Silver Clusters of Five Atoms as Highly Selective Antitumoral Agents Through Irreversible Oxidation of Thiols

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Low atomicity clusters present properties dependent on the size, due to the quantum confinement, with well-defined electronic structures and high stability. Here it is shown that Ag$_5$ clusters catalyze the complete oxidation of sulfur to S$^{6-}$. Ag$_5$ catalytic activity increases with different oxidant species in the order O$_2$ $<$ H$_2$O$_2$ $<$ OH•. Selective oxidation of thiols on the cysteine residues of glutathione and thioredoxin is the primary mechanism human cells have to maintain redox homeostasis. Contingent upon oxidant concentration, Ag$_5$ catalyzes the irreversible oxidation of glutathione and thioredoxin, triggering apoptosis. Modification of the intracellular environment to a more oxidized state to mimic conditions within cancer cells through the expression of an activated oncogene (HRAS$^{	ext{G12V}}$) or through ARID1A mutation, sensitizes cells to Ag$_5$ mediated apoptosis. While cancers evolve to evade treatments designed to target pathways or genetic mutations that drive them, they cannot evade a treatment that takes advantage of aberrant redox homeostasis, which is essential for tumor progression and metastasis. Ag$_5$ has antitumor activity in mice with orthotopic lung tumors reducing primary tumor size, and the burden of affected lymphatic nodes. The findings suggest the unique intracellular redox chemistry of Ag$_5$ may lead to new redox-based approaches to cancer therapy.

1. Introduction

The mitochondrial electron transport chain is indispensable for cell proliferation,$^{[1-4]}$ generating as by-product reactive oxygen species (ROS).$^{[5,6]}$ H$_2$O$_2$ sensors measure H$_2$O$_2$ concentration by a redox-based mechanism and set H$_2$O$_2$ specific scavengers' expression, maintaining the H$_2$O$_2$ concentration below a toxic threshold.$^{[7]}$ Thiol redox status within mitochondria, is determined by the mitochondrial glutathione (GSH) and thioredoxin (Trx) systems, critical for cell viability and function.$^{[8]}$ Simultaneous and irreversible inhibition of these pathways could constitute an effective antitumor treatment.$^{[9]}$ Of interest, Au clusters with 5–10 atoms can catalyze the aerobic oxidation of thiophenol to disulfide (S$^{-2}$ to S$^{-1}$) at room temperature and 5 bar O$_2$ with similar catalytic activity to the sulfhydryl oxidase.$^{[10]}$ Metal clusters of just a few atoms can display unique properties that are not observed...
in larger nanoparticles.[11–14] Reducing cluster size from the bulk material to the subnanometric cluster scale alters the particle properties which are greatly affected by strong quantum confinement. Those cluster properties depend strongly on the number of atoms. Here we show that contrary to smaller Ag3 clusters, which do not bind to thiols but intercalate into DNA,[14,15] Ag5, with only two more atoms, binds to thiols and display exceptional catalytic activities for their complete oxidation. Contingent upon oxidant concentration, Ag5 catalyzes the oxidation of cysteine (Cys), GSH, and Trx successively to sulfoxide (S−1), sulfenic (S−2), sulfonic (S−4), and finally irreversibly detaches the sulfur atom from the rest of the molecule and oxidizing it to sulfate (S−6). Having shown that Ag5 affects thiol-based antioxidants, we hypothesized that this could be effectively employed for novel pharmacological applications where redox state is important, such as in cancer.[16,17] We present here that Ag5, depending on the intracellular redox state, catalyzes the oxidation of redox-active Cys residues, leading to the activation of programmed cell death. We further describe driven mutations frequently found in human cancers that render cells sensitive to Ag5.

2. Results and Discussion

2.1. Ag5 Catalyze the Oxidation of Cysteine

Ag5 was synthesized by a modification of a previously developed electrochemical method for the synthesis of Ag3.[14,15] As we describe in the Experimental Section, we increased the voltage with respect to the preparation of Ag3 clusters. This allows to focus the kinetics to produce Ag5 clusters mainly. When the voltage is further increased (see the Experimental Section) larger clusters are obtained. Interestingly such larger clusters do not display the catalytic activities here described for Ag5 but act specifically in the lysosomes and do not cause cell mortality as it will be reported elsewhere.

Ag clusters were fully characterized by high-angle dark-field scanning transmission electron microscopy (HAADF-STEM) (see Characterization of Ag5 and Figures S1 and S2, Supporting Information), UV–Vis, and fluorescence spectroscopy (see Characterization of Ag5 and Figures S3 and S4, Supporting Information), electrospray ionization time-of-flight (ESI-TOF) mass spectrometry (see Characterization of Ag5 and Figures S5 and S6, Supporting Information), X-ray absorption near edge structure (XANES) (see Characterization of Ag5 and Figure S7, Supporting Information). All characterization techniques indicate that samples contain mainly Ag5, with a small percentage (≤10%) of noncatalytic smaller Ag3 and Ag2 clusters, because they do not bind S groups, as we reported before.[14,15]

Using X-ray absorption near-edge structure at the sulfur K edge (S-K XANES), we assessed the effect of Ag5 on the oxidation of the sulfur groups of free Cys, GSH, and recombinant Escherichia coli Trx. The R-SH groups of these three species were oxidized to S−6 (sulfate) in the presence of Ag5 at very low concentration (1000 times less than R-SH, [R-SH]/[Ag5] =1000) after 10 min of reaction (Figure 1a). The gradual increase in the S−6 peak relative to the S−2 peak with increasing Ag5 concentration (Figure 1b) is indicative of the SH oxidation reaction dependence on the Ag5 concentration (see XANES study of Ag5 catalysis, Table S1 and Figures S8–S10, Supporting Information). Thus, Ag5 catalyzes R-SH overoxidation until a biological irreversible state (S−5 or S−6).[18] When the concentration of Ag5 is reduced ([R-SH]/[Ag5] < =10 000) Ag5 still catalyzes the oxidation of sulfur groups but only to form sulfoxides (S−1), at the same time of reaction. As far as we know, there is no catalyst that can totally oxidize sulfur groups with atmospheric oxygen, indicating the high catalytic activity of Ag5 clusters. Moreover, the catalytic activity of Ag5 for the partial sulfur oxidation to disulfide is (TOR ≈ 1000–10 000), at the same reaction time, we obtain a population of different oxidation species, namely, S−1, S−2, S−4, and S−6 (see XANES study of Ag5 catalysis and Figure S11, Supporting Information).

We used density-functional theory (DFT) to determine the possible mechanism by which S-containing compounds are oxidized catalytically by Ag5 (Figure 1c,d). First, we found that Ag5 can adsorb oxygen molecules, activating some of them to superoxide-like species (see Theoretical Calculations, Table S2 and Figures S12–S19, Supporting Information). Using such activated oxgens, Ag5 forms, in a fast step, a disulfide (Figure 1c). Subsequently, as we can see in Figure 1d, the activated oxgens are able to break the S–S bond with the formation of R-(SO)2-Ag5 sulfinic species (I3, S−2) attached to the cluster. Such sulfinic species can be further catalytically oxidized to sulfite R-(SO)3-Ag5 (I5, S−4) and finally, detaching the S from the rest of the organic molecule, (SO4)2-Ag5 (I6), to sulfate (SO4)2-Ag5 (P, S−6) species.

2.2. Other Oxidants Involved in Ag5 Catalysis

Oxidants other than O2 could also be involved in the catalytic activity of Ag5 under biological conditions and understanding their impact could help elucidate whether Ag5 has clinical applications.[19] Therefore, we investigated the catalytic activity by XANES of Ag5 against recombinant E. coli Trx in the presence of H2O2 or the hydroxyl radical (OH•). Due to the higher oxidation activity of these species,

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we carried out the experiments, at 10 min of reaction time, with concentrations of oxidants 10 times less than oxygen and a ratio \([\text{R-SH}] / [\text{Ag}_5] = 5000\). For comparison purposes (Figure 1e), at the same conditions, when exogeneous oxygen is present with clusters we observe the formation of ≈4% of the S in an oxidation state \(> -1\) (1.6% \(S^{+4}\) and 2.4% \(S^{+6}\), i.e., 16% and 24% without concentration correction, displayed in Figure 1e for a better visualization) versus 0% without clusters. We show also that \(\text{H}_2\text{O}_2\) without clusters oxidizes 60% (25% \(S^{+4}\) and 22% \(S^{+6}\)) of recombinant \(\text{E. coli Trx}\), while with clusters this proportion increases to 92% (31% \(S^{+4}\) and 26% \(S^{+6}\)). When using \(\text{OH}^-\) the oxidized proportion is 88% (34% \(S^{+4}\) and 29% \(S^{+6}\)) while with clusters is 92% (12% \(S^{+4}\) and 50% \(S^{+6}\)). Therefore, we can conclude that the catalytic activity of \(\text{Ag}_5\) increases with different oxidant species in the order \(\text{O}_2 \ll \text{H}_2\text{O}_2 < \text{OH}^-\).

**2.3. Cellular \(\text{Ag}_5\) Uptake**

To assess the ability of \(\text{Ag}_5\) to enter cancer cells, we incubated human acute lymphoblastic leukemia MOLT-4 cells with \(\text{Ag}_5\) at 37 °C. After 5 min incubation at 37 °C, 14% ± 1.72 SEM of the total silver offered to the cells was found inside the cell (see Figure S20a,b, Supporting Information). To elucidate the mechanism of \(\text{Ag}_5\) transport across the plasma membrane, we compared the uptake of \(\text{Ag}_5\) in MOLT-4 cells at 4 and 37 °C. There was no difference with temperature indicating \(\text{Ag}_5\) transport is a passive rather than an active process (see Figure S20c, Supporting Information). Moreover, modification of the mitochondrial membrane potential did not alter the amount of silver in isolated mitochondria after \(\text{Ag}_5\) treatment (see Figure S20d, Supporting Information). Together, these data suggest that \(\text{Ag}_5\) can enter cells and mitochondria by passive diffusion as an uncharged species.

**2.4. \(\text{Ag}_5\) Increases Intracellular Thiol Oxidation**

As \(\text{Ag}_5\) oxidizes thiols in vitro and can diffuse into cells we sought to demonstrate that \(\text{Ag}_5\) could oxidize intracellular thiols. We observed a significant reduction (≈20%) in the...
intracellular GSH/GSSG ratio in whole-cell lysates of A549 cells treated with Ag₅ for 1 h (see Figure S21, Supporting Information). Glutathione reductase (GR) maintains the ratio of GSH/GSSG present in the cell by catalyzing the reduction of GSSG to GSH. Western blotting of whole-cell lysates of A549 cells revealed a rapid and substantial increase in the oxidized GR form following stimulation with Ag₅ (Figure 2a). This suggests that Ag₅ may accelerate thiol oxidation by targeting sensitive thiols on redox enzymes, thereby tipping the balance toward the oxidative state. To elucidate time-dependent changes of thiol oxidation by Ag₅, we used a redox sensitive glutaredoxin-1 (Grx1)-roGFP biosensor designed to specifically measure the GSH redox potential. The addition of Ag₅ to A549 cells led to a prompt oxidative response with the maximum signal observed after 7 min (Figure 2b) revealing Ag₅ rapidly mediated thiol oxidation catalysis.

2.5. Ag₅ Increases the Oxidation of Thioredoxin-Dependent Peroxiredoxin 3 (PRDX3) in A549 Lung Carcinoma Cells Mitochondria

As shown by XANES experiments, Ag₅ increases oxidation of thiol groups of E. coli recombinant Trx. Furthermore, QM/MM calculations confirmed the binding of Ag₅ in the Cys-X-X-Cys motif of the Trx (see Theoretical Calculations, Supporting Information). Additionally, in an in vitro assay, Ag₅ strongly inhibited the Trx/thioredoxin reductase (TrxR) system (Figure 2c) (see Ag₅ catalyze Trx and TrxR and Figure S22, Supporting Information). Grx1 also possesses the Trx motif Cys-X-X-Cys, so the observed activation in the Grx1-roGFP biosensor might also be due to the direct effect of Ag₅ mediated oxidation of the Cys residues in Grx1. Therefore, we explored whether Ag₅ also affected the Trx antioxidant system in cells. Trx oxidation depends on H₂O₂ and PRDX3, an enzyme located exclusively in the mitochondrial matrix that reacts rapidly with H₂O₂ and dimerizes via a disulfide. Mitochondria isolated from Wistar rats’ heart and liver were incubated with Ag₅ at different doses for 5 min at 37 °C. As shown in Figure 2d, PRDX3 oxidation was dependent on the Ag₅ concentration (40–240 × 10⁻⁹ m). In contrast, Ag₅ at the largest concentration of Ag₅ used did not affect the PRDX3 redox state. This finding confirms the membrane permeability of Ag₅, as it must have crossed the inner mitochondrial membrane to oxidize PRDX3. Furthermore, 15 min after intact cells were exposed to Ag₅, PRDX3 was oxidized (Figure 2e), confirming that Ag₅ rapidly diffuses into the cell, enters the mitochondria, and increases the oxidation of PRDX3.

2.6. Ag₅ is Cytotoxic for Human Cancer Cells

A chemical disrupting redox homeostasis would be expected to induce cell death. Treatment of A549 human lung adenocarcinoma cells and A2780 human ovarian cancer cell line with Ag₅ increased mortality (Figure 2f) (see Figure S23a, Supporting Information). Consistent with cell death being mediated through thiol oxidation by Ag₅, this toxic effect could be abrogated by the thiol-reducing agents, dithiothreitol (DTT) and N-acetylcysteine (NAC) (Figure 2f). These results were corroborated in various human cancer cell lines (see Figure S23a–e, Supporting Information).

To further implicate the GSH/GR system in the intracellular catalytic activity of Ag₅, we treated A549 and A2780 cells with various chemicals known to inhibit GSH synthesis. Buthionine-[S,R]-sulfoximine (BSO) erasin, and sulfasalazine (SSZ) increased Ag₅ cytotoxicity (see Figure S24a–d, Supporting Information). Erasin and SSZ are pharmacological inhibitors of the cystine transporter SLC7A11, required for GSH synthesis. Gene silencing of ARID1A significantly increases cell sensitivity to Ag₅ (Figure 2g), the estimated ED50 for HCT116 Arid1A was 232 × 10⁻⁹ m, and for HCT116 Arid1A the ED50 value was 192 × 10⁻⁹ m. Thus, known ARID1A mutations in human cancers may indicate potential for Ag₅ treatment.

Furthermore, PRDX3 oxidation corresponds to cytochrome c release and caspase activation, so we examined the Ag₅ effect on apoptotic biomarkers: PARP cleavage, Annexin-V / FITC and 7AAD staining and caspase-3/7 activation (see Figure S25a,b and Movie S1, Supporting Information). All were activated by Ag₅, indicating Ag₅ is triggering apoptotic cell death and concordant with PRDX3 oxidation. If true, pretreatment with auranofin (AF), a TrxR inhibitor that causes the oxidation of PRDX3 could be expected to potentiate Ag₅ cytotoxicity. The combination of AF and Ag₅ present greater cytotoxicity than either treatment alone (see Figure S25c,d, Supporting Information). Conversely, if PRDX3 oxidation is relevant for Ag₅ action, increasing the levels of PRDX3 should reduce Ag₅ efficacy. Transient overexpression of PRDX3 in HEK293 consistently increased the Ag₅ ED50 from 177 ± 12.9 to 193 ± 14.6 × 10⁻⁹ m (Figure 2h).

PRDX3 plays an important role in mitochondrial redox control in cancer cells thus, we studied Ag₅ cytotoxicity across a wide range of tumor cell types to see whether it correlates with PRDX3 expression. To this end, an ED50 value for Ag₅ was obtained for each cell line of a panel of 85 human tumor cell lines (see Figure S26, Supporting Information). PRDX3 mRNA levels and ED50 value are inversely correlated (Figure 2i), pinpointing PRDX3 as a primary mediator of Ag₅ action.

2.7. The Cytotoxic Activity of Ag₅ Depends on the Intracellular Redox State

Contingent upon H₂O₂ concentration, Ag₅ catalyzes the irreversible oxidation of GSH and Trx. Thus, we reasoned that cancer cells with an increased generation or reduced disposal of H₂O₂ will be more sensitive to Ag₅. H₂O₂ is generated continuously in cells that consume oxygen and it is well established that during proliferation cell respiration is increased and thus, could be used to target tumor redox vulnerability (see Figure S27a, Supporting Information). Therefore, we explored whether the increased oxidative stress necessary for tumor cell division is sufficient for Ag₅ to trigger cell demise. First, we confirmed that proliferation increased the A549 intracellular oxidative state, assessing the fluorescence due to 2′,7′-dichlorofluorescein (DCF) oxidation, compared
to that of nonproliferating A549 cells, consistent with an increased oxidative state (see Figure S27b, Supporting Information). In good agreement, Ag5 did not affect the viability of confluent non-proliferating cells; however, proliferating A549 cells viability decreased (Figure 3a). Likewise, serum starvation, a condition widely used to induce reversible cell

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**Figure 2.** Ag5 increases intracellular thiol oxidation affecting the Trx and GSH antioxidant systems. 

- **a)** Western blot analysis showing GR oxidation in A549 cells 15 m after the addition of Ag5. The results are representative of three independent experiments. 
- **b)** A549 cells expressing Grx1-roGFP2 were incubated with Ag5 (0.78 × 10^{-6} m) analyzed by confocal microscopy. Ratios of the fluorescence excitations at 405/488 nm were recorded for 20 min every 10 s. In each experiment, the ratio was quantified for nine individual cells and plotted against time. Data are representative of three independent experiments. 
- **c)** Inhibition of the Trx system in the presence of Ag5. Data are shown as the mean ± SD (n = 3), 2-tailed Student’s t-test. 
- **d)** Western blot analysis showing PRDX3 oxidation in mitochondria from the heart (top) and liver (bottom) after Ag5 (44.5 × 10^{-9}, 89 × 10^{-9}, and 222.6 × 10^{-9} m) and Ag^+ (222.6 × 10^{-9} m) treatment. The results are representative of three independent experiments. 
- **e)** Western blot analysis showing PRDX3 oxidation in A549 cells 15 m after the addition of Ag5. The results are representative of three independent experiments. 
- **f)** Viability of proliferating A549 cells cotreated with DTT (0.5 × 10^{-3} m) and Ag5 ((0.45–2.3) × 10^{-6} m) for 1 h or pretreated with NAC (5 × 10^{-3} m) for 1 h and then Ag5 ((0.45–2.3) × 10^{-6} m) for an additional 1 h. Data are shown as the mean ± SD (n = 3), 2-tailed Student’s t-test. 
- **g)** Viability of HCT116-ARID1A WT and HCT116-ARID1A KO cells treated with Ag5 ((0.25–0.13) × 10^{-6} m) for 1 h. Data are shown as the mean ± SD (n = 3), 2-tailed paired t-test. p = 0.0233. 
- **h)** Viability of HEK293 cells transfected with pcDNA3.1 empty vector or pcDNA3.1-PRDX3 and treated with Ag5 ((0.22–0.12) × 10^{-6} m) for 1 h. Data are shown as the mean ± SD (n = 3), 2-tailed paired t-test. p = 0.0374. 
- **i)** Scatterplot of ED50 values measuring sensitivity to Ag5 versus mRNA expression of PRDX3 for 85 cell lines, Spearman’s rank correlation test. p = −0.30, p = 0.005. For all the panels, cell viability was determined by the MTT assay.
Figure 3. The effects of Ag₅ are dependent on the intracellular redox potential. a) Viability of proliferating and non-proliferating A549 cells exposed to different concentrations of Ag₅ ((0.74–2.2) × 10⁻⁶ M). The results are representative of three independent experiments. b) Viability of proliferating and non-proliferating A549 cells treated with Ag₅, H₂O₂, or both. Data are shown as the mean ± SD (n = 3), 2-tailed Student's t-test. c) Proliferating A549 cells were treated with mito-TEMPO (20 × 10⁻⁶ M) for 1 h and then Ag₅ ((0.16–0.45) × 10⁻⁶ M) for 1 h. Data are shown as the mean ± SD (n = 3), 2-tailed Student's t-test. d) Proliferating A549 cells were treated with Ebselen (25 × 10⁻⁶ M) for 1 h and then treated with Ag₅ ((0.45–2.3) × 10⁻⁶ M) for an additional 1 h. Data are shown as the mean ± SD (n = 3), 2-tailed Student's t-test. e) Viability of proliferating A549 cells treated with 2DG (20 × 10⁻³ M) for 1 h and then Ag₅ (0.45–2.3) × 10⁻⁶ M) for 1 h. Data are shown as the mean ± SD (n = 3), 2-tailed Student's t-test. f) Indirect immunofluorescence images of HEK293T cells stained with an anti-NRF2 antibody (red) and an anti-Keap1 antibody (green). Nuclei were counterstained with Hoechst (blue). Merged images show the nuclear location of NRF2 after 30 min of treatment with Ag₅ (0.52 × 10⁻⁶ M). Scale bars 50 µm. g) Indirect immunofluorescence images of A549 cells stained with an anti-MTF1 antibody (red) and counterstained with Hoechst (blue). Merged images show the nuclear location of MTF1 after 2 h of treatment with Ag₅ (0.78 × 10⁻⁶ M). Scale bars 50 µm. h) Expression of phospho-Jnk (p-Jnk) and total Jnk in U251 cells treated with Ag₅ (0.45 × 10⁻⁶, 0.54 × 10⁻⁶, and 0.72 × 10⁻⁶ M). H₂O₂ (200 × 10⁻⁶ M) was used as a positive control, and α-tubulin was used as the loading control. Representative results from three independent experiments are shown. i) Expression of p-Jnk in A2780 cells 30 min and 2 h after Ag₅ (0.1 × 10⁻⁶ M) and Ag₅ treatment. Representative results from three independent experiments are shown. j) Survival fraction of A549 cells 8 days after treatment with Ag₅ (2.2 × 10⁻⁶ M) and X-ray irradiation (0, 2, 4, and 6 Gy). Data are shown as the mean ± SD (n = 3), 2-tailed Student's t-test. For all the panels, cell viability was determined by the MTT assay.
that various effectors can mediate apoptosis triggered by Ag5. (see Figure S27e, Supporting Information). Further confirmed both serum-starved and confluent nonproliferating A549 toxicity of Ag5 compared to that with Ag5 alone in A549 cells (Figure 3c), indicating that mitochondrial H2O2 levels are important for Ag5 activity. Conversely, Ebselen has been reported to decrease ROS levels[34] and it caused a dose-dependent reduction in the cytotoxic effect of Ag5 on proliferating A549 cells and A2780 cells (see Figure S27d, Supporting Information). To further evaluate the role of the intracellular redox environment in the intracellular catalytic activity of Ag5, we treated A549 and A2780 cells with 2-deoxyglucose (2DG) that produce oxidative stress[35] and, accordingly increased Ag5 cytotoxicity (Figure 3e) (see Figure S27e, Supporting Information). Further confirmation of the link between Ag5 action and the intracellular oxidative state comes from comparing the cell oxidative state, as seen by DCF fluorescence, among the A549 and A2780 cell lines. A2780 cells shown an increased fluorescence and are more sensitive to Ag5. (see Figure S27i, Supporting Information). In summary, in the presence of an intracellular oxidative environment, Ag5 accelerates oxidative reactions, leading to programmed cell death. However, in a reductive intracellular environment, Ag5 has no biological effects.

2.8. Ag5 Activate Redox Signaling Pathways

Ag5 mediated oxidation of GR and PRDX3 should lead to oxidative stress and, consequently, activation of redox signaling pathways. Among the most well-known redox signaling pathways are ASK1,[36] NRF2-KEAP1,[37] and MTF1[38] pathways. NRF2 and MTF1 are nuclear-cytoplasmic shuttling proteins that accumulate in the nucleus upon activation. In the presence of Ag5, we observed the nuclear accumulation of NRF2 (Figure 3f) (see Figure S28a,b, Supporting Information) and MTF1 (Figure 3f) (see Figure S28c, Supporting Information). Confirming previous findings, we observed a significant increase in the nuclear accumulation of MTF1 and NRF2 in HEK293T cells treated with Ag5 in the presence of DTT (0.5 × 10⁻³ m) (see Figure S28h,c, Supporting Information). Moreover, qPCR confirmed the overexpression of genes under NRF2 transcriptional regulation, HMOX1 and GCLM, and genes transcriptional regulated by MTF1, MTF2, and MTF5 in a human multiple myeloma MM.1S cell line (Table S3, Supporting Information). Furthermore, activation of the ASK1 pathway was assessed by analyzing its downstream effector c-Jun N-terminal kinase (JNK).[39] Ag5 treatment caused a dose- and time-dependent increase in JNK phosphorylation, indicating ASK1 activation (Figure 3h,i, respectively). JNK phosphorylation activates mitochondrial death factors such as Bim and Bax, resulting in cytochrome c release and initiation of apoptosis[40] suggesting that various effectors can mediate apoptosis triggered by Ag5.

2.9. X-Ray Irradiation Enhances the Intracellular Catalytic Activity of Ag5

Radiotherapy forms free radicals, notably OH•, which then damages the DNA. XANES experiments suggested that the increase of OH• in irradiated cells could sensitize them to Ag5. Of relevance, Ag5, in combination with X-ray irradiation at even the lowest absorbed dose, 2 Gy, decreased cell viability, indicating X-ray irradiation cooperates to increase Ag5 cytotoxicity (Figure 3j) (see Figure S29a, Supporting Information). Importantly, X-ray irradiation-induced potentiation of the effect of Ag5 on cell death was not due to a concomitant increase in phosphorylation of the histone H2AX (γ-H2AX), a marker for DNA damage (see Figure S29b, Supporting Information), suggesting that Ag5 acts through a different cell death mechanism, probably using OH• generated by X death-ray irradiation to accelerate irreversible Cys oxidation, as XANES experiments suggests.

2.10. Ag5 Preferentially Cause Cell Death in RAS-Transformed Cells and Hypoxic Cells

Activated oncogenes cause metabolic changes that give rise to the accumulation of ROS.[41–43] We therefore reasoned that compared to non-transformed cells, transformed cells would be preferentially killed by treatment with Ag5. To evaluate this hypothesis, we introduced a doxycycline-inducible activated allele of HRAS (HRASG12V) into non-transformed immortalized mouse fibroblasts (W3T3 cells) and confirmed inducible HRAS expression by western blotting (Figure 4a). Interestingly, cells expressing HRAS had greater DCF fluorescence, indicating increased oxidation (Figure 4b) and were more sensitive to cell death induced by Ag5 activity than noninduced cells (Figure 4c). These effects were inhibited by DTT coadministration, confirming that HRAS-induced redox changes are responsible for the increased effects of Ag5. (see Figure S30, Supporting Information).

Metabolic phenotypes in tumors are both heterogeneous and flexible[44–45] and thus may affect Ag5 therapeutic efficacy. Conditions within tumoroids or multicellular tumor spheroids (MCTSs) resemble the pathophysiological conditions within human tumor tissue in many ways and are widely used for drug testing.[46] Like human tumors, large MCTSs, more than 600 μm diameter, are characterized by a heterogeneous cell subpopulation, with actively proliferating cells on the periphery and quiescent, hypoxic, and necrotic cells in the inner regions.[47] Therefore, to assess the effects of the Ag5 on hypoxic cells, we developed A549 cell MCTSs as an ex vivo tumor model. The MCTSs were treated with Ag5 (4.5 × 10⁻⁶ m) four times (days 0, 2, 4, and 6, with the first day of treatment set as day 0) or vehicle (control) and examined daily. Ag5 treatment decreased the area of the MCTSs over the seven-day period (see Figure S31, Supporting Information). Notably, a less dense region was observed in the center of the MCTSs treated with Ag5, but not the control MCTSs, suggesting decreased cellularity that appears to be more pronounced in hypoxic centrally located cells of the MCTSs (Figure 4d, red arrow). Comparable results were obtained in MCTSs formed from HCT116,
Figure 4. Ag₅ preferentially induce cell death in RAS-transformed and hypoxic cells and show antitumoral activity in vivo. a) W3T3 cells carrying a doxycycline inducible RASV12 allele were exposed to doxycycline (red bars) or vehicle (white bars) for 24 h and then treated with different concentrations of Ag₅ (2.6–7.5 × 10⁻⁶ M). RASV12 expression was assessed by western blotting, b) DCFA oxidation induction was measured by flow cytometry, and c) cell viability was determined by MTT assay 24 h later. Data are the mean ± SEM of four independent experiments: two-way ANOVA with Bonferroni's correction. d) Images of control and Ag₅-treated MCTSs formed from A549 cells showing differences in size and cellular density in the central region (red arrows). Scale bar 100 μm. e) Optical sections of live HCT116 cell-derived MCTSs treated with Ag₅ obtained with light sheet fluorescence microscopy (LSFM). Nuclei were stained using Hoechst, dead cells using propidium iodide (PI) and apoptotic cells using the CellEvent Caspase-3/7 green detection reagent. Scale bar 200 μm. f) Confocal images of live A549 cell derived MCTSs treated with Ag₅. Hypoxic regions were stained using Image-iT green hypoxia reagent. Nuclei were stained by Hoechst. Red asterisks point to agarose that remained adhered to the spheroid surface. Scale bar 200 μm. g) Tumor growth in mice with orthotopic lung cancer was measured in vivo by luminescence (IVIS Spectrum). Black arrows indicate days on which treatments were administered. h) Luciferase activity was quantified ex vivo in lung and mediastinal lymph nodes. i) Immunohistochemical staining of lung tumors with a monoclonal antibody specific for human cytokeratin 7 allowed us to differentiate the morphology of the tumor and nontumor tissues. Red arrows indicate tumor nodules. Experimental groups: CDDP (4 mg kg⁻¹), Ag₅ (0.25 mg kg⁻¹), and control (no treatment). Data represents the mean ± SD. Error bars represent standard deviation; n = 5 mice per group. Mann-Whitney test.
indicating that cellular distribution rather than the cell line, was responsible for the effect (Figure 4e). The presence of these Ag₅ sensitive inner regions in the MCTSs after Ag₅ treatment could be related to the ability of the Ag₅, given their small size and neutral charge, to penetrate the MCTSs and reach these central hypoxic regions commonly found in human tumors.[48]

Then, because both hypoxia and low-glucose conditions increase intracellular oxidation,[33] we hypothesized that they would sensitize cells to Ag₅ activity. To assess this hypothesis, we first measured the degree of hypoxia in the tumors using a fluorescent probe to confirm the presence of hypoxic conditions and clearly observed in a 1000-cell tumoroid (Figure 4f, top row). Interestingly, exposure of the tumoroids to Ag₅ at increasing doses caused a dose-dependent reduction in hypoxic cells (Figure 4f). Nevertheless, a fraction of the cells in the tumoroid is resistant to Ag₅, which we tentatively ascribed to nonproliferating cells with a higher intracellular reductive state; further characterization is now needed to establish whether cellular resistance is permanent or can be reversed when cells proliferate again.

2.11. Ag₅ Show Antitumoral Activity In Vivo

To determine whether the in vitro results described above would be confirmed through in vivo experiments, we evaluated the anti-tumoral activity of Ag₅ in vivo by using an orthotopic model of metastatic lung cancer[49] in which metastatic cells can be detected in the mediastinal lymph nodes 13 days after the injection of cancer cells into the lung. Therefore, to evaluate the effects of Ag₅ on an advanced cancer in which metastasis has occurred, we intravenously administered Ag₅ 20 days after the implantation of cancer cells in the lung, when lymph node metastases were already evident. Tumor growth was measured over time by in vivo bioluminescence imaging, and the cancer cell burden from both lymph node metastases and the primary tumor was assessed ex vivo at the time of sacrifice. Cisplatin (CDDP), a first-line treatment for metastatic lung cancer, was used as positive control for tumor growth inhibition. As shown in Figure 4g, treatment with Ag₅ significantly reduced tumor growth compared to that in untreated animals, in which the tumors grew exponentially. Additionally, compared to control mice, Ag₅-treated mice exhibited significantly reduced luciferase activity in both the primary tumor and mediastinal lymph nodes at the time of sacrifice (Figure 4h). Further immunohistochemical analysis of lung sections from the mice confirmed the antitumor effect of Ag₅ (Figure 4i). Additionally, no signs of toxicity, either by body weight loss or by blood cell counts, were observed in the mice after treatment with Ag₅ at the therapeutic doses used, suggesting their potential safety in humans (Table S4 and Figure S32, Supporting Information). Together, these results show the ability of Ag₅ to significantly decrease both the primary tumor and metastatic cell burden in an advanced metastatic cancer.

3. Conclusion

There are very few redox active therapeutic modalities. Here we have shown that the unique intracellular chemistry of Ag₅ can selectively target and disrupt cell metabolism in cells with elevated redox stress, such as cancer cells. Treating tumors based on their unique genetic profile has proved difficult, exposed the patients to severe toxicity when either monotherapy or combination therapy has been used, and acquired resistance is common.[90] As an alternative, targeting metabolic vulnerabilities shared by tumors has generated growing interest in recent years. Here we show that Ag₅ triggers tumor cell death by acting synergistically with the elevated levels of redox stress in tumor cells. Importantly, in most normal resting cells which have a low level of redox stress, so H₂O₂ levels are low, while the oxidation of their thiols is accelerated by Ag₅, the unstressed antioxidant systems in these cells can maintain homeostasis. However, if H₂O₂ levels increase above a threshold level, for example in tumors, antioxidant systems are overwhelmed because they cannot counteract the increased oxidation rate caused by the presence of Ag₅. Also, if the antioxidant systems are affected, the necessary amount of H₂O₂ to produce cell death in the presence of Ag₅ is lower. Therefore, Ag₅ does not induce cell death in the balanced redox environment in normal cells, but when the redox balance is skewed in the presence of elevated intracellular H₂O₂ production, Ag₅ can catalyze the irreversible oxidation of thiol groups in key molecules of both the GSH and Trx antioxidant pathways, particularly in the mitochondria that ultimately leads to oxidative stress and cell death. Of relevance, our results indicate that X-ray irradiation forming free radicals sensitized cells to Ag₅; the combination of Ag₅ with X-ray irradiation, at even at the lowest absorbed dose, improves the therapeutic ratio of radiation, which now deserves further studies.

Hypoxia is strongly associated with poor prognosis[51] and is a leading cause of therapy resistance,[52] the finding that Ag₅ was effective in reducing hypoxic regions of MCTS is of interest as a potential new approach for the treatment of tumor hypoxic cells. Therapeutic efficacy of Ag₅ was demonstrated in an orthotopic model of metastatic lung cancer in which Ag₅ was shown to inhibit the growth of both the primary tumor and the mediastinal lymph node metastases. These findings are of particular interest because high ROS levels have not only been described in proliferating tumor cells, but also in metastatic settings. Our findings suggest the unique intracellular redox chemistry of quantum clusters may lead to new redox-based approaches to cancer therapy.

4. Experimental Section

Synthesis of Ag Clusters: Unless otherwise specified, all reagents were purchased from Sigma Aldrich, Co., Spain. Thioredoxin (TRX-01 Thioredoxin1, E. coli (native)) was purchased from IMCO Corp Ltd AB. Silver sheets (99%) were purchased from Goodfellow Cambridge Ltd., Huntingdon, UK. Alumina nanoparticles (average size ≈ 50 nm) and cloth pads were purchased from Buehler, Düsseldorf, Germany. Sandpaper (1000 grit) was supplied by Wolfcraft España S.L, Madrid, Spain. All aqueous solutions were prepared with MilliQ-grade water using a Direct-Q8UV system from Millipore (Millipore Ibérica S.A., Madrid, Spain). Mica sheets (Grade V-1 Muscovite) were purchased from SPI Supplies, West Chester, PA, USA. The synthesis was carried out with a Biologic VMP300 potentiostat (Seyssinet-Pariset, France). A Methrom thermally insulated three-electrode electrochemical cell, deoxygenated just prior to the synthesis, was used with a hydrogen electrode as a reference and two Ag foils (17.5 cm² surface area) as working electrodes.
as counter and working electrodes. The experimental conditions were similar to those reported before,[41,56] but the voltage was increased with respect to the preparation of Ag7 clusters (10 V) because in this way larger clusters are produced. This (besides the electrode’s polishing, which determines the surface’s roughness) is crucial for achieving the desired cluster’s size, as it will be reported elsewhere. It has to be noted that Ag clusters with larger sizes were also synthesized (mainly consisting in Ag9 and Ag11, see inset in Figure S4, Supporting Information) increasing further the voltage, to compare their biological properties with the ones reported here. Prior to the synthesis, both silver electrodes were polished with sandpaper followed by alumina (50 nm), washed thoroughly with MilliQ water and sonicated. An ion-selective electrode was used to verify that, after synthesis, the concentration of remaining Ag ions are always less than 3 mg L⁻¹ after precipitation with NaCl, and various techniques were used to characterize the synthesized Ag clusters (see also below).

The samples were finally concentrated at 50 °C using a rotary evaporator (Buchi Rotavapor R-210 at a pressure of 2 mbar) (Massó Anàlitica S.A., Barcelona, Spain) to a final concentration of ~30 mg L⁻¹ as determined by flame atomic absorption spectroscopy.

Cluster’s Characterization Methods: In Ag clusters’ characterization methods, both UV-vis and fluorescence spectroscopy experiments were performed at room temperature using a 1 cm path length Hellma quartz cuvettes (Hellma GmbH & Co. KG, Müllheim, Germany). UV-vis spectra were recorded with an Analytik Jena Specord S600 spectrometer (Analytik Jena AG, Jena, Germany) with a diode array detector, and fluorescence spectra were recorded with a Cary Eclipse Varian fluorimeter (Agilent Technologies Spain, S.L., Madrid, Spain).

Electron microscopy studies were performed on a FEI Titan Themis 60-300 Double Aberration Corrected microscope operated at 200 kV. The aberrations of the condenser lenses were corrected up to fourth order using the Zernike table to obtain an astigmatism electron probe. A condenser aperture of 50 µm yielding an electron probe with a convergence angle of 20 mrad was used. To avoid sample modification under the electron probe a beam current of 0.025 nA was used. STEM samples were prepared by depositing one drop of Ag clusters’ solution onto holey carbon coated Cu grids. After preparation, samples were maintained under vacuum conditions.

Electrospray ionization time-of-flight (ESI-TOF) mass spectrometry measurements were performed using a Bruker MicroTOF mass-spectrometer operating in negative ionization mode. Temperature control and nitrogen drying gas (1 µL/min) in ESI source were employed to assist the ionization process. The ESI source conditions were as follows: source voltage -4.5 kV, heated capillary temperature 275 °C, capillary voltage –35 V and sheath gas auxiliary gas S and 2 (N₂, arbitrary units). For full-scan MS analysis, the spectra were recorded in the range of m/z 100 to 1000 with a scan speed of 1 scan/s. All spectra were acquired in reflectron mode of the TOF mass spectrometer equipped with multistep detection to obtain maximum sensitivity. The mass resolution was set at 15000 FWHM. The instrument was calibrated using a calibration solution according to the manufacturer’s instructions. Due to the difficulty to charge the uncharged clusters[53] a systematic investigation was carried out to obtain the maximum sensitivity for the detection of Ag clusters. As it will be published elsewhere, concentrations of NaCl and formic acid are crucial to achieve this, approaching 0.1 ppm sensitivity for the optimal conditions. 2 µL of Ag clusters’ solutions diluted 300 times to contain 25 × 10⁻³ M of NaCl (which is the optimum NaCl concentration for Ag clusters’ detection) was directly injected into a mobile phase of acetonitrile containing 0.1% formic acid at a flow rate of 0.2 mL min⁻¹. Details of the analysis of Ag clusters’ peaks are given in the Supporting Information.

XANES Characterization of Sulfur Oxidation States: S K-edge (2470 eV) and L3 Ag-edge (3351 eV) X-ray absorption near edge structure (XANES) experiments were performed at de SXS beamline at the Laboratório Nacional de Luz Sincrotron (LNLS, Campinas, Brazil) which was equipped with an InSb (111) double crystal monochromator with slit aperture of 1 mm, to achieve a resolution of about 0.5 eV. Details of the experimental setup of the SXS beam line were published elsewhere.[33] X-ray absorption spectra were recorded in fluorescence mode, collecting the emitted X-ray from the S K-edge, (at 2309.5 and 2308.4 eV, respectively) and Ag L₂ (at 2928.7 eV) emission lines for each measured edge. Absorption experiments were performed either in a vacuum of 10⁻⁶ mbar at room temperature, and in a special liquid sample holder designed ad hoc for the experiment with reactive oxygen species, at room temperature and atmospheric pressure, allowing us to determine initial reactions intermediaries as well as final compounds. The photon energy was calibrated by assigning the value 2481.5 eV to the highest maximum of Na₂SO₃ (corresponding to the so-called inner sphere), in accordance with the criteria previously reported by Vairavamurthy.[34] The final XANES spectra were obtained after background subtraction and normalization to the post edge intensity, following usual procedure described elsewhere. XANES quantification was performed with Athena software and subsequent analysis on Origin lab software. For GSM characterization, a fraction of solution was deposited by drop casting on carbon disks (Ted Pella, Inc) to have an Ag or S concentration in a detectable value. For Trx samples, they were carefully mounted in a liquid sample holder, designed ad hoc for this experiment. Phosphate buffered saline (PBS) solution was used as the solvent in all the reaction mixes with Trx, with the purpose to reproduce the same intracellular pH and ionic strength. The hydroxyl radical solutions were prepared by Fenton reaction, with H₂O₂ and FeCl₃. Concentration values are indicated in the Table S1, Supporting Information.

Theoretical Calculations: DFT and QM/MM calculations were performed on the systems shown in the Supporting Information, with full geometry optimization, using the B3LYP[55,56] functional, the 6-31G(d,p)[57,58] basis set for H, C, N, O, S atoms, and Lanl2dz[59] basis set for Ag. The ONIOM[100] method was used for two layers QM/MM calculations and the UFF[80] force field was used in the lower layer. The implicit water solvent was considered through the C-PCM[201] method. Transition state structures were found by using the synchronous transit guided quasi-Newton method.[202] Frequency calculations, within the harmonic oscillator approximation, were performed on the optimized geometries, to confirm that they corresponded to minimum energy structures (all real vibration frequencies) or transition states (one imaginary vibration frequency). All calculations were performed by the Gaussian09[84] program package.

Ag7 Biological Action: The following reagents were used: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT (Sigma, M2128), hydrogen peroxide solution, H₂O₂ (Sigma, 516813), d-cysteine (DTT) (Fluka, 43815), 2-deoxy-d-glucose, 2DG (Sigma, D6134), l-buthionine-sulfoximine, BSO (Sigma, B2515), N-acetyl-l-cysteine, NAC (Sigma, A9765), Erastin (Sigma, E7781-SMG), Sulforalisone (Sigma, S0883-10G), Ebselen (Enzo, ALX-270-097-M005), Auranofin (Sigma, A6733) CM-H₂DCFDA (Molecular Probes, C6827), CellEvent Caspase 3/7 Reagent (Molecular Probes, C10423), Annexin V-FITC (BD Pharmingen, 550911), 7-amino actinomycin D, 7-AAD (Molecular Probes, I14834), Premo Cellular Redox Sensor Gx1-1roGFP (Molecular probes, P23648), Hoechst 33258 (Molecular Probes, 10778483), Fluoroshoield Mounting Medium (Sigma, F6182), The following antibodies were used: anti-phospho-histone H2A.X (Ser 139) FITC conjugate (Millipore, 16-202A), anti-MTF-1 (Santa Cruz Biotechnology, sc-48775), anti-Nrf2 (Santa Cruz Biotechnology,
screening of the cell panel was carried out by OncoLead (OncoLead GmbH & Co. KG, Zugspitzstr. 5, 85757 Karlsfeld, Germany).

Mammalian Mitochondrial Preparations: Mitochondria isolation from rat liver and rat heart was prepared as described previously.8,9 Bovine heart mitochondrial membranes (BHM) were prepared as described previously.65,66 Protein concentration was determined using the bicinchoninic acid (BCA) assay with BSA as a standard.

Ag5: Uptake: Cellular uptake: MOLT-4 (6 × 10^6) cells were incubated with Ag5 (557 ± 10^−12 M) for 5 min at 37 or 4 °C. After that, cells were centrifuged for 5 min at 1000 rpm. Supernatant was collected, and cellular pellet was washed with cold PBS and centrifuged at 4 °C for 5 min at 1000 rpm.

Mitochondrial uptake: 1 mg mL^−1 of isolated mitochondria from rat liver were incubated for 5 min at 37 °C with Ag5 (333 ± 10^−9 M) and either rotenone (4 µg mL^−1); rotenone (4 µg mL^−1) and succinate (5 × 10^−3 M); rotenone (4 µg mL^−1), succinate (5 × 10^−3 M), and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (500 × 10^−9 M). Alternatively, FCCP was added after the incubation with the previous mixture. Then, mitochondria were centrifuged at full speed for 1 min and pellet was collected.

Both, cellular and the mitochondrial pellets were digested in HNO3 at 90 °C for 4 h. Ag concentration was measured in a NexION 300X ICP-MS, Perkin Elmer.

Cell Viability Assays: Cells were seeded in a 96-well plate (A549 (4 × 10^4 cells per well), MCF7 (4 × 10^4 cells per well), U87-Luc (5 × 10^3 cells per well), HCT116 (5 × 10^3 cells per well), HEK293 (5 × 10^3 cells per well), and A2780 (6 × 10^3 cells per well)). For proliferating cells, 24 h later cells were treated under different conditions: 1) with Ag5 at the desired concentrations for 1 h, 2) with H2O2 (200 × 10^−6 M) alone or in combination with Ag5 for 1 h, 3) preincubated with NAC (5 × 10^−3 M) or Ebselen (25 µL) for 1 h or with 2DG (20 × 10^−6 M) or BSO (20 × 10^−3 M) for 24 h and then treated with Ag5 at desired concentrations for 1 h more, and 4) treated with DTT (0.5 × 10^−6 M) alone or with Ag5 and DTT (0.5 × 10^−6 M) for 1 h. Then, the medium was replaced with complete medium for 24 h more and cell death was measured by the MTT assay. 10 µL of MTT solution (5 mg mL^−1) were added to each well and incubated at 37 °C protected from light. 4 h later, 100 µL of solubilization solution (SDS:0.1N HCl) were added and samples were incubated for 18 h at 37 °C. Absorbance was measured at 595 nm using a CLARIOstar microplate reader. For nonproliferating cells, proliferation was stopped using two approximations: 1) confluency: cells were allowed to grow during 96 h in medium-10% of FBS to reach the confluence and 2) serum starvation: 24 h after seeding medium was replaced with medium-0.05% of FBS for 72 h. Then, cells were treated with 1) Ag5 for 1 h and incubated in complete medium for 24 h more or 2) with H2O2 (200 × 10^−6 M) alone in combination with Ag5 for 1 h. Then, the medium was replaced with complete medium for 24 h more and cell death was measured by the MTT assay as described above. Trx and TrxR Activity: Trx and TrxR activity was assessed using the Kit for assays of mammalian Tlx and TrxR (IMCO) following manufacturer’s instructions.

To measure Tlx activity, Tlx and TrxR were incubated with different doses of Ag5, for 30 min following manufacturer’s instructions. Then, absorbance was measured at 412 nm using a CLARIOstar plate reader, BMG Labtech. The effect of Ag5 in Tlx activity was determined by the inhibition of TBN signal. Tlx activity was measured using two electron acceptors: 1) Selenocysteine (protocol adapted from Cuniff et al.85). TlxR from rat liver (100 ng dissolved in PBS) was incubated with Ag5 (1.3 × 10^−6 M) or Ag^+ (the same Ag concentration) for 10 min in a 96-well plate. After that, a solution of NAPDH and Selenocysteine was added to each well at a final concentration of 400 × 10^−6 M. Immediately, NAPDH consumption was measured at 340 nm for 30 min using a SpectraMax plate reader, Molecular Devices. 2) H2O2 (protocol adapted from Zhao et al.86). TlxR from rat liver (17 × 10^−9 M, dissolved in Tris−Cl pH 7.5 5 × 10^−3 M, EDTA 1 × 10^−3 M) was incubated for 30 min with Ag5 (1.15 × 10^−6 M) in a 96-well plate. Then, H2O2 (0.5, 1, 2, and 5 × 10^−3 M) was added. Finally, NAPDH (dissolved in Tris 50 × 10^−3 M, EDTA 1 × 10^−3 M) was added to each well
at a final concentration of 100 × 10⁻⁶ M. Immediately, the plate was read at 340 nm in a CLARIOstar plate reader, BMG Labtech.

**Insulin Oxidation:** For the aerobic conditions, 210 × 10⁻³ m HEPEs pH 7.6, 790 × 10⁻³ m insulin and 20 × 10⁻³ m EDTA were mixed and 19.2 µL of the solution were incubated in TE buffer with 5 µL of 0.5 m DTT and 5 µL of 12.3 × 10⁻¹ m Ag⁺ as indicated in 50 µL final volume for 30 min. Then, 200 µL of 1 × 10⁻³ m DTNB supplemented with 7.2 m GuHCl in 0.18 m Tris–Cl pH 8.0 and 9.95% ethanol was added to each well and absorbance was measured at 412 nm using a micro plate reader in a CLARIOstar plate reader, BMG Labtech.

For the anaerobic conditions, insulin was incubated in TE buffer and DTT as described above in a Baker Ruskinn Invivo2 200 hypoxic chamber (0.2% O₂ and 5% CO₂) prior deoxygenation of samples for 30 min. Then, insulin sample was tested in the presence of DTT (control) or passed through a Illustra MicroSpin G-25 Column, GE Healthcare containing Sephadex as manufacturer’s instructions to remove DTT. After that, 5 µL of deoxygenated Ag⁺ (9.6 × 10⁻⁴ m) were added and after a 30 min incubation in hypoxic conditions, DTNB was incorporated and absorbance was measured as described above.

**GSH Oxidation in Living Cells:** A549 cells (2.5 × 10⁴ cells) were seeded in 35 mm plate dishes (Mattek) and transduced with the Premo Cellular Redox Sensor (C-1-GFP) following manufacturer’s instructions. After that, Ag⁺ (IC₉₀) were added to the dish and cells imaged during 10 min. Images were taken every 10 s using a Leica TCS SP8 confocal microscope. Fluorescence intensity emitted by each cell was measured at 405 and 488 nm and emission was collected at 500–530 nm to calculate the ratio images. Images were processed using the ImageJ software. Ratio images were created using the RatioPlus Plugging and the ImageJ Look Up table “green fire blue” was used to create false-color ratio images.

**GSH/GSSG Ratio:** A549 cells (6 × 10⁴ cells) were seeded in 96-well clear bottom white microplates and 24 h later treated with Ag⁺ (IC₉₀) for 1 h. Then, oxidized, and total glutathione were measured using the GSH/GSSG-Glo Assay according to the instructions of the kit using a CLARIOstar microplate reader.

**PRX3 Transfection:** HEK293 cells were transfected with pcDNA3.1-PRX3 (GenScript, clone ID OHu14665) or the empty vector using calcium phosphate. At 24 h, the cells were counted and seeded on 96-well plates previously covered with polylysine. After one day, Ag⁺ was added at the indicated amounts for 1 h, washed and cells were allowed to grow for additional 24 h. Cell viability was measured by the MTT assay. PRX3 overexpression was checked by western blot.

**Correlation Between PRDX3 mRNA Expression and Ag⁺ Sensitivity:** An EDSG value was obtained for each cell line by fitting a two-parametric log-logistic model with lower limit at 0 and upper limit at 1 using the drm package⁴⁹ to cell viability values relative to the negative control, as a function of drug concentration. Gene expression data were obtained from https://depmap.org/portal/download, Version 20Q2⁵⁰ and available for 85 of our cell lines. Their correlation was assessed using Spearman’s rank correlation implemented in the cor.test function of R.

**Determination of ROS Using Fluorescent Dyes: H₂DCFDA staining:** A549 (3 × 10⁴ cells per well) and A2780 (3.5 × 10⁴ cells per well) were seeded in 24-well plates and allowed to grow for 24 or 96 h for proliferating and nonproliferating cells. W3T3 RasV12 (5 × 10⁴ cells per well) were seeded in 12-well plates. Then, cells were trypsinized, washed with PBS and incubated with CM-H₂DCFDA for 30 min at 37 °C. Stained cells were analyzed using the Guava EasyCyte flow cytometer and processed with the Flowjo program.

**Immunoblotting:** Unless specified, protein extracts from cultured cells were extracted using RIPA buffer with protease inhibitor cocktail (Sigma). After the electrophoresis proteins were transferred to PVDF membranes and blotted against the indicated antibodies.

**Caspase 3–7 Assay:** A549 cells (3 × 10⁴) were seeded in 24-well plate and 24 h later cells were treated with Ag⁺ (IC₉₀), in the presence of the CellEvent Caspase 3/7 reagent (8 × 10⁻⁶ M). Time-lapse images were acquired every minute during 1 h using an inverted Leica DMi600B Wide-Field microscope equipped with an Okolab incubator controlled by an Okolab temperature controller and a DFC360FX Camera. Images were processed with the LAS AF software.

**Annexin-V Assay:** A549 (6 × 10⁴) cells were seeded in 12-well plate and 24 h later treated with Ag⁺ (IC₉₀) for 30 min. Then, cells were washed twice with PBS and stained with Annexin V-FITC and 7-AAD following manufacturer’s instructions. Images were obtained using Olympus IX51 microscope equipped with an Olympus DP72 camera and CellSens Imaging Software and processed with ImageJ.

**Cell Irradiation:** Cells were irradiated with 6 MV X-Ray beams generated by a Varian Medical Systems (Palo Alto, California) CLINAC 2100 C/D High energy linear accelerator at Radiation Physics Laboratory of University of Santiago de Compostela (http://www.usc.es/rpl). Absorbed dose to cells was determined by ionometric dosimetry corrected by Monte Carlo simulations. Irradiations were carried out by placing cells in 24-well plates surrounded by water equivalent plastic for electronic build up. A549 (3 × 10⁴ cells per well) were seeded in 24-well plates and allowed to grow for 96 h until they reach confluence (nonproliferative state). Then, cells were treated with Ag⁺ (1.5 × 10⁻⁶, 2 × 10⁻⁶, 2.2 × 10⁻⁶ m) for 10 min and irradiated. Target absorbed doses (between 2 and 6 Gy) were delivered by single fractions at a rate of 2 Gy min⁻¹. After irradiation, cells were incubated with Ag⁺ until complete 1 h of treatment and then trypsinized and resuspended for colony formation. 8 days later, cultures were fixed with crystal violet and colonies (>50 cells) were counted. Survival fractions (SF) were fitted to the linear quadratic model using GraphPad Prism 5 software (GraphPad Prism Software Inc., La Jolla, CA, USA).

**DNA Damage Assay:** DNA damage following irradiation was determined by the extent of phosphorylation of the histone protein γH2AX. A549 cells (3 × 10⁴ cells) were seeded in 24-well plate. After heating at 95 °C for 5 min. Protein was determined by western blotting with anti-PRDX3. Membrane was visualized with an Odyssey CLx.
96 h, cells were treated with Ag₂ (1.5 × 10⁻⁶, 2 × 10⁻⁶, 2.2 × 10⁻⁶ m) for 10 min and irradiated. Then, cells were collected and DNA damage was measured using the p-H2AX (ser 139) FITC-conjugate antibody as described previously.[14] Stained cells were analyzed on Guava EasyCyte flow cytometer using InCyte program.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR (qRT-PCR): MM.1S cells (4 × 10⁵) were seeded in 60 mm culture dishes and treated with Ag₂ (IC₅₀) for 30 min. Then, treatment was removed and 4 h later cells were collected, and their RNA isolated using the kit NucleoSpin ARN (Macherey-Nagel) following the manufacturer instructions. Reverse transcription was performed using the M-MLV reverse transcriptase, according to the manufacturer’s instructions and analyzed by qRT-PCR using the TaqMan chemistry. Relative changes in gene expression were determined using the 2^-ΔΔCt method.[7]

Multicellular Tumoroids: A549 and HCT116 multicellular tumoroids were generated by the hanging-drop method as described previously.[7] 500 cells/20 µL were dispensed into each well of a MicroWell MiniTray (Nunc). At day 5 upon the inversion of the tray, spheroids were transferred to a 96-well plate coated with agarose (1%) and treated with Ag₂ four times on alternate days. To monitor spheroids growth images of each spheroid were taken every day using an Olympus IX51 microscope. Spheroid’s area was measured using ImageJ. At the end of the experiment, A549 spheroids were stained with Image-IT Green Hypoxia Reagent 5 × 10⁻⁶ m and Hoechst (1 µg mL⁻¹) for 1 h. Images of control and treated spheroids were taken on a Leica AOBBS-SPP confocal microscopy and analyzed using ImageJ software. HCT116 spheroids were stained with CellEvent Caspase 3/7 reagent (8 × 10⁻⁶ m), Propidium iodide (1 µg mL⁻¹) and Hoechst (1 µg mL⁻¹) for 30 min. Then the spheroids were mixed with low-melt agarose solution (1%), the mixture was sucked into 1 mL syringes (diameter 1 mm) and allowed to gel at room temperature. After that the agarose blocks containing spheroids were imaged using Light Sheet Fluorescence Microscopy (LMSF Carl Zeiss Microscopy GmbH) and the images were processed using the Imaris Cell Imaging Software.

Statistical Analysis: All statistical analyses were performed with GraphPad Prism Version 5.0 software (GraphPad Software, Inc., La Jolla, USA). The differences were considered significant for p < 0.05. A two-way ANOVA with Bonferroni’s correction was used for multiple group comparisons. Comparisons between two groups were analyzed using the 2-tailed Student’s test. Mann–Whitney test was used for mice experiments.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
M.A.L.Q. and F.D. are scientific advisors and shareholders of Arjuna Therapeutics. M.A.L.Q., F.D., and D.B. have patents on cluster synthesis and therapeutic applications.

Data Availability Statement
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords
cancer therapy, catalysis, low atomic clusters, silver clusters, sulfur oxidation

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