the terminal acceptor. In *N. gonorrhoeae* and mitochondria, these are Complex III (ubiquinol:ferricytochrome *c* oxidoreductase) and Complex IV (cytochrome *c* oxidase, Figure 2A).[18]

We have since extended our work to include *Escherichia coli* and confirmed that the Complex I homologue in this bacterium was also inhibited by Cu(gtsm) (Djoko and McEwan, manuscript in preparation). Given the potential applications of Cu(gtsm) and related Cu(btsc) complexes for human use, we sought to examine if they also acted as inhibitors of mitochondrial Complex I.

RESULTS AND DISCUSSION

Cu(gtsm) inhibits mitochondrial respiration *via* Complex I. ***(1) Inhibition of respiration in intact mitochondria.*** Rates of respiration in intact mitochondria were measured in the presence of glutamate and malate as a source of NADH for Complex I (Figure 2A) under conditions where oxidative phosphorylation was active (State 3).[19] Compared to the vehicle (DMSO) control, addition of Cu(gtsm) (100 μM) led to a decrease in the rate of O_2 consumption (Figure 3). This result confirmed that Cu(gtsm) can act as an inhibitor of the mitochondrial respiratory pathway *via* Complex I (Figure 2A). By contrast, Cu(atsm) did not appear to affect respiration significantly (Figure 3). We have also observed that Cu(atsm) did not suppress respiration in intact, live *N. gonorrhoeae* but this compound did suppress NADH oxidation in isolated bacterial membrane vesicles.[2]

(2) Inhibition of respiration in submitochondrial particles (SMPs). Inhibition of mitochondrial Complex I by Cu(gtsm) was investigated further using SMPs, which are inside-out vesicles composed of the inner mitochondrial membrane, including all components of the electron transport chain from NADH to O_2 (Figure 2A). Our SMP preparations catalysed the

oxidation of NADH at a rate of 180 ± 12 nmol NADH min^{-1} mg $protein^{-1}$ (Table 1). The rate of O_2 reduction was 78 ± 3 nmol O_2 min^{-1} mg $protein^{-1}$, consistent with the expected NADH : O_2 stoichiometry of ~ 2. Respiration was inhibited by rotenone, Antimycin A and azide, which are inhibitors of Complex I, Complex III and Complex IV, respectively (Figure 2A). The uncoupler CCCP and the ATP synthase substrate ADP did not stimulate oxidation of NADH in the presence of phosphate (Table 1), indicating that electron transport from NADH to O_2 was independent of proton translocation and oxidative phosphorylation, respectively. This activity is hereby referred to as 'NADH:O_2 oxidoreductase (OR) activity'. It involves electron transport *via* Complex I, Complex III and Complex IV, and uses endogenous ubiquinone as the electron carrier (Figure 2A).

Compared to the DMSO control, addition of Cu(gtsm) or Cu(atsm) led to decreases in NADH:O_2 OR activity. The degree of inhibition was variable and was sensitive to the time and temperature of incubation of the compounds in the buffer *before* addition of SMPs. This behaviour was characteristic of compound aggregation. Aggregation of Cu(atsm), which is more hydrophobic than is Cu(gtsm)[5], was also indicated by a time-dependent decrease in the intensity of the charge transfer band at 457 nm and a concomitant increase in the spectral baseline (Supplementary Figure 1). Yellow-orange precipitates were visible within 30 min.

To maintain solubility of Cu(btsc) complexes, organic solvents such as DMSO are typically used at a final concentration of 20 – 30 (v/v) %. However, this high amount of DMSO disrupts the structural integrity of lipid bilayers[20] such as those in SMPs, thus it was incompatible with our measurements (Table 1). Moreover, speciation of Cu(btsc) complexes in organic-aqueous solvent systems may not closely represent intracellular conditions.[21] Our preferred approach to prevent aggregation of Cu(btsc) was to add bovine serum albumin (BSA, 1 mg mL^{-1}) to all assay buffers. BSA binds strongly to lipids and lipophilic molecules including Cu(btsc)[22] but did not affect NADH:O_2 OR activity in SMPs (Table 1). Thus, BSA

inhibits nonspecific aggregation of Cu(btsc) complexes and also prevents adventitious binding of the compounds to SMPs. Under these conditions, no precipitation or aggregation of Cu(atsm) was detected (Supplementary Figure 1).

In the presence of BSA, both Cu(gtsm) and Cu(atsm) suppressed NADH:O_2 OR activity reproducibly and the extent of inhibition increased with compound concentration. Dose-response curves for Cu(gtsm) estimated an average I_{50} value of 15.6 μM (Figure 4 and Supporting Table 1). By contrast, addition of Cu(atsm) only led to minor losses of activity (Figure 4). Due to poor solubility of Cu(atsm) at concentrations above 100 μM, an I_{50} value could not be extrapolated reliably. The relative inhibitory powers of Cu(gtsm) and Cu(atsm) matched our earlier observations in intact mitochondria (Figure 3) and in whole, live bacteria.[2] We have reported previously that Cu(atsm) also strongly suppressed the activity of NADH dehydrogenases in *N. gonorrhoeae.*[2] However, this effect was likely due to aggregation of Cu(atsm) in the BSA-free buffer. Consequently, inhibition of the gonococcal NADH dehydrogenases by Cu(atsm) was removed upon addition of BSA (data not shown). Taken together, these results confirmed that Cu(gtsm) was a more effective inhibitor of NADH:O_2 OR activity than was Cu(atsm). Based on these findings, subsequent experiments were performed with Cu(gtsm) only.

(3) Complex I as the specific target of inhibition. High concentrations of Cu(gtsm) (up to 100 μM) did not significantly suppress the rates of succinate oxidation (succinate:O_2 OR activity, Figure 5). Similarly, treatment with Cu(gtsm) led to only a small but detectable loss in the rate of succinate respiration in intact mitochondria (Figure 3). Oxidation of NADH and that of succinate share a common pathway *via* Complex III and Complex IV (Figure 2). As Cu(gtsm) inhibited oxidation of NADH but only slightly affected oxidation of succinate, it was likely that Complex I was the primary target of Cu(gtsm) action.

Due to relative ease of manipulation, the mechanism of inhibition of bacterial and

mitochondrial Complex I by Cu(gtsm) was examined further in this work using the mitochondrial homologue as a model enzyme.

Release of bioavailable Cu ions is not responsible for the inhibition of Complex I by Cu(gtsm). ***(1) The Cu centre in Cu(gtsm) is required for an inhibitory effect.*** The observed contrast between the inhibitory powers of Cu(gtsm) and Cu(atsm) (Figure 4) appeared to correlate well with the "copper-boosting" model (Figure 1B). The gtsm ligand binds tightly to Cu^{II} ($K_D \leq 10^{-17}$ M^{-1}) and Cu^{I} ($K_D \sim 10^{-13}$ M^{-1}).[8] These affinities are comparable to those displayed by the atsm ligand. However, Cu^{II}(gtsm) displays a less negative Cu^{II}/Cu^{I} reduction potential (by ca. 160 mV) compared to Cu^{II}(atsm). These thermodynamic considerations lead to generation of bioavailable Cu^{I} ions from Cu(gtsm) but not Cu(atsm). Here we confirmed that the Cu centre in Cu(gtsm) was indeed required for the inhibition of NADH:O_2 OR activity, as the non-metallated H_2gtsm ligand was not inhibitory (Figure 6).

(2) Comparison with solvated Cu^{2+}_{aq} ions and other Cu^{II} chelates. Due to the high affinity of the gtsm ligand to Cu^{I}, dissociation of Cu^{I} ion from Cu(gtsm) occurs only in the presence of a strongly competing ligand such as protein thiols.[8] Using Ellman's reagent, we estimated a total of ca. 700 nM of SMP thiols in each NADH:O_2 OR activity assay mixture. This amount of thiols would not be sufficient to drive significant reduction and dissociation of Cu^{I} from Cu(gtsm), which was used typically at 10 – 50 μM. Consistently, there was no decrease in the intensity of the charge transfer band of Cu(gtsm) at 478 nm to indicate reduction of Cu^{II} to Cu^{I} (Supplementary Figure 2). In addition, generation of Cu^{I} was not detected using excess bicinchoninic acid or bathocuproine disulfonate as colorimetric indicators for Cu^{I} (Supplementary Figure 2), which report Cu^{I} concentrations at the low micromolar levels ($[Cu^{I}(Bca)_2]^{3-}$: λ_{max} 562 nm, ε, 8000 M^{-1} cm^{-1}; $[Cu^{I}(Bcs)_2]^{3-}$: λ_{max} 483 nm, ε, 13000 M^{-1} cm^{-1})[23].

Nevertheless, to test the possibility that release of bioavailable Cu^{I} ions caused inhibition of Complex I, the inhibitory effects of Cu(gtsm) were compared to those of Cu^{2+}_{aq} salts supplied as $CuCl_2$ or $Cu(NO_3)_2$. The standard reduction potential of Cu^{2+}_{aq} salts is positive (E_m +150 mV *vs.* SHE). More importantly, the resulting Cu^{1+}_{aq} ions are not stabilised by an external ligand. Like Cu(gtsm), Cu^{2+}_{aq} salts also inhibited NADH:O_2 OR activity (Figure 6). The dose-response curves estimated an average I_{50} value of 16.2 μM, which was comparable to that of Cu(gtsm) (Supporting Table 1). However, at low concentrations below 10 μM, Cu^{2+}_{aq} salts were *less* inhibitory than was Cu(gtsm) (Figure 6). Curve fitting with the Hill equation reproducibly yielded n = 2.7 for Cu^{2+}_{aq} salts and n = 1.1 for Cu(gtsm) (Supporting Table 1), indicating that the mechanisms of inhibition by solvated Cu^{II} ions and Cu^{II}(gtsm) complex were not equivalent. Furthermore, Cu^{2+}_{aq} salts were nonspecific and were also effective inhibitors of succinate:O_2 OR activity while Cu(gtsm) was selective for NADH:O_2 OR activity (Figure 5).

Inhibition of NADH:O_2 OR activity by Cu^{2+}_{aq} salts was relieved by chelation with the high-affinity Cu^{II} ligand Egta (K_D $10^{-17.7}$ M^{-1}; Figure 7A)[23]. Correspondingly, high concentrations of the pre-formed Cu(Egta) complex (up to 200 μM) had no detectable effect on NADH:O_2 OR activity (Figure 7B). These observations were inconsistent with the model in Figure 1B. Due to a more positive reduction potential (by ca. 170 mV, E_m -290 mV *vs.* SCE or -40 mV *vs*. SHE)[24] and a lower affinity to Cu^{I} (K_D >> 10^{-13} M^{-1})[8], reduction of Cu^{II} and release of bioavailable Cu^{I} from Cu(Egta) would be thermodynamically more favourable than that from Cu(gtsm). Yet, unlike Cu(gtsm), the Cu(Egta) complex was not an effective inhibitor of NADH:O_2 OR activity (Figure 7B).

The inhibitory effects of Cu^{2+}_{aq} salts as observed in Figure 6 were likely due to bioavailable Cu^{II} ions (K_D for solvated Cu^{2+}_{aq} ions ~ 10^{-6} M^{-1}), which were effectively removed from solution by Egta. However, Egta did not protect against inhibition by Cu(gtsm)

(Figure 7), consistent with the expectation that bioavailable Cu^{II} ions would not dissociate from the gtsm ligand ($K_D \leq 10^{-17}$ M^{-1})[8] under our experimental conditions. Taken together, all of our results thus far suggested that the release of bioavailable Cu ions, either as Cu^{II} or Cu^{I}, did not play a dominant role in the inhibition of Complex I by Cu(gtsm).

(3) Comparison with the Zn(gtsm) analogue. In our O_2-rich experimental system where thiols were limiting, reduction and subsequent re-oxidation of Cu(gtsm), albeit undetectable (Supplementary Figure 2), may occur without dissociation of the Cu centre. The Cu-free, redox-inactive analogue Zn(gtsm) also caused a concentration-dependent inhibition of NADH:O_2 OR activity (Figure 8). Zn(gtsm) may transmetallate with trace amounts of Cu that may be present in the assay buffer or in SMPs to generate Cu(gtsm)[25], which would subsequently cause inhibition of NADH:O_2 OR activity. However, no Cu was detected in our assays using bicinchoninic acid as a colorimetric reporter for Cu^{I} in the presence of ascorbate. In addition, the characteristic charge transfer band at 478 nm for Cu(gtsm) was not observed in all assays containing Zn(gtsm). Taken together, these observations suggested that potential redox activity associated with Cu(gtsm) and transmetallation with trace Cu did not contribute significantly to the inhibition of Complex I.

The action of the intact form of Cu(gtsm) as an inhibitor of Complex I. Based on the available experimental data, we propose that Complex I was inhibited by the intact Cu(gtsm) molecule. This may follow a classical enzyme inhibition model, in which noncovalent binding between the target enzyme and the inhibitor results in an inactive or unproductive ternary complex. This enzyme-inhibitor interaction is usually dictated by structural considerations.

Steric factors may indeed account for the relative inhibitory powers of Cu(gtsm) and Cu(atsm). Bulky methyl substituents in Cu(atsm) in place of protons at equivalent positions in Cu(gtsm) (Figure 1A) may prevent efficient binding of the former to Complex I. Likewise,

the estimated average I_{50} value for Zn(gtsm) was 36.4 μM, ca. 2 times higher than that of Cu(gtsm) (Figure 8 and Supporting Table 1). Comparison of X-ray crystal structures of several available Cu and Zn analogues in the gtsm family revealed that, while the Cu complexes are planar, the Zn complexes are tetrahedral.[26-28] This structural difference may rationalise the small but reproducible increase in I_{50} value for Zn(gtsm) compared to Cu(gtsm).

(1) Cu(gtsm) is a reversible inhibitor. One hallmark of classical enzyme inhibition is reversibility, which was tested here using a dilution approach. A concentrated stock of SMPs (100 μg mL^{-1}, *i.e.* 10x the amount in routine assays) was incubated with 50 μM Cu(gtsm) (above the I_{50} value, *cf.* Figure 4) for 30 min in the absence of NADH. NADH:O_2 OR activity was subsequently assayed in two ways. In the first set, NADH was added to undiluted (neat) samples. In the second set, samples were diluted 20x into assay buffer containing NADH to generate final SMP and Cu(gtsm) concentrations of 5 μg mL^{-1} (0.5x the amount in routine assays) and 2.5 μM (below the I_{50} value, *cf.* Figure 4), respectively. While neat samples displayed suppressed NADH:O_2 OR activity as expected, diluted samples regained their activity (Figure 9A), suggesting that inhibition was in fact fully reversible.

(2) Cu(gtsm) is not an aggregation-based inhibitor. Given the propensity of Cu(gtsm) and related complexes to aggregate in solution (Supplementary Figure 1), Cu(gtsm) may behave as an aggregation-based inhibitor. The extent of aggregation-based inhibition typically shows a dependence on the concentration of target enzymes and incubation time.[29] However, the I_{50} value for the inhibition of NADH:O_2 OR activity by Cu(gtsm) did not change with the amount of SMPs over a ten-fold range (Figure 10A). In addition, progress traces NADH oxidation in the presence of Cu(gtsm) were linear over 5 min (Supplementary Figure 3). Correspondingly, pre-incubation of Cu(gtsm) with SMPs for 20 min prior to addition of NADH did not produce a shift in the dose-response curve within experimental

error (Figure 10B). Likewise, recovery of NADH:O_2 OR activity upon dilution did not require an initial incubation time (Figure 9B). These results confirmed that Cu(gtsm) is not an aggregation-based inhibitor.

(3) The site of inhibition of Complex I by Cu(gtsm). Global fitting of the dose-response curves in Figure 4 using Eq. 1 (see Methods) yielded a Hill coefficient of near unity (n = 1.1, Supporting Table 1), consistent with the presence of a single inhibition site or binding site of Cu(gtsm) to Complex I. Most Complex I inhibitors disrupt catalysis at or near the NADH and ubiquinone (Q) binding sites, which are solvent-accessible, while intramolecular electron transfer between the seven solvent-inaccessible Fe-S clusters usually remains unperturbed.[17]

To identify the site of inhibition by Cu(gtsm), NADH oxidation was decoupled from O_2 consumption using azide, a Complex IV inhibitor (Figure 2A and Table 1). Complex I function was subsequently monitored using artificial electron acceptors (Figure 11A). Ferricyanide (FeCN) receives electrons directly from the flavin cofactor in Complex I (FMN). Thus NADH:FeCN OR activity involves electron transfer processes between NADH and FMN only (Figure 11A).[17] Similarly, the ubiquinone analogue decylubiquinone (DQ) receives electrons from the N2 Fe-S cluster, which is downstream of FMN.[17] Therefore, NADH:DQ OR activity involves electron transfer processes between NADH, FMN and all seven Fe-S clusters (Figure 11A).[17]

Cu(gtsm) (40 μM) inhibited NADH:O_2 OR (Figure 11B) and NADH:DQ OR (Figure 11C) activity but it did not affect NADH:FeCN OR activity (Figure 11D). Therefore, Cu(gtsm) disrupted Complex I function at or near the ubiquinone binding site but did not impact upstream processes associated with the flavin group. Although unlikely, we could not definitively rule out an effect on the solvent-inaccessible Fe-S clusters. This action of Cu(gtsm) as a specific inhibitor of decylubiquinone reduction is reminiscent of many

Complex I inhibitors, as exemplified by rotenone (Figure 11D). Rotenone is thought to act as a non-competitive inhibitor, *i.e.* it inhibits the rates of ubiquinone reduction but does not block access by ubiquinone to its reduction site.[30] Unlike rotenone, preliminary kinetic analyses indicated that Cu(gtsm) may be a competitive inhibitor of Complex I (Supplementary Figure 4). In this case, Cu(gtsm) and ubiquinone may compete for the same binding site. However, the precise mechanism of Complex I inhibition by Cu(gtsm) must await further studies.

Like Cu(gtsm), curve-fitting of the dose-response curve for Zn(gtsm) in Figure 8 yielded n = 1.1 (Supporting Table 1). Thus, it is likely that Cu(gtsm) and Zn(gtsm) share the same binding site. However, due to significant spectral overlap with NADH (Supplementary Figure 5), the effect of Zn(gtsm) on NADH:DQ and NADH:FeCN activities was not examined in this work.

CONCLUSION

The results reported here add to the growing body of evidence that the intact complex may be important in the overall cellular activity of Cu(btsc) complexes and thus must not be overlooked. Although the present work has focused on mitochondrial Complex I, we have confirmed our findings using Complex I homologues from *N. gonorrhoeae* and *E. coli* (manuscript in preparation).

Notably, the electron transport chain in bacteria is located in the cytoplasmic membrane and thus it is readily accessible to the environment. Indeed, exposure to Cu(gtsm) led to an immediate inhibition of aerobic respiration in intact live bacteria and eventually loss of bacteria viability.[2] By contrast, in eukaryotes such as cancer and neuronal cells, Cu(gtsm) must cross the plasma membrane, the cytosol and the outer mitochondrial membrane to

finally access Complex I in the inner mitochondrial membrane. While nanomolar concentrations of Cu(gtsm) are highly toxic to bacteria[2,3], micromolar concentrations are required to achieve a toxic effect in eukaryotic cells.[10]

The intracellular fate of Cu(gtsm) and related derivatives in eukaryotes appears to be cell-specific but the reasons for these variations are not fully understood.[12,21,31-34] Nevertheless, interaction of Cu(btsc) with the mitochondria inside live cells has been reported previously. For instance, intracellular reduction of Cu(atsm) and Cu(ptsm) (R^1 = H, R^2 = CH_3 in Figure 1A, E_m -280 mV *vs*. SHE) in tumour cells was shown to occur specifically in the mitochondria and not in the cytosol.[33,34] Whole-cell imaging of a fluorescent Zn analogue in ovarian tumour cells has also identified a co-localisation with mitochondria.[32] In addition, treatment of neuronal cells with Cu(gtsm) but not Cu(atsm) led to decreases in metabolic activity as detected by MTT assay[35] and this is not inconsistent with impairment of mitochondrial function. While we have shown inhibition of respiration in isolated mitochondria (Figure 3), whether Cu(gtsm) inhibits respiration in whole live cells remains to be established.

Interestingly, inhibition of Complex I in eukaryotic cells by rotenone stimulates the production of superoxide in mitochondria and subsequently triggers apoptosis.[36] Although there is a wealth of evidence that Cu(gtsm) treatment does lead to cell death in both mammalian model systems and bacteria, it is not known whether this effect is associated with superoxide production. To the best of our knowledge, there has been no report or measurement of superoxide production in Cu(gtsm)-treated cells of either eukaryotic or bacterial origins. This may be an important line of future investigation given the potential applications of Cu(gtsm) as therapeutics in the treatment of cancer and bacterial infections.

EXPERIMENTAL

Reagents. Analytical-grade reagents were purchased from Sigma and used as supplied. Concentrations of NADH were estimated using the solution absorbance at 340 nm (ε, 6220 M^{-1} cm^{-1}). Stocks of Cu^{2+}_{aq} salts were prepared in deionised water and calibrated as described elsewhere.[37]

Stocks of Cu(atsm) and Cu(gtsm) were prepared in neat DMSO and their concentrations were estimated using the solution absorbance in PBS containing 30 (v/v) % DMSO (Cu(atsm): λ_{max} 457, ε 7200 M^{-1} cm^{-1}; Cu(gtsm): λ_{max} 478, ε 8700 M^{-1} cm^{-1}).[8] Stocks of Zn(gtsm) and H_2(gtsm) were also prepared in DMSO and their concentrations were estimated using the solution absorbance of their corresponding Cu(gtsm) forms following incubation with a stoichiometric excess of $CuCl_2$.

Cu(Egta) complex was generated *in situ* using $CuCl_2$ and 2 molar equivalent of Egta. The free Egta ligand without any $CuCl_2$ was used as control but it had no detectable effect on NADH oxidase activity.

Isolation of intact mitochondria. Intact mitochondria were isolated from rat liver tissues. Liver tissues were obtained from 6- or 7-week-old male Wistar rats. The following steps were performed at 4 ^{o}C in Buffer A (Na-Hepes (20 mM), Tris-Cl (4 mM), mannitol (220 mM), sucrose (70 mM), Edta (1 mM), pH 7.4). Tissues were washed with BSA (1 mg mL^{-1}) to remove blood and fatty contaminants, homogenised also in the presence of BSA, and finally centrifuged (600 *g*, 30 min) to remove unhomogenised tissues and nuclear debris. The supernatant fraction was collected and re-centrifuged (10,000 *g*, 30 min). The brown, mitochondria-rich pellet was washed twice without BSA and resuspended finally in BSA-free buffer to a final concentration of 40 mg protein mL^{-1}. Mitochondrial preparations were kept on ice and used immediately for respiratory assays.

Mitochondrial respiration. Respiration rates were measured at 35 ^{o}C using an S1/Mini Clark-type oxygen electrode (Hansatech Instruments) in conjunction with an Oxytherm control unit. Each assay contained Tris-Mops buffer (10 mM, pH 7.4), KCl (125 mM), Egta (1 mM), potassium phosphate (100 μM), and BSA (1 mg mL^{-1}). Sodium glutamate, sodium malate and sodium succinate (2.5 mM each) were used as required. Cu(atsm) and Cu(gtsm) (100 μM each) were added to the assay mixture prior to addition of mitochondria. Consumption of O_2 was initiated by addition of mitochondria (1.5 mg mL^{-1}). ADP was added to a final concentration of 100 μM.

Preparation of SMPs. The following steps were performed at 4 ^{o}C. Isolated intact mitochondria were sonicated (10 x 30 s bursts at 200 W with 1 – 2 min intervals) in Buffer B (Buffer A + 10 mM $MgCl_2$). Following removal of insoluble debris by centrifugation (10,000 *g*, 1 h), the resulting supernatant was incubated with NADH (500 μM) for 30 min and re-centrifuged (100,000 *g*, 1 h). The dark-red pellet was washed once with Buffer C (Buffer A + 10 mM $MgCl_2$, adjusted to pH 9 with 2.5 M Tris base) and once more with Buffer B. The resulting pellet was homogenised in Buffer D (Buffer A without any Edta) to a final concentration of 8.7 mg protein mL^{-1} and stored as aliquots at -80 ^{o}C until further use. No loss of NADH:O_2 OR activity was detected after one cycle of freezing and thawing.

Activity assays. Frozen SMPs were thawed on ice immediately before use and kept on ice throughout the assay. NADH:O_2 OR activity of SMPs remained stable for at least 8 h under these conditions. Oxidation of NADH was monitored spectrophotometrically at 340 nm (ε, 6220 M^{-1} cm^{-1}) while reduction of O_2 was measured by polarography using a Clark-type O_2 electrode. Assays were carried out at 35 ^{o}C. Each reaction contained Na-Hepes (50 mM, pH 7.4), BSA (1 mg mL^{-1}) $MgCl_2$ (10 mM), NADH (50 μM), and the desired inhibitor (0 – 100 μM). DMSO was used as a vehicle control. The final concentration of DMSO in all assays was < 1 (v/v) %. Oxidation of NADH was initiated by addition of SMPs (10 μg mL^{-1}).

The initial velocity (within 1 – 2 min) was used to calculate NADH:O_2 OR activity (nmol NADH min^{-1} mg $protein^{-1}$). Where required, NADH:O_2 OR activity was presented as a percentage of the DMSO-treated control.

NADH:DQ OR activity and NADH:FeCN OR activity were assayed as above but in the presence of sodium azide (10 mM) to block electron transfer to O_2 *via* Complex IV. decylubiquinone (DQ, 33 μM) or ferricyanide (FeCN, 150 μM) was used as electron acceptors. SMPs were used at a final concentration of 6 and 1.5 μg mL^{-1} for NADH:DQ OR and NADH:FeCN OR activity assays, respectively.

Succinate:O_2 OR activity was assayed as above but in the presence of sodium succinate (1 mM) in place of NADH. Rates of succinate oxidation were obtained by monitoring O_2 consumption using the Clark-type electrode.

Where required, the appropriate concentrations of Cu(gtsm) and Cu(atsm) were used to zero the spectrophotometer prior to assays. Due to significant spectral overlap between NADH and Zn(gtsm) (Supplementary Figure 5), activity assays in the presence Zn(gtsm) were performed using the O_2 electrode only.

Curve fitting. Dose-response curves were fitted globally to the Hill equation in Eq. 1 with floating parameters *m* and *n*, where $m = I_{50}$ value (in μM) and *n* = Hill coefficient (Hill slope). Fits were performed in KaleidaGraph 4.1 (Synergy Software) using at least five data points. Each data point was generated by averaging the results of three replicates.

- Eq. 1

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TABLES

Table 1. NADH:O_2, NADH:FeCN and NADH:DQ oxidoreductase activities of SMPs. All activities were measured in the presence of 1 mg mL^{-1} BSA.

Activity		Treatment	Rate[a]
NADH:O_2	O_2 reduction[b]	-	78 (3)
	NADH Oxidation[c]	-	180 (12)
		Without BSA	173 (13)
		Rotenone (100 nM)	n.d.
		Antimycin A (1 μg/mL)	4 (2)
		Azide (10 mM)	37 (2)
		ADP (100 μM)	189 (13)
		CCCP (10 μM)	162 (8)
		DMSO (30 (v/v) %)	n.d.[d]
NADH:FeCN[c]		-	3840 (180)
NADH:DQ[c]		-	342 (13)

[a]Averaged from at least three replicates. Standard deviations were shown in brackets.

[b]Measured using a Clark-type O_2 electrode. Rate was shown in nmol O_2 min^{-1} mg $protein^{-1}$.

[c]Measured spectrophotometrically at 340 nm. Rates were shown in nmol NADH min^{-1} mg $protein^{-1}$.

[d]n.d. not detectable

FIGURES

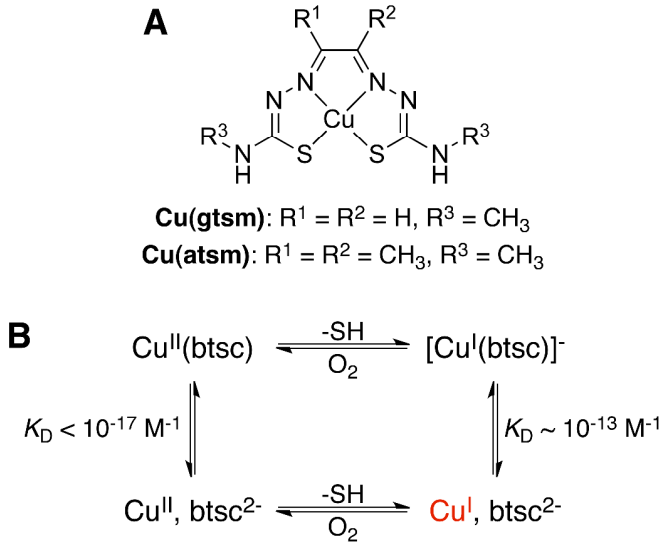

Figure 1. A. Structure of Cu(btsc). **B.** "Copper-boosting" action of Cu(btsc), which relies on dissociation of the Cu centre as bio-available Cu^{I} ions (in red).

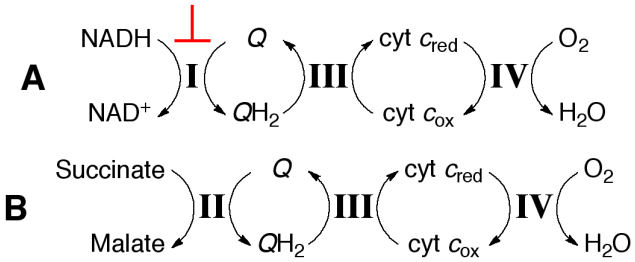

Figure 2. The mitochondrial electron transport chain to O_2. A. Electron transport from NADH *via* Complex I. The site of inhibition by Cu(gtsm) as identified in this work is indicated in red. **B.** Electron transport from succinate *via* Complex II.

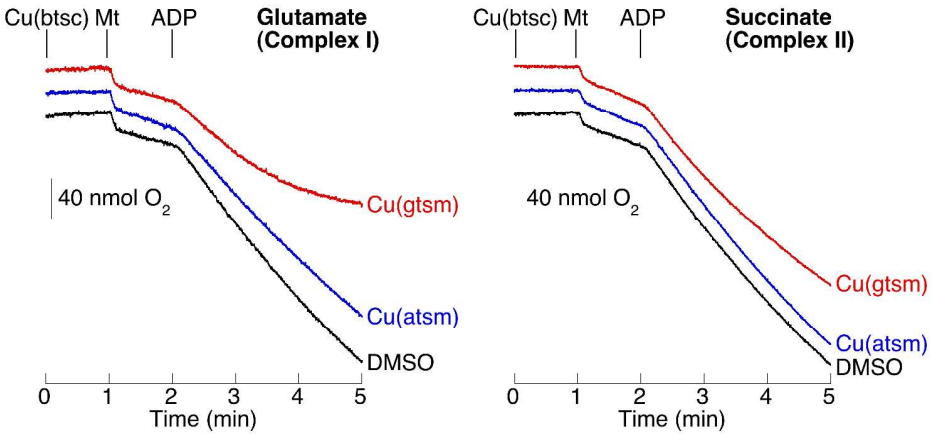

Figure 3. Inhibition of mitochondrial respiration by Cu(gtsm). Consumption of O_2 was measured in the presence of glutamate-malate (2.5 mM, left panel) or succinate (2.5 mM, right panel) and DMSO (black traces), Cu(atsm) (100 µM, blue traces), or Cu(gtsm) (100 µM, red traces). Cu(btsc), mitochondria (1.5 mg mL^{-1}, Mt) and ADP (200 µM) were added to the assay as indicated. Vertical scale bars represent 40 nmol O_2.

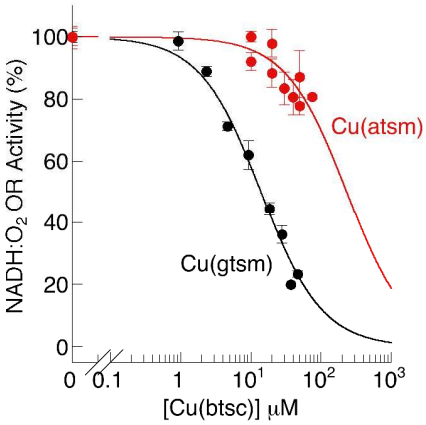

Figure 4. Inhibition of NADH:O_2 OR activity by Cu(gtsm) (black trace) and Cu(atsm) (red trace).

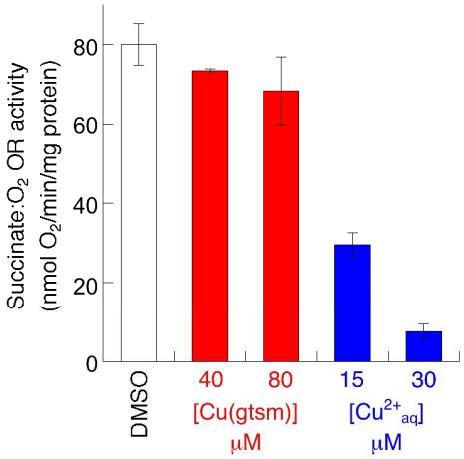

Figure 5. Inhibition of succinate:O_2 OR activity by Cu(gtsm) (red columns) and Cu^{2+}_{aq} salts (blue columns).

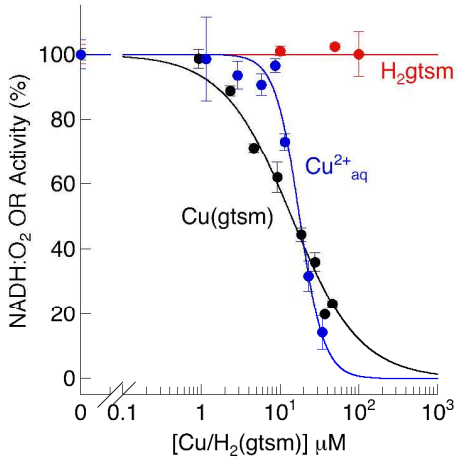

Figure 6. Inhibition of NADH:O_2 OR activity by Cu(gtsm) (black trace), H_2gtsm (red trace) and Cu^{2+}_{aq} salts (blue trace).

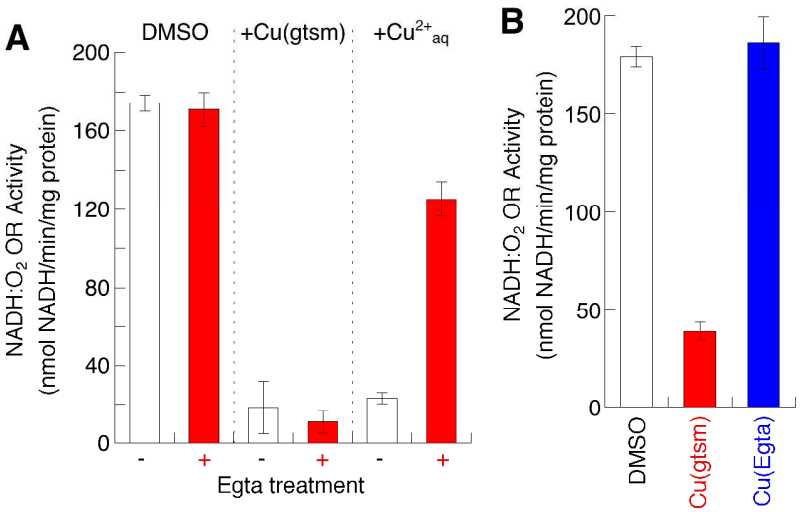

Figure 7. Protective effects of Egta. A. Egta relieved inhibition by Cu^{2+}_{aq} salts but not by Cu(gtsm). NADH:O_2 OR activity was measured in the presence of DMSO, Cu(gtsm) (50 μM), or Cu^{2+}_{aq} salts (30 μM). Water (-, white columns) or Egta (100 μM, +, red columns) was added to the assay after 1 min of turnover. **B.** Pre-formed Cu(Egta) complex was not inhibitory. NADH:O_2 OR activity was measured in the presence of DMSO, Cu(gtsm) (50 μM), or Cu^{2+}_{aq} salts (30 μM). All assay buffers contained Egta (100 μM).

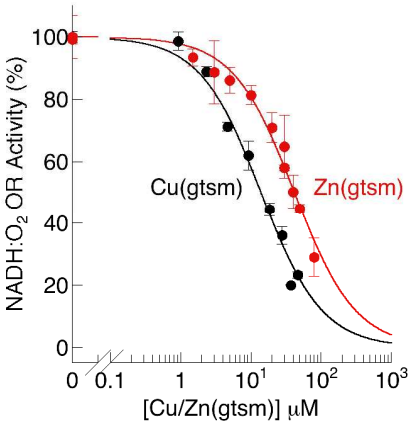

Figure 8. Inhibition of NADH:O_2 OR activity by Cu(gtsm) (black trace) and Zn(gtsm) (red trace).

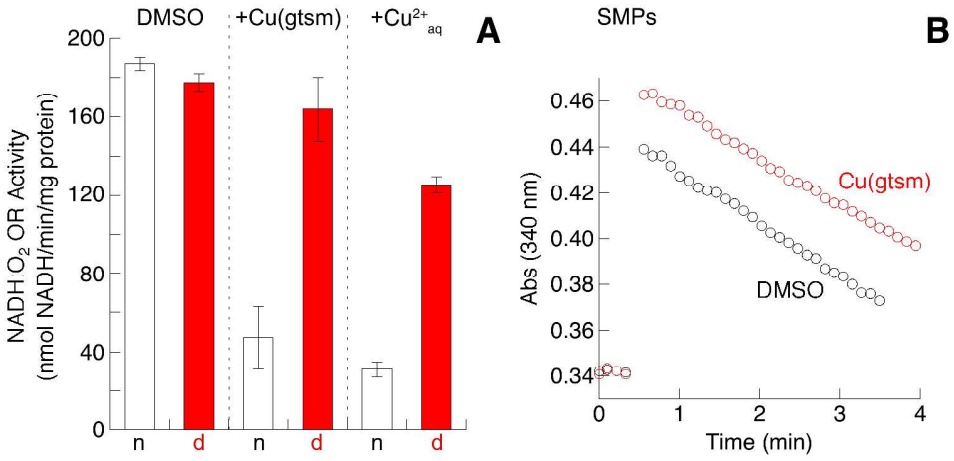

Figure 9. Reversible inhibition of NADH:O_2 OR activity by Cu(gtsm). A. SMPs (100 μg mL^{-1}) were incubated with DMSO, Cu(gtsm) (50 μM), or Cu^{2+}_{aq} salts (30 μM) in the absence of NADH. The mixture was used directly (n, white columns) or was diluted (d, red columns) for NADH:O_2 OR activity measurements. **B.** Progress curves for diluted samples. DMSO-treated (black circles) or Cu(gtsm)-treated (red circles) SMPs were diluted by addition to assay buffer containing NADH (50 μM) as indicated. The decrease in absorbance at 340 nm was monitored for up to 4 min.

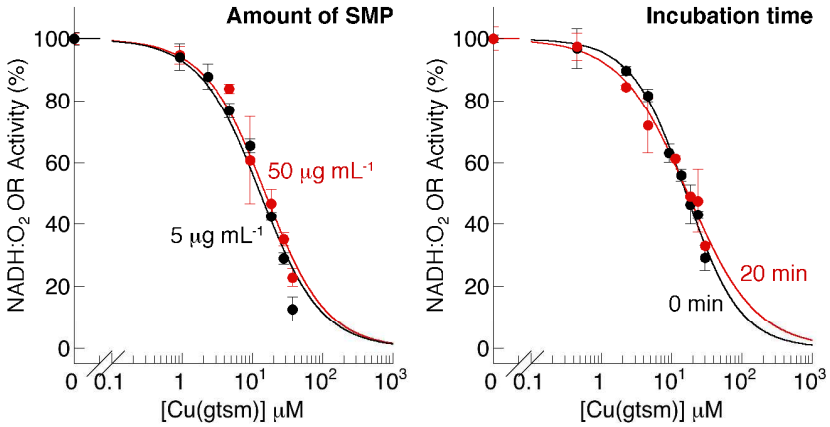

Figure 10. Cu(gtsm) is not an aggregation-based inhibitor. Left panel: effect of SMP concentrations on the inhibition of NADH:O_2 OR activity by Cu(gtsm). The assay was performed using either 5 (black trace) or 50 (red trace) μg mL^{-1} of SMPs. Right panel: effect of incubation time. SMPs (10 μg mL^{-1}) were incubated with Cu(gtsm) for 0 (black trace) or 20 (red trace) min prior to addition of NADH.

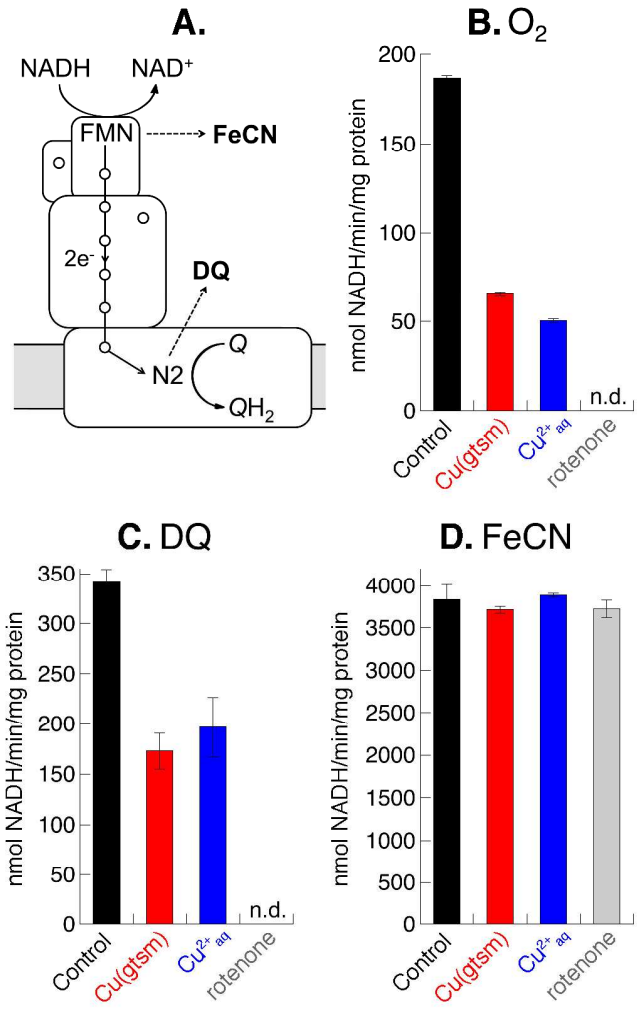

Figure 11. Site of Complex I inhibition by Cu(gtsm). A. The architecture of Complex I. Circles represent Fe-S clusters. Solid arrows indicate direction of electron transfer. Dashed arrows indicate transfer of electrons to artificial acceptors in the solvent. **B-D.** The effects of DMSO (black columns), Cu(gtsm) (40 μM, red columns), Cu^{2+}_{aq} salts (30 μM, blue columns) and rotenone (1 μM, grey columns) on **B.** NADH:O_2, **C.** NADH:DQ, and **D.** NADH:FeCN OR activities. n.d., not detectable.