Chemotherapeutic induction of mitochondrial oxidative stress activates GSK-3α/β and Bax, leading to permeability transition pore opening and tumor cell death

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Survival of tumor cells is favored by mitochondrial changes that make death induction more difficult in a variety of stress conditions, such as exposure to chemotherapeutics. These changes are not fully characterized in tumor mitochondria, and include unbalance of the redox equilibrium, inhibition of permeability transition pore (PTP) opening through kinase signaling pathways and modulation of members of the Bcl-2 protein family. Here we show that a novel chemotherapeutic, the Gold(III)-dithiocarbamate complex AUL12, induces oxidative stress and tumor cell death both favoring PTP opening and activating the pro-apoptotic protein Bax of the Bcl-2 family. AUL12 inhibits the respiratory complex I and causes a rapid burst of mitochondrial superoxide levels, leading to activation of the mitochondrial fraction of GSK-3α/β and to the ensuing phosphorylation of the mitochondrial chaperone cyclophilin D, which in turn facilitates PTP opening. In addition, following AUL12 treatment, Bax interacts with active GSK-3α/β and translocates onto mitochondria, where it contributes to PTP induction and tumor cell death. These findings provide evidence that targeting the redox equilibrium maintained by mitochondria in tumor cells allows to hit crucial mechanisms that shield neoplasms from the toxicity of many anti-tumor strategies, and identify AUL12 as a promising chemotherapeutic compound.

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Neoplasms acquire the capability to grow in a rapid and deregulated way under the strong selective pressure of stressful conditions, as they adopt flexible molecular strategies to overcome the multiplicity of apoptotic signals and environmental constraints they encounter.1,2 This high degree of biological plasticity makes cancer cells an elusive target for most therapies, and urges to define specific biological traits of malignant cells that could be utilized for tailored pharmacological approaches.

Several biochemical changes that crucially contribute to neoplastic transformation take place in mitochondria. In most cases, it is in mitochondria that cell commitment to death reaches the point of no return. Opening of the mitochondrial permeability transition pore (PTP), a large channel located in the inner mitochondrial membrane, results in mitochondrial depolarization, swelling, Ca2+ release, rupture of the outer membrane and delivery of proteins that trigger the executioner phase of apoptosis.3 Release of these effector proteins from mitochondria can also occur following the insertion/oligomerization in the outer mitochondrial membrane of pro-apoptotic members of the Bcl-2 family protein, such as Bax or Bak.4 Mitochondria of tumor cells undergo changes that contribute to the build-up of solid anti-apoptotic defences. Expression levels of Bcl-2 family proteins are altered, or they are targeted by a myriad of post-translational modifications driven by oncogenic signaling pathways,5-8 with an increase of anti-apoptotic or a decrease of pro-apoptotic functions. A reduced sensitivity of mitochondrial PTP to diverse stress stimuli was also observed in neoplastic cells,3,6 and we and others have recently found that PTP opening is enhanced by a GSK-3α/β-dependent phosphorylation of the chaperone cyclophilin D (CyP-D), a well-known regulator of the PTP, and that this pathway is inhibited in cancer cells.7-9

During the progression to malignancy, neoplastic accrual overgrows the supply of oxygen and nutrients provided by surrounding blood vessels.10 To avoid the noxious effect of

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Abbreviation: AUL12, ([AuIIIBr2(ESDT)]; ESDT, ethylsarcosinedithiocarbamate); BIP, Bax inhibiting peptide; CRC, Ca2+ retention capacity; CsA, cyclosporin A; CyP-D, cyclophilin D; HK II, hexokinase isofrom II; IND, indirubin-3’-oxime; NAC, N-acetyl-L-Cysteine; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PTP, permeability transition pore; RC, respiratory chain; ROS, reactive oxygen species

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these hypoxic conditions, cells in the tumor mass interior enhance glucose utilization (the Warburg effect), with a concomitant decrease of mitochondrial respiration. This inhibition of respiratory chain (RC) complexes can increase reactive oxygen species (ROS) production, and above a certain threshold, oxidative stress can damage a variety of cellular structures and eventually prompt cell death by triggering PTP opening through poorly defined mechanisms. Cancer cells are therefore forced to concomitantly decrease mitochondrial respiration, and cell death induction in the same ambit of drug dose and time (Figure 1a, b). In RWPE cells, K-Ras transformation significantly enhanced cell death induced by AUL12 (compare Supplementary Figure 2a and 2b). Pre-treating cells with the anti-oxidant N-acetyl-L-Cysteine (NAC) prevented mitochondrial depolarization (Figure 1a) and cell death (Figure 1b and Supplementary Figure 2a and 2b), demonstrating the ROS-dependency of AUL12 toxicity. By contrast, the reference drug cisplatin failed to induce cell death after a 3-h incubation (Supplementary Figure 3a), whereas it triggered a NAC-insensitive cell death after 24 h (Supplementary Figure 3b). Taken together, these results indicate that the two metal-based compounds damage cells through different mechanisms, and that AUL12 prompts a rapid cell death by increasing mitochondrial ROS levels.

AUL12 inhibits RC complex I and elicits ROS production. Mitochondrial RC complexes I, II and III are among the main sources of intracellular ROS, and their targeting by AUL12 could inhibit oxygen consumption rate (OCR) and boost ROS levels in tumor cells. We observed that a 15-min pretreatment with AUL12 inhibited in a dose-dependent fashion both coupled respiration and total mitochondrial OCR, up to a complete abrogation of any mitochondrial oxygen consumption (Figure 2a, right and left panel).

To dissect the effect of AUL12 on respiration, we directly tested maximal RC complex activity under conditions where complexes are made accessible in mitochondria or permeabilized cells. AUL12 exerted a modest inhibitory effect on RC complex II/III activity both in mitochondria and permeabilized cells (Figures 2c and d). The lack of an additive effect on the rise of mitochondrial ROS levels between the complex I inhibitor rotenone and AUL12 (Supplementary Figure 1b) further indicates that AUL12 increases ROS by targeting complex I.

AUL12 sensitizes the permeability transition pore to opening. It is postulated that ROS increase intracellular Ca++, which in turn would augment ROS generation, in a feed-forward circuit eventually leading to PTP opening and cell death. In cancer cell models, PTP opening is made more difficult by a constitutive GSK-3β inhibition, which acts as a solid survival mechanism.

In order to investigate whether AUL12 influences the pore, we used a whole-cell Ca++ retention capacity (CRC) assay, which evaluates the modulation of PTP opening through the assessment of the amount of Ca++ taken up by mitochondria of digitonin-permeabilized cells. A 3-h treatment with AUL12 elicited a dose-dependent CRC shortening, that is, an induction of PTP opening, both on cells (Figures 3a and b) and on isolated liver mitochondria (Figures 3c and d). This induction was totally prevented by the anti-oxidant NAC (Figures 3e and f), whereas the addition to permeabilized cells of cyclosporin A (CsA), an inhibitor of the pore regulator CyPD, markedly increased the amount of Ca++ required to open the PTP (Figures 3a and b).

GSK-3β activation mediates the biological effects of AUL12. These data indicate that AUL12 can rapidly induce inhibition of RC Complex I and oxidative stress, unlocking the

Results

AUL12 induces dose-dependent cell death. In order to understand the mechanism of cytotoxicity of AUL12, we first characterized its effects on viability in: (a) a model of highly aggressive cancer cells, the human osteosarcoma SAOS-2 cells, characterized by loss of p53 activity; (b) the human epithelial prostate cells RWPE-1, which are immortalized but lack any tumorigenic potential; and (c) the RWPE-2 cells, which are made tumorigenic by expression of K-Ras in RWPE-1 cells. AUL12 treatment resulted in a rapid (3 h) dose- and time-dependent raise of mitochondrial superoxide levels in SAOS-2 cells (Supplementary Figure 1a), which was paralleled by a massive mitochondrial depolarization and cell death induction in the same ambit of drug dose and concentration. AUL12 was paralleled by a massive mitochondrial depolarization and cell death induction in the same ambit of drug dose and concentration.

Here we have characterized the mechanism of action of AUL12, finding that it inhibits RC complex I, raising ROS levels and activating GSK-3β. Active GSK-3β prompts tumor cell death, both facilitating PTP opening and causing Bax redistribution to mitochondria. Our data indicate that a survival platform that functionally connects RC complexes, the redox balance, kinase signaling and mitochondrial death executioners can be targeted in neoplastic cells in order to obtain their selective clearing.

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mitochondrial PTP of tumor cells in a ROS-dependent way. In tumor cells, a condition that markedly induce the PTP is GSK-3α/β activation.6 We found that increasing doses of AUL12 elicit a marked and NAC-sensitive raise of GSK-3α/β activity20 (Figure 4a). Furthermore, cell pre-incubation with the GSK-3α/β inhibitor indirubin-3’-oxime before AUL12 treatment both abrogated PTP sensitization (Figure 4b) and protected cells from apoptosis (Figure 4c). Therefore, AUL12 activates GSK-3α/β in a ROS-dependent fashion, and GSK-3α/β activation in turn induces PTP opening. GSK-3α/β inhibition by activation of survival kinase signaling favors the recruitment of the isoform II of hexokinase (HK II) onto the mitochondrial surface of tumor cells,31,32 where it diminishes PTP sensitivity to stress stimuli.29 However, we found that the level of HK II bound to mitochondria was not changed by AUL12 treatment (data not shown). Another possible mechanism of pore regulation by GSK-3α/β was phosphorylation of the PTP regulator CyP-D by the mitochondrial fraction of the kinase, thus enhancing PTP opening. Accordingly, we found that AUL12 activates mitochondrial GSK-3α/β (Figure 4d) and induces CyP-D phosphorylation (Figure 4e).

GSK-3α/β also triggers cell death through mitochondrial circuits alternative to PTP opening, such as mitochondrial translocation of the pro-apoptotic Bcl-2 family member Bax,33 which leads to permeabilization of the outer mitochondrial membrane. Co-immunoprecipitation experiments revealed that Bax and active, Tyr-phosphorylated GSK-3α/β interact in cells treated with AUL12, and this interaction was abrogated by cell pre-treatment with either indirubin-3’-oxime or the antioxidant NAC (Figure 5a and b). Moreover, we observed that AUL12 induces Bax translocation to mitochondria, and this process is inhibited by the GSK-3α/β inhibitor indirubin-3’-oxime (Figure 5c). Notably, cell pre-treatment with a selective Bax-inhibiting peptide (BIP) could partially inhibit pore opening induced by AUL12 (Figure 5d) and protected tumor cells from AUL12 lethality (Figure 5e). Taken together, these findings indicate that the increased intracellular ROS levels elicited by AUL12 activate GSK-3α/β, which in turn triggers cell death through mitochondrial membrane permeabilization mediated both by PTP opening and by Bax recruitment on mitochondria, which could contribute to pore induction.

**Discussion**

In the present work, we have characterized the biochemical mechanisms sustaining the anticancer action of AUL12, a new chemotherapeutic of the Gold(III)-dithiocarbamato family.
We have found that AUL12 strongly inhibits the activity of the RC Complex I and causes a rapid raise in ROS levels, which in turn lead to mitochondrial PTP opening and cancer cell apoptosis. The role played by oxidants in neoplastic transformation is complex and not fully understood. ROS may contribute in stimulating proliferation, invasion and metastasis and in inhibiting apoptosis. By inducing genomic instability, ROS accelerate the rate of mutations and lead to further neoplastic alterations, such as dysfunctions in energy metabolism, with leakage of electrons during the oxidative phosphorylation process and the ensuing generation of superoxide and subsequently hydrogen peroxide. Activation of several oncogenes, such as Ras, Bcr-Abl and c-Myc or loss of functional p53 increase ROS production by enhancing glycolysis and inhibiting oxidative phosphorylation. These alterations are postulated to make tumor cells vulnerable to a further oxidative stress. Accordingly, we have found that human osteosarcoma SAOS-2 cells, which lack a functional p53, undergo a massive apoptosis after 3 h of AUL12 treatment.

However, the interplay between pro-neoplastic signal transduction and redox regulation can be complex, as in the case of the K-Ras oncogene: a low level of K-Ras expression promotes a ROS detoxification program required for tumor initiation, proliferation and survival, but a marked increase in its activity boosts mitochondrial ROS, which stimulate cellular proliferation and anchorage-independent growth, and drugs such as lanperisone or erastin induce mitochondrial release of ROS, oxidative cell death and tumor growth suppression in oncogenic model harboring oncogenic K-Ras mutations. Consistent with a K-Ras sensitization to oxidative stress, AUL12 is more effective in inducing apoptosis in the K-Ras-transformed RWPE-2 prostate epithelial cells than in the non-transformed parental RWPE-1 cells, although these are more sensitive to a variety of apoptogenic stimuli. In addition, AUL12 inhibits the xenographic growth of prostate tumor cells in vivo, causing minimal systemic toxicity. However, above a certain threshold, oxidative stress may induce a prolonged opening of the mitochondrial PTP, irreversibly committing cells to death. A rise in ROS levels

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**Figure 2** AUL12 inhibits OCR and RC complex I. (a) On the right, representative traces of OCR measurements performed on monolayers of living SAOS-2 cells treated with AUL12. Subsequent additions of the ATP synthase inhibitor oligomycin, of the uncoupler FCCP, of the RC complex I inhibitor rotenone and of the RC complex III inhibitor antimycin A were carried out. AUL12 at the reported concentrations was pre-incubated 15 min before starting the experiments. Bar graphs on the right show the coupled mitochondrial respiration, which was calculated by subtracting the uncoupled mitochondrial OCR from the basal OCR (after and before oligomycin addition, respectively) after having deducted the non-mitochondrial component of OCR (the rotenone/antimycin insensitive OCR fraction). The enzymatic activity of RC complexes II–III (b) or of RC complex I (c) was evaluated on mitochondria isolated from SAOS-2 cells in the presence or absence of different concentrations of AUL12 added 5 min before starting the experiment. In (d), the activity of RC complex I was analyzed on permeabilized SAOS-2 cells pre-treated or not for 90 min with 40 μM AUL12. All data of RC complex activity were normalized to the citrate synthase activity and are mean ± S.D. values (n = 6). Statistical significance was measured with a Student’s t-test and is indicated by asterisks (**P < 0.005)
can trigger a feed-forward loop involving a progressive Ca\(^{2+}\) surge, a further ROS increase and prolonged PTP openings,\(^{16,27,28}\) but the molecular mechanisms promoting this process and their possible deregulation in cancer remain poorly defined. Most of the ROS generated in intact mitochondria are contributed by RC Complex I,\(^{44}\) a NADH:Quinone Oxidoreductase endowed with an extremely complex structure.\(^{45}\) A fine regulation of Complex I activity occurs during tumorigenesis. For instance, K-Ras-transformed fibroblasts decrease Complex I content and activity, as compared with a control counterpart.\(^{46}\) The quinone analog rotenone increases superoxide production from NAD\(^{+}\)-linked substrates, and interferes with the formation of a stabilized ubisemiquinone in the matrix part of the complex.\(^{34}\) As we observe that ROS induced by AUL12 are not further increased by rotenone, we suspect that the two drugs act on the same biochemical process within Complex I. Notably, rotenone-dependent oxidative stress elicits autophagic death in cancer cells.\(^{47}\)

ROS recruit and tune the activity of signaling molecules in a highly compartmentalized fashion within the cell.\(^{48}\) ROS-sensitive proteins regulate a variety of cellular functions that are relevant in tumor onset and development.\(^{35}\) We observe that the oxidative stress elicited by AUL12 causes phosphorylation of the Tyr(279/216) residue on GSK-3\(^{a, b}\), which activates the enzyme and favors its subcellular redistribution.\(^{53}\) A ROS-dependent activation of GSK-3\(^{a}\) was observed in several cell models, including cardiomyocytes through sustained ERK\(^{50}\) or mTOR\(^{51}\) activation, mesangial cells, where a ROS-GSK-3\(^{a}\) signaling pathway induces autophagy,\(^{52}\) or neuronal cells, in which oxidative stress elicits a marked activation of GSK-3\(^{b}\) that antagonizes survival signals.\(^{53}\) GSK-3\(^{a}\) can favor PTP opening either by promoting HK II detachment from mitochondria,\(^{19,31,35}\) or by...
and in neoplasms, several oncogenic signals converge on mitochondrial GSK-3α/β to maintain the PTP locked. Here we determine that ROS-dependent GSK-3α/β activation does not change the HK II-binding equilibrium with mitochondria, but it phosphorylates CyP-D, functionally connecting AUL12-dependent GSK-3α/β activation with PTP induction. In parallel, we have observed that ROS raised by AUL12 induce an interaction between GSK-3α/β and the pro-apoptotic Bcl-2 family protein Bax, with a dramatic relocation of Bax on mitochondria. This result is in accordance with previous observations, showing that GSK-3α/β can activate Bax either directly, by phosphorylation on Ser-163 or by promoting its pS3-induced expression. Mitochondrial Bax induces cell death, prompting permeabilization of the outer membrane and the subsequent release of apoptogenic factors. However, functional connections between Bax and PTP regulation have also been proposed, as it was shown that Bax blocks a voltage-dependent K⁺ channel termed Kv1.3 in the inner mitochondrial membrane, leading to rapid ROS production and PTP opening. Accordingly, we observe that Bax inhibition with a selective peptide partially rescues PTP opening and cell death elicited by AUL12. The mechanisms by which AUL12 targets the unbalanced homeostatic redox equilibrium of malignant cells allow to shed light into the mitochondrial machinery that orchestrates neoplasm survival. The functional connection we observe among Complex I, enhanced ROS production, activation of GSK-3α/β and Bax and unlocking of the PTP offers multiple, promising therapeutic targets. Treatment-derived toxicity, cell-insensitivity to drugs and lack of therapeutic selectivity are still major issues in developing strategies leading to cancer cure. Given its ability to selectively kill cancer cells in vivo and in vitro without systemic toxicity, AUL12 could...
constitute a leading compound in the development of chemotherapeutics that target the unique neoplastic alterations of the cell redox equilibrium.

Materials and Methods

Chemicals and reagents. FITC-conjugated Annexin-V was from Roche (Indianapolis, IN, USA); Calcium Green-5N and tetramethylrhodamine methyl ester (TMRM) were from Molecular Probes (Eugene, OR, USA); PD 98059 and Indirubin-3-monoxime were from Calbiochem (San Diego, CA, USA); all other chemicals (oligomycin, antimycin, rotenone, N-acetyl-L-Cysteine (NAC)) were from Sigma (St. Louis, MO, USA). The mouse monoclonal anti-actin antibody (input) was from Sigma; the mouse monoclonal anti-GSK-3β (97 kDa) antibody was from Cell Signalling (Beverly, MA, USA); FITC-conjugated secondary antibodies were from Sigma. N-acetyl-L-Cysteine (NAC), was added 1 h before starting apoptosis induction by AUL12, whereas IND was added 3 h before.

Flow cytometry analysis of apoptosis induction. Flow cytometry recordings of apoptotic changes were performed, as described. Briefly, after induction of apoptosis, cells were resuspended in 135 mM NaCl, 10 mM HEPES, 5 mM CaCl2 and incubated for 15 min at 37 °C in FITC-conjugated Annexin-V, TMRM (20 nM) and propidium iodide (PI, 1 μM). Mitochondrial Ca2+ uptake in the various conditions is compared with that measured in unstimulated cells. (e) Death induction on SAOS-2 cells exposed to different concentrations of AUL12 with or without pre-incubation with the BIP (60 μM) is analyzed by cytofluorimetry with a propidium iodide/Annexin V-FITC double staining, as in Figure 1b. Viable cells reported in the bar graph are negative for both fluorophores. All along the figure, bar graphs report mean ± S.D. values (n = 3). Statistical significance was measured with a Student’s t test and is indicated by asterisks (*P<0.01, **P<0.005)

Figure 5  AUL12 induces an interaction between phosphorylated GSK-3β/α and Bax, which translocates onto mitochondria and contributes to PTP induction and cell death. (a) On the left, GSK-3α/β immunoepitop in SSO cells treated or not for 2h with 40 μM AUL12; where indicated, cells were pre-incubated with IND (2 μM, 3 h) or NAC (1 mM, 1 h). The Western immunoblot was probed with Bax, whereas β-catenin is used as a positive control of co-immunoprecipitation with GSK-3α/β in starved cells (STV); NC, negative control. On the right, p(Y216)GSK-3α/β immunopotentiation in SAOS cells treated or not for 2 h with 40 μM AUL12. The Western immunoblot was probed with GSK-3α/β and Bax. In both panels, IgG is used as a loading control. (b) In both panels, IgG is used as a loading control. (c) Western immunoblot with antibodies conjugated to protein A- or protein G-Sepharose beads (Sigma) at

Cell lysis, fractionation and western immunoblot analysis. Total cell extracts were prepared at 4 °C in 140 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% glycerol and 1% Triton X-100 in the presence of phosphatase and protease inhibitors (Sigma). To separate mitochondria from the cytosolic fraction, cells were placed in an isolation buffer (250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA-Tris, pH 7.4) and homogenized at 4 °C. Mitochondria were then isolated by differential centrifugation (three times, first at 700 x g and twice at 9000 x g, all at 4 °C, 10 min) in mitochondrial isolation buffer.

For immunoprecipitations, 2000–3000 μg of proteins per reaction were incubated with antibodies conjugated to protein A- or protein G-Sepharose beads (Sigma) at 4 °C overnight. Negative controls were performed by incubating lysates on...
conjugated beads in the absence of primary antibodies. Samples were then separated in reducing conditions on SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham, Little Chalfont, UK). Primary antibodies were incubated for 16 h at 4 °C, and horseradish peroxidase-conjugated secondary antibodies were added for 1 h. Western immunoblots were carried out under standard conditions, and proteins were visualized by enhanced chemiluminescence (Millipore, Billerica, MA, USA).

Isolation of mouse mitochondria. Mitochondria were isolated from livers of wild-type, BALBc mice, through sequential centrifugations, as described. All procedures were carried out at 4 °C.

Measurement of mitochondrial Ca2+ retention capacity. The CRC assay was used to assess PTP opening following trains of Ca2+ pulses and measured fluorimetrically at 25 °C in the presence of the Ca2+ indicator Calcium Green-5N (1 μM; λ exc: 505 nm; λ em: 535 nm; Molecular Probes). We performed CRC experiments either on isolated mitochondria or on whole cells placed in an isotonic buffer (130 mM KCl, 1 mM Pi-Tris (pH inorganic phosphate), 10 mM Tris/MOPS, 10 μM EGTA/Tris, 5 mM glutamate/2.5 mM malate, 10 μM cytochrome c, pH 7.4). Whole cells CRC was carried out after plasma membrane permeabilization with the non-ionic detergent digitonin, which is highly selective for cholesterol-enriched membranes and does not damage the mitochondrial membranes. Cells were washed twice in a buffer composed of 130 mM KCl, 1 mM Pi-Tris, 10 mM Tris/MOPS, 1 mM EGTA/Tris, pH 7.4 and then permeabilized with 150 μM digitonin (15 min, 4 °C) in the same buffer with modified EGTA/Tris, (1 mM, pH 7.4). Digitonin was then washed away by spinning cells twice in the washing buffer with 0.1 mM EGTA/Tris, and the number of cells carefully assessed before starting each experiment. Mitochondria (0.5 mg ml−1) or permeabilized cells (7 × 106 cells per experiment) were then placed in the presence of the Ca2+ indicator Calcium-Green-5N, which does not permeate mitochondria, and exposed to Ca2+ spikes (10 μM and 5 μM, respectively). Fluorescence drops were used to assess mitochondrial Ca2+ uptake. PTP opening was detected as a fluorescence increase.

Determination of mitochondrial respiratory complex activity. To measure the enzymatic activity of RC complexes, mouse liver mitochondria were homogenized with an electric potter (Sigma) in a buffer composed by 250 mM sucrose, 10 mM Tris/HCl, 0.1 mM EGTA-Tris, pH 7.4, Percoll 10%, protease and phosphatase inhibitors and mitochondria isolated, as described above with different centrifugations. The activities of mitochondrial respiratory complex I and II/III were determined spectrophotometrically, as described. Briefly, for complex I mouse liver mitochondria (40 μg ml−1) were placed in an assay medium composed by Tris 10 mM pH 8, bovine serum albumin 3 mg ml−1, alamethicin 5 μM, sodium azide 5 mM, antymycin 1 μM, CoQ 6,5 μM and the absorbance change (340 nm, 37 °C) with or without AUL12(10 min) incubation was recorded for 1 min. Then NADH (0.1 mM) was added, and the NADH oxidoreductase activity was measured for 5 min before the addition of rotenone (20 μM) after which the activity was measured for an additional 3 min. Complex I activity is the rotenone-sensitive NADH oxidoreductase activity.

For the determination of complex II/III activities, mouse liver mitochondria (40 μg ml−1) were placed in a medium composed of KH2PO4 25 mM, bovine serum albumin 1 mg ml−1, sodium azide 5 mM, succinate 10 mM, alamethicin 5 μM, ATP 0.1 mM with or without AUL12 (10 min incubation). Then cytochrome c 50 μM was added and the decrease of absorbance (550 nm, 37 °C) recorded for 10 min. Finally, the data were corrected for the activity of citrate synthase:mouse liver mitochondria (40 μg ml−1) were placed in a medium composed by Tris/HCl 100 mM, pH = 8, DTNB 0.1 mM, acetyl CoA 0.3 mM and oxaloacetate 0.5 mM. The increase of absorbance (412 nm, 37 °C) was then recorded for 10 min.

Oxygen consumption rate (OCR) experiments. The rate of oxygen consumption was assessed in real-time with the XF24 Extracellular Flux Analyzer (Seahorse Biosciences), which allows to measure OCR changes after up to four sequential additions of compounds. Cells (5 × 104 per well) were plated the day before the experiment in a DMEM/10% serum medium; experiments were carried out on confluent monolayers. Before starting measurements, cells were placed in a running DMEM medium (supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium Pyruvate, and without serum) and pre-incubated for 1 h at 37 °C in atmospheric CO2. OCR values were then normalized for the protein content of each sample. An accurate titration with the uncoupler FCCP was performed, in order to utilize the FCCP concentration (20 nM) that maximally increases OCR.

Statistical analysis. Data were collected by investigators blinded to the experimental setup and were statistically analyzed by parametric Student’s t-test. In all graphs, mean ± S.D. (standard deviation of the mean) are shown. P-values 0.01 were considered to be statistically significant. Statistical analysis was performed with Statgraphics Centurion XVI, version 16.1.12 (StatPoint Technologies, Inc. Warrenton, VA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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