Application Note

O_2 Concentration of a Simulated Tumor Environment Affects Metabolic Response to Chemotherapeutics

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Key Features

- The tumor microenvironment contains metabolically aberrant conditions that are difficult to reproduce *in vitro* and as a result:
    - Physiologically relevant sample types are often expensive and time consuming to obtain.
    - Metabolic studies conducted *in vitro* are often carried out at ambient oxygen concentrations, which differ from that of the tumor microenvironment.
- HCT116 colon carcinoma cells and myeloid-derived tumor suppressor cells were cultured at different O_2 concentrations to simulate a component of the tumor microenvironment.
- Mitochondrial stress, fatty acid oxidation stress, and mitochondrial fuel flexibility tests reveal how metabolic phenotype can vary greatly based on culture conditions, illustrating the importance of controlling O_2 concentration in metabolic studies.

Introduction

Within the tumor microenvironment (TME) exists a unique metabolic milieu consisting of low O_2 concentration, low pH, and high fatty acid concentrations. These conditions, which are often dictated by the tumor and tissue type from which it is derived, can be difficult to reproduce under *in vitro* conditions.[1] Immunosuppressive myeloid-derived suppressor cells (MDSCs) occupy the TME and aid in tumor growth by suppressing T cells, which would normally attack the tumor.[2] MDSCs that are localized to the TME are thought to undergo an alteration in their metabolic phenotype. This allows them to utilize the high concentrations of fatty acids found in the TME as their primary carbon source, thus becoming reliant on fatty acid oxidation (FAO). It has been previously proposed that the chemotherapeutic agents oxaliplatin and SN-38 (the active metabolite of irinotecan) can inhibit FAO, sensitizing cells to the TME.[3,4] Here we show the effects on metabolic function after treatment with SN-38, oxaliplatin, and the known FAO inhibitor etomoxir. Both HCT116 human colon carcinoma cells and primary human MDSCs cultured at different O_2 concentrations (to simulate a component of the TME *in vitro*) demonstrate altered metabolic phenotypes.

Methods

Cell Culture

HCT116 colon carcinoma cells were cultured in DMEM supplemented with 10% FBS, 1 mM glutamine, and pen/strep at 37°C/5% before plating on an XF96 plate (1 x 10^5 cells/well) with continued culture at ambient O_2 or 1% O_2/5% CO_2 in DMEM supplemented as above, unless otherwise noted.

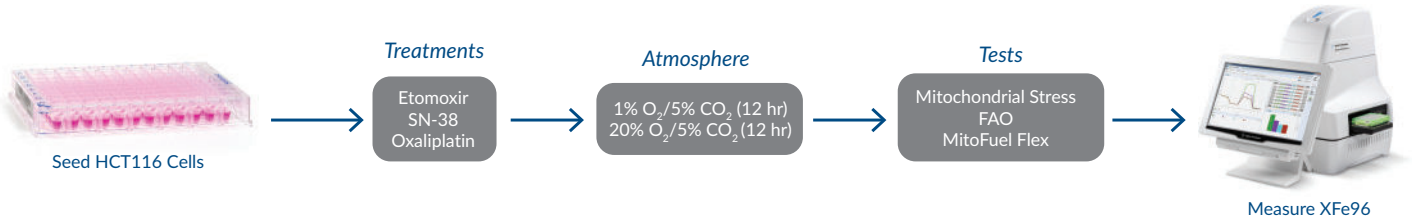


Figure 1. Method workflow for mitochondrial stress test, fatty acid oxidation stress test, and mitochondrial fuel flex test.

Mitochondrial Stress Test

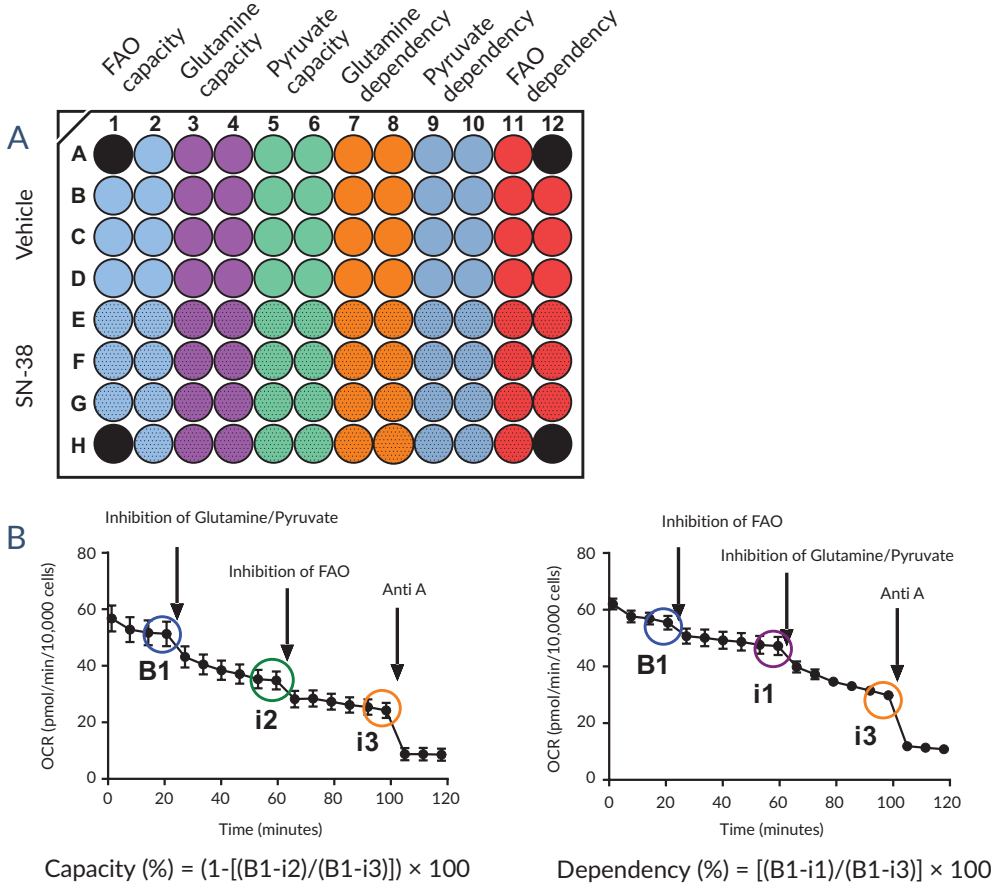
HCT116 cells were seeded at 1 x 10^5 cells/well and allowed to settle for 1 hour before treatment with compounds or vehicle. Cells that were not treated with compounds or vehicle were challenged during the assay with the FAO inhibitor etomoxir. Cells were cultured overnight in DMEM supplemented with 10% FBS, 1 mM glutamine, and pen/strep at 37°C/5% CO_2 at ambient O_2 or 1% O_2. The following morning, complete DMEM was removed and replaced with XF96 DMEM supplemented with 10 mM glucose, 1 mM glutamine, and 1 mM pyruvate (Agilent Technologies). Cells were degassed and imaged using the Cytation™ 5 Imaging Multi-Mode Plate Reader (BioTek Instruments) before performing all experiments. Cells were challenged with buffer/100 µM etomoxir (Injection A) followed by 1 µg/ml oligomycin (Injection B) to inhibit ATP synthesis, 1 µM FCCP (1 µM BAM15 for MDSCs and DCs) (Injection C) to uncouple the ETC from OXPHOS to maximize OCR, and a combination of antimycin A, a complex III inhibitor, and Hoechst stain (Injection D) to aid in normalization. At the conclusion of all XF96 experiments cell normalization was accomplished using XF96 Cell Normalization Software (Agilent Technologies).

Fatty Acid Oxidation Stress Test

In order to maximize the effects of SN-38 and oxaliplatin on FAO, cells were cultured for 24 hours in low-nutrient, low-serum media in the presence of carnitine, maximizing the metabolic dependence on exogenous fatty acids, thereby upregulating FAO. Chemotherapeutic agents were added for the last 12 hours of the culture period and cultured at 21% or 1% O_2. Following the treatment period, cells were placed in a modified low-glucose Krebs-Henseleit buffer containing carnitine and supplemented with BSA:palmitate as a substrate for FAO. The mitochondrial stress test was performed to measure mitochondrial function utilizing FAO.

Mitochondrial Fuel Flex Test

To determine mitochondrial fuel flexibility, cells were cultured in the presence or absence of SN-38 (30 nM) at 21% or 1% O_2. Following the treatment period, cell culture media was exchanged for XF96 DMEM supplemented with 10 mM glucose, 1 mM glutamine, and 1 mM pyruvate (Agilent Technologies). Oxygen consumption rate (OCR) was measured using the plate layout shown in **Figure 2A**. Capacity and dependency for FAO, glutamine, and pyruvate were determined by injecting different inhibitors to inhibit transporters of each pathway. OCR values were used at points of equilibrium indicated in **Figure 2B**, and a rate was calculated for each inhibitor combination.[5] Example traces for FAO capacity and FAO dependency, as well as equations used for calculations for untreated cells are shown in **Figure 2B**. Inhibitors and final concentrations utilized were as follows: Etomoxir (2 µM) to inhibit CPT1 (FAO), UK 5099 (4 µM) to inhibit mitochondrial pyruvate oxidation, and BPTES (3 µM) to inhibit glutamine oxidation.



Capacity (%) = (1-[(B1-i2)/(B1-i3)]) × 100

Dependency (%) = [(B1-i1)/(B1-i3)] × 100

Figure 2. A. Plate map. **B.** Example traces, equations for % capacity and % dependency used for determining mitochondrial fuel flexibility.

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MDSC and DC Isolation and XF96 experiments

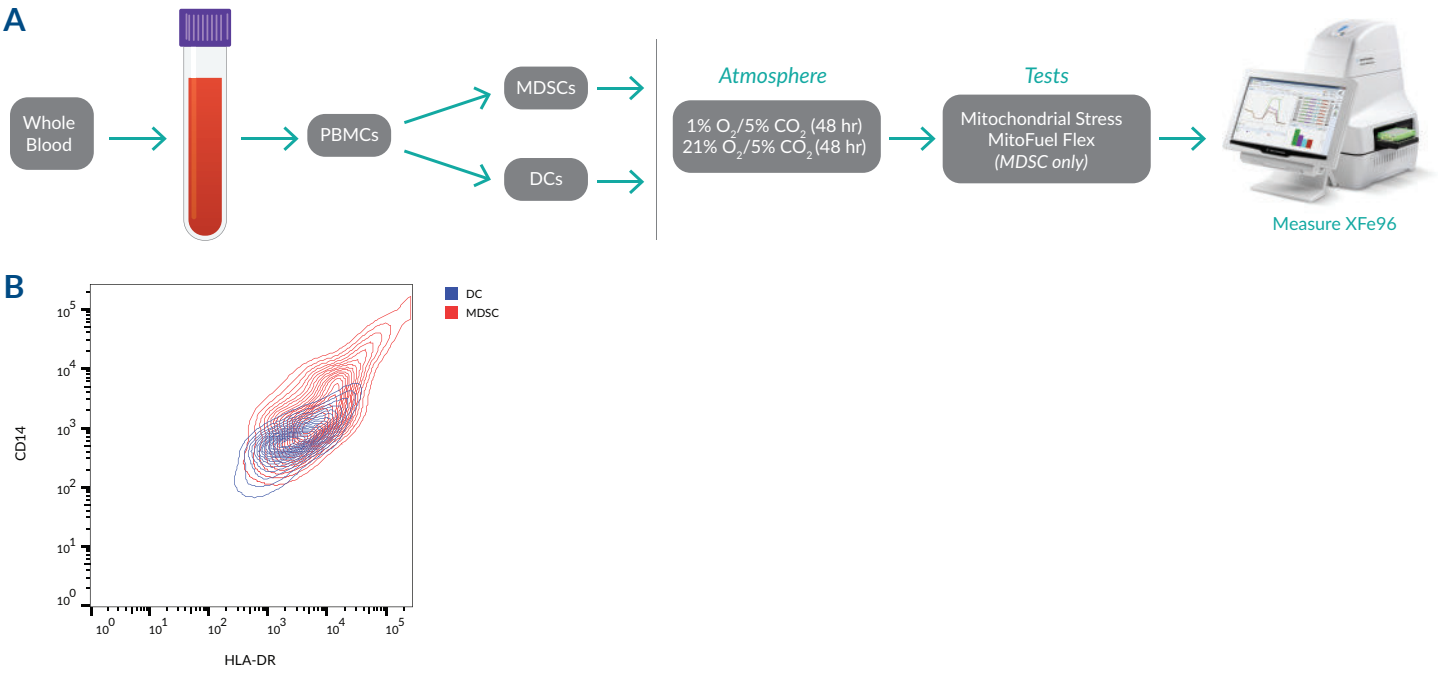


Figure 3. A. Workflow for conducting MDSC and DC experiments. **B.** Flow cytometry trace used to identify DC and MDSC subsets.

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood. Immediately following PBMC isolation, untouched monocytes were isolated from PBMCs using the Dynabeads® Untouched™ Human Monocytes Kit (Thermo). The untouched monocytes were stimulated with IL-4 and GM-CSF to facilitate differentiation into dendritic cells (DCs) or IL-4 and GM-CSF plus 1 µM prostaglandin E_2 to facilitate differentiation into MDSCs (**Figure 3A**). After 4 days, cells were placed at 1% O_2 or maintained at 21% O_2 for the remainder of the differentiation period (6 days total). Following differentiation, cells were stained for CD14 and HLA-DR to determine characteristics of the two cell types (**Figure 3B**).

The remainder of cells were plated in XF96 RPMI, pH 7.4, supplemented with 10 mM glucose, 1 mM glutamine, and 1 mM pyruvate (Agilent Technologies) at 2 x 10^5 cells/well on poly-L-lysine coated XF96 plates. Plates were centrifuged for 1 minute at 200 x g with the brake off. Cells were degassed and imaged using the Cytation™ 5 Imaging Multi-Mode Plate Reader (BioTek Instruments) before XF96 experiments were performed. The mitochondrial stress test and mitochondrial fuel flex test were carried out as previously described. At the conclusion of all experiments, OCR values were normalized to cell number using XF96 Cell Normalization Software (Agilent Technologies).

Results

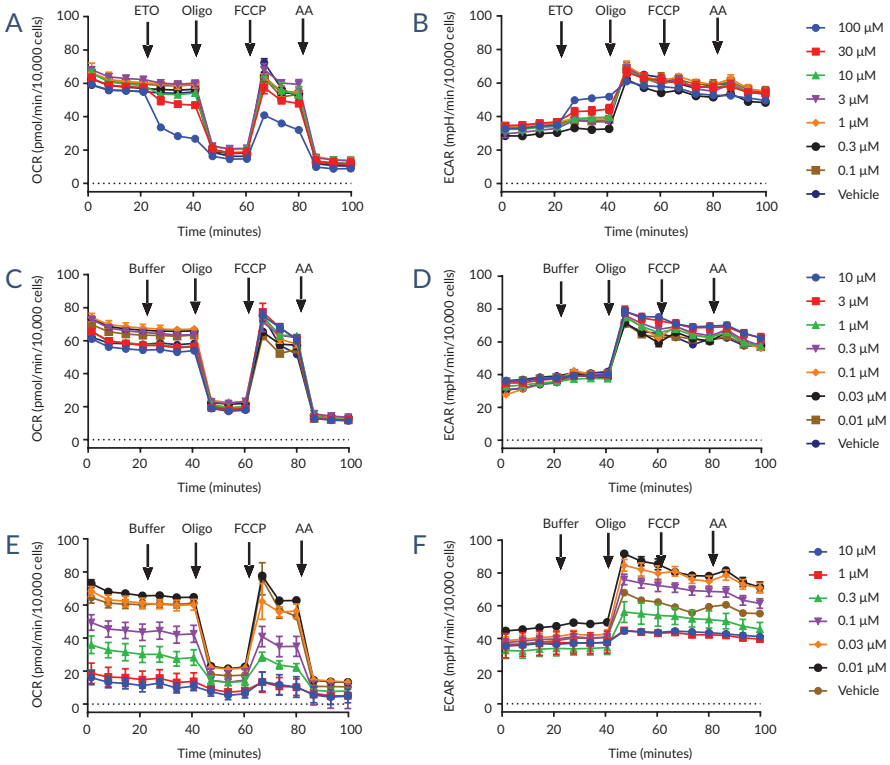
To compare the effects of chemotherapeutic agents to FAO inhibitors in tumor cells, HCT116 cells were pretreated for 12 hours with SN-38 and oxaliplatin. The CPT1 inhibitor etomoxir was added at the first injection point during experiment (**Figure 4A**). Cells were then cultured at 21% and 1% O_2. Following treatment, a mitochondrial stress test was performed in order to determine the effect of each condition on the cell's mitochondrial function.

Treatment with the CPT1 inhibitor etomoxir, at 21% O_2 inhibited OCR at concentrations of 30 µM and above (**Figure 4A**). However, since the oligomycin induced OCR remains largely unchanged compared with vehicle, inhibition is only observed when a demand is placed on the ETC (*e.g.*, ATP synthesis or uncoupling), suggesting a reduction in metabolite availability rather than a direct inhibition of the ETC. Additionally, an increase in ECAR was observed upon the addition of etomoxir (**Figure 4B**). This indicates an increase in glycolysis to meet the ATP deficit left by FAO inhibition. Treatment with SN-38 at concentrations between 0.03-1 µM slightly increased basal OCR without causing uncoupling, as evidenced by the identical OCR rates following the addition of oligomycin (**Figure 4C**). The response of glycolysis (ECAR) to the ATP synthase inhibitor oligomycin does not appear to be affected by SN-38 (**Figure 4D**), indicating the SN-38 does not inhibit glycolysis. Treatment with oxaliplatin at concentrations greater than 0.3 µM inhibited both OCR and glycolysis (**Figure 4E and F**), indicating cellular toxicity. Cells cultured at 1% O_2 exhibited an overall decrease in OCR and a slightly higher ECAR as would be expected at lower O_2 concentrations. It is worth noting that at 1% O_2, cells appeared more sensitive to etomoxir compared to those cultured at 21% (**Figure 4G and H**). Cells cultured at 1% O_2 and treated with SN-38 and oxaliplatin (**Figure 4I and J, Figure 4K and L,** respectively) behaved in a similar fashion as those cultured at 21% O_2 albeit with lower OCR rates due to the difference in O_2 concentration.

FAO Stress Test

In order to gain a better understanding of the effects of O_2 concentration, SN-38, and oxaliplatin on FAO, an FAO stress test was performed to directly measure the OCR rates resulting from FAO. Etomoxir treatment resulted in a profile similar to the standard mitochondrial stress test, showing inhibition at concentrations of 100 and 30 µM (**Figure 5A**) with ECAR following suit (**Figure 5B**). This was also observed at 1% O_2, but only with lower OCR and higher ECAR values than expected. Under normoxic conditions, SN-38 had minimal effects on OCR apart from a slight increase over vehicle with the addition of FCCP (**Figure 5C**). No differences in ECAR were immediately apparent with SN-38 treatment (**Figure 5D**). Treatment with oxaliplatin, as with the standard mitochondrial stress test, resulted in inhibition of OCR, suggesting cellular toxicity at the concentrations tested (**Figure 5E**). This effect was mirrored in ECAR (**Figure 5F**). Given the similarities between the standard and FAO stress tests, these data suggest that FAO is an important metabolic pathway for HCT116 cells at 21% O_2. Based on these observations, oxaliplatin does not affect FAO, but rather resulted in cellular toxicity. Because of this, SN-38 (30 nM) was used as the primary focus of future experiments.

21% O_2



1% O_2

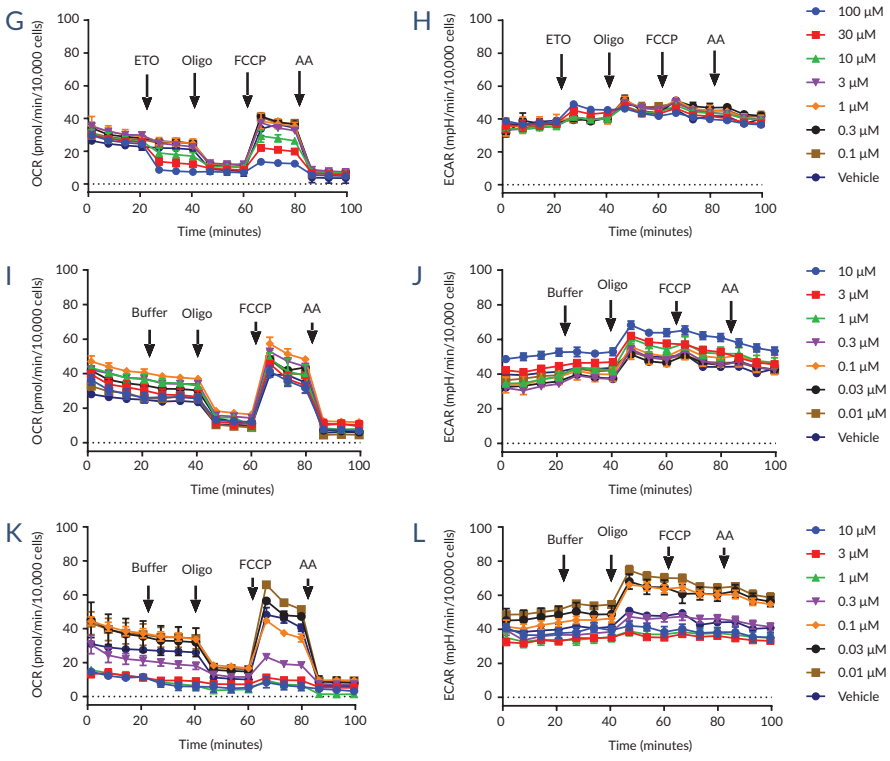
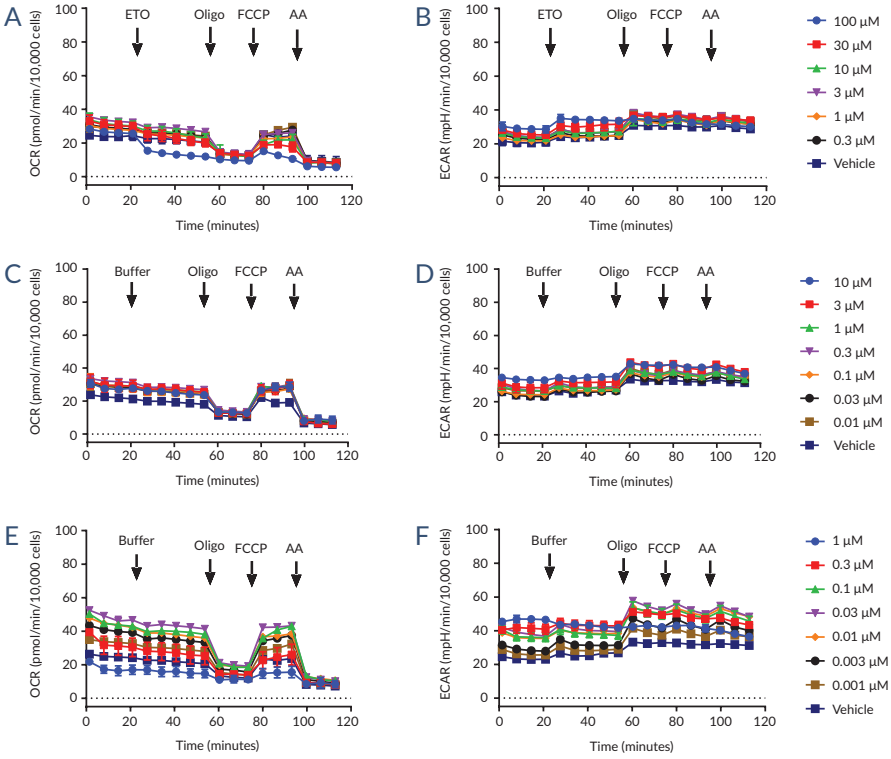


Figure 4. A-F. Respective OCR and ECAR responses of HCT116 cells cultured at 21% O_2 to acute treatment with etomoxir (**A-B**), prolonged treatment with SN-38 (**C-D**), and prolonged treatment with oxaliplatin (**E-F**). G-L. HCT116 cells cultured at 1% O_2 response to acute treatment with etomoxir (**G-H**), prolonged treatment with SN-38 (**I-J**), and prolonged treatment with oxaliplatin (**K-L**). For all experiments, final concentrations of inhibitors were as follows: Oligomycin (1 µg/ml), FCCP (1 µM), antimycin A/Hoechst (10 µM/100 µM). Data are presented at means ± standard error (n=3).

21% O_2



1% O_2

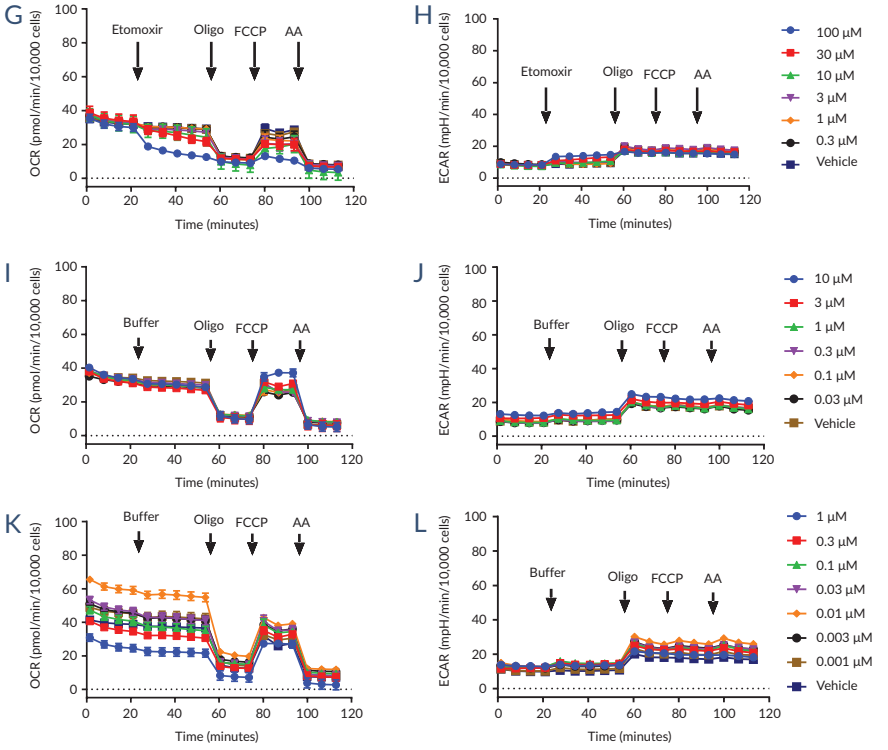
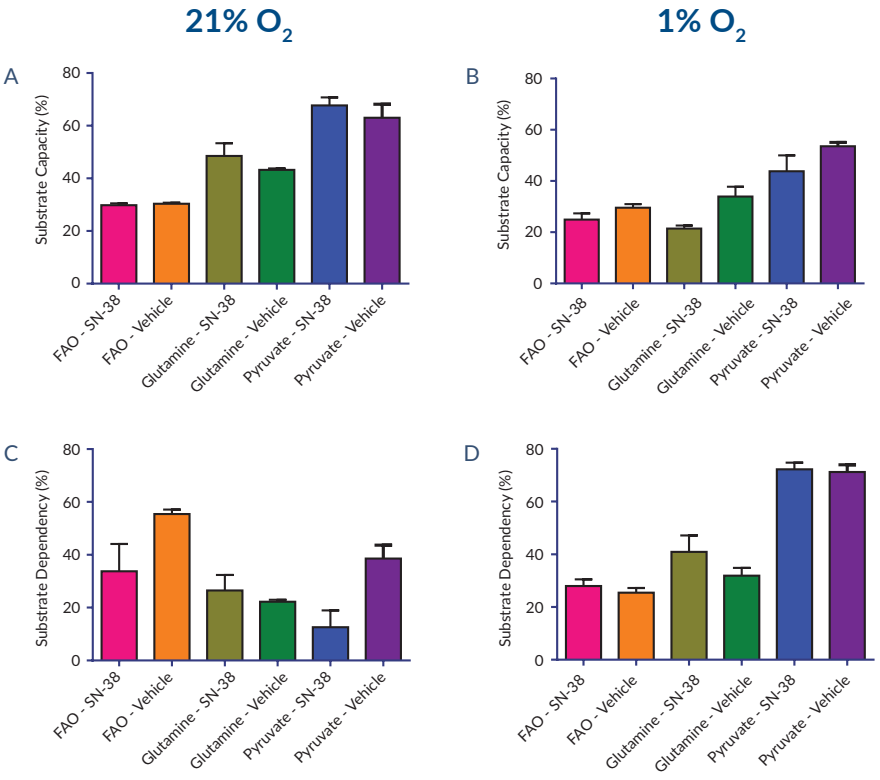


Figure 5. An FAO mitochondrial stress test was carried out at 21% and 1% O_2 as described in the methods. **A-B**, **G-H**. Etomoxir. **C-D**, **I-J**. SN-38. **E-F**, **K-L**. Oxaliplatin. For all experiments, final concentrations of inhibitors were as follows: Oligomycin (1 µg/ml), FCCP (1 µM), antimycin A/Hoechst (10 µM/100 µM). Data are presented at means ± standard error (n=3).

In order to examine this further, a mitochondrial fuel flex test was performed under each incubation condition. Performing mitochondrial fuel flex tests allows for the determination of substrate capacity and substrate dependency for pyruvate oxidation, glutamine metabolism, and FAO for each condition. When treated with SN-38 for 12 hours at 21% O_2, HCT116 cells exhibited no changes in substrate capacity (**Figure 6A**), yet decreased substrate dependency is observed for both FAO and pyruvate (**Figure 6C**). At 1% O_2, a decrease in glutamine capacity is observed with minimal change in glutamine dependency (**Figure 6B**). When 1% O_2 was compared to 21% O_2, an increase in pyruvate dependency was observed, which was expected under hypoxic conditions since upregulated glycolysis would increase pyruvate availability.

In cells acutely treated with SN-38 at 21% O_2, a decreased glutamine capacity was observed in the presence of SN-38, as well as a slight decrease in pyruvate capacity (**Figure 6E**). Additionally, a slight increase in FAO capacity in the presence of SN-38 was also observed. When looking at substrate dependency, FAO dependency was greatly decreased in the presence of SN-38 with a slight increase in pyruvate dependency (**Figure 6G**). When comparing these data to long-term treatments, an effect of SN-38 was observed on FAO dependency (**Figure 6C**). Since this effect was observed under both treatment conditions, the data suggest that SN-38 had an immediate effect, and that this was not the result of long-term changes to the cell's metabolism. As with 1% O_2 in the 12-hour SN-38 treatment, an increase in pyruvate dependency was observed (**Figure 6H**). Taken together, these data show that at 21% O_2, HCT116 cells have increased dependency on FAO. This increase in dependency is prevented by the addition of SN-38. At 1% O_2, FAO dependency is already lowered, and the addition of SN-38 had minimal effect, suggesting that at 1% O_2 this cell line can switch its metabolic phenotype to be more reliant on pyruvate. These data provide evidence that while SN-38 is capable of inhibiting FAO, HCT116 cells are less dependent on fatty acids as substrates at lower O_2 concentration.

12-hour Treatment



Acute Treatment

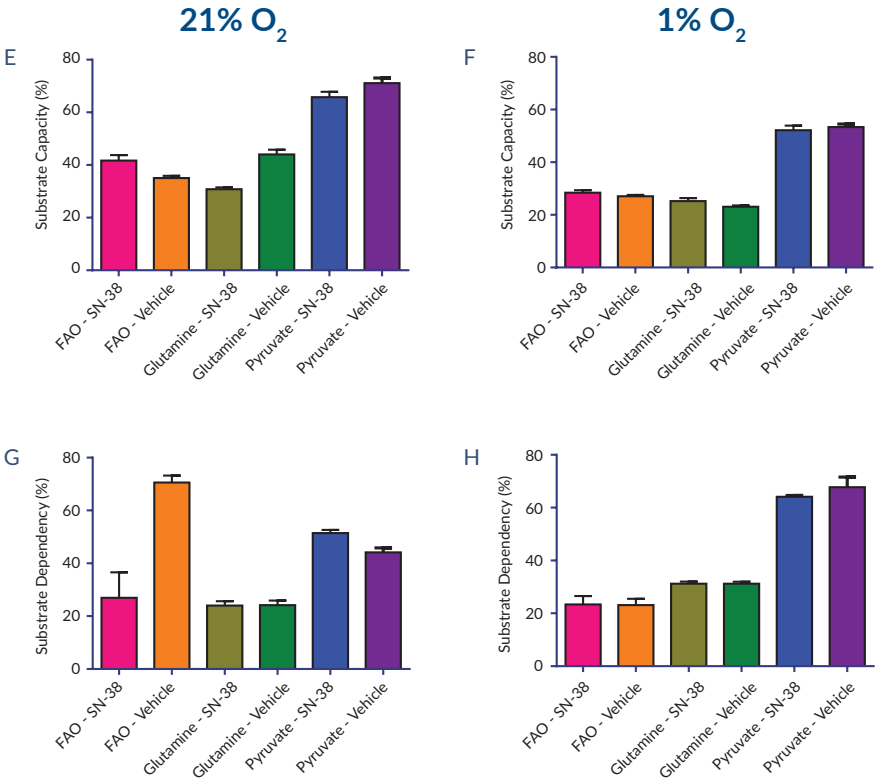


Figure 6. Substrate capacity and substrate dependency were calculated using the equations in **Figure 2B**. **A-D**. Calculated substrate capacities and dependencies for a 12-hour treatment with 30 nM SN-38 at 21% and 1% O_2. **E-H**. Calculated capacities and dependencies for an acute treatment of 30 nM SN-38 at 21% and 1% O_2. Data are presented as means ± standard error.

Since MDSCs are thought to occupy the TME, potentially suppressing T cell function, it was proposed that exposure to low O_2 concentration would affect metabolic phenotype in a similar manner as the HCT116 cells. In order to examine this, mitochondrial stress tests and mitochondrial fuel flex tests were performed on primary human MDSCs and dendritic cells (DCs) cultured at 21% and 1% O_2 for the final two days of differentiation. A mitochondrial stress test was performed on MDSCs and DCs cultured as described above (**Figure 7**). OCR values, normalized to cell number, show DCs differentiated at 21% O_2 were expectedly more aerobic than those cultured at 1% O_2, possessing a higher basal OCR (**Figure 7A**). MDSCs behave in a similar manner, with those differentiated at 21% O_2 having a higher basal OCR compared to those differentiated under 1% O_2. ECAR values for DCs are consistent with OCR values in that cells differentiated under 1% O_2 have higher ECAR values, showing higher rates of glycolysis (**Figure 7B**). Interestingly, MDSCs do not appear to follow this pattern. Upon normalization to baseline, it becomes apparent that MDSCs, regardless of O_2 concentration, have an increased response to uncoupler (BAM15) when compared to DCs, indicating a different metabolic phenotype (**Figure 7C**). To further investigate this, a mitochondrial fuel flex test was performed on MDSCs differentiated at 1% and 21% O_2. The data show that MDSCs that were differentiated at 1% O_2 had an increased dependency on FAO, suggesting that lower O_2 concentration has the potential to alter the metabolic phenotype of MDSCs.

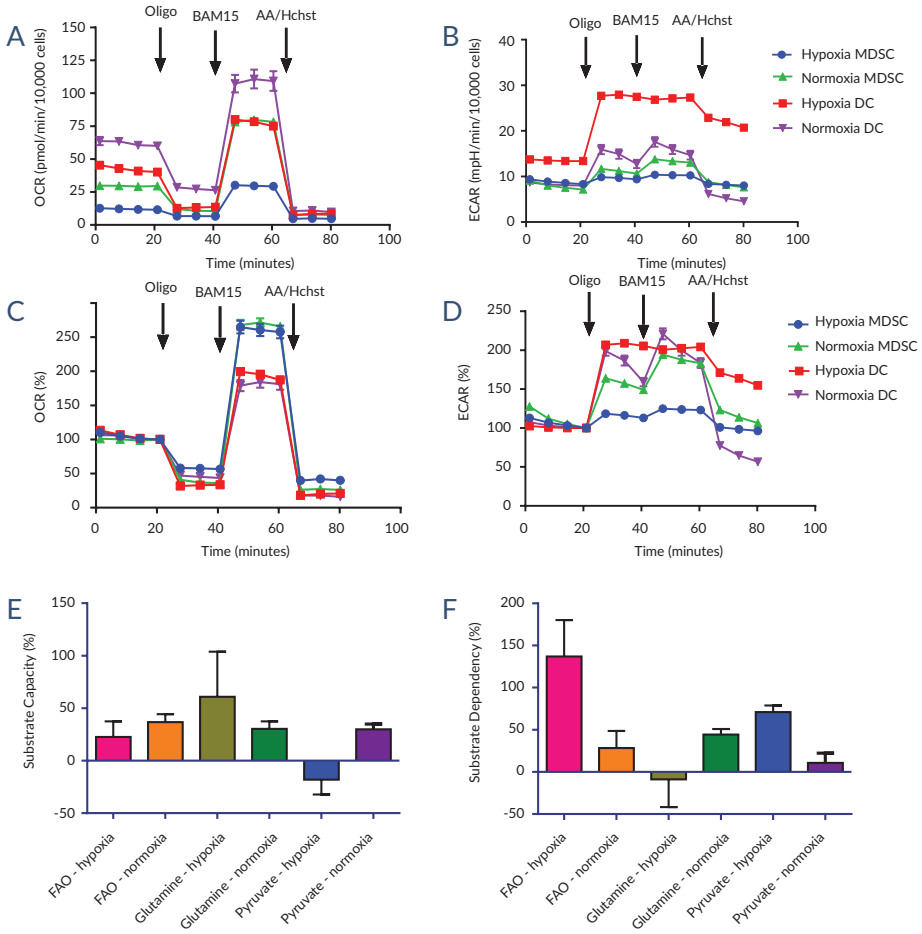


Figure 7. A-B. MDSCs and DCs that were differentiated at 21% and 1% O_2 were subjected to a mitochondrial stress test. **C-D.** Data were normalized to baseline to illustrate % change in OCR independently of cell number. **E-F.** MDSCs differentiated at 21% and 1% O_2 were subjected to a mitochondrial fuel flex test.

Conclusions

While minimal changes were observed when looking at the effects of O_2 concentration on mitochondrial function and FAO in the presence of SN-38 and oxaliplatin, a more drastic change was observed when looking at the metabolic phenotype of HCT116 cells and MDSCs. The mitochondrial fuel flexibility data show that MDSCs and HCT116 cells exhibit different substrate dependencies at 1% O_2 compared to 21% O_2. Under ambient oxygen concentrations, HCT116 cells were shown to be more dependent on FAO. This dependency can be diminished through treatment with SN-38, or by culturing cells at 1% O_2. In contrast to this, MDSCs cultured at 21% O_2 appeared to have increased dependency on glutamine. However, at 1% O_2, a substrate dependency shift in favor of FAO was observed. Taken together these data indicate that culture conditions such as O_2 concentration can have a significant impact on metabolic substrate utilization.

References

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