



OPEN Metformin treatment impairs the adenine nucleotide translocator activity and energy metabolism in human clear cell renal carcinoma cells

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Although metformin (MET), the well-known antidiabetic drug, exhibits clear antineoplastic effects and is reported to target mitochondria, several issues are still open in this regard, thus limiting its utilization as an anticancer drug alone or in combination with other molecules. Here a functional investigation was carried out to reveal how MET impacted on mitochondrial functions and cell energy metabolism in human cultured clear cell renal carcinoma cells (ccRCCs), in which the anticancer effect of MET is already known. The in vitro effect of increasing MET concentrations on cell viability, necrosis and apoptosis of ccRCCs was checked and compared to normal immortalized HK2 cells. At the same time, the effect of MET on mitochondrial functions, ATP synthesis via oxidative phosphorylation, cellular ATP level, L-lactate (L-LAC) production and export, glucose consumption and key mitochondrial and cytosolic enzyme activities was also investigated in cancer cells. MET affected ccRCC viability and impaired mitochondrial respiration, membrane potential generation and ATP production by targeting complex I (CI), III and IV of the respiratory chain at a concentration near to the IC₅₀ value (25 mM). Importantly, we first identified a significant inhibition of the adenine nucleotide translocator (ANT) activity in response to MET treatment. Notably, the sensitivity of ANT and CI activity to increasing MET concentrations differed markedly, the former being considerably inhibited already at a low, near-clinically relevant concentrations, while the latter only at concentrations ≥ 1 mM. The drug also induced a glycolytic shift in ccRCCs and increased the activity of the mitochondrial flavoenzymes succinate dehydrogenase (SDH) and D-lactate dehydrogenase (D-LDH), and of the key enzymes of the pay-off phase of glycolysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK) and pyruvate kinase (PK). Nevertheless, cellular ATP level dropped markedly, and the intracellular L-LAC amount was almost doubled in the presence of MET. Interestingly, MET-induced glycolytic shift showed a drug concentration dependence similar to that seen for CI inhibition, suggesting not ANT but rather CI inhibition may be the trigger for metabolic rewiring. These findings give new insights into MET mechanisms of action which may potentially improve its application and outcome in cancer as well as in other pathologies.

Keywords Clear cell renal cell carcinoma, Metformin, Mitochondrial respiratory chain complexes, Oxidative phosphorylation, Adenine nucleotide translocator, Glycolysis

Abbreviations

AMPK AMP-activated protein kinase

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ANT	Adenine nucleotide translocator
Ap5A	P1,P5-Di(adenosine-5) pentaphosphate
ASC	Ascorbate
ATP-ds	ATP-detecting system
ATR	Atractyloside
ccRCC	Clear cell renal cell carcinoma
CN ⁻	Potassium cyanide
CS	Citrate synthase
CSF	Cell soluble fraction
D-LDH	D-lactate dehydrogenase
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
FCCP	Carbonylcyanide p-trifluoromethoxy phenylhydrazine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
GLU	Glutamate
HK	Hexokinase
L-LAC	L-lactate
L-LDH	L-lactate dehydrogenase
MAL	Malate
MALO	Malonate
mTOR	Mammalian target of rapamycin
MET	Metformin
MMEF	Mitochondrial membrane enriched fraction
mGPDH	Mitochondrial glycerophosphate dehydrogenase
MRC	Mitochondrial respiratory chain, OLIGO, oligomycin
OAA	Oxaloacetic acid
OXPHOS	Oxidative phosphorylation
PGK	Phosphoglycerate kinase
PK	Pyruvate kinase
pRCC	Digitonin-permeabilized ccRCC Shaw cells
PEN2	Presenilin enhancer 2
ROT	Rotenone, SUCC, succinate
SDH	Succinate dehydrogenase
T2DM	Type 2 diabetes mellitus
TMPD	N, N, N, N-tetramethyl-p-phenylenediamine

Common feature of different types of cancer is their metabolic flexibility and/or plasticity, i.e. the ability to use different metabolic substrates and/or to catabolize the same nutrient in different ways, respectively¹. This peculiarity derives from both changes in the expression of genes regulating enzyme activities and fluctuations in metabolite levels inducing or repressing specific enzyme reactions and metabolic pathways. The metabolic plasticity and flexibility of cancer cells are key to both survival and growth regardless of the surrounding environmental changes and to overcome challenges imposed by anticancer drugs, thus generating drug-resistance². For this reason, in order to target different metabolic/signaling pathways, cancer research and therapy preferably apply drug combinations with different molecular mechanisms of action at the same time³. This strategy is the more effective the more detailed is the knowledge of the mechanisms of action of the involved drugs.

Metformin (MET), widely used for glycemic control in type 2 diabetes mellitus (T2DM)⁴ is one of the metabolic drugs being repurposed to enhance the antitumor effect of established anticancer drugs. In fact, MET is known to lower the incidence of neoplastic disease in T2DM patients^{5–7} and to have antineoplastic effects in a wide variety of cancers by acting as a multitarget metabolic modulator^{8–10}. Different ways of action have been reported for MET in human cancers and numerous ongoing clinical trials, in almost all type of cancers, report on the potential of this drug as an enhancer of classic anticancer chemotherapy or as an adjuvant/neoadjuvant chemotherapy agent¹¹. However, the mode of action underpinning these effects of MET is still not fully understood and a matter of controversy, limiting the elaboration of effective combinatorial therapies¹².

The main targets of MET appear to be mitochondria¹³. Complex I (CI) of the mitochondrial respiratory chain (MRC) and the mitochondrial glycerophosphate dehydrogenase (mGPDH), both involved in the oxidation of the cytosolic NADH for mitochondrial ATP production and glycolysis maintenance, are targeted by MET, explaining the significant energetic stress and cell redox status alteration associated to MET treatment^{14–17}. The consequent augmentation of AMP/ATP ratio has been used to explain MET-dependent activation of AMP-activated protein kinase (AMPK), which in turn inhibits anabolism and triggers catabolism causing cell cycle arrest and apoptosis in cancer¹⁷. In certain types of cancer, MET-dependent AMPK activation lacks, while the anti-proliferative effect is still maintained^{18,19}, drawing attention to AMPK-independent ways of MET action, too. The role of the similarly important adenine nucleotide translocator (ANT) in the turnover, which catalyzes the free ATP and free ADP exchange across the inner mitochondrial membrane²⁰, has not yet been investigated in the context of MET.

MET is generally accepted as a mitochondrial inhibitor. However, its mechanism upon mitochondrial activity as well as its primary mitochondrial target(s) either in normal or in cancer cells, are still elusive. To increase this uncertainty, it has been recently demonstrated that the antiproliferative effect of MET on different cancer cell models was independent from CI inhibition²¹. Moreover, clinically relevant MET doses (1.5–2 g per day) yield

only 10–40 μM plasma levels^{22,23}. Though 40–80 μM concentration was detected in the portal vein^{22,23}, these concentrations are still unable to influence the AMP/ATP ratio in primary hepatocytes, while AMPK activity is maintained^{18,24}. Recently, the lysosomal AMPK pathway has been shown to be a possible target of low-dose MET through presenilin enhancer 2 (PEN2), explaining how AMPK can be activated by the drug without affecting cellular AMP levels²⁵. Overall, several issues remain open regarding MET's action and molecular targets, both in cancer and diabetes.

The experimental model of clear cell renal cell carcinoma (ccRCC), the most common and severe type of renal carcinomas that usually does not respond to conventional chemo- and radiotherapy²⁶, is generally used. A correlation has been shown between worsened prognosis and a metabolic shift including increased pentose phosphate shunt, glutamine transport and fatty acid production, with decreased AMPK protein level and Krebs cycle gene upregulation²⁷. Recently, the metabolic reprogramming in human ccRCC was studied *in vivo* by the infusion of [^{13}C]-glucose in patients with primary ccRCC tumors undergoing nephrectomy or partial nephrectomy, and enhanced glycolysis and suppressed glucose oxidation in the tumors were found compared to matched, adjacent kidney tissues²⁸. Both *in vitro* and *in vivo* studies using xenograft murine models demonstrated that MET inhibits ccRCC cell growth leading to cell cycle arrest in G_0/G_1 phase. The process involved the activation of AMPK and the inhibition of mammalian target of rapamycin (mTOR), a central regulator of protein synthesis and cell growth²⁹. Consistently, daily treatment of mice with MET prevented ccRCC growth in a xenograft model³⁰. Yet, the inhibition of ccRCC proliferation and the proapoptotic effect of MET were not associated with MET-dependent AMPK activation, *in vitro*³¹.

To achieve a better understanding of the molecular mechanisms of action of MET at the mitochondrial level, the mitochondrial bioenergetics in ccRCC cells was investigated to evaluate the response to the drug and to detect how cancer cell metabolism adapts to such exposure. It was found that MET affected ccRCC cell viability in a concentration-dependent manner and significantly impaired mitochondrial functions when used at a supra-physiological dose near to MET's IC_{50} for these cells. On the contrary, the key enzymes of the pay-off phase of glycolysis as well as the mitochondrial flavoenzymes succinate dehydrogenase and D-lactate dehydrogenase (D-LDH), were activated following MET treatment, likely to compensate for the fall of ATP level originating from mitochondrial dysfunction. Importantly, here we first identified the occurrence of ANT inhibition at low, clinically relevant drug concentrations at which CI is not affected and the glycolytic shift does not occur.

Methods

Cell cultures and materials

ccRCC Shaw^{32,33} and Elthem cells (RCC BA85#21), purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK), are human renal carcinoma cell lines derived from primary kidney tissue explants. Both are aggressive drug-resistant cell lines showing impaired stabilization and activation of wildtype p53³². HK-2 cells are immortalized human proximal tubule epithelial cells³⁴ purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in a culture medium consisting of RPMI-1640 medium (GIBCO/BRL) supplemented with 10% inactivated fetal bovine serum (GIBCO/BRL), 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) (GIBCO/BRL). Cells were collected by trypsinization, washed with phosphate buffered saline (PBS) medium containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 15 mM KH_2PO_4 (pH 7.4), seeded at a density of $0.35\text{--}0.4 \times 10^6$ cells/ml into 15–10 cm Petri dishes or 96-well plates and then cultured for about 24 h to obtain 60–70% confluence.

All reagents used were from Sigma-Aldrich unless otherwise specified and were of analytical grade.

MET treatment

MET (1,1-dimethylbiguanide hydrochloride) was dissolved at the indicated concentrations in the growth medium the day of the experiment and then used to replace the culture medium of plated cells.

Viable cell counting

Viable cells were detected by Trypan blue exclusion test. Cells, incubated for 24 h in the absence or presence of increasing MET concentrations, were trypsinized, washed in PBS, stained with Trypan blue and counted in Countess 3 FL automated fluorescence cell counter (Thermo Fisher Scientific) within 5 min after staining.

Detection of cell necrosis and apoptosis

Released L-lactate dehydrogenase (L-LDH) activity was assayed photometrically at 340 nm as a marker of cell necrosis, essentially as described in³⁵. The culture medium of cells incubated for 24 h in the absence or presence of increasing MET concentrations, was centrifuged (1300 g for 3 min, 4 °C) to precipitate any cell contamination and 0.1 ml of the obtained supernatant was added to 1.9 ml of standard medium consisting of 0.3 M sucrose, 10 mM KCl, 1 mM MgCl_2 , and 20 mM Hepes-Tris (pH 7.0), at 25 °C, in the presence of 0.2 mM NADH. Then, 1 mM pyruvate was added to start L-LDH reaction. L-LDH activity, expressed as nmol/min/ 10^6 cells, was reported as percent of L-LDH activity/ 10^6 cells found in the medium of control cells (2.0 ± 0.3 nmol/min/ 10^6 cells) (100%).

Apoptotic specific assay was performed using the Cell Event Caspase 3/7 Green Flow Cytometry Assay Kit (Thermo Fisher™) according to the manufacturer's instruction, which enables the flow cytometric detection of activated caspase-3 and caspase-7 in apoptotic cells³⁶. Briefly, 60–70% confluent cells were incubated in the absence or presence of increasing MET concentrations (0, 1, 2, 5, 10, 20, 40 and 60 mM) and harvested 24 h later. Then, 1×10^6 cells were resuspended in PBS, incubated with appropriate reagents according to the manufacturer's recommendations and analyzed with a Attune NxT flow cytometer (Thermo Fisher™). Negative

control cells (both for treatments and for cytometric reagents) were used to set cytometric parameters. For each sample, apoptotic cell number is reported in graph as percentage of the number of counted cells (100%).

Cell permeabilization and citrate synthase assay

Permeabilized ccRCC Shaw (pRCC) cells were gained by the addition of digitonin (0.01% w/v) to cell suspension (0.4 mg cell protein) for 5 min, at room temperature. For the detection of mitochondrial mass variation, citrate synthase (CS), a mitochondrial matrix marker enzyme, was assayed in pRCC cells essentially as in³⁷. pRCC cells (0.4 mg) were incubated in a final volume of 2 ml of respiration medium consisting of 210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 20 mM Tris-HCl pH 7.4, 3 mM MgCl₂, 5 mM KH₂PO₄/K₂HPO₄ (pH 7.4) plus 1 mg/ml BSA and then added with acetyl-CoA (0.3 mM), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, 0.2 mM) and oxaloacetic acid (OAA). CS reaction was detected as absorbance increase at 412 nm over time after the addition of the membrane detergent Triton x100 (Tx100, 0.2% v/v) able to disrupt mitochondrial membranes in pRCC cells. No significant activity was found before Tx100 addition, indicative of mitochondrial intactness. No variation in the activity of CS was found between ccRCC + MET and MET-untreated cells, indicating similar amounts of mitochondria in the two samples.

Isolation of the mitochondrial membrane enriched fraction (MMEF) and the cell soluble fraction (CSF)

MMEF and CSF were prepared as described earlier³⁸. Briefly, after trypsinization, ccRCC cells were precipitated by centrifugation (1300 g for 5 min, 4 °C) and washed with ice cold PBS. The cell pellet was dried and frozen at -80 °C to favor cell membranes rupture and then the pellets were suspended in about 1 ml (final density of about 5×10^6 cells/ml) of ipotonic medium consisting of 10 mM Tris/HCl (pH 7.5) plus 1 mg/ml BSA, exposed to ultrasound energy for 15 s at 0 °C³⁹ and centrifuged at 1,500 g for 5 min at 4 °C to remove intact cells, cellular debris and nuclei. The supernatant was subsequently centrifuged at 14,000 g for 10 min at 4 °C. The resulting supernatant represents the CSF (about 1.2 mg protein/ml). The pellet was washed twice with 1 ml of cold PBS to eliminate any CSF contamination. The final pellet (MMEF) was suspended in 0.5 ml of respiration medium (pH 7.4). In each experiment, the absence of cytosolic contamination in MMEF was confirmed by excluding the presence of the cytosolic marker glucose-6P-dehydrogenase (G6PDH) assayed as reported in⁴⁰. The activity of G6PDH was not affected by MET treatment.

Measurement of mitochondrial respiratory chain (MRC) complex and D-lactate dehydrogenase (D-LDH) activities in MMEF

The photometric measurement of the mitochondrial respiratory chain (MRC) complex and D-LDH activities was carried out in MMEF (0.1 mg protein), essentially as in³⁵. Briefly, MRC complexes were analyzed by three assays which rely on the sequential addition of reagents to measure the activities of: (i) NADH: ubiquinone oxidoreductase (complex I, CI) followed by ATP synthase (complex V, CV) ($\lambda = 340$ nm, $\epsilon = 6.22$ mM⁻¹ × cm⁻¹), (ii) succinate: ubiquinone oxidoreductase (complex II, CII) ($\lambda = 600$ nm, $\epsilon = 21.0$ mM⁻¹ × cm⁻¹) and (iii) cytochrome c oxidase (complex IV, CIV) followed by cytochrome c oxidoreductase (complex III, CIII) ($\lambda = 550$ nm, $\epsilon = 19.1$ mM⁻¹ × cm⁻¹)⁴¹. A JascoV-550 spectrophotometer was used in all assays. MMEF were incubated in a final volume of 1 ml of respiration medium (pH 7.4), at 37 °C, and then added with the following reaction mixtures: (i) ubiquinone (100 μM) and potassium cyanide (CN⁻, 400 μM) for CI detection, followed by rotenone (2 μg/10 μl), phosphoenolpyruvate (2 mM), MgCl₂ (5 mM), pyruvate kinase/L-LDH coupled enzymes (0.30 U) and P(1)P(5)-diadenosine-5'-pentaphosphate (Ap5A, 50 μM), inhibitor of adenylate kinase⁴², for the detection of the ATPase activity of CV; (ii) rotenone (2 μg/10 μl), CN⁻ (400 μM), ubiquinone (100 μM) and 2,6-dichloroindophenol (DCIP, 40 μM), for CII detection; (iii) reduced cytochrome c (10 μM) for CIV detection, followed by CN⁻ (400 μM), ATP (0.5 mM), malonate (MALO, 5 mM), EDTA (2 mM) and oxidized cytochrome c (10 μM) for CIII detection. MRC complex reactions were started by the addition of 0.2 mM NADH (CI), 0.5 mM ATP (CV); 0.01 mM reduced cytochrome c (CIV); 0.05 mM reduced coenzyme Q (CIII); 5 mM succinate (CII). The following specific inhibitors were used to stop the reaction of each MRC complex: rotenone (2 μg/10 μl) (CI), MALO (10 mM) (CII), 0.8 μM antimycin A (CIII), CN⁻ (400 μM) (CIV) and oligomycin A (OLIGO, 5 μM) (CV). D-lactate dehydrogenase (D-LDH) activity was assayed similarly to CII with the addition of 10 mM D-lactate and inhibited by 10 mM oxalate³⁸. When present, inhibitor-insensitive rates of absorbance change were subtracted as nonspecific rates. MRC complex activities were expressed as nmol/min × mg of MMEF protein.

Enzyme activity assays

The activities of the cytosolic hexokinase (HK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), pyruvate kinase (PK), L-LDH and glucose-6-phosphate dehydrogenase (G6PDH) were assayed photometrically, essentially as described earlier³⁵, in CSF (0.05 mg protein) obtained from ccRCC Shaw cells incubated for 24 h in the absence (control) or presence of 25 mM MET (ccRCC + MET). A JascoV-550 spectrophotometer set to 340 nm wavelength, at 37 °C, was used in all assays. A final volume of 2 ml of respiration medium (pH 7.4) for PK, standard medium (pH 7.0) for HK, PGK, L-LDH, and G6PDH, and phosphate buffer 100 mM (pH 7.0) for GAPDH, were added with the following reaction mixtures: ADP (1 mM), NADH (0.2 mM), MgCl₂ (5 mM) and L-LDH from rabbit muscle (5 U) for PK detection; NADH (0.2 mM) for L-LDH detection; MgCl₂ (5 mM), G6PDH from baker's yeast (0.5 U), NADP⁺ (0.25 mM) and glucose (10 mM) for HK detection; NAD⁺ (1 mM), ADP (1 mM), Ap5A (10 μM) and PGK from rabbit muscle (15 U) for GAPDH detection; ATP (0.5 mM), Ap5A (10 μM), NADH (0.2 mM), MgCl₂ (5 mM), GAPDH (0.7 U) for PGK detection; NADP⁺ (0.25 mM) for G6PDH detection. Enzyme reactions were started by the addition of: 1 mM phosphoenolpyruvate (PK), 1 mM pyruvate (L-LDH); 1 mM ATP (HK), 3mM glyceraldehyde 3-phosphate (GAPDH); 5 mM 3-phosphoglycerate (PGK); 10 mM glucose-6-phosphate (G6PDH).

Measurement of cellular ATP and L-lactate level

Cells were seeded at a density of 3.0×10^6 cells in 10 cm plates, cultured for 24 h and then incubated with or without MET (25 mM) for additional 24 h. Then control and ccRCC + MET cells ($9.0 \pm 0.8 \times 10^6$ and $5.0 \pm 0.5 \times 10^6$ cells/plate, respectively) were trypsinized, collected, washed and suspended in PBS for the counting. Cells were then precipitated, dissolved in 0.5 ml of boiling water and centrifuged at 14,000 g for 10 min at 4 °C to obtain cell extracts (supernatants)⁴³. L-lactate (L-LAC) and ATP content were measured fluorimetrically ($\lambda_{\text{em}} = 334\text{--}456$ nm), as described in^{35,44}, by incubating cell extracts (250 μ l) in a final volume of 2 ml of glycine/hydrazine buffer (pH 9.5) or standard medium (pH 7.0), respectively, at 25 °C, in the presence of bovine heart L-LDH (10 U) for L-LAC detection and the ATP-ds (ATP-detecting system), consisting of glucose (2.5 mM), HK (2 U), glucose-6-phosphate dehydrogenase (G6PDH) (1 U) and NADP⁺ (0.25 mM), plus Ap5A (10 μ M) for ATP detection. The assay was started by the addition of NAD⁺ (1 mM) or glucose (2.5 mM), respectively. L-LAC and ATP standard solutions with known increasing concentrations were subjected to the same experimental procedure described above, to obtain standard curves for the calculation of L-LAC and ATP concentration in cell extracts. The amount of L-LAC and ATP found in cell extracts was expressed as nmol/10⁶ cells.

L-lactate and glucose level assay in the cell culture medium

Cells were seeded at a density of 3.0×10^6 cells in 10 cm plates, cultured for 24 h and then incubated with 10 ml of RPMI medium containing or not MET at the indicated concentrations, for additional 24 h. Then, 1 ml of culture medium was taken from each plate, centrifuged (1300 g for 3 min, 4 °C) to precipitate any cell contamination and used for extracellular L-LAC and glucose assay. Control and MET-treated cells in each plate were collected and counted. Viable control cells were about $9.0 \pm 0.8 \times 10^6$ cells/plate, whereas the number of viable MET-treated cells decreased with increasing MET concentrations by the percentages reported in Fig. 1a, ranging from 8.5 ± 0.5 to $2.0 \pm 0.3 \times 10^6$ cells/plate. Both L-LAC and glucose assays were carried out photometrically at the wavelength of 340 nm, at 25 °C, measuring the absorbance increase due to NAD(P)H formation. Either MET-treated or control cell culture medium, or commercial RPMI medium, were used.

L-LAC was assayed in 10 μ l of culture medium by the L-LDH method described above. A standard curve was obtained by assaying known concentrations of sodium L-LAC dissolved in RPMI medium. Moreover, the nonspecific absorbance variation (ΔA) found with commercial RPMI assayed for L-LAC under the same experimental conditions (nonspecific $\Delta A = 0.15 \pm 0.01$), was subtracted from the ΔA values found with MET-treated or control cell culture medium. The total amounts of extracellular L-LAC (81.0 ± 6.0 μ mol L-LAC in control plates) were normalized to the number of cells in the plates and expressed as μ moles L-LAC/10⁶ cells (9.0 ± 0.8 μ mol L-LAC/10⁶ cells in controls). Where indicated, the normalized L-LAC amounts found in MET-treated cell plates were expressed as % of those found in control plates, set as 100%.

For glucose assay, 10 μ l of culture medium was added to a final volume of 2 ml of standard medium (pH 7.0) containing ATP (1 mM), NADP⁺ (0.25 mM), HK (2 IU) and glucose-6-phosphate dehydrogenase (G6PDH) (1 IU). To obtain the amount of glucose consumed by cells, the amount of glucose found in each plate was expressed as μ mol glucose/ml and subtracted from that measured in commercial RPMI medium (equal to 9.6 ± 0.4 μ mol glucose/ml). The resulting value was then referred to the total volume of culture medium in the plate (10 ml), normalized to the cell number and expressed as μ mol glucose/10⁶ cells. Where indicated, the normalized glucose consumption was expressed as % of that found with MET-untreated cells (45.0 ± 4.0 μ mol glucose consumed/control plate; 5.0 ± 0.3 μ mol consumed glucose/10⁶ control cells), set as 100%. Standard curves obtained with known glucose concentrations were used for the calculation of glucose concentration in all samples. Both externally added L-LAC and the glucose content of RPMI were assayed in commercial RPMI added or not with MET at the same concentrations used for cell incubations. MET proved not to interfere with both substrate assays (not shown).

Measurement of mitochondrial ATP synthesis and efflux

ATP synthesis via oxidative phosphorylation (OXPHOS) and efflux into the extramitochondrial phase mediated by ANT, were determined as described earlier³⁹. Permeabilized ccRCC + MET or control cells (0.4 mg) were incubated at 37 °C in 2 ml of respiration medium (pH 7.4) in the presence of the ATP-ds plus 10 μ M Ap5A⁴², and ADP (0.5 mM). Care was taken to use sufficient HK/G6PDH-coupled enzymes (about 5 U) to ensure a non-limiting ADP-regenerating system for the correct measurement of ATP efflux rate. NADPH formation revealing ATP appearance was monitored as absorbance increase at 340 nm (A_{340}), following the addition of the CI- CII- or CIV-respiratory substrates MAL + GLU (5 mM each), SUCC (5 mM) or ASC+TMPD (5 mM and 0.2 mM), respectively. Rotenone (2 μ g/10 μ l), MALO (10 mM) or CN⁻ (1 mM) were then added to inhibit CI-, CII- or CIV-dependent ATP production, respectively. OLIGO (5 μ M), used to inhibit ATP synthase³⁸, also proved to block ATP appearance in all cases, as expected (not shown).

Measurement of ANT activity

MET-treated and control pRCC cells (0.4 mg) were preincubated at 37 °C in 2 ml of respiration medium (pH 7.4) in the presence of the ATP-ds plus Ap5A (10 μ M) and SUCC (5 mM) for 2 min. After that, ADP was added to start the reaction at the reported concentrations. Where indicated, the non-penetrant inhibitor of ANT atractyloside (ATR) was added to MET (25 mM)-treated or control pRCC 2 min before ADP (0.5 mM) addition. To check MET's direct effect on ANT activity, MET was dissolved in respiration medium (pH 7.4) without BSA and then added to permeabilized ccRCC cells (0.4 mg) in 2 ml of respiration medium (pH 7.4), at 37 °C, for 5 min before ANT assay. Rates were expressed as nmol NADPH/min x mg cell protein.

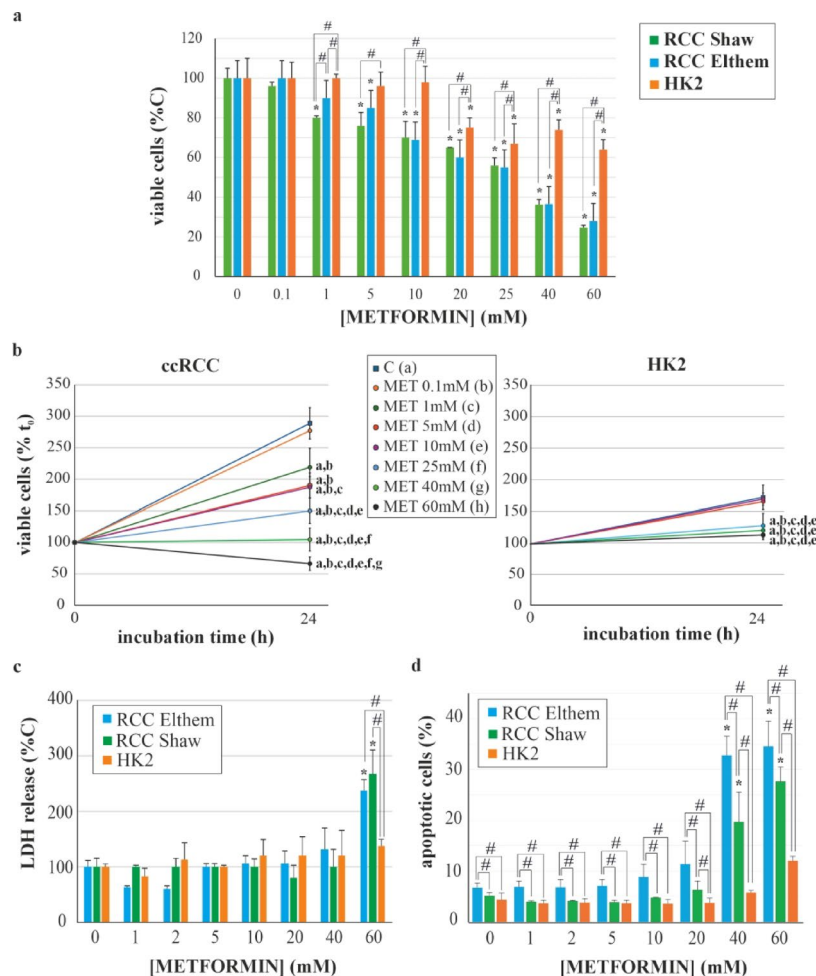


Fig. 1. The effect of increasing MET concentrations on HK2, ccRCC Shaw and ccRCC Elthem cell viability, necrosis and apoptosis. **(a)** HK2, ccRCC Shaw and ccRCC Elthem cells were incubated for 24 h in the absence or presence of MET at the reported concentrations. Trypan Blue exclusion method assay was used to determine cell viability reporting the number of viable cells as % of the respective values found in MET-untreated cells, set as 100% (C). All values represent means $\pm$ SD ($n = 5$). * $P < 0.05$, significantly different from untreated cells; # $P < 0.05$, significantly different from each other (Two-Way ANOVA and Tukey's post-hoc test). In **(b)**, the mean number of viable ccRCC Shaw or HK2 cells reported in **(a)**, incubated in the absence (C) or presence of the increasing MET concentrations for 24 h, was expressed as % of the mean number of seeded viable cells (t_0), set as 100%. The letters besides the lines identify statistically different values ($P < 0.05$, One-way ANOVA and Tukey's post-hoc test). **(c)** L-LDH activity was assayed photometrically in the culture medium of HK2, ccRCC Shaw and ccRCC Elthem cells incubated for 24 h in the absence or presence of MET at the reported concentrations, as described in the Methods section. The data in the histogram represent means $\pm$ SD ($n = 4$) and are expressed as percent of L-LDH activity found in the medium of untreated cells after 24 h of incubation, set as 100%. * $P < 0.05$, significantly different from untreated cells (Student's t-test). # $P < 0.05$, significantly different from each other (One-way ANOVA and Tukey's post-hoc test). **(d)** Apoptosis detection was carried out by flow cytometric analysis. For each sample the number of apoptotic cells, identified as green labelled cells, was reported as percentage of the total cell number (6×10^4 cells) set as 100%. All values are means $\pm$ SD ($n = 3$). * $P < 0.05$, significantly different from untreated cells; # $P < 0.05$, significantly different from each other (Two-Way ANOVA and Tukey's post-hoc test).

Measurement of oxygen consumption in pRCC cells

Mitochondrial respiration in pRCC cells (0.5 mg/ml) was measured in respiration medium (pH 7.4) at 37 °C. Uncoupled substrate-dependent respiration, in the presence of 1.25 μ M carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP), was measured by the following additions: 5 mM malate (MAL) plus 5 mM glutamate (GLU), 1 μ M rotenone (CI inhibitor), 5 mM succinate (SUCC), 0.8 μ M antimycin A (CIII inhibitor), 5 mM ascorbate (ASC) plus 0.3 mM TMPD, 5 mM azide (CIV inhibitor). For each substrate, specific inhibitor-insensitive rate of oxygen consumption was also measured and subtracted. Oxygen consumption rates were expressed as nmol O₂/min $\times$ mg cellular protein.

Measurement of mitochondrial membrane potential ($\Delta\psi$) generation

Mitochondrial membrane potential ($\Delta\psi$) generation was measured at 37 °C by monitoring the fluorescence changes of the probe safranin O at the excitation-emission wavelengths of 520–570 nm, respectively, essentially as in³⁸. Control and ccRCC + MET cells were trypsinized, precipitated, washed in PBS and finally suspended in 1 ml of respiration medium (pH 7.4) without BSA, at room temperature. Then cells (1 mg protein) were added to the experimental cuvette in the presence of 10 μ M safranin O and digitonin (0.01% w/v), in a final volume of 2 ml respiration medium (pH 7.4). After 5 min, GLU plus MAL (5 mM each), rotenone (2 μ g/10 μ l) plus SUCC (5 mM), or rotenone (2 μ g/10 μ l), 0.8 μ M antimycin A, ASC (5 mM) and TMPD (0.3 mM) were added to induce $\Delta\psi$ generation. Finally, the uncoupler FCCP (1.25 μ M) was added to abolish $\Delta\psi$.

Statistical analysis and computing

Each experiment was performed at least in triplicate and results expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were conducted using Student's t-test. For comparisons among multiple groups, One-Way or Two-Way analysis of variance (ANOVA) was applied as appropriate, followed by Tukey's HSD (honestly significant difference) post-hoc test. Threshold for statistical significance was set to $P < 0.05$. Experimental plots were obtained by Graft software (Erithacus).

Results

MET hampers ccRCC cell viability in a concentration-dependent manner

The effect of MET on cell viability (Fig. 1a) was checked by incubating ccRCC Shaw, ccRCC Elthem and HK2 cells with increasing drug concentrations for 24 h. A dose-dependent reduction of the percentage of viable cells was found for all cell lines (Fig. 1a and Supplementary Fig. 1), being significantly reduced starting from 1 mM to 5 mM MET in ccRCC Shaw and ccRCC Elthem cancer cell lines, respectively. It is, however, notable that the two ccRCC cell lines were more sensitive to MET than HK2 cells, as the viability of the former cell types was reduced at MET concentrations lower than that effective on HK2 cell viability (20 mM) (Fig. 1a).

Reporting the number of viable ccRCC Shaw cells after 24 h of incubation with increasing MET concentrations as % of the number of seeded cells, set as 100%, we found that the percentage increase in cancer cell number was significantly reduced by MET in a concentration-dependent manner, as compared to control, starting from 1 mM MET (Fig. 1b). However, the percentage increase in cell number was either abolished or decreased below the seeding density only at 40 or 60 mM MET, respectively (Fig. 1b), thus indicating that cancer cell death occurs only at very high MET concentrations. Similar results were obtained for ccRCC Elthem cells (not shown), while MET effect on the percentage increase in viable HK2 cell number over time was far less pronounced, with no MET concentration able to abolish or reverse it (Fig. 1b).

To confirm the above observation, it was checked whether necrosis or apoptosis occurred after 24 h of cell incubation with increasing MET concentrations. Released L-lactate dehydrogenase (L-LDH) activity, a marker enzyme of cell necrosis, was assayed in the culture medium of different cell types (Fig. 1c and Supplementary Fig. 2). In agreement with the results reported in Fig. 1b, significant L-LDH release occurred only at extreme MET concentration (60 mM) in the case of the two cancer cell types, whereas no L-LDH release was observed from HK2 cells at any MET concentration (Fig. 1c and Supplementary Fig. 2). Similarly, significant apoptotic cell death, detected by cytometric specific assay, occurred in both ccRCC Shaw and Elthem cells only at very high MET concentrations (≥ 40 mM), but not in HK2 cells (Fig. 1d and Supplementary Fig. 2). The decrease in cell viability starting at 1–5 mM MET (Fig. 1a) and the occurrence of cell death at 40–60 mM MET (Fig. 1b and c) together suggest that cancer cell proliferation is impaired by MET treatment at low doses (1–25 mM), while cell death is induced at higher drug concentrations.

Although accumulated data show the beneficial effect of MET on RCC patients²⁹ and in RCC tumor xenografts³⁰ when used at clinically relevant concentrations, probably linked to the long-time exposition to the drug, our results show that in vitro experiments MET exerts its antitumor effects at high (mM) concentrations (Fig. 1), in agreement with data reported by several studies carried out on different cancer cell types, including ccRCC³⁰. For this reason, to investigate the molecular targets of MET, we used supra-physiological concentrations of the drug. Hence, unless otherwise noted, ccRCC Shaw cells were incubated for 24 h in the absence or presence of 25 mM MET (ccRCC + MET), a concentration near to the IC_{50} value of MET for these cells (28.0 ± 2.0 mM) and able to reduce cancer cell viability by about 40% after 24 h (Fig. 1a).

Metformin affects mitochondrial respiratory chain, and leads to increased glycolysis and decreased ATP level in ccRCC cells

Since MET can target MRC and induce glycolysis activation^{45,46}, the effect of MET (25 mM) on the activity of both the MRC complexes, glycolytic enzymes key to ATP production, and glucose-6-phosphate dehydrogenase (G6PDH), catalyzing the rate-limiting step of the pentose phosphate pathway, was first investigated in ccRCC cells. To this aim, mitochondrial membrane enriched fractions (MMEF) and cell soluble fractions (CSF) obtained from the same control and ccRCC + MET cells were used. The method for the detection of MRC complex activity in MMEF free from any cytosolic contamination, due to the use of specific substrates and inhibitors allows to study each MRC complex separately from the others, thus providing information on the effect of MET on the given single complex (Fig. 2a). CI activity was remarkably inhibited by MET compared to controls (7.2 ± 1.2 and 29.7 ± 2.0 nmol NADH/min x mg MMEF protein, respectively, $P < 0.01$). Additionally, a significant reduction of CIII (8.9 ± 2.0 and 12.6 ± 3.0 nmol cyt c/min x mg MMEF protein, respectively, $P < 0.05$), and CIV (6.0 ± 1.0 and 10.0 ± 2.0 nmol NADH/min x mg MMEF protein, respectively, $P < 0.05$) activity were also detected in ccRCC + MET. On the contrary, a slight increase in the activity of both succinate dehydrogenase (CII) (12.0 ± 2.0 and 10.0 ± 2.0 nmol/min x mg MMEF protein, respectively, $P < 0.05$) and D-lactate dehydrogenase (D-LDH)

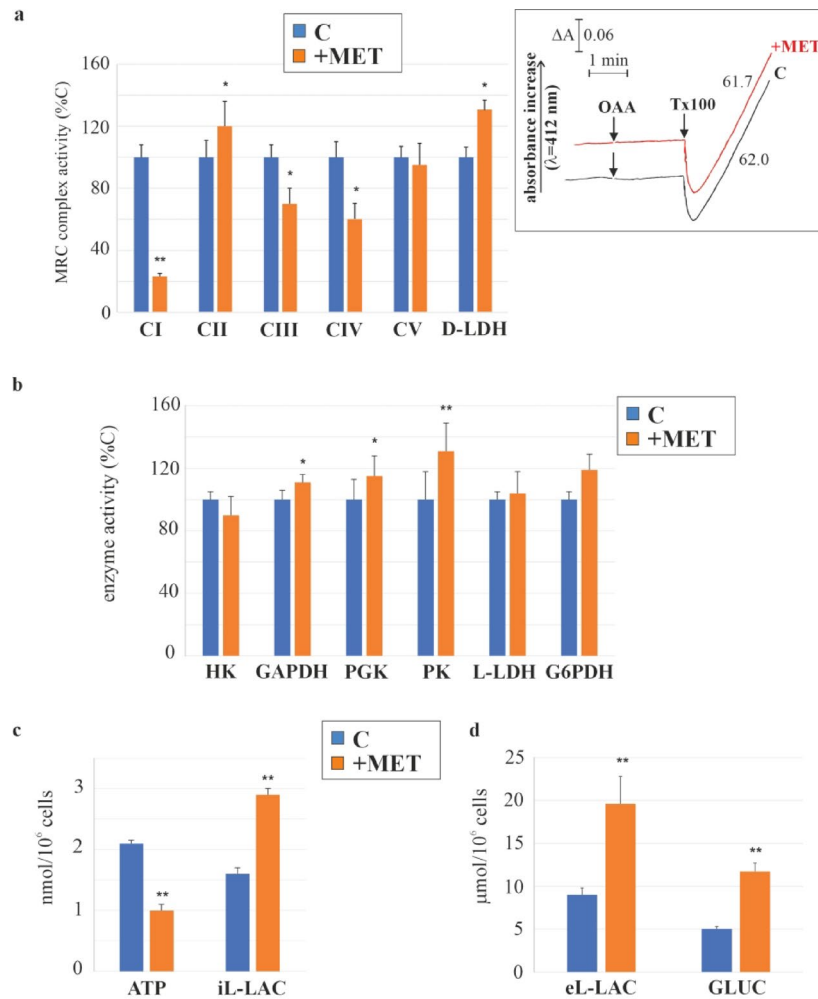


Fig. 2. MET's effect on MRC complex and enzyme activities and ATP, L-LAC and glucose levels. MMEF, CSF, cell extracts and culture medium were obtained from ccRCC Shaw cells incubated for 24 h in the absence (C) or presence of MET (+MET, 25 mM). **(a)** The activity of the MRC complexes I-V and D-LDH was assayed photometrically in MMEF. Values represent mean rates $\pm$ SD ($n=4$) expressed as nmol/min $\times$ mg MMEF protein and then reported in the histogram as % of the mean activities found in MMEF of untreated (control, C) cells. * $P<0.05$, ** $P<0.01$, significantly different from control cell activities, set as 100% (Student's t-test). In the inset, CS activity was assayed photometrically at 412 nm in permeabilized ccRCC Shaw cells (0.4 mg cell protein) incubated in the absence (C) or presence of 25 mM (+MET) for 24 h. Where indicated oxaloacetate (OAA, 0.5 mM) and Tx100 (0.2% v/v) were added to the experimental cuvette. Numbers along traces are specific enzyme activities expressed as nmol/min $\times$ mg cell protein. **(b)** The activity of HK, GAPDH, PGK, PK, L-LDH and G6PDH was assayed photometrically in CSFs. Values represent mean rates $\pm$ SD ($n=4$) expressed as μ mol or nmol/min $\times$ mg MMEF protein and then reported in the histogram as % of the mean specific activities found in CSFs of untreated (control, C) cells, set as 100%. * $P<0.05$, ** $P<0.01$, significantly different from control cell activities (Student's t-test). **(c,d)** Cell extracts **(c)** or culture medium **(d)** were used for the detection of cellular ATP and L-LAC (iL-LAC) levels **(c)** or extracellular L-LAC (eL-LAC) and consumed glucose amounts (GLUC) **(d)**, respectively, as described in the Methods. Values represent means $\pm$ SD ($n=4$) expressed as nmol/10⁶ cells **(c)** or μ mol/10⁶ cells **(d)**. * $P<0.05$, ** $P<0.01$, significantly different from control values (Student's t-test).

(5.3 ± 0.6 and 4.0 ± 0.6 nmol/min $\times$ mg MMEF protein, respectively, $P<0.01$) was found in ccRCC + MET compared to controls. To detect whether the findings on MRC complex activity could have been affected by a variation in mitochondrial mass induced by MET (25mM) treatment, citrate synthase (CS) activity was assayed as mitochondrial matrix marker enzyme in permeabilized ccRCC + MET and control cells added with the membrane detergent Tx100, able to disrupt mitochondrial membranes. No significant change in CS activity was detected (63.3 ± 1.6 and 61.8 ± 0.8 nmol/min $\times$ mg cell prot in ccRCC + MET and control cells, respectively), thus excluding variations in the mitochondrial mass (Fig. 2a inset).

A slight but significant increase in the activity of the pivotal enzymes of the pay-off phase of glycolysis was detected (Fig. 2b). GAPDH (1.20 ± 0.07 and 1.40 ± 0.07 μ mol NADH/min $\times$ mg CSF protein, in control and ccRCC+MET cells, respectively, $P<0.05$), PGK (732 ± 120 and 843 ± 98 nmol NAD⁺/min $\times$ mg CSF protein,

in control and ccRCC+MET cells, respectively, $P < 0.05$), and PK (2.5 ± 0.3 and 3.3 ± 0.5 $\mu\text{mol NAD}^+/\text{min} \times \text{mg CSF protein}$, in control and ccRCC+MET cells, respectively, $P < 0.01$) showed an elevation of enzymatic activity. On the contrary, no significant changes were measured in the enzymatic activity of HK (43.0 ± 2.0 and 42.0 ± 4.0 $\text{nmol NADPH}/\text{min} \times \text{mg CSF protein}$, in control and ccRCC+MET cells, respectively), L-LDH (4.7 ± 0.2 and 4.0 ± 0.6 $\mu\text{mol NAD}^+/\text{min} \times \text{mg CSF protein}$, in control and ccRCC+MET cells, respectively) and G6PDH (116.0 ± 6.0 and 138.0 ± 14.0 $\text{nmol NADPH}/\text{min} \times \text{mg CSF protein}$, in control and ccRCC+MET cells, respectively) (Fig. 2b).

To check the effect of MET treatment upon the overall cell energy status and glycolysis flux, cellular ATP and L-LAC (Fig. 2c) and extracellular L-LAC and glucose (Fig. 2d) levels were determined. As shown, the ATP level dropped to half, whereas the intracellular L-LAC amount was almost doubled in ccRCC+MET cells compared to controls (1.0 ± 0.1 and 2.1 ± 0.05 $\text{nmol ATP}/10^6$ cells, and 2.9 ± 0.1 and 1.6 ± 0.1 $\text{nmol L-LAC}/10^6$ cells, respectively). A similar increase occurred in extracellular L-LAC and consumed glucose amounts in the cell culture medium of ccRCC+MET cells compared to controls (9.0 ± 0.8 and 19.6 ± 3.2 $\mu\text{mol L-LAC}/10^6$ cells, and 5.0 ± 0.3 and 11.7 ± 1.0 $\mu\text{mol glucose}/10^6$ cells, respectively). Thus, glycolysis was activated upon MET treatment, but this metabolic rewire was not sufficient to reestablish normal ATP levels in ccRCC cells.

MET impairs mitochondrial function

The impact of MET on mitochondrial functions in ccRCC cells was checked by measuring ATP production via OXPHOS (Fig. 3), mitochondrial respiration (Fig. 4), $\Delta\psi$ generation (Fig. 5).

Inhibition of mitochondrial ATP synthesis and export by MET

To investigate the effect of MET (25 mM) on mitochondrial ability to produce ATP via OXPHOS, a spectroscopic approach was used for the continuous monitoring of newly synthesized mitochondrial ATP appearance in the extramitochondrial space, where the ATP-ds can detect it. ATP synthesis and export was measured in response to the addition to permeabilized cells of externally added respiratory substrates and ADP that allow ATP synthesis and efflux from mitochondria through ANT-mediated transport (Fig. 3)³⁹. As shown by the experimental traces in Fig. 3, following the addition of malate (MAL) plus glutamate (GLU) (Fig. 3a), succinate (SUCC) (Fig. 3b) or ascorbate (ASC) plus N, N, N, N-tetramethyl-p-phenylenediamine (TMPD) (Fig. 3c), an increase in absorbance due to NADPH formation, reflecting ATP appearance, was observed at a rate (10.0 ± 0.6 , 8.8 ± 1.2 and 8.5 ± 0.9 $\text{nmol}/\text{min} \times \text{mg}$, respectively, $n = 4$) almost halved in ccRCC + MET (4.3 ± 0.5 , 4.0 ± 0.8 and 3.8 ± 0.4 $\text{nmol}/\text{min} \times \text{mg}$, respectively, $n = 4$) compared to controls ($P < 0.001$) regardless of the respiratory substrate added, thus showing that CI-, CII- and CIV-dependent mitochondrial ATP production and export from mitochondria were all impaired by MET. These findings were in line with the observed CI and CIV inhibition but contrasted with the increased CII and unaffected CV activities that occurred in ccRCC + MET as compared to controls (Fig. 2a), thus prompting us to check whether ANT activity could be impaired in the presence of MET.

ANT activity is inhibited in ccRCC incubated with MET

Since both CV-mediated ATP production via OXPHOS and ANT-mediated ADP/ATP exchange contribute to the rate of appearance of ATP in the extramitochondrial space, we checked which of the two steps was rate-limiting of the whole process thus determining the rate of ATP appearance. To achieve this aim, permeabilized ccRCC + MET or control cells were incubated in the absence or presence of increasing concentrations of the ANT's inhibitor ATR, which cannot cross the inner mitochondrial membrane, thus being unable to inhibit CV-mediated ATP synthesis. Then the rates of ATP appearance, measured as in Fig. 3b, were plotted in a Dixon plot (Fig. 4a). In accordance with the control strength criterion^{39,47,48}, the coincidence of the y-intercepts of lines fitting the experimental points obtained in the presence of ATR with that gained in its absence (Fig. 4a) showed that the rate of absorbance increase was that of the ADP/ATP exchange in both ccRCC + MET and control cells. To exclude the presence of any additional ATP sources, for instance adenylate kinase activity, insensitive to ATR, the data reported in Fig. 4a were used to calculate the fractional inhibition i (i equals to $1 - v_i/v_o$ where v_i and v_o are the rates of ATP efflux in the presence and in the absence of ATR, respectively), which was then plotted as $1/i$ against $1/[\text{ATR}]$ (Fig. 4a, inset)⁴⁸. In both ccRCC + MET cells and controls, the y-intercepts were 1, indicating that no ATP production occurred in a manner insensitive to the ANT inhibitor in both cases.

Then, the dependence of ANT activity on increasing ADP concentrations was studied in pRCC cells incubated with or without MET (25 mM) for 24 h. The means of rates were plotted in either a Michaelis-Menten (Fig. 4b) or a double-reciprocal plot (Fig. 4b, inset). The K_m and $V_{\max}$ (maximum rate) values for ANT were 0.8 ± 0.1 μM and 9.2 ± 1.0 $\text{nmol}/\text{min} \times \text{mg}$ of cell protein, respectively, in controls, and changed to 1.5 ± 0.1 μM and 4.1 ± 0.7 $\text{nmol}/\text{min} \times \text{mg}$ of cell protein values in the presence of MET. The double-reciprocal plot gives a clear graphical indication of MET-dependent mixed-type inhibition of ANT⁴⁸, resulting in both $V_{\max}$ reduction and K_m increase (Fig. 4b, inset).

MET affects mitochondrial respiration and membrane potential generation

Mitochondrial respiration was checked under FCCP-induced uncoupled state 3, reflecting the maximal rate of RC complex-driven oxygen consumption (Fig. 5a). CI- and CIV-driven respiration measured after the addition of MAL plus GLU or ASC plus TMPD, respectively, occurred at a significantly lower rate in ccRCC + MET with respect to controls, especially for CI (CI: 1.6 ± 0.08 versus 8.0 ± 1.6 $\text{nmol O}_2/\text{min} \times \text{mg cell protein}$, respectively, $P < 0.05$; CIV: 13.5 ± 1.7 versus 17.4 ± 1.5 $\text{nmol O}_2/\text{min} \times \text{mg cell protein}$, respectively, $P < 0.05$) (Fig. 5a). On the contrary, SUCC addition to permeabilized cells incubated in the presence of rotenone, induced CII-dependent oxygen consumption at a rate about 35% higher in ccRCC + MET with respect to controls (12.9 ± 1.2 and 8.4 ± 2.1 $\text{nmol O}_2/\text{min} \times \text{mg cell protein}$, respectively, $P < 0.05$) (Fig. 5a), in good agreement with the results reported in Fig. 2a.

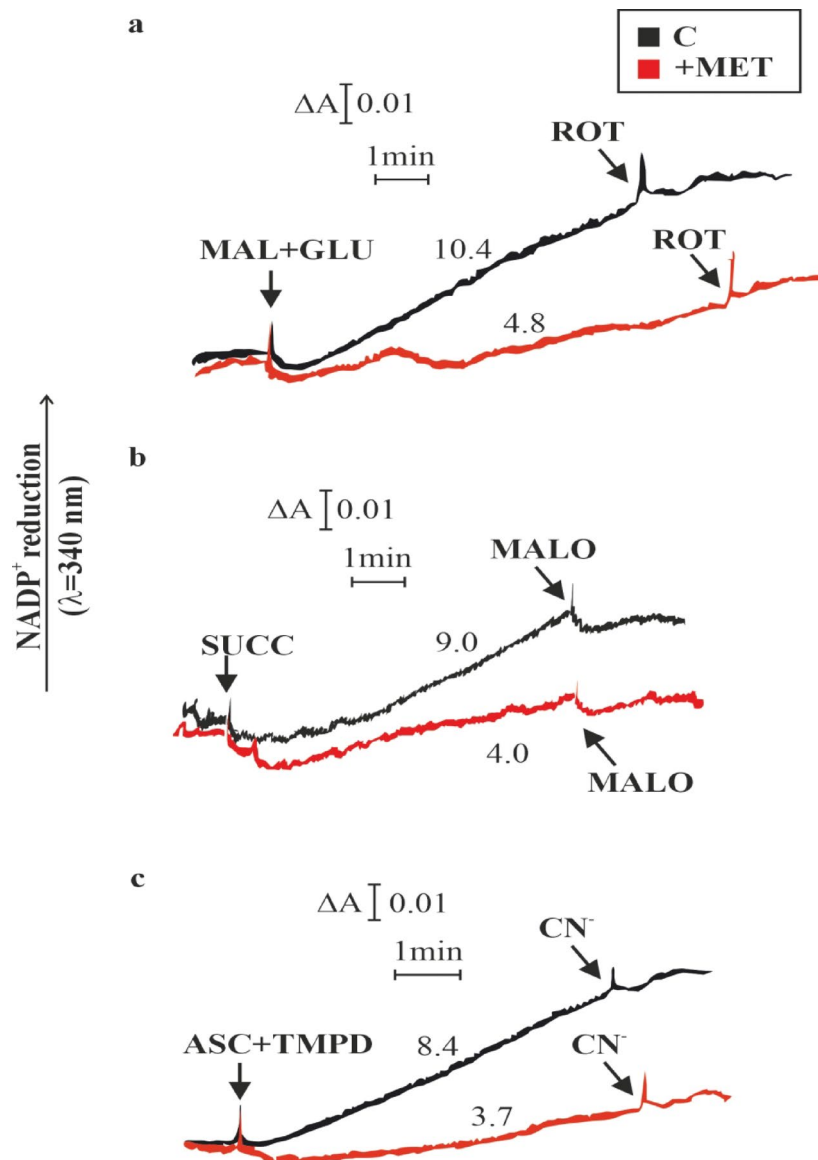


Fig. 3. The effect of MET treatment on mitochondrial ATP synthesis via OXPHOS and efflux from mitochondria. The ccRCC Shaw cells were incubated for 24 h in the absence (C) or presence of MET (+MET, 25 mM) and then permeabilized with digitonin, as described in the Material and methods. The appearance of ATP was monitored in pRCC cells (0.4 mg) incubated with ADP (0.5 mM), Ap5A (10 μM) and ATP-ds, by following NADP⁺ reduction as absorbance increase at 340 nm. MAL + GLU (5 mM each) (a), SUCC (5 mM) (b) or ASC+TMPD (5 mM and 0.2 mM, respectively) (c) were added to start CI-, CII- or CIV-dependent ATP production and efflux from mitochondria, respectively. Where indicated rotenone (ROT, 2 μg/10 μl), malonate (MALO, 10mM) or cyanide (CN⁻, 1 mM) were added to inhibit CI-, CII- or CIV-dependent ATP appearance, respectively. The numbers along curves indicate NADPH formation rates measured as tangents to the initial tract of the experimental traces and expressed as nmol of NADPH/min x mg of cell protein.

Finally, energy generation in the form of mitochondrial membrane potential ($\Delta\Psi$) was evaluated both in control and ccRCC + MET cells (Fig. 5b). As shown by the typical traces, the rate and extent of $\Delta\Psi$ generation due to either MAL plus GLU (Fig. 5b, a) or ASC plus TMPD (Fig. 5b, c) were significantly lower in ccRCC + MET cells compared to controls, while a slight stimulation of SUCC-dependent $\Delta\Psi$ generation rate and extent was observed (Fig. 5b, b), in agreement with the previously shown results.

ANT activity is inhibited in the presence of low MET concentrations that are not effective on CI activity and do not induce the glycolytic shift

The ability of MET treatment to inhibit ANT was never investigated before. Therefore, ANT inhibition was further analyzed with the comparative examination of its dependence upon increasing MET concentration. Its behavior under the influence of MET was collated to that of CI. To do so, ccRCC cells were incubated for

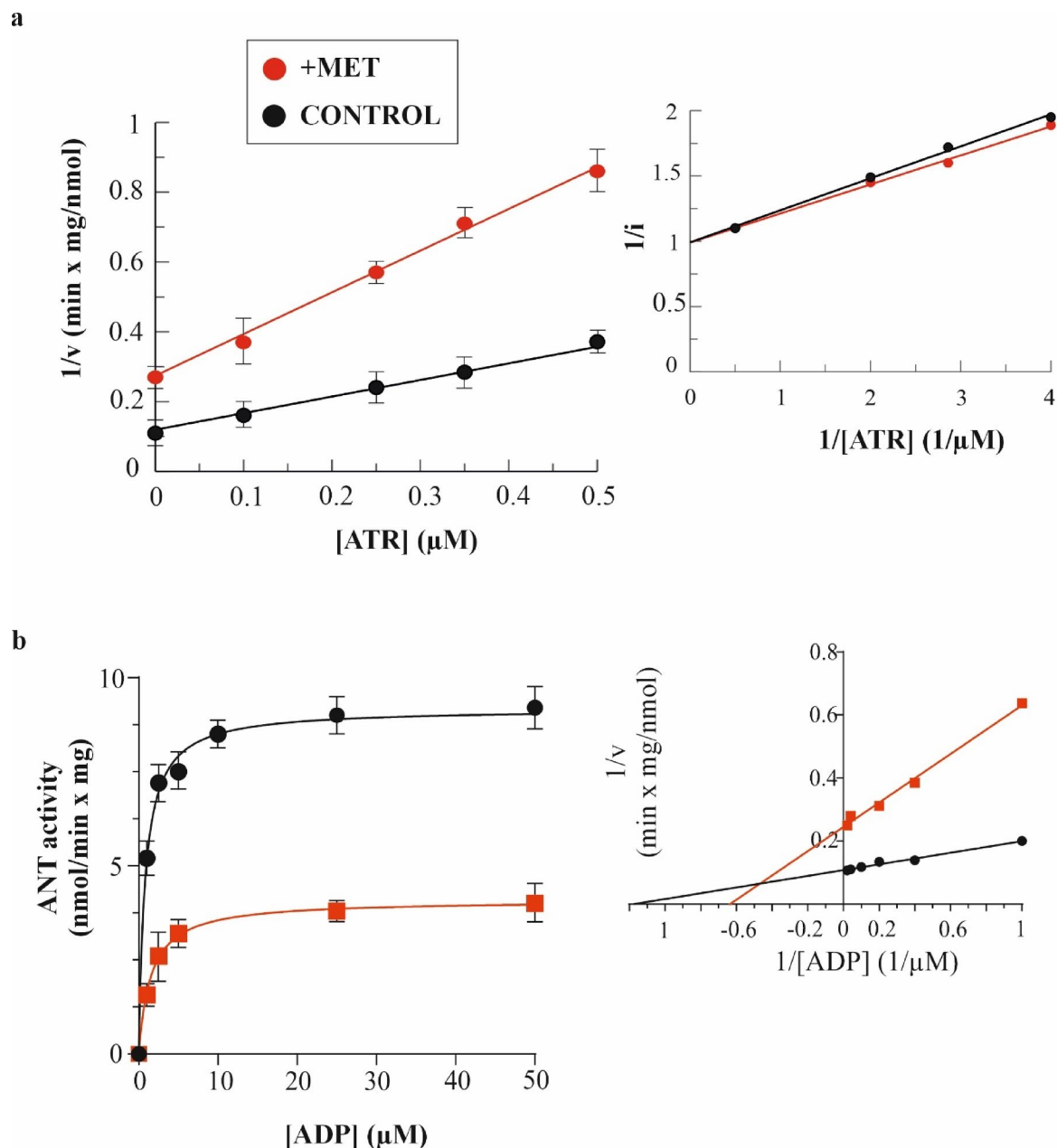


Fig. 4. Dixon plot of the inhibition by atractyloside of ATP appearance rate. Kinetic analysis of ANT. **(a)** Dixon plot of the inhibition by atractyloside (ATR) of the rate of ATP appearance in the extramitochondrial phase. pRCC cells (0.4 mg cell protein) were incubated for 2 min either in the absence or presence of ATR at the indicated concentrations, in a final volume of 2 ml of respiration medium (pH 7.4) containing the ATP-ds plus Ap5A (10 μM) and ADP (0.5 mM), at 37 °C. The rate of ATP appearance in the extramitochondrial phase was measured as in Fig. 5B, after the addition of SUCC (5 mM). The rate (v), measured as the tangent to the initial part of the progress curve, is expressed as nmol of NADPH formed/min x mg cell protein. All values are means $\pm$ SD ($n = 3$). The inset is a replot of the mean values reported in (a), where $i = 1 - v_i/v_0$ (see the text for details). **(b)** Michaelis-Menten and double reciprocal plots (inset) of ANT-mediated ATP/ADP exchange. ADP was added to pRCC cells (0.4 mg cell protein) at the indicated concentrations in the presence of 5 mM SUCC and ATP appearance rate, indicative of ANT activity, measured as in (a) and expressed as nmol NADPH/min x mg cell protein. All values are means $\pm$ SD ($n = 3$). The inset is a replot in a double reciprocal plot of the mean values reported in (b).

24 h with increasing MET concentrations and then divided into two aliquots, one to be permeabilized for the measurement of ANT activity, while the other to be sonicated to obtain MMEF for CI activity assay. As shown, ANT activity was already halved at 50 μM MET concentration, a concentration near to that found in the plasma of MET-treated diabetic patients^{22,23} and at which neither HK2 nor ccRCC cell viability and proliferation were

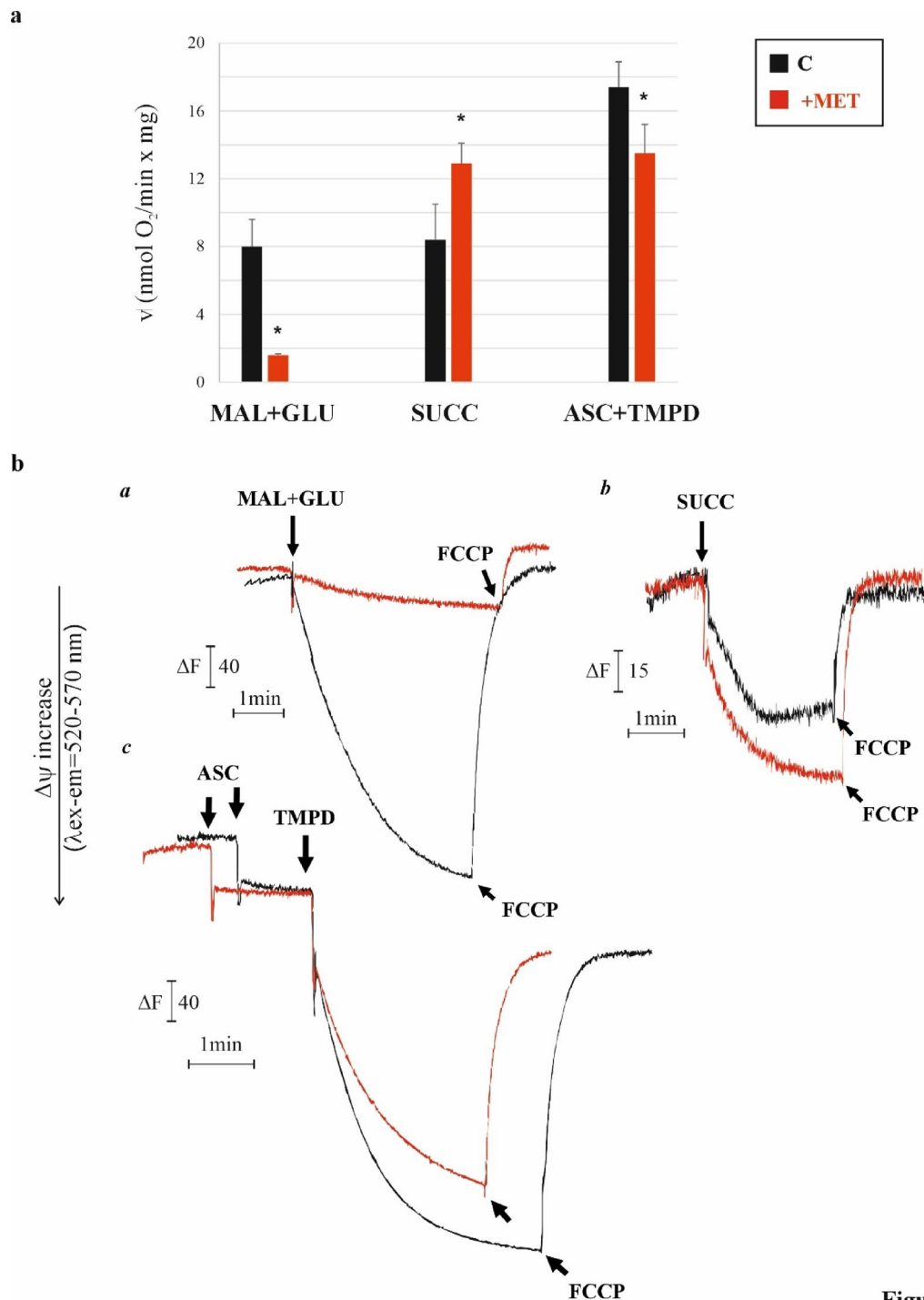


Fig. 5. The effect of MET treatment on mitochondrial respiration and $\Delta\psi$ generation. The ccRCC Shaw cells were incubated for 24 h in the absence (C) or presence of MET (+MET, 25 mM) and then permeabilized with digitonin, as described in Materials and methods. **(a)** pRCC cells were added with 1.25 μ M FCCP to obtain uncoupled respiration measured after the addition of the CI, CII and CIV respiratory substrates MAL plus GLU (MAL + GLU, 5 mM each), SUCC (5 mM) and ASC (5 mM) plus TMPD (0.3 mM) (ASC+TMPD), respectively. The respiration rates (V), corrected of the rate of specific inhibitor-insensitive oxygen consumption, were reported as means $\pm$ SD ($n = 3$). * $P < 0.05$, significantly different from control values (Student's t-test). **(b)** ccRCC Shaw cells (1 mg of cell protein) were permeabilized with digitonin directly in the experimental cuvette containing 2 ml of respiration medium (pH 7.4) and 10 μ M safranin, in the presence of 2 μ g/10 μ l rotenone in the case of SUCC, or 2 μ g/10 μ l rotenone plus 0.8 μ M antimycin A in the case of ASC plus TMPD. Then safranin O fluorescence was measured as a function of time at the indicated excitation (ex) and emission (em) wavelengths. GLU plus MAL (5 mM each) **(a)**, SUCC (5 mM) **(b)** or ASC (5 mM) and TMPD (0.3 mM) **(c)** were added to induce $\Delta\psi$ generation, detected as fluorescence decrease, which was finally abolished by the uncoupler FCCP (1.25 μ M).

affected by MET (Fig. 1a, b). ANT activity remained essentially unchanged even if MET concentrations were elevated (Fig. 6a). Unlike the inhibition of ANT, CI activity was only inhibited by MET when its concentrations were ≥ 1 mM (about 20% of inhibition) (Fig. 6a), stressing different sensitivities of these activities toward MET. Notable is, the higher the dose of MET, the greater the inhibition of CI activity (Fig. 6a), an effect not seen for ANT.

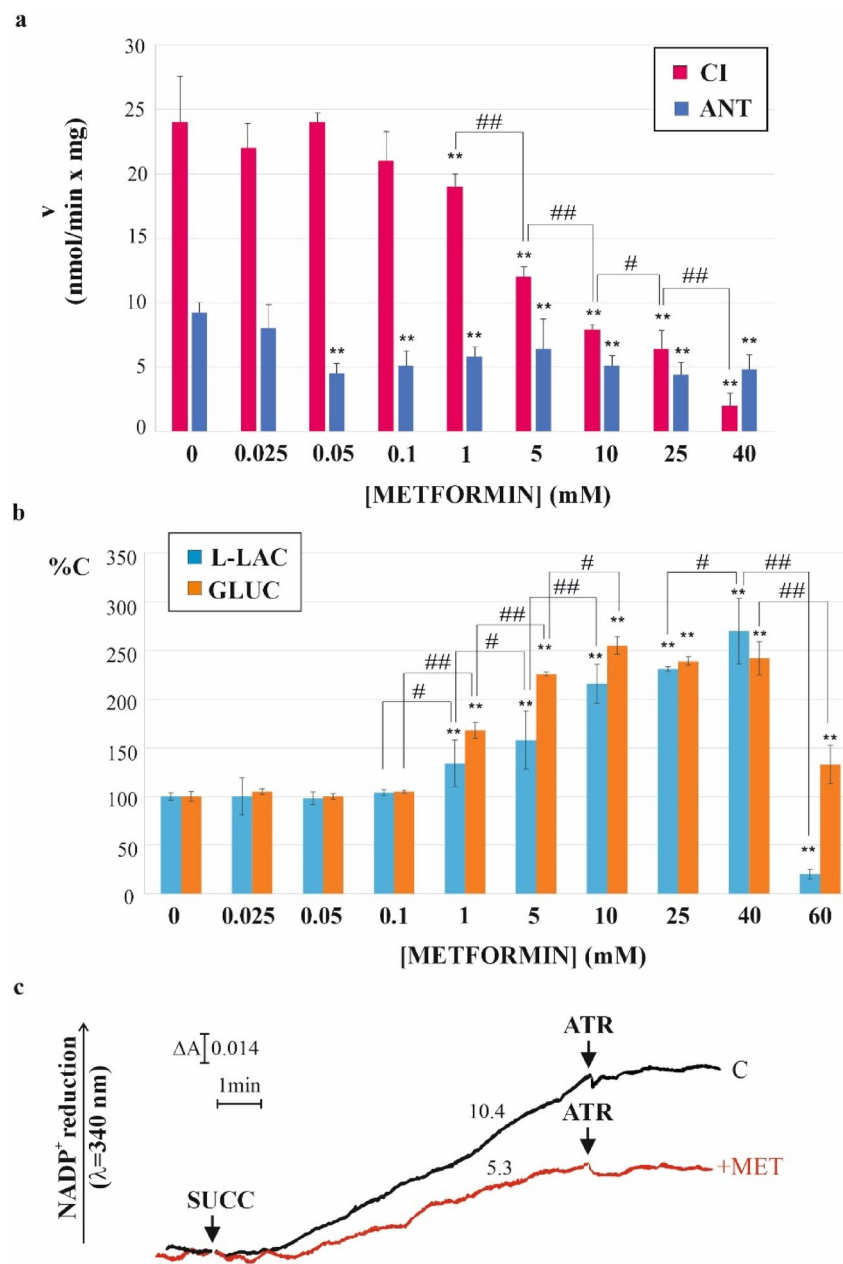


Fig. 6. The dependence of CI and ANT activity and the glycolytic shift on increasing MET concentrations. **(a)** CI and ANT activities were assayed as in Figs. 2A and 3B in MMEF and pRCC, respectively, obtained from the same ccRCC Shaw cells incubated for 24 h in the absence (C) or presence of the reported MET concentrations (+ MET). Values reported in the histogram are means $\pm$ SD ($n=3$). **(b)** The cell culture medium or commercial RPMI medium were used for L-LAC or glucose assay as in Fig. 2c, d. Numbers reported in the graph represent means $\pm$ SD ($n=4$) of normalized extracellular L-LAC and consumed glucose amounts (GLUC) expressed as % of those found in MET-untreated cells, set as 100% (C). The significance of MET increasing concentration's effect on CI and ANT activity **(a)** and L-LAC and GLUC amounts **(b)** has been assessed separately for each variable within the same cell line, using One-Way ANOVA and Tukey's post-hoc test. * $P < 0.05$, ** $P < 0.01$ indicate significant difference from control values; # $P < 0.05$, ## $P < 0.01$ indicate significant difference between the indicated values. **(c)** pRCC cells (0.4 mg) were preincubated for 5 min, at 37 °C, in the absence (C) or presence of MET (50 μM) (+ MET). At the arrows SUCC (5 mM) and ATR (100 μM) were added. Values along the experimental traces are rates of NADP⁺ reduction expressed as nmol/min x mg cell proteins.

To further substantiate the ability of MET to cause metabolic rewiring, the glycolytic shift in ccRCC cells was investigated at increasing MET concentrations by determining the released L-LAC and consumed glucose amounts into the culture medium of the same cells used for ANT and CI activity assay. The results were normalized to cell numbers and expressed as % of the values found for untreated control cells (Fig. 6b). In the concentration range higher than 0.1 mM MET a significant increase in L-LAC release and glucose consumption was observed, reaching peak values for L-LAC and for glucose at 40 mM and 10 mM of MET, respectively. This kind of profile of glucose consumption is eerily similar to CI inhibition profile (Fig. 6a). At MET concentrations higher than 40 mM, the amount of released L-LAC and consumed glucose drastically decreased in the medium, reflecting the arisen cell death (compare to Fig. 1).

Finally, to check whether the observed decrease in ANT activity after 24 h of incubation with MET (Fig. 6a) could be due to a direct inhibitory effect of MET on the carrier, we measured ANT activity in permeabilized ccRCC control cells incubated for 5 min in the absence or presence of low MET concentration (50 μ M), added directly into the experimental cuvette (Fig. 6c). As shown by the typical traces, the rate of ATP appearance was almost halved in the presence of MET compared to control (5.0 ± 0.6 and 9.7 ± 0.8 nmol/min $\times$ mg of cell protein, respectively. $P < 0.001$, $n = 3$) and abolished in both cases by ATR (100 μ M), as expected. Thus, a direct inhibition of ANT by MET might occur.

Discussion

The central issue for cells regardless of their type is to sustain their functional and structural integrity via a continuous flow of matter as well as energy. This rule applies to tumor cells even more emphatically as those are in a continuous proliferation, even if with different rate, as a result of which their susceptibility to drugs affecting cellular bioenergetics differ. Cell division is a complex, sophisticated, high energy demand process⁵⁰. From the above, it is not a surprise that one of the targets of cancer chemotherapy is to abolish/inhibit the ability of tumors to produce energy and one of the anticancer agents is MET mainly targeting mitochondrion, the powerhouse of the cells^{16,51,52}. In the present study, the above aspect was investigated to gain a new insight into the molecular mechanisms of action of MET and its overall effect on mitochondrial functions as well as energy metabolism in ccRCC as model.

Our results show that MET affected ccRCC cell viability in a concentration-dependent manner, starting from 1 to 5 mM MET (Fig. 1a, b). The observed reduction of viable cell number could be a result of impaired proliferation or depend on the induction of cell death by MET. However, MET was found to induce cell apoptosis or necrosis only at extremely high (40–60 mM) concentrations (Fig. 1c, d), thus indicating that cancer cell proliferation is impaired by MET treatment at lower doses (1–25 mM), in line with previous studies indicating the potential of MET to treat ccRCC³⁰. Data also shows that tumor cells were more susceptible to MET than HK2 cells (Fig. 1).

High dose MET treatment affected functional mitochondria of ccRCC cells in several ways. In detail, the specific activities of the mitochondrial respiratory complexes CI, CIII and CIV were reduced by MET, whereas a mild enhancement of specific activities of FAD/FMN-dependent enzymes, CII and D-LDH, was observed in the same cells (Fig. 2a), likely bypassing the severe impairment of MET-induced NADH oxidation. It should be noted that, similarly to L-LDH, assayed here as a key glycolytic enzyme, D-LDH, a mitochondrial enzyme acting as a MRC complex, could play a role in cancer metabolism as suggested by the existing correlation between D-LDH gene expression increase and better survival rate in ccRCC patients²⁷. It is notable that others did not find MET inhibition while investigating these complex activities⁴⁶, whereas others reported on CI inhibition by MET⁵³.

The effect of MET treatment on mitochondrial ATP synthesis via OXPHOS in ccRCC permeabilized with digitonin confirmed the results of activity measurements since MET inhibited ATP production using MAL + GLU and ASC + TMPD as substrates for CI- and CIV-complexes, respectively, by more than 50% (Fig. 3a and c). It is, however, intriguing that using SUCC, substrate for CII in the same model, a similar degree of inhibition was detectable (Fig. 3b).

The effect of MET on MRC complexes was further supported by mitochondrial respiration and $\Delta\Psi$ generation experiments. Both CI- and CIV-driven mitochondrial respiration (Fig. 5a) and $\Delta\Psi$ generation (Fig. 5b, a, b and c) were decreased by MET, whereas both mitochondrial functions were enhanced when driven by CII (Fig. 5b, b). While the inhibition of respiratory complex activities and O_2 consumption by MET are in general accepted in the literature^{17,45,46,52}, nothing is known upon MET's effect on ANT which provides a continuous supply of ADP to mitochondria and proved to be the rate limiting step of the global process consisting of ATP synthesis via OXPHOS and export from ccRCC mitochondria (Fig. 4a), as already demonstrated in other experimental systems^{39,48}.

Although other experimental approaches, such as the reconstitution of purified ANT in proteoliposomes, direct binding or biophysical interaction assays, are needed to ascertain whether ANT is a primary target of MET, here we show several indications that MET could directly inhibit ANT. Indeed, in this study ANT activity was supported by SUCC as respiratory substrate. Since neither SUCC-dependent $\Delta\Psi$ generation (Fig. 5b, b), nor CII activity (Fig. 2a) and CII-dependent mitochondrial respiration (Fig. 5a) were affected by high dose MET treatment, the observed inhibition of ANT activity in the presence of MET cannot be accounted for by respiratory chain impairment and drop in $\Delta\Psi$. Consistently, as shown by the Dixon plot, the rate of SUCC-dependent ATP synthesis and efflux from mitochondria was limited by ANT activity both in the absence or presence of MET (Fig. 4a). Furthermore, low dose MET (50 μ M), which did not affect CI activity, was able to inhibit significantly ANT activity when added to control ccRCC cells in the experimental cuvette for 5 min (Fig. 6c), thus excluding the involvement of MET-activated signalling molecules in ANT inhibition and supporting the idea that ANT might be a primary target of MET. However, further experiments will be required to clearly ascertain the direct

effect of MET on ANT and definitively exclude that the observed inhibition could be secondary to altered mitochondrial energetics.

A limitation of the present study is the lack of information on the potential contribution of AMPK which is activated under conditions of low energy and can phosphorylate specific enzymes and growth control nodes to increase ATP generation and decrease ATP consumption. Indeed, AMPK activation is known to occur in vitro upon MET treatment when used at mM concentrations in several ccRCC cell lines^{30,54}. Therefore, some of the findings reported here, such as the effect of MET on cell viability, as well as MET-dependent glucose consumption increase and activation of certain catabolic enzymes, could be mediated by AMPK, thus contributing to the observed antitumor effects of MET⁵⁵.

Interestingly, lysosomal AMPK activation has also been shown to occur by low MET concentration (5 μ M), without any perturbation of AMP/ADP levels in primary hepatocytes²⁵. Consistently, we found that neither CI nor ANT activity are inhibited at MET concentrations lower than 50 μ M (Fig. 6a). However, MET anticancer effect requires additional mitochondrial targeting, as supported by the evidence that the drug becomes effective on cancer cell viability only at higher concentrations affecting mitochondrial functions (Fig. 1). Thus, MET could act on both AMPK and cell energy metabolism in different ways. This view is supported by the fact that, at physiologic glucose levels, cells that are defective in AMPK signalling are actually hypersensitive to MET since MET-induced energetic stress cannot be properly compensated by AMPK activation⁵⁶. Interestingly, AMPK is often less activated in human cancer tissues than in corresponding normal tissue⁵⁷. This also applies to renal carcinoma²⁷. Hence, in these cancers, MET could be more effective than compounds that activate AMPK without inducing energetic stress⁵⁷.

In our experimental model, MET reduced both the $V_{\max}$ and the affinity of the carrier for its substrate ADP (increased K_m value), thus showing a mixed-type inhibition of ANT (Fig. 4b). Thus, it can be claimed that MET can bind both the free carrier and the carrier-substrate complex⁴⁹. This could explain why ANT activity was almost halved even by low MET concentration (50 μ M) (Fig. 6a) near to that found in the plasma of patients treated with MET²⁵, at which neither the inhibition of HK2 and ccRCC cell viability and proliferation (Fig. 1a, b) and CI activity (Fig. 6a), nor the induction of the glycolytic shift (Fig. 6b) occurred. Taken together, it is strongly suggested that in cells exposed to low MET concentrations, meeting clinically relevant concentrations, it is not the ability of mitochondria to generate ATP compromised, but rather the rate at which ATP is made available in the cytosol for anabolic reactions. This could explain the cytostatic effect of MET on cancer cells, associated with a reduction in tumor cell viability and proliferation but not with cell death (Fig. 1). Moreover, since the rate of ATP efflux from the mitochondrial matrix in exchange with cytosolic ADP is affected, increased cytosolic ADP/ATP ratio could occur even at low MET concentrations, explaining how clinically relevant doses of MET can activate AMPK and at the same time decrease liver gluconeogenesis, even in the absence of CI-dependent ATP synthesis inhibition, a still debated topic^{24,25}.

A further response of ccRCC to MET treatment was the enhancement of glycolysis, shown by the remarkable increase of L-LAC formation and glucose consumption (Figs. 2c and d and 6b). Despite this, only a slight, but significant increase in the specific activity of the key enzymes of the pay-off phase of glycolysis was detected, while HK and L-LDH activity, as well as G6PDH, were not affected by MET (Fig. 2b). These results seem to support the idea that the glycolytic capacity and the pentose phosphate pathway were already boosted in ccRCC cells²⁷ even in the absence of MET, perhaps due to HIF1 α over-activation⁵⁸. Indeed, in vivo experiments showed enhanced glycolysis and suppressed glucose oxidation in ccRCC tissue compared to matched adjacent kidney tissue²⁸.

In this work, it was also found that despite the glycolytic shift, MET treatment halved the cellular ATP level in ccRCC cells compared to controls (Fig. 2c), directing attention to the centrality of mitochondrial function to maintain adequate ATP levels in these cells.

Finally, the dependence of the glycolytic shift (Fig. 6b), cell viability and proliferation reduction (Fig. 1a and b) and CI inhibition (Fig. 6a) on MET concentration, compared with that of ANT inhibition (Fig. 6a), clearly indicate a higher sensitivity of ANT to MET. However, from these data it can be suggested that CI, but not ANT inhibition is responsible for the activation of glycolysis and the impairment of cell division in ccRCC, since these events do not occur when ANT but not CI is inhibited, while becoming significant with CI activity reduction (Fig. 6).

Conclusions

A deeper knowledge of the mechanisms of action of complex metabolism-modifying drugs such as MET could improve the design and application of new drug combinations in cancer as well as in other disorders. The present study gives evidence of how MET targets ccRCC energy metabolism and how ccRCC cells respond to MET treatment by increasing glycolysis and the activity of mitochondrial flavoenzymes as SDH and D-LDH to partially compensate MET-induced mitochondrial energy deficit (Fig. 7). Our findings also show that ANT activity is impaired in ccRCC cells treated with MET at low concentrations, near to those found in the plasma of MET-treated patients, demonstrating the involvement of ATP transport across the mitochondrial inner membrane. Hence, we propose that, by reducing the rate at which mitochondrial ATP is made available to cytosolic biosynthetic processes, MET induces an “energy-saving” phenotype, leading to the observed cytostatic effect, which could be one of the possible explanations of how MET reduces the cancer risk in diabetic patients⁵⁹, still not inducing lactic acidosis thus being overall safe for patients. Although further studies are needed to support this hypothesis, the results in this paper give a new insight into MET mechanism of action in renal carcinoma and into the metabolic responses that occur in these cells challenged to the drug, data that could be exploited for MET use in combination with other antitumor drugs with known mechanism of action. It is also

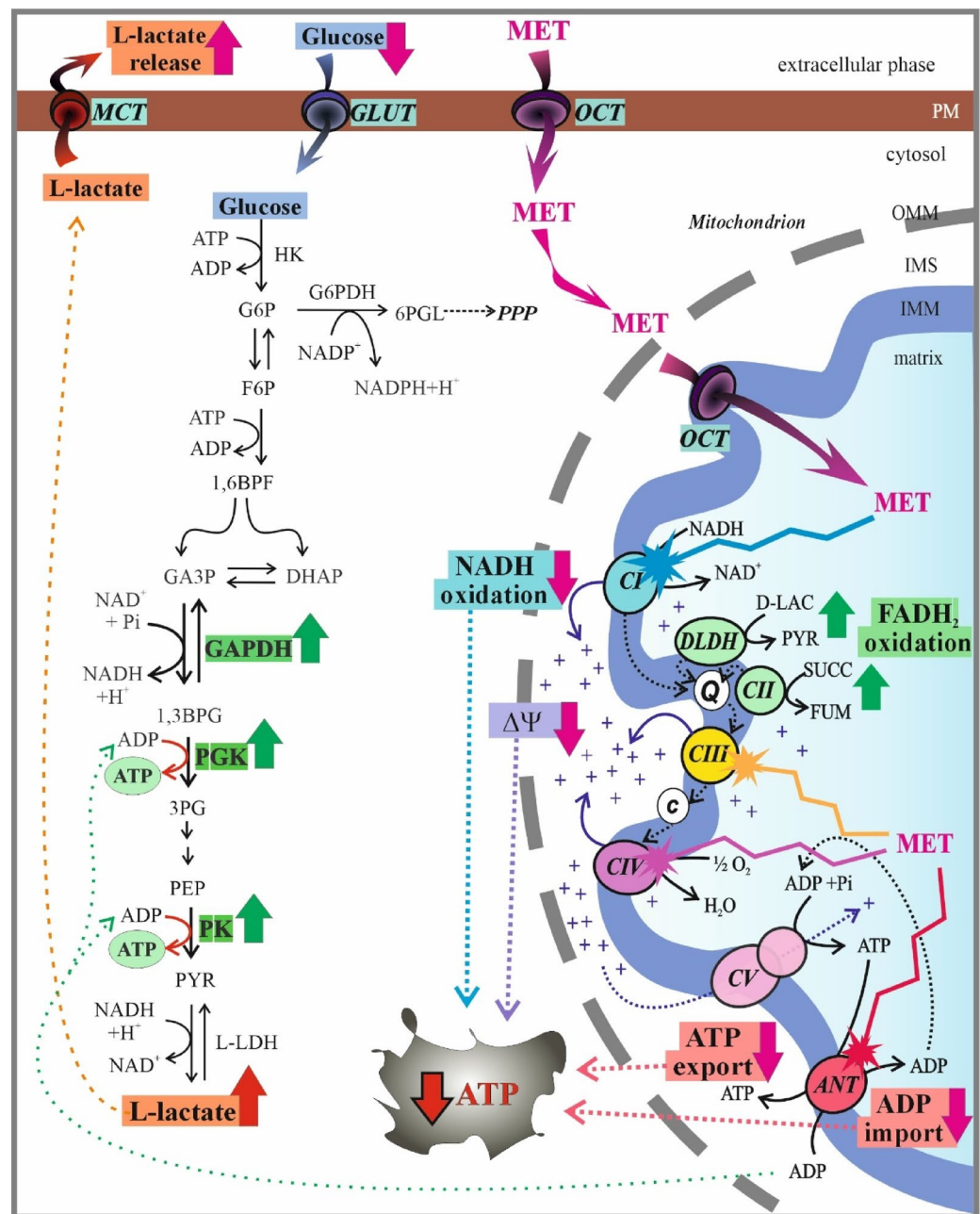


Fig. 7. Overview of the effect of MET on ccRCC cell energy metabolism and mitochondrial functions. MET can enter ccRCC cells and mitochondria by the organic cation translocator (OCT) located at both plasma membrane (PM) and inner mitochondrial membrane (IMM)⁶⁰. MET impairs mitochondrial functions (oxygen consumption, $\Delta\Psi$ generation and ATP synthesis and export) in ccRCC, by affecting the activity of both complexes I (CI), III (CIII) and IV (CIV) and ANT, this latter identified here for the first time as a MET's target. MET-treated ccRCC cells undergo a partial activation of succinate dehydrogenase (CII) and D-lactate dehydrogenase (D-LDH), both flavin-dependent and IMM-associated enzymes, probably to bypass CI-dependent impairment of NADH oxidation. A marked glycolytic shift also occurs in MET-treated ccRCC cells, as shown by the increase in cellular L-lactate production/release and extracellular glucose consumption, accompanied by the activation of the key glycolytic enzymes of the "payoff phase" of glycolysis, namely GAPDH, PK and PGK. However, despite this metabolic rewiring, MET treatment drastically reduced the whole cellular ATP level, this ultimately affecting ccRCC cell viability and proliferation. Other abbreviations: ATR, atractyloside; 1,6BPF, fructose 1,6-bisphosphate; 1,3BPG, 1,3-bisphosphoglycerate; c, cytochrome c; DHAP, dihydroxyacetone phosphate; D-LAC, D-lactate; F6P, fructose 6-phosphate; G6P, glucose 6 phosphate; 6PGL, 6-phosphogluconolactone; G6PDH, glucose 6-phosphate dehydrogenase; GA3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; IMM, inner mitochondrial membrane; IMS, intermembrane space; PEP, phosphoenolpyruvate; Q, coenzyme Q; OMM, outer mitochondrial membrane; PYR, pyruvate.

believed that the data in this study might be useful at exploiting ANT modulation by MET in other experimental models and in pathologies other than cancer.

Data availability

All data generated or analyzed during this study are included in this published article. Further inquiries can be directed to the corresponding author.

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Author contributions

Conception and design of the work, supervision and figure preparation: L.d.B.; investigations and interpretation of data: L.d.B., M.F.C., G.P., M.C. and M.P.K; original draft preparation: L.d.B.; substantial revision and editing: M.P.K, L.d.B., M.F.C., G.P., C.A., T.A., and A.S. All authors read and approved the submitted manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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