Chemotherapeutic drug screening in 3D-Bioengineered human myobundles provides insight into taxane-induced myotoxicities

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Highlights
Taxane chemotherapies are linked to chronic myotoxicities and metabolic risks
Potential mechanisms were investigated using 3D-bioengineered human myobundles
Taxane treatment disrupted microtubule acetylation and cytoskeletal architecture
Taxane treatment disrupted glucose metabolism and mitochondrial bioenergetics

Torres et al., iScience 25, 105189
October 21, 2022 © 2022
https://doi.org/10.1016/j.isci.2022.105189
Chemotherapeutic drug screening in 3D-Bioengineered human myobundles provides insight into taxane-induced myotoxicities

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SUMMARY

Two prominent frontline breast cancer (BC) chemotherapies commonly used in combination, doxorubicin (DOX) and docetaxel (TAX), are associated with long-lasting cardiometabolic and musculoskeletal side effects. Whereas DOX has been linked to mitochondrial dysfunction, mechanisms underlying TAX-induced myotoxicities remain uncertain. Here, the metabolic and functional consequences of TAX ± DOX were investigated using a 3D-bioengineered model of adult human muscle and a drug dosing regimen designed to resemble in vivo pharmacokinetics. DOX potently reduced mitochondrial respiratory capacity, 3D-myobundle size, and contractile force, whereas TAX-induced acetylation and remodeling of the microtubule network led to perturbations in glucose uptake, mitochondrial respiratory sensitivity, and kinetics of fatigue, without compromising tetanic force generation. These findings suggest TAX-induced remodeling of the microtubule network disrupts glucose transport and respiratory control in skeletal muscle and thereby have important clinical implications related to the cardiometabolic health and quality of life of BC patients and survivors.

INTRODUCTION

Breast cancer (BC) is the most common cancer diagnosis among women in the United States, with 85% of cases having no family history. One in eight women will confront the disease in their lifetime and over 300,000 new cases are diagnosed each year (AmCancSoc, 2020). More encouraging, however, are epidemiological data indicating that 10-year disease-free survival rates have increased to 85–90%, owing in large part to advances in BC screening, cancer treatments, and chemotherapeutics. For women with node-positive BC, and in a subset of women with hormone-positive, node-negative disease and a high risk of recurrence, adjuvant or neoadjuvant chemotherapies commonly include a taxane (TAX) (e.g., docetaxel, paclitaxel), which is often prescribed in combination with Adriamycin (doxorubicin, DOX) (Waks and Winer, 2019). Whereas these drugs can be lifesaving, the potent antineoplastic properties of anthracycline and taxane drugs are associated with long-lasting cardiovascular and neuromuscular side effects that not only reduce the quality of life, but also raise all-cause mortality rates (Kirkham et al., 2019; Simon et al., 2018). The staggering high incidence of the disease coupled with increasing survival rates have together led to a fast-expanding population of long-term BC survivors who are at an elevated risk of cardiometabolic disorders owing to prior exposure to noxious chemotherapeutic agents (AmCancSoc, 2020).

The cardiotoxic side effects of DOX have been investigated for decades (Singal and Iliskovic, 1998). Clinical use of this drug is dose-restricted across the lifespan owing to cumulative, progressive actions that compromise left ventricular function, leading to long-term and sometimes irreversible cardiac remodeling and heart failure (Tan et al., 2015). By contrast, TAX-based chemotoxicities, which are relatively understudied, have been associated with fatigue (Berger et al., 2009), arthralgia and myalgia (Chiu et al., 2017), and peripheral neuropathy (Rivera et al., 2018). Moreover, a number of reports have linked TAX therapy delivered to early-stage BC patients (with no pre-existing conditions) to impaired glucose homeostasis and other components of the metabolic syndrome, not only during but also long after cancer treatments were completed (Dieli-Conwright et al., 2016; Fredslund et al., 2019; Thomson et al., 2009). Adding to the health concerns of this patient population, chemotherapy exposure has also been associated with clinically relevant decrements in cardiorespiratory fitness (Caslia et al., 2015; Jones et al., 2012; Peel et al., 2014;
Simon et al., 2018; Zagar et al., 2016). For example, BC survivors of 40–50 years of age present mean VO2max values that are 15–27% lower than healthy, sedentary, age-matched controls with no history of BC (Casla et al., 2015; Jones et al., 2012; Peel et al., 2014; Yu et al., 2020). Notably, this decline in physical fitness is equivalent to two decades of healthy aging. Indeed, cardiovascular disease has become the most important competing cause of death in BC survivors (Bradshaw et al., 2016; Kirkham et al., 2019).

The foregoing health statistics underscore an urgent need for studies that investigate the molecular mechanisms underlying TAX-associated toxicities, not only in rapidly dividing cancer cells but also in terminally differentiated off-target cells. Taxanes oppose tumor growth by disrupting the normal turnover of cellular cytoskeletal structures known as microtubules (MT), which are comprised of polymerized heterodimers of the proteins α- and β-tubulin (Alushin et al., 2014; McGrogan et al., 2008). In addition to playing a critical role in cell proliferation, MT dynamics and lysine acetylation of α-tubulin (AcK40) have been implicated as important regulators of glucose transport and mitochondrial function, raising the possibility that chemotherapeutic agents impose a two-hit threat to cellular energy homeostasis. Importantly, however, the metabolic impact of TAX on tissues with high-energy demands, such as skeletal muscle and heart, remains poorly studied and understood.

Given the current knowledge gaps and the limitations of conventional two-dimensional skeletal muscle cultures, we sought to elucidate the molecular, metabolic, and functional consequences of TAX exposure by leveraging a new three-dimensional, bioengineered human skeletal muscle system (3D-myobundle). Generated from primary human skeletal muscle progenitor cells, these 3D-myobundles successfully recapitulate structural hallmarks of native adult muscle (Madden et al., 2015); including multinucleated and striated myofibers, an aligned architecture, and contractile activity in response to electrical/chemical stimuli (Davis et al., 2017; Truskey et al., 2013). For the current study, TAX was administered to 3D-myobundles with and without DOX, using a dosing regimen designed to resemble clinical pharmacokinetics. The resulting impact of these drugs on markers of cytoskeleton dynamics, glucose metabolism, mitochondrial function, and myobundle force generation shed light on the mechanisms by which TAX-based chemotherapies disrupt skeletal muscle bioenergetics and raise the risk of adverse cardiometabolic health outcomes.

**RESULTS**

**TAX increases α-tubulin acetylation and disrupts glycolytic and oxidative metabolism in human primary myotubes**

Considering the widely reported neuromuscular side effects of TAX, we sought to determine whether exposure to the drug would alter MT dynamics and/or cellular metabolism in fully differentiated, non-dividing myocytes. To this end, primary human skeletal muscle progenitor cells were expanded in 2D-culture and terminally differentiated into multinucleated skeletal myotubes. Following 4 days of differentiation, myotubes were exposed to vehicle or TAX at doses of 0.1, 1, 10, or 100 nM for 3 h per day, over a period of three consecutive days. This regimen was designed to mimic chemotherapy infusions received by BC patients in the clinic. The highest dose (100 nM) approximates peak concentrations in the blood during each treatment (Brunsvig et al., 2007).

In rapidly dividing cells, TAX exposure leads to robust lysine acetylation of α-tubulin, the fundamental protein unit of MTs. This prominent post-translational protein modification (PTM) is closely linked to MT stabilization (Roll-Mecak, 2015). In the current study, total α-tubulin levels in human skeletal myotubes trended higher upon TAX exposure, reaching significance (p = 0.05) at the highest dose (Figure 1A); whereas acetylated α-tubulin at lysine residue 40 (AcK40) increased markedly in a dose-dependent manner (up to 5.5-fold increase with TAX 100 nM (p <0.0005) (Figure 1B). The ratio of AcK40α-tubulin/α-tubulin was also elevated after TAX exposure (Figure 1C), whereas expression levels of γ-tubulin remained unaltered (Figure 1D). Notably, the drug-induced changes in acetylated α-tubulin were still evident one week after the last treatment cycle (Figure 1E), reflecting persistent perturbations in MT dynamics in these terminally differentiated myotubes.

Lactate efflux rates decreased upon TAX exposure in a dose- and time-dependent manner, reaching a 25% drop (p < 0.05) 24 h after the third cycle (shift day S7) with TAX doses of 10 and 100 nM (Figure 1F). A similar decline in lactate efflux was also evident when 10-nM TAX was administered in combination with 10-nM DOX (Figures S1A and S1B). TAX-induced reductions in lactate efflux and 3H-2-Deoxy-glucose uptake...
Figure 1. Nanomolar concentrations of TAX alter α-tubulin acetylation, glucose homeostasis, and cellular respiration in human primary myotubes

(A–D) Western blot analysis and representative blots of total α-tubulin (A), acetylated K40 α-tubulin (B), the calculated ratio (C), and γ-tubulin (D) were performed using lysates of primary myotubes exposed to a regimen of 3-h treatments with TAX concentrations of 0, 0.1, 1, 10, or 100 nM, for three consecutive days. Each 24-h period is considered as one cycle.

(E) Representative blots showing TAX-induced acetylation of α-tubulin persists up to 7 days post-treatment.

(F) Lactate efflux rates calculated from sampled media collected from well (each containing four bundles) after each 24-h TAX cycle.

(G) Time course of oxygen consumption rates, OCR (top), and extracellular acidification rate, ECAR (bottom), during a mitochondrial stress test in primary myotubes 24 h after treatment.

(H and I) (H) Basal mitochondrial OCR (basal OCR – OCR Rotenone/Antimycin A) and (I) maximal respiratory capacity (OCR FCCP – OCR Rotenone/Antimycin A) are expressed as the % relative to CTL owing to the variability between cell batches.
Figure 1. Continued

(Figure S1A) occurred without changes in protein abundance of the GLUT1 glucose transporter (Figure S1F), whereas TAX + DOX (but not DOX alone) caused a slight decrease in GLUT1 expression. These results suggest potential disruption of glucose metabolism, specifically upon exposure to TAX.

Lastly, a mitochondrial stress test using the Seahorse Extracellular Flux Analyzer (Figure 1G) revealed that basal oxygen consumption rates (OCR) were lower in myotubes treated with TAX doses as low as 1 nM (Figure 1H, p < 0.05). Higher doses of TAX (100 nM; Figures 1I and 1J) led to diminished maximal and reserve respiratory capacity, whereas OCR linked to ATP synthesis (Figure 1K) was reduced at TAX concentrations ≥1 nM (p < 0.0005). In addition, proton leak was 40 and 46% higher with 1- and 100-nM TAX, respectively (Figure 1L), potentially reflecting a reduction in coupling efficiency (Figure 1M). Overall, the results show that TAX-induced alterations in tubulin acetylation were accompanied by perturbations in mitochondrial function.

Toxicity studies using 3D-myobundles and a drug regimen that mimics in vivo pharmacokinetics

We next sought to examine the adverse effects of these drugs in 3D bioengineered human muscles. Generation of 3D-myobundles from human primary skeletal muscle progenitor cells was performed according to the workflow detailed in Figure 2A, as previously described (Davis et al., 2017; Madden et al., 2015). Briefly, myoblasts were expanded and seeded (300,000 cells per bundle) for hydrogel molding, compacted to the workflow detailed in Figure 2A, as previously described (Davis et al., 2017; Madden et al., 2015). Mature 3D-myobundles were thus exposed to either one of four treatment regimens: control (CTL), TAX alone, TAX + DOX, or DOX alone. In patients, TAX and DOX infusions reach peak plasma concentrations of 1 μM 3 h post-infusion, decreasing to 10 nM for TAX (Brunsvig et al., 2007) and 100 nM for DOX (Barpe et al., 2010) after 24 h. To closely mimic the pharmacokinetics observed in the plasma of patients in the 24 h following a chemotherapy infusion, mature myobundles were treated with daily cycles consisting of a 3-h exposure to 1 μM TAX, followed by 21 h exposure to 10 nM TAX. This cycle was applied for three consecutive days (S6–S8). For the groups also receiving DOX, the dose was (3 h at 0.5 μM + 21 h at 50 nM), administered one time only, on S7. It is noteworthy that a pilot study to optimize drug exposure revealed that higher doses of DOX resulted in dramatic reductions in myobundle expression levels of key contractile and structural proteins (myosin heavy chain (MHC) and γ-tubulin, Figures S3A and S3B), along with a significant decline in cross-sectional area (data not shown). For this reason, the DOX dose selected for peak treatment was slightly below the systemic exposure reported in patients (0.5 μM instead of 1 μM), although still consistent with the reported 1/10 decline in plasma exposure after 24 h (0.5 μM × 3 h + 50 nM × 21 h, compared with pharmacokinetic reports of 1 μM at 3 h to 100 nM at 24 h (Barpe et al., 2010)).
Figure 2. Experimental workflow of 3D-myobundle generation and treatment with pharmacokinetic-mimicking regimens

(A) Study design overview. Human myogenic cells isolated from biopsied muscle samples are expanded in 2D-culture (myoblasts), and utilized to generate 3D-myobundles using a hydrogel-molding technique (300,000 cells per myobundle) in PDMS molds as previously described (Madden et al., 2015). Shift days S6–9 refer to the number of days after switching from growth media to differentiation media. Once compacted, myobundles anchored to the frames are removed for free-floating culture and drug treatments. Following 5 days of differentiation, 3D-myobundles were exposed to either one of four treatment regimens, CTL, TAX alone, TAX + DOX, or DOX alone. The regimens consisted of three cycles with a 3 h peak-dose, followed by a 21 h low-dose treatment.
Cytotoxicity assessed by adenylate kinase (AK) leak in media 24 h after treatments (at S9). Data are means ± SEM. 

Immuno-staining of 3D-myobundles: (1) fibroblasts expressing vimentin are shown in the periphery of the bundle in green; (2) transverse myobundle cross-section showing vimentin from fibroblasts (in green), dense uniformly distributed, sarcomeric alpha-actinin (in red), and nuclei (in blue). Scale bars represent 100 μm; (3) F-actin (in green); and (4) sarcomeric α-actinin (in red) contractile myofibers within myobundle. Scale bars represent 25 μm.

Immuno-staining of 3D-myobundles at S9, after treatments depicted in panel A. For each group, transverse myobundle cross-sections are show on the right, with dense uniformly distributed, sarcomeric α-actinin (in red), and nuclei (in blue). Scale bars represent 100 μm.

Cytotoxicity assessed by adenylate kinase (AK) leak in media 24 h after treatments (at S9). Data are means ± SEM, n = 9 per group, where each data point is a homogenate of two myobundles. *p < 0.05 vs CTL from one-way ANOVA analysis followed by Dunnett’s test. Experiments were performed using myoblasts derived from two different donors, as detailed in the STAR Methods. See also Figures S2 and S3.

**TAX treatment induces acetylation and cellular remodeling of α-tubulin**

Similar to the foregoing observations in primary myotubes (Figures 1A–1C), exposure of 3D myobundles to TAX (± DOX) led to robust increases (p < 0.0005) in total α-tubulin, AcK40α-tubulin, and the AcK40α-tubulin/α-tubulin ratio (Figures 3A–3C), reflecting marked stabilization of MTs. Confocal imaging of total α-tubulin and AcK40α-tubulin (Figure 3D) showed that exposure to TAX caused dramatic re-organization of the MT network, with AcK40α-tubulin shifting from a diffuse cellular localization pattern to a peri-nuclear co-localization. DOX alone appeared to lower the signal intensities of both total and AcK40α-tubulin (Figure 3D, bottom); however, these changes were not detected by western blot analyses using myobundles pooled from three independent experiments (Figures 3A–3C). The cellular distribution of α-tubulin after treatment with TAX + DOX was comparable to TAX alone. Treatment with TAX alone led to a 47% increase in expression levels of MHC (Figure 3E), whereas the structural protein, γ-tubulin, and mitochondria marker, VDAC, were unaffected (Figure 3F). At the lower, less toxic doses used in these experiments, DOX did not affect myobundle levels of VDAC or the structural and contractile proteins (Figures 3E and 3F).

**TAX disrupts glucose metabolism in 3D-myobundles**

At day 9 of differentiation (S9), 24 h after completion of the last treatment cycle with TAX, glucose consumption rates decreased to approximately half (p < 0.05) the value measured in the CTL (Figure 4A). Likewise, TAX exposure decreased lactate efflux by 40% (p < 0.005) at S9, regardless of the presence of DOX (Figure 4B). Lactate efflux correlated (R = 0.71) with glucose uptake (Figure 4C) and approximated (slope = 1.73 ± 0.2, the predicted 2:1 ratio of lactate produced relative to glucose consumed. TAX exposure caused a downward/leftward shift in this plot, consistent with a limitation at the level of glucose transport. GLUT1 expression decreased (−33%, p < 0.05) when TAX was administered alone, remained lower (−20%) although not statistically significant vs CTL with TAX + DOX, and was unaffected with DOX alone treatment (Figure 4D). Thus, in aggregate, TAX but not DOX led to overt changes in myobundle glucose metabolism.

To further evaluate the impact of TAX on fuel metabolism, mass spectrometry-based metabolite profiling was performed on a separate set of bundles treated ± TAX. These assays revealed a TAX-induced decline in myobundle content of acetyl-carnitine (a commonly used proxy of acetyl CoA) of −28% (Figure 4E), along with lower levels of citrate and succinate, two prominent intermediates of the tricarboxylic acid (TCA) cycle (Figure 4F). Western blot analysis of the enzyme that catalyzes the conversion of pyruvate to acetyl CoA, pyruvate dehydrogenase (PDH), showed that both total and phosphorylated PDH (which inhibits enzyme activity) were unaffected by TAX (Figure 4G). By contrast, several even chain acyl-carnitines, which are derived as byproducts of incomplete fatty acid oxidation, were elevated after TAX exposure (Table S1). Levels of most amino acids were unaffected by TAX treatment, with the exception of a decrease in proline (Table S1). Collectively, the metabolite profile of 3D-myobundles is suggestive of TAX-induced limitations in glucose uptake and glycolytic flux along with a potential shift toward increased dependence on fatty acid catabolism, all of which were accompanied by reductions in the mitochondrial pools of acetyl units and some TCA cycle intermediates.

**TAX and DOX cause distinct perturbations in respiratory function**

Before examining the impact of these drugs on myobundle respiratory function, a protocol for myobundle permeabilization with digitonin (0.3 mg/mL) was optimized to ensure the preservation of both mitochondrial integrity and maximal JC-1 responses measured with the Oroboros high-resolution respirometry O2K system (data not shown). Respiratory function in permeabilized 3D-myobundles (PMB) was assessed with pyruvate plus malate (P/M), followed by the addition of saturating ADP levels and then succinate. The combinations of P/M and P/M + succinate were selected to elicit carbon and electron fluxes through pyruvate...
dehydrogenase and Complex I of the electron transport chain (ETC), as well as succinate dehydrogenase (CII), respectively. Cytochrome c (CytC) was added at the end of the assay to assess mitochondrial membrane integrity after cell membrane permeabilization. The respiratory profile of PMB exposed to TAX + DOX was surprisingly unremarkable. By contrast, maximal respiratory capacity (JO2) of PMBs treated with DOX alone was decreased by 25% (p < 0.05) in the context of P/M (C I-linked substrates), and 30% (p < 0.005) upon the addition of succinate (C I + II-linked substrates) (Figure 5A). In the CTL and TAX + DOX groups, the addition of CytC elicited a modest (10%) increase in respiration; whereas the respiratory response to CytC was 2.5-fold greater in PMBs exposed to DOX alone (Figure 5B), suggesting a higher degree of DOX-induced mitochondrial toxicity when TAX was absent. Of note, expression levels of voltage-dependent anion channel (VDAC), a marker of mitochondrial content, were not significantly affected by the drug treatments (Figure 3G), at least early upon exposure.

In a parallel set of experiments, mitochondrial respiratory sensitivity was measured in PMBs subjected to an ADP titration. Results of this test (Figure 5C) corroborated the foregoing finding that maximal JO2 was
preserved with TAX while also revealing a biphasic kinetic response to increasing ADP concentrations (a rise in metabolic demand). Inverse plots of Figure 5C, which show in Figures 5D–5G, were used to determine mitochondrial sensitivity to ADP (i.e. $K_{M_{ADP}}$). In CTL PMBs, the $K_{M_{ADP}}$ was 145 $\mu$M, which closely matches values previously reported for human permeabilized muscle fibers in the presence of creatine (Perry et al., 2011). Treatment with TAX alone resulted in a two-phase linearity plot (Figure 5E); the first component had a $K_{M_{ADP}}$ of 120 $\mu$M (comparable with CTL), whereas the second component exhibited a $K_{M_{ADP}}$ of 390 $\mu$M (2.5-fold greater than CTL). Treatment with DOX alone lowered the $K_{M_{ADP}}$ to 80 $\mu$M (Figure 5G). The abundance of protein subunits belonging to mitochondrial OXPHOS complexes remained largely unchanged in response to either TAX or DOX treatment alone, whereas TAX + DOX tended to lower a marker of complex I (−27%, $p < 0.05$) (Figures 5H and 5I). Together, these findings imply that the two drugs impose distinct effects on cellular bioenergetics.

**TAX exposure diminishes respiratory sensitivity tested under physiological energy demands**

Conventional respirometry approaches provide crude information pertaining to mitochondrial quality and remodeling because these assays are typically performed in the context of physiologically irrelevant thermodynamic conditions in which extramitochondrial ADP concentrations far exceed ATP levels. To achieve more physiologically relevant thermodynamic constraints, we used the creatine kinase (CK) energetic clamp technique (Fisher-Wellman et al., 2018; Glancy et al., 2008). In this assay, a large excess of CK added together with defined concentrations of phosphocreatine (PCr), Cr, and ATP, provide excess capacitance to maintain the ATP/ADP ratio in a range that better approximates energetic demands in vivo. Each

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**Figure 4. TAX disrupts glucose homeostasis in 3D-myofibers**

(A and B) Glucose consumption determined as 2-DOG uptake (A) and lactate efflux rates (B) were determined from media sampled every 24 h following each treatment cycle.

(C) Linear relationship between glucose consumption and lactate efflux rates is shown at S9 after treatments. $N = 5$–6 per group, where each data point represents a sampled well containing four myofibers.

(D) Western blot analysis of GLUT1 expression. Data are means ± SEM, $n = 8$ per group, where each data point is a homogenate of two myofibers. $^*p < 0.05$, $^{**}p < 0.005$ vs CTL from one-way/two-way ANOVA analysis followed by Dunnett’s test.

(E and F) Mass spectrometry-based metabolite profiling of myofibers at S9 after respective treatments, showing levels of acetyl-carnitine (E), and TCA cycle intermediates (F).

(G) Western blot analysis of total and phosphorylated pyruvate dehydrogenase (PDH), expressed as the ratio of $p$-PDH/total PDH. $N = 4$ per group, where each data point being homogenates of two myofibers. $^*p < 0.05$ vs CTL from one-way ANOVA followed by Dunnett’s test for all panels, or $t$-test (F, G).

Experiments A–D, G versus E–F were performed using myofibers derived from two different donors, as detailed in the STAR Methods. See also Table S1.
Figure 5. TAX and DOX disrupt mitochondrial function in 3D-myobundles via distinct mechanisms

(A) Oxygen consumption (JO₂) determined via OroborO2K respirometry after the addition of 10-mM pyruvate, 2.5-mM malate, sequential additions of ADP (50 μM–4 mM), 10-mM succinate, and 20 μM cytochrome c.

(B) Respiratory response (% JO₂) following cytochrome c addition.

(H) Fold change vs CTL

(J) Oxygen consumption (JO₂) determined via OroborO2K respirometry after the addition of 10-mM pyruvate, 2.5-mM malate, sequential additions of ADP (50 μM–4 mM), 10-mM succinate, and 20 μM cytochrome c.

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(B) Respiratory response (% JO₂) following cytochrome c addition.
To test the latter possibility we employed an in vitro approach wherein isolated mouse skeletal muscle mitochondria were incubated with free tubulin at 37°C. Some degree of polymerization. In the current study, we found that tubulin added to the O2K chamber at a concentration of 0.05 mg/mL will not polymerize at 37°C. However, polymerized MTs, rather than free tubulin, might bind to and disrupt VDAC-mediated transport of adenylates.

Whereas the current results suggest stabilized MT might impact mitochondrial bioenergetics, previous reports have suggested that free tubulin (rather than its polymerized form) interacts with and inhibits mitochondrial VDAC (Gurnev et al., 2011; Noskov et al., 2013), altering respiratory control in striated muscle (Guzun et al., 2012; Rostovtseva et al., 2008). To test the latter possibility we employed an in vitro approach wherein isolated mouse skeletal muscle mitochondria were incubated with free tubulin at 37°C in a 1:1 tubulin:mitochondria stoichiometric ratio, which far exceeds physiologically-relevant conditions. The polymerization of tubulin was observed under conditions that simulate physiological conditions, exhibiting a 12% decrease in respiratory conductance (i.e. sensitivity to a change in demand). TAX treatment compromised mitochondrial respiratory control when evaluated under physiologically relevant thermodynamic conditions, exhibiting a 12% decrease in respiratory conductance (i.e. sensitivity to a change in demand).

The slope of the relationship between JO2 and ΔGATP provides a measure of overall respiratory conductance (i.e. sensitivity to a change in demand). TAX treatment compromised mitochondrial respiratory control when evaluated under physiologically relevant thermodynamic conditions, exhibiting a 12% decrease in respiratory conductance (p < 0.005), likely attributable to a decrease in ADP sensitivity (Figures 5D–5G). The phenotype of preserved maximal respiratory capacity but diminished sensitivity upon TAX exposure implies increased resistance at Complex V and/or the adenine nucleotide transporter.

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DOX decreases tetanic force generation whereas TAX alters fatigue kinetics in 3D-myobundles

The functional impact of TAX ± DOX treatments was determined via measurement of 3D-myobundle tetanic force generation upon electrical stimulation. Notably, the myobundle size was not changed by TAX alone, whereas DOX alone or in combination with TAX caused a 22–24% decrease (p < 0.05) in the myobundle diameter and the calculated cross-sectional area (Figure 6A). Force-time plots and peak force-normalized force-time plots are shown in Figures 6B and 6C. The total force produced during the experiment (presented as the tension time integral, TTI in Figure 6D) was unaffected by TAX and trended lower with DOX (p = 0.06). Maximal peak force (Figure 6F) was reduced by DOX + TAX (~30%, p < 0.05) and DOX alone (~36%, p < 0.05). This effect appeared to be related to the DOX-induced decrease in the myobundle size (Figure 6A), as differences between groups were diminished when force was normalized to cross-sectional area (Figure 6G). The combination of TAX + DOX increased the time to reach peak force (Figure 6H) by 63% (p < 0.005); and surprisingly, the rate of fatigue, assessed by the T1/2 fatigue (time to reach half the peak force), was increased by TAX but not DOX (Figure 6I). Furthermore, the slope of the first 30-s post-maximal force in the peak force-normalized plot (Figure 6J) was lower in the TAX groups but unaffected by DOX. In the context of the specific protocol tested, these results suggest DOX-mediated myotoxicity is associated with mitochondrial dysfunction, myobundle atrophy, and corresponding decrements in tetanic force generation. By contrast, the TAX agent tended to alter the kinetics of fatigue by increasing...
the time to reach peak force and slowing the fatigability process, which might relate to altered bioenergetics and/or fuel selection.

**DISCUSSION**

Mounting clinical and epidemiological evidence linking BC survival to increased risk of chronic cardiometabolic diseases has exposed a need for new model systems that can offer insights into mechanisms by which chemotherapeutic agents compromise vital organ systems. Whereas DOX is known to cause severe cardiotoxicities (Bowles et al., 2012; Singal and Iliskovic, 1998; Tan et al., 2015), TAX-based therapies are frequently associated with neuromuscular and musculoskeletal symptoms (Berger et al., 2009; Chiu et al., 2017; Bao et al., 2016; Rivera et al., 2018). In patients with metastatic BC receiving first-line taxane

**Figure 6.** DOX but not TAX decreases tetanic force generation in 3D-myobundles

Experiments were performed 24 h after drug treatments at S9, with electrical stimulation for 50 s at 20 Hz.

(A) Myobundle cross-sectional area was calculated from the diameter measured from the top view during experiment.

(B and C) Tetanic force-time traces, and (C) force-time traces normalized to the peak tetanic force.

(D and E) Calculated tension time integral (area under the curve) from force-time plots, and (E) the respective values normalized to the myobundle cross-sectional area.

(F and G) (F) Peak tetanic force (maximal force) and (G) peak force normalized to the myobundle cross-sectional area.

(H–J) (H) Time to reach peak tetanic force. Analysis of fatigue included (I) $T_{1/2}$ fatigue calculated as delta time to reach 50% of the peak force during a continuous tetanic contraction, and (J) fatigue slope from Figure 6C in the 30 s following peak force. Data are means ± SEM, $n = 5$ CTL, and $n = 6$ bundles per treatment group, with each data point specific to individual 3D-myobundles. *$p < 0.05$, **$p < 0.005$ vs CTL from one-way ANOVA analysis followed by Dunnett’s test. Experiments were performed using myoblasts derived from a single donor, as detailed in the STAR Methods.
Chemotherapy, parameters of skeletal muscle density and quality have been shown to associate with toxicity symptoms, hospitalization, and survival rates (Shachar et al., 2017). To advance a basic understanding of TAX-induced myotoxicities, the current study employed 3D-bioengineered human myobundles. This in vitro 3D-myobundle system provides a valuable translational model for toxicological screening (Madden et al., 2015) and afforded a unique opportunity for first-time studies to examine metabolic, architectural, and functional perturbations caused by exposure to TAX in the absence and presence of DOX. The drug dosing regimen administered to the myobundles was designed to approximate pharmacokinetics observed in BC patients (Barpe et al., 2010; Brunsvig et al., 2007; de Almeida and Rosa, 2018), and to mimic cyclic treatment schedules proven to reduce 10-year risk of cancer recurrence (Early Breast Cancer Trialists’ Collaborative, 2019). Herein, we provide compelling evidence that TAX disrupts MT architecture in mature skeletal myofibers while also reducing glucose uptake, lactate efflux, and mitochondrial respiratory sensitivity (Figure 7). The current work also corroborates previous reports that DOX compromises mitochondrial respiratory capacity, and extends those findings by showing that DOX-induced toxicities are accompanied by the reduced 3D-myobundle size and contractile force. Together, these findings highlight drug-specific mechanisms by which these agents alter muscle metabolism and function.

**Figure 7. Working model of TAX-induced myotoxicity**

TAX-mediated stabilization of the microtubule network leads to marked acetylation of α-tubulin, impaired glucose uptake and metabolism, along with dysregulated ATP/ADP transport, possibly via the blockage of VDAC.

Chemotherapy, parameters of skeletal muscle density and quality have been shown to associate with toxicity symptoms, hospitalization, and survival rates (Shachar et al., 2017). To advance a basic understanding of TAX-induced myotoxicities, the current study employed 3D-bioengineered human myobundles. This in vitro 3D-myobundle system provides a valuable translational model for toxicological screening (Madden et al., 2015) and afforded a unique opportunity for first-time studies to examine metabolic, architectural, and functional perturbations caused by exposure to TAX in the absence and presence of DOX. The drug dosing regimen administered to the myobundles was designed to approximate pharmacokinetics observed in BC patients (Barpe et al., 2010; Brunsvig et al., 2007; de Almeida and Rosa, 2018), and to mimic cyclic treatment schedules proven to reduce 10-year risk of cancer recurrence (Early Breast Cancer Trialists’ Collaborative, 2019). Herein, we provide compelling evidence that TAX disrupts MT architecture in mature skeletal myofibers while also reducing glucose uptake, lactate efflux, and mitochondrial respiratory sensitivity (Figure 7). The current work also corroborates previous reports that DOX compromises mitochondrial respiratory capacity, and extends those findings by showing that DOX-induced toxicities are accompanied by the reduced 3D-myobundle size and contractile force. Together, these findings highlight drug-specific mechanisms by which these agents alter muscle metabolism and function.

**Taxanes block cell division by interfering with the normal turnover of MT filaments that play an essential role in spindle formation during mitosis.** MTs are hollow, cylindrical polymers formed of α/β-tubulin dimers. Each tubulin monomer binds and hydrolyzes GTP, a process that regulates MT assembly. When tubulin monomers are bound to taxanes, the drug allosterically mimics a GTP-bound-like state that promotes polymerization and hyper-stabilization of the MT network (Alushin et al., 2014). In humans, there are eight combinations of α-tubulin and β-tubulin isoforms that differ in their carboxyl-terminal tails (Redeker, 2010). These Ct tails exhibit isotype-specific responses to MT stabilizing agents (Parker et al., 2018), and thereby tune MT dynamics (Vemu et al., 2017). Interestingly, BC tumors have increased expression of β-tubulin isoforms I and IVb (Leandro-Garcia et al., 2010), the same isoforms that are expressed abundantly in heart and skeletal muscle tissues (Leandro-Garcia et al., 2010). Together, these observations imply that the degree of taxane-induced MT stabilization in BC cells might be similar to that occurring in off-target muscle tissues wherein disruption of MT dynamics could affect critical processes such as nutrient transport and organelle migration (Fletcher et al., 2000; Parker et al., 2019; Tepp et al., 2014).
MTs are decorated by several post-translational modifications (PTM) (Strzyz, 2016). Interestingly, however, α-tubulin acetylation of K40 is the only PTM that occurs on the luminal side of MTs (Roll-Mecak, 2015), and occurs predominately upon MT stabilization. In agreement with previous studies performed in proliferative mammalian cells (Fernandez et al., 2012; Han et al., 2016), exposure to TAX led to marked acetylation of K40-α-tubulin in both human primary skeletal myotubes and bioengineered 3D-myobundles. The MT network plays a key role in vesicle trafficking, including translocation of the GLUT1 and GLUT4 glucose transporters (Fletcher et al., 2000; Parker et al., 2019). In the present study, we found that TAX-induced acetylation and stabilization of the MT network were accompanied by diminished glucose uptake and lactate efflux, raising the possibility that TAX might disrupt GLUT1 and/or GLUT4 processing and/or motility. Moreover, the metabolite profile of the 3D-myobundles is consistent with a TAX-induced limitation in the contribution of glucose to the mitochondrial pools of acetyl-CoA and TCA cycle intermediates, and a potential shift toward increased dependence on fatty acid catabolism.

Alterations in MT acetylation have also been linked to impaired motility of organelles, including mitochondria (Godena et al., 2014). The interplay between MT dynamics and mitochondrial biology has been recognized for decades, but the functional relevance of this connection remains poorly characterized. The MT network appears to modulate mitochondrial shape and function (Anesti and Scorrano, 2006), fission-fusion (Woods et al., 2016), and biogenesis (Karbowski et al., 2001). Moreover, tubulin is presumed to interact with VDAC, which is localized on the outer mitochondrial membrane and regulates membrane permeability to ATP and ADP (Noskov et al., 2013; Rostovtseva et al., 2008). Whereas previous reports have suggested that free, non-polymerized tubulin can block VDAC and thereby modulates respiratory control and electron leak in muscle cells (Guzun et al., 2012; Ramos et al., 2019), the current investigation found no evidence that free tubulin inhibits respiration in semi-purified mitochondria isolated from mouse skeletal muscles. These observations lead us to speculate that the polymerized form of tubulin has more impact on mitochondrial bioenergetics than the free form. In agreement, two recent studies found that in vitro treatment of rodent myofibers with paclitaxel increased α-tubulin-VDAC2 interactions and altered bioenergetics via blockage of ATP/ADP transport (Kerr et al., 2015; Ramos et al., 2019). Thus, the biphasic phenomenon observed in ADP kinetics upon TAX exposure could be indicative of two distinct subpopulations of VDAC (one open and another blocked), which would result in a second, higher ΚMADP value.

The current study provides multiple lines of evidence linking TAX-induced stabilization of the MT network to a shift in mitochondrial ADP sensitivity. First, in human myotubes grown conventionally using a 2D-culture model, nanomolar concentrations of TAX led to dose-dependent decrements in respiratory performance and capacity. By contrast, myobundle exposure to TAX did not affect maximal JO2. In a similar fashion, Ramos et al. reported no changes in respiration of skeletal muscle fibers treated with paclitaxel in vitro (Ramos et al., 2019). However, myobundle exposure to TAX did induce a biphasic response to ADP, which was remarkably similar to prior reports in isolated mitochondria incubated with micromolar concentrations of tubulin (Monge et al., 2008; Rostovtseva et al., 2008). Moreover, the CK clamp assay revealed a subtle TAX-induced reduction in overall respiratory conductance in the myobundles. This points to a shift in the “force-flow” relationship (Figure 5K) between energy demand and JO2, which reflects the collective efficiency and resistance to energy transfer at various steps in oxidative metabolism (e.g. mitochondrial enzymes, the ETC, ATP synthesis and transport) (Fisher-Wellman et al., 2018; Glancy et al., 2013). The lower conductance observed in TAX-treated myobundles provides evidence of increased resistance at a site distal to the ETC, possibly reflecting compromised ATP turnover owing to limitations at the level of adenylate exchange and transport. Taken together, our findings show that 3D-myobundle exposure to three cycles of TAX affected respiratory thermodynamics without imposing kinetic limitations on electron flux.

Despite clear shifts in glucose metabolism and respiratory sensitivity, TAX exposure did not compromise tetanic force generation. Although surprising, this result agrees with another recent study showing that a single dose of TAX administered intravenously to healthy mice did not impair force production in hindlimb muscles (Chaillou et al., 2017). In the current experiments, contractile function was tested for 24 h following three consecutive days of TAX treatment. Thus, changes in muscle force generation might occur after more prolonged exposures to the drug, as typically occurs during BC treatment. However, TAX treatment did affect the myobundle time to peak force and the rate of fatigability, suggesting a potential link between altered bioenergetics and fatigue kinetics. Notably, a recent study in rodent muscle (Coleman et al., 2021) showed that MTs enriched in acetylated α-tubulin increase cytoskeletal stiffness and viscoelastic
resistance. Thus, from a mechanical standpoint, TAX-induced acetylation and polymerization of MTs in 3D-myobundles could be responsible for extending the time to peak force and slowing fatigue. By contrast, DOX treatment compromised both mitochondrial function and peak force while also eliciting myobundle atrophy and several markers of myotoxicity. These results are consistent with previous reports in rodent muscle (Gilliam et al., 2011, 2016), and numerous studies describing multiple mechanisms of DOX-mediated mitochondrial dysfunction (reviewed in (Gorini et al., 2018)). Lastly, evidence from clinical studies suggests taxanes can aggravate DOX-induced cardiotoxicity (Salvatorelli et al., 2006). Thus, prolonged disruption of both oxidative and glycolytic energy metabolism during regimens involving both drugs is likely to have a detrimental impact on heart and skeletal muscle function.

In conclusion, when the adverse systemic effects of abrasive chemotherapies are combined with those stemming from chronic anti-estrogen treatments, the potential long-term consequences on musculoskeletal and cardiometabolic wellness are substantial, concerning, and deserving of attention. The current report builds on emerging clinical evidence that TAX-based chemotherapies disrupt cytoskeletal architecture and energy metabolism in skeletal muscle, now demonstrated directly in human myotubes and 3D-bioengineered myobundles. The findings raise new questions regarding the chronic health consequences of a hyper-stabilized MT network and its broad impact on cardio-oncology rehabilitation, metabolic fitness, muscle function, and overall quality of life in BC survivors. Importantly, clinical use of taxanes extends beyond BC to tumors affecting the lung, stomach, head and neck, and prostate. This report provides an impetus for further mechanistic work in bioengineered muscle models while also offering a strong rationale for advancing this line of investigation toward clinical studies in cancer patients and survivors.

LIMITATIONS OF THE STUDY
Owing to the limited yield of cells obtained from each donor, it was necessary to use cells from four different donors to complete these studies. Most experiments were performed using cells from a single donor, which remained consistent across all but one experiment as noted in the figure legends. The cell lots used for this study were selected in a random, unbiased manner without consideration of donor characteristics (e.g. BMI, sex, age). Because the cells are obtained from discarded muscle tissue procured during surgery, we do not collect subject data on each donor.

Whereas the use of a 3D culture system moves closer to muscle tissue in vivo and permits assessment of contractile function, we recognize that this model has inherent limitations and did not reproduce several important features of the chemotherapy treatment, including the precise treatment schedule and provision of adjuvant drugs that are administered along with the chemotherapies. Moreover, although the drug treatment regimens used in the current investigation were designed to approximate drug pharmacokinetics in cancer patients, we do not know precise intramuscular concentrations of TAX and DOX in patients receiving chemotherapy as compared with those that occur in myobundles. Additionally, exposure of myobundles to an abbreviated 3-day cycle rather than the typical 3-week cycle could result in more severe cytotoxicity. Lastly, we did not assess the drug clearance kinetics in vitro as compared with in vivo. It is therefore important to consider that the clearance kinetics occurring in vitro in the context of a 3-day cycle could lead to supraphysiological levels of these agents, resulting in more severe effects on myocyte metabolism and function.

Among the major findings of this study is that TAX appears to disrupt mitochondrial respiratory control and bioenergetics. Whereas the mechanistic basis for these observations was not fully elucidated, strong circumstantial evidence implies that TAX might alter the interplay between mitochondria and MTs. To this point, it is important to consider that the state of the MTs after myobundle permeabilization was not evaluated in this study. The process of permeabilizing human myobundles might have disrupted and/or destabilized the MT network, which could have dampened the observed effects of TAX on respiratory control. This might explain why TAX-induced changes in respiratory kinetics were more robust when measured in intact 2D myotubes and compared with permeabilized 3D myobundles.

STAR METHODS
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2-D culture of human myotubes
Preparation of 3D-bioengineered human myobundles

METHOD DETAILS

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2-Deoxy-glucose uptake in primary myotubes
Maximal oxygen consumption in primary myotubes
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High-resolution O2K respirometry in 3D-myobundles
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In vitro incubations of murine muscle mitochondria with α-tubulin, and analysis of ADP kinetics

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105189.

ACKNOWLEDGMENTS

We thank Dr Olga Ilkayeva and the Sarah W. Stedman Center Metabolomics and Biomarkers Core Laboratory at Duke University for performing the metabolomics assays. The DMPI Metabolomics Core Laboratory is supported by Diabetes and Endocrine Research Center grant P30 DK124723. This work was supported by the American Heart Association (AHA 19POST34370054 [M.J.T.], the Duke Cancer Institute as part of the P30 Cancer Center Support Grant (NIH CA014236 [D.M.M. and G.A.T.]), and a Duke CCPS Pilot Study Award, NIH grant UH3TR002142 (G.A.T), and funding support from the Mordecai family [D.M.M., and G.A.T.].

AUTHOR CONTRIBUTIONS

Conceptualization, D.M.M., M.J.T., and G.A.T.; methodology, M.J.T., X.Z., D.H.S., T.R.K., H.P., D.M.M., and G.A.T.; investigation, M.J.T., X.Z., D.H.S., and H.P.; writing – original draft, M.J.T. and D.M.M.; writing – review and editing, M.J.T, D.M.M., T.R.K., X.Z., and G.A.T.; visualization, M.J.T., X.Z., D.H.S., and H.P.; supervision, D.M.M., T.R.K., and G.A.T.; funding acquisition, D.M.M., M.J.T., and G.A.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 27, 2021
Revised: May 23, 2022
Accepted: September 20, 2022
Published: October 21, 2022

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Sarcomeric myosin heavy chain | DSHB | Cat# MF 20; RRID:AB_2147781 |
| α-Tubulin | Cell Signaling | Cat# 2125; RRID:AB_2619646 |
| Ac-K40-α-Tubulin | Cell Signaling | Cat# 5335; RRID:AB_10544694 |
| Gamma-Tubulin | Millipore Sigma | Cat# T6557; RRID:AB_477584 |
| VDAC | Abcam | Cat# ab15895; RRID:AB_2214787 |
| OXPHOS cocktail | Abcam | Cat# ab110413; RRID:AB_2629281 |
| GLUT 1 | Abcam | Cat# ab6552; RRID:AB_305540 |
| IRDye® 680RD Secondary Antibodies | LI-COR Biosciences | Cat# 925-68072; RRID:AB_2814912; Cat# 925-68071; RRID:AB_2721181; Cat# 925-68078; RRID:AB_2814915 |
| Sarcomeric α-actinin | Millipore Sigma | Cat# A7811; RRID:AB_476766 |
| Vimentin | Millipore Sigma | Cat# V6389; RRID:AB_609914 |
| α-bungarotoxin | ThermoFisher | Cat# B13422 |
| Phalloidin | ThermoFisher | Cat# R415 |
| Pyruvate dehydrogenase (PDH) | Millipore Sigma | Cat# ab110330; RRID:AB_10858459 |
| Phospho-PDH (PDH-E1(pSer306)) | Millipore Sigma | Cat# AP1064; RRID:AB_10618090 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Docetaxel pharmaceutical secondary standard | Millipore Sigma | CAS #148408-66-6 |
| Doxorubicin hydrochloride | Millipore Sigma | CAS #25316-40-9 |
| DMEM low glucose | Invitrogen | Cat# 11885 |
| XF Assay Medium Modified DMEM | Millipore Sigma | Cat# D5030 |
| Glutamax | GibCO | Cat# 35050061 |
| Fetal bovine serum | HyClone | Cat Cytiva# SH30070.03 |
| Dexmethasone | Millipore Sigma | Cat# D4902; CAS# 50-02-2 |
| Fetauin | Millipore Sigma | Cat# F3385; CAS# 9014-81-7 |
| Horse serum | Millipore Sigma | Cat# H1138 |
| L-Carnitine HCl | Millipore Sigma | Cat# C0283; CAS# 6645-46-1 |
| Gentamycin | GibCO | Cat# 15710064 |
| Amphotericin B | GibCO | Cat# 15290018 |
| Human recombinant Epidermal growth factor | VWR | Cat# 10005-454 |
| Trypsin | Millipore Sigma | Cat# T4799; CAS# 9001-51-8 |
| Matrigel | Corning | Cat# 354234 |
| Polydimethylsiloxane mold | Teflon Masters | Cat #102092-312 (VWR) |
| CereX frames (9.2 × 9.5 mm outer dimensions, 6.8 × 8.3 mm inner dimensions) | CereX Advanced Fabrics Inc | PBN II 4.0 |
| Thrombin | Millipore Sigma | Cat# T7513; CAS# 9002-04-4 |
| Fibrinogen | Millipore Sigma | Cat# F6330; CAS# 9001-32-5 |
| 6-aminocaproic acid | Millipore Sigma | Cat# A7824 ; CAS# 60-32-2 |
| Goat serum | Gibco | 16210064 |
| Paraformaldehyde | Anaer | J61899-AP |
| DAPI | Millipore Sigma | Roche 10236276001; CAS# 28718-90-3 |

(Continued on next page)
## REAGENT or RESOURCE | SOURCE | IDENTIFIER
---|---|---
Protease Inhibitor Cocktail | MilliporeSigma | Cat# P8340
Phosphatase Inhibitor Cocktail 2 | MilliporeSigma | Cat# P5726
Phosphatase Inhibitor Cocktail 3 | MilliporeSigma | Cat# P0044
4%–15% Criterion TGX Stain-Free Protein Gel, 18well | Biorad | Cat# 5678084
10X Tris Glycine SDS Running Buffer | Biorad | Cat# 1610732
10X Tris Buffered Saline | Biorad | Cat# 1706435
Cell Lytic M | MilliporeSigma | Cat# C2978
Fish Gelatin | MilliporeSigma | Cat# G7765
Casein | MilliporeSigma | Cat# C0626
Sodium Nitrate | MilliporeSigma | Cat# 58032
Thiamine Pyrophosphate | MilliporeSigma | Cat# C8754; CAS# 154-87-0
Pyridoxal 5’-phosphate | MilliporeSigma | Cat# P9255; CAS# 853645-22-4
Trichostatin A | MilliporeSigma | Cat# T8552; CAS# 58880-19-6
Nicotinamide | MilliporeSigma | Cat# N3376; CAS# 98-92-0
Lactate dehydrogenase | MilliporeSigma | 61311
Hydrazine sulfate | MilliporeSigma | Cat# 216046 CAS# 10034-93-2
Nicotinamide Adenine Dinucleotide (NAD*) | MilliporeSigma | Cat# N1636; CAS# 53-84-9
Digitonin | MilliporeSigma | CAS# 11024-24-1
EDTA | MilliporeSigma | Cat# E0270; CAS# 65501-24-8
EGTA | MilliporeSigma | Cat# E4378; CAS# 67-42-5
Potassium Chloride | MilliporeSigma | Cat# P5405; CAS# 7447-40-7
Sodium Chloride | MilliporeSigma | Cat# S7653; CAS# 7647-14-5
Magnesium Chloride Hexahydrate | MilliporeSigma | Cat# M2670; CAS# 7791-18-6
Potassium Dihydrogen Phosphate | MilliporeSigma | Cat# P9791; CAS# 7778-77-0
HEPES | MilliporeSigma | Cat# H3375; CAS# 7365-45-9
Potassium Pyruvate | Combi-Blocks | QA-1116; CAS# 4151-33-1
Rotenone | MilliporeSigma | Cat# R8875; CAS# 83-79-4
Carbonil-cyanide p-triflouromethoxy-phenyl-hydrazone (FCCP) | MilliporeSigma | CAS# 370-86-5
Antimycin A | MilliporeSigma | Cat# A8674; CAS# 1397-94-0
Oligomycin | MilliporeSigma | Cat# 75351; CAS# 579-13-5
Cytochrome C from equine heart | MilliporeSigma | Cat# C2506; CAS# 9007-43-6
Creatine Kinase from Rabbit Muscle | MilliporeSigma | Cat# 10736988001
Creatine Monohydrate | MilliporeSigma | Cat# C3630; CAS# 6020-87-7
Phosphocreatine Tris salt | MilliporeSigma | Cat# P4635; CAS# 108321-17-1
Na-F | MilliporeSigma | Cat # A13019.08
Glycerol | MilliporeSigma | Cat# G9012; CAS# 56-81-5
Malic Acid (Malate) | MilliporeSigma | Cat# M1000; CAS# 97-67-6
Glutamic Acid (Glutamate) | MilliporeSigma | Cat# G1501; CAS# 6382-01-0
Succinic Acid (Succinate) | MilliporeSigma | Cat# S3674; CAS# 110-15-6
Adenosine Diphosphate (ADP) | MilliporeSigma | Cat# A5285; CAS# 72696-48-1
D- (+)-Glucose Solution | MilliporeSigma | Cat# G8769; CAS# 50-99-7
2-deoxyglucose | MilliporeSigma | Cat# D6134; CAS# 54-17-6
[2-3H] deoxyglucose | PerkinElmer | Cat# NET328A250UC
2[14C]deoxyglucose | PerkinElmer | Cat# NEC720A

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Deborah M. Muoio (muoio@duke.edu).

**Materials availability**
This study did not generate new or unique reagents.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Cytochalasin B       | Millipore Sigma | Cat # C2743 |
| Sodium dodecyl sulfate | Millipore Sigma | Cat# 436143; CAS# 151-21-3 |
| UltimaGold Scintillation Fluid | PerkinElmer | CAS# 6013371 |
| α-Tubulin protein (>99% Pure) from porcine brain | Cytoskeleton Inc | Cat# T240-A |
| Acetonitrile; Optima LC/MS | Fisher Scientific | A955-500 |
| Formic Acid | Millipore Sigma | 399388 |
| Water; Optima LC/MS | Fisher Scientific | W6 |
| Methanol; Optima HPLC | Fisher Scientific | A454-4 |
| 96-well Plates, Caplugs | Evergreen | 222-8032-01R |
| **Critical commercial assays** | | |
| Pierce BCA protein assay kit | ThermoFisher | Cat#23225 |
| MemCode Reversible protein Stain Kit for PVDF membranes | ThermoFisher | Cat# 24585 |
| Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes | Thermo Fisher Scientific | Cat# 24580 |
| Glucose Assay Kit | Millipore Sigma | MAK181 |
| ToxiLight Cytotoxicity BioAssay Kit | Lonza Rockland | Cat# LT07-217 |
| **Experimental models: Cell lines** | | |
| Human skeletal myotubes from 4 donors. Expanded as described in (Madden et al., 2015; Zhang et al., 2018). | Obtained from surgical waste under Duke University IRB approved protocol Pro00063964 | Exempt from IRB review, category 45CFR46.101b. |
| **Software and algorithms** | | |
| Image acquisition software for Leica confocal Sp5 microscopes | LEICA | LAS AF v2.7.3.9723 |
| Image Studio version 3.1 | LICOR Biosciences | RRID:SCR_013715 |
| WorkOut 2.0 software (for luminescence measures) | Dazdaq Solutions Ltd. | NA |
| ImageJ-Fiji plugin (Tracking>TrackMate) to quantify Particle image velocimetry (PIV) for Ecoflex displacement | https://imagej.net/plugins/trackmate/ | As described in (Zhang et al., 2018). |
| MassLynx 4.1 for mass spectrometry analysis | Waters (Milford, MA) | RRID:SCR_014271 |
| GraphPad Prism 9 | GraphPad Software LLC | RRID:SCR_002798 |
| **Other** | | |
| Seahorse XFe24 plate | Agilent | Cat# 100867-100 |
| 24-well collagen-coated plates | Corning | Cat# 356408 |
| Custom Viton O-ring | McMaster Carr | 70 BUNA, Size 24 1” ID |
**Data and code availability**

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. All data produced in this study are included in the published article and its supplementary information, or are available from the lead contact upon request. This paper does not report original code.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Isolation of human myogenic progenitor cells**

Human skeletal muscle samples were obtained from surgical waste under Duke University IRB approved protocol (Protocol ID Pro00063964, exempt from IRB review, category 45CFR46.101b). Satellite cells from four different donors were expanded as previously described (Madden et al., 2015; Zhang et al., 2018). Briefly, muscle samples were minced, washed in PBS, and enzymatically digested in 0.05% trypsin for 30 min. Muscle was collected by centrifugation, pre-plated for 2 h, and transferred to a matrigel coated flask for attachment. Cells were expanded in growth media containing low glucose DMEM, supplemented with EGF, fetuin, dexamethasone, and gentamicin without insulin, with 10% fetal calf serum. Myogenic cells were cryopreserved in 90% growth medium with 5% fetal calf serum and 5% DMSO at passage 1 or 2, and used at passage 3–5 for the generation of myobundles.

**2-D culture of human myotubes**

Cells were cultured in 2D human growth media containing low-glucose (LG; 1 g/L glucose) DMEM supplemented with 8% fetal bovine serum, 0.4 μg/mL dexamethasone, 10 ng/mL EGF, 50 μg/mL Fetuin, 0.1% Gentamycin (1X) and 0.1% Amphotericin B (1X). Myoblasts were either differentiated into myotubes for 2D-cultured cell experiments or used for the generation of 3D myobundles as previously described (Davis et al., 2017). After the myoblasts reached ~80% confluence, they were switched to differentiation media (hDM), consisting of LG-DMEM supplemented with 2% adult horse serum (HS), 0.05% BSA, 100 μM car- nitine, and 1 μL per 100 μL (1X) antibiotic-antimycotic. Myotubes were differentiated for 4 days, and then enzymatically removed from the well plate using 0.025% trypsin-EDTA, centrifuged, and re-suspended in hDM.

**Preparation of 3D-bioengineered human myobundles**

The 3D-myobundle assembly was performed as previously described (Davis et al., 2017; Madden et al., 2015; Zhang et al., 2018). Briefly, myoblasts encapsulated in a fibrin/Matrigel matrix were seeded on a nylon frame pinned on the PDMS (polydimethylsiloxane) mold. The matrix was fabricated by mixing a cell solution (11 μL, 300,000 cells per bundle in culture media containing 4 U/mL thrombin, the stock thrombin solution was 50U/ml in PBS with 0.1% BSA) and an ice-cold gelling solution (4.4 μL Matrigel +4.4 μL of 20 mg/mL fibrinogen in PBS+ 2.2 μL PBS); then pipetting the mixture (total 22 μL per bundle) in a custom-made PDMS mold placed between the beams of the frame. The myobundles polymerized in the mold anchored to the frame for 30 min at 37°C. Once compacted, myobundles within the mold were cultured in a 6-well plate with 2 mL/well of 3D growth media (2D growth media +1.5 mg/mL 6-aminocaproic acid, ACA) on a rocker (0.33 Hz) at 37°C. After 4 days, the nylon frames containing 4 myobundles each were peeled off, for free-floating culture and differentiation in 3D shift media (LG-DMEM with GIBCO N2 supplement, 0.1% Gentamycin 1X and 0.1% Amphotericin B 1X + 2 mg/mL ACA, for 5 days before drug treatments. The 3D human myobundles provided for these studies were generated from human myoblasts derived from four different donors based on availability in the BME cell bank at the time of the experiment. Most experiments were performed with myoblasts from a single donor, which remained consistent across all but two experiments (S. Figures 2, 6E and 6F), as noted in the Figure Legends. The cell lots used for this study were selected in a random, unbiased manner without consideration of donor characteristics (e.g. BMI, sex, age) and the investigator executing the experiments was blinded. Because the cells are obtained from discarded muscle tissue procured during surgery, subject characteristics of each donor are not recorded. Most donors are pediatric girls undergoing surgery for scoliosis.

**METHOD DETAILS**

**Chemotherapy treatments in 2D-cultured primary myotubes**

Beginning on day three of differentiation, myotubes were treated for three consecutive days (differentiation days S4-S6) with daily 3 h cycles of 0, 0.1, 1, 10, 100 nM TAX ± 10 nM DOX. After the completion of
the last treatment cycle, myobundles were switched to clean differentiation media alone for another 24 h before trypsinization and use for experiments.

**Pharmacokinetic-mimicking drug treatments in 3D-myobundles**

TAX and DOX reach peak plasma concentrations of 1 μM 3 h post-infusion, and 10 nM TAX (Brunsvig et al., 2007) and 100 nM DOX (Barpe et al., 2010), after 24 h. TAX is commonly administered weekly (Hainsworth, 2004; Palmeri et al., 2008), whereas DOX alone (Anampa et al., 2015) or in combination with TAX (Baltali et al., 2001) may be given in 3 weeks intervals. Here, a 3-week regimen was recapitulated in vitro with a 3-day regimen of 3 cycles, maintaining a 3:1 TAX/DOX ratio of exposure. Following five days of differentiation, 3D-myobundles were exposed to either one of 4 treatment regimens: CTL, TAX alone, TAX + DOX or DOX alone. The regimens consisted of a 3 h peak-dose, followed by a 21 h low-dose treatment with TAX +/- DOX, for 3 consecutive days (S6-S8). TAX dose was (1 μM x 3 h + 10 nM x 21 h) x 3 days; and DOX dose (0.5 μM x 3 h + 50 nM x 21 h) x 1 day. The single dose of DOX was administered in the second cycle only (at S7). The group receiving DOX alone received a single dose on S7, and fresh media the remaining days. TAX stock solutions (0.62 mM) prepared in DMSO and DOX stock solutions (1.8 mM) prepared in water were stored at −80°C and fresh aliquots were used for each treatment. Both stocks were serially diluted in culture medium to reach the foregoing final concentrations ranging from 10 nM to 1 μM. All drugs were removed and myobundles were fed with fresh media 24 h prior to each experiment, to permit a recovery period.

**2-Deoxy-glucose uptake in primary myotubes**

2-Deoxy-glucose uptake was measured in 2D-cultured human skeletal muscle myoblasts as previously reported (Pender et al., 2005). Briefly, myoblasts were cultured in 24-well collagen-coated plates and allowed to differentiate to myotubes for four days prior to drug treatments. 24 h after the last cycle of chemotherapy, cells were pre-incubated with hDM for 15 min at 37°C. Cytochalasin B (final 40 μM) was added to select wells to determine nonspecific incorporation of label. 10 μL of uptake media was added per well (final concentration: 10 μM cold 2-DG, 1 μCi/mL 2-Deoxy-[3H]glucose) followed by 60 min of incubation at 37°C. Cell uptake of glucose was terminated by rinsing each well 3 times with ice-cold PBS containing 20 mM D-glucose. Myotubes were then solubilized in 500 μL 0.1% SDS and cells were lysed in an orbital shaker for 30 min at room temperature. 300 μL of the lysate aliquots was added to 4 mL of scintillation fluid and counted in a scintillation counter. The remaining lysates were used to determine protein concentration, measured by BCA protein assay.

**Maximal oxygen consumption in primary myotubes**

Oxygen consumption rates in primary human myotubes were measured with a Seahorse Flux Analyzer XFe24 (Seahorse Bioscience, Billerica, MA). Myoblasts were plated (40,000 cells/well) on a Seahorse XFe24 plate and then differentiated for 4 days with differentiation media (LG-DMEM supplemented with 2% adult horse serum, 0.05% BSA, 100 μM carnitine, and 100 mg/mL Pen/Strep. On S7, 24 h after termination of the 3 cycles of drug treatments (S4-S6), media was switched to XF Assay Medium Modified DMEM (Sigma D5030) (pH = 7.4) containing added glucose (2.5 M, final 2 mM), pyruvate (100 mM, final 1 mM), and glutamax (200 mM, final 2 mM). Following a 1 h equilibration at 37°C in a CO2-free incubator, plates were transferred to the XFe24 analyzer for measurement of cellular respiration in a mitochondrial stress test. Each measurement cycle in the protocol consisted of a 3 min mixing step, a 2 min wait period, and a 3 min extracellular O2 flux measure. This “mix, wait, measure” phase of each measurement cycle was performed three times during the basal, oligomycin (1 μM), carbonil-cyanide-p-trifluoromethoxy-phenyl-hydrazone (FCCP, 1 μM), rotenone (2 μM), and antimycin A (1 μM) steps of the test. At termination of the experiment, media was aspirated, and 100 μL Cell Lytic M containing protease inhibitors was added to the wells, followed by a 30 min incubation on an orbital shaker. Total protein quantification by BCA assay was performed for normalization of OCR data.

**Immuno-fluorescent staining and confocal microscopy of 3D-myobundles**

Following 24 h after drug treatments (at S9), myobundles were fixed with 4% PFA for 1 h, washed in PBS, permeabilized with 0.2% Triton X-100 for 30 min, and blocked in 10% goat serum overnight, followed by a 24 h incubation with the appropriate antibodies for immuno-staining: myosin heavy chain ½/4/6 (1:200), sarcomeric α-actinin (1:200), and vimentin (1:100), α-Tubulin, Ac-K40-α-Tubulin. DAPI was used to stain nuclei (1:1000). Corresponding fluorescently labeled secondary antibodies used: α-bungarotoxin...
High-resolution O2K respirometry in 3D-myobundles

High-resolution O2 consumption measurements were conducted using the Oroboros Oxygraph-2K (Oroboros Instruments), including a custom Viton O-ring added to the chamber to prevent interaction of the myobundles with the stir bar as previously described (Davis et al., 2017). All experiments were carried out in buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K2HPO4, 5 mM MgCl2-6H2O, 0.5 mg/mL BSA, pH = 7.1) at 37°C, in a 2 mL chamber. During drug treatments, each well contained a nylon frame with 4 myobundles, which was cut in half to introduce in the O2K chamber. The set of two myobundles (attached to half the frame (Figure 5L)) were rinsed with buffer Z to eliminate residual culture media and positioned in the O2K chamber between the O-ring and stopper. 0.3 mg/mL digitonin was added in the chamber for permeabilization, following addition of 10 mM pyruvate and 2.5 mM malate, increasing concentrations of ADP (42 μM–5 mM), 10 mM succinate and 20 μM cytochrome c (for assessment of integrity of mitochondrial membrane). At the conclusion of each experiment, myobundles were rinsed with PBS and flash-frozen for western blot analysis.

Western blot analysis

Each set of two myobundles was homogenized in 100 μL of 2x sample lysis buffer containing 20 mM Tris-HCl, 4% SDS, 10 Mm NaF, 1 mM EDTA, 20% glycerol, supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktails, 10 mM nicotinamide and 1 μM Trichostatin A (deacetylase inhibitor) using a Potter-Elvehjem tissue grinder. Samples were sonicated and centrifuged for 20 min at 13,000 × G for 20 min at room temperature. Supernatants were transferred into clean tubes and stored at −80°C. 20 μL of sample containing β-mercaptoethanol and bromophenol blue were loaded onto a 4–15% Criterion TGX Stain Free SDS-PAGE gel (Bio-Rad), transferred to a nitrocellulose membrane via the Bio-Rad Turbo-Blot Transfer system. Because the number of cells utilized to make each myobundle was consistent, no significant differences in protein concentration across the samples were expected. Nonetheless, equal protein loading was controlled by Pierce Reversible Stain MEMcode or Ponceau staining. Membranes were then blocked in fish gelatin with tris-buffered saline (TBS) for 1h at room temperature and incubated with primary antibodies overnight at 4°C. Following incubation with primary antibodies for Gamma-Tubulin, α-Tubulin, Ac-K40-α-Tubulin, myosin heavy chain, VDAC, OXPHOS cocktail, GLUT 1, or PDH, membranes were washed in TBS +0.05% Tween, incubated with appropriate secondary antibody diluted in fish gelatin in TBS, and imaged using the Odyssey Imager (LI-COR Biosciences). Band intensities were determined using Image Studio version 3.1 and ImageJ where appropriate. Memcode and Ponceau stains were quantified using Image lab software from BioRad. VDAC and OXPHOS proteins were normalized using a single Memcode/Ponceau band at ~24KDa to avoid interference from large matrix proteins that were abundant in the 3D culture. Images of Memcode/Ponceau staining for each membrane are provided in the supplemental information along with original western blotting images.

Mitochondrial thermodynamic force-flow relationship

Steady-state oxygen consumption rates (J02) ranging from 95% of maximal state 3 (i.e. exercise) to state 2 (i.e. resting) were determined using a modified version of the creatine kinase (CK) energetic clamp (Glancy et al., 2008; Messer et al., 2004) in the presence of excess CK, and known amounts of ATP, creatine (Cr), and phosphocreatine (PCr). Buffer Z was supplemented with 5 mM Cr, 1.5 mM PCr, 20 U/ml CK, 2.5 mg/mL fatty-acid free BSA, and 1 mM EGTA (pH 7.2). Stabilization of respiration after addition of 0.3 mg/mL digitonin, was followed by additions of respiratory substrates (10 mM pyruvate, 2.5 mM malate, 10 mM succinate), and ATP (5 mM). Sequential additions of PCr (1.5–10.5 mM) progressively shift the CK equilibrium, altering energy demand (ATP/ADP ratio) and gradually lowering J02. The free energy of ATP hydrolysis (ΔGATP) after each PCr addition was calculated using the online tool (https://dmpio.github.io/bioenergetic-calculators/) (Fisher-Wellman et al., 2018), which utilizes the equation: ΔGATP = ΔGATP° – 2.3 RT*K*2.3*log([PCr]/[Cr][Pi]), where ΔGATP° is the standard ΔGATP (~−7.592 kcal/mol), R is the gas constant (1.987 cal K−1 mol−1), and T is the temperature (310 K). The slope of the linear portion of J02 vs ΔGATP (the force-flow relationship) represents a measure of the overall “conductance” of the system.
**Glucose, lactate and adenylate kinase leak in media**

Media from each well (containing a single frame of four myobundles) was sampled every 24 h after each treatment cycle, for spectrophotometric/fluorometric quantification of lactate, glucose and AK leak per standard methodologies. Lactate was measured kinetically by following the reduction of 11 mM NAD⁺ at 340nm in assay buffer containing 175 mM hydrazine sulfate, 68 mM glycine, 2.9 mM EDTA, and 50 U/ml lactate dehydrogenase. Glucose was determined fluorometrically using the Glucose Assay Kit. Adenylate kinase release from 3D myobundles was assessed using the ToxiLight® BioAssay Kit according to the manufacturer’s instructions. Luminescence was measured using a Perkin Elmer Victor3 V plate reader and WorkOut 2.0 software.

**In situ electrical stimulation and force testing of 3D-myobundles**

Force testing of 3D-myobundles was performed as previously described (Zhang et al., 2018), following stretching of the Ecoflex films by microbead displacement recorded through a stereomicroscope. The in situ stimulation system consisted of Ecoflex membranes (platinum-catalyzed silicones) with microbeads added at a final concentration of 1.25% (w/w), aligned in series with myobundles and attached to nylon frames. Myobundles were stimulated for 50 seconds at 20 Hz using a force stimulator. Stretching of the Ecoflex films was recorded as video through a stereomicroscope. Particle image velocimetry (PIV) was performed using the ImageJ-Fiji plugin (Tracking>TrackMate) to quantify Ecoflex displacement. Force contraction was analyzed via particle displacement and the strain of Ecoflex film (Videos S1 and S2). The direct relation between myobundle force production and Ecoflex film stretch was previously validated (Zhang et al., 2018).

**Mass spectrometry-based metabolite profiling of 3D-myobundles**

Following treatments, 3D myobundles were rinsed in ice-cold PBS and two bundles were pooled and snap-frozen in liquid nitrogen. Bundle metabolites were extracted in 0.5ml of 50% acetonitrile containing 0.3% formic acid. Samples were processed and analyzed using flow injection ESI tandem mass spectrometry by the Duke Metabolomics Core Laboratory using previously published methods for amino acids and acyl-carnitines and organic acids (An et al., 2004; Koves et al., 2008). Data were generated with a Waters Triple Quad detector equipped with AcquityTM UPLC using MassLynx 4.1 (Waters, Milford, MA).

**Murine skeletal muscle mitochondrial isolation**

Animal studies were approved by the Duke University Institutional Animal Care and Use Committee (IACUC protocol #A096-18-04) and conducted in Assessment and Accreditation of Laboratory Animal Care-associated facilities. Male mice on a C57BL/6NJ background were used for experiments shown in Figure S4. Gastrocnemius, quadriceps and soleus muscles were excised and immediately placed in ice-cold phosphate buffered saline (PBS) supplemented with 10 mM EDTA (pH 7.4). Skeletal muscle mitochondria isolation was performed by differential centrifugation as previously described (Williams et al., 2020). Protein content of the final mitochondrial prep was determined using the Pierce BCA protein assay. 35 mg of total mitochondrial protein were loaded in the O2K chamber for mitochondrial function assessment.

**In vitro incubations of murine muscle mitochondria with α-tubulin, and analysis of ADP kinetics**

α-Tubulin protein was reconstituted in 0.5 mg/mL in G-PEM buffer (80 mM PIPES, 2 mM MgCl2, 0.5 mM EGTA, 1 mM GTP, pH 6.9). Mitochondria were either pre-incubated with α-tubulin for 30 min at room temperature in KMEM mitochondrial isolation buffer or tubulin was added once in the O2K chamber. In both experiments, the final mitochondrial protein:tubulin ratio achieved was 1:1 (i.e. 35 µg of tubulin was added together with 35 µg of total mitochondrial protein in the O2K chamber). ADP titrations were performed in the presence of a hexokinase (HK) clamp to allow for stable ATP/ADP concentrations (Gouspillou et al., 2011; Lark et al., 2016). Experiments were conducted in buffer Z supplemented with 5 mM glucose and 1 U/ml HK. The respiratory substrates were 10 mM glutamate + 2.5 mM malate, followed by sequential additions of ADP.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are presented as means ± SEM. Statistical analyses were preformed using one-way (or two-way when appropriate) ANOVA with Dunnett multiple comparisons tests for analysis of significance among groups versus control group. Statistics and figures were prepared using GraphPad Prism (Version 9.0). The level of significance was set at p ≤ 0.05.