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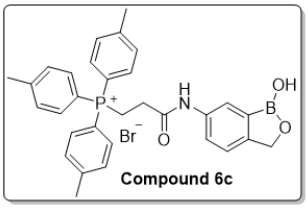

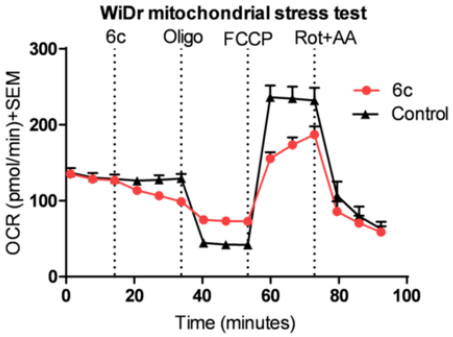

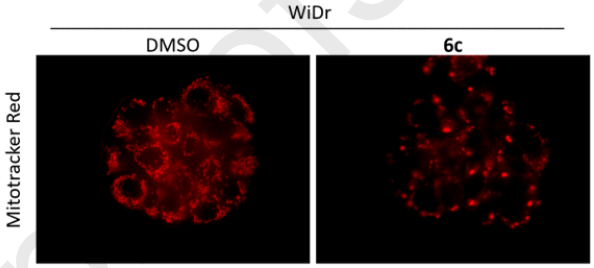

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ABSTRACT

Arylphosphonium-benzoxaborole conjugates have been synthesized as potential mitochondria targeting anticancer agents. The synthesized compounds have been tested for their effects on cell viability in various solid tumor cell lines including breast cancer 4T1 and MCF-7, pancreatic cancer MIAPaCa-2 and colorectal adenocarcinoma WiDr. Compound **6c** is designated as a lead compound for further studies due to its enhanced effects on cell viability in the above-mentioned cell lines. Seahorse Xfe96 based metabolic assays reveal that the lead candidate **6c** inhibits mitochondrial respiration in 4T1 and WiDr cell lines as evidenced by the reduction of mitochondrial ATP production and increase in proton leak. Epiflourescent microscopy experiments also illustrate that **6c** causes significant mitochondrial fragmentation in 4T1 and WiDr cells, morphologically consistent with programmed cell death. Our current studies illustrate that arylphosphonium-benzoxaborole conjugates have potential to be further developed as anticancer agents.

Elevated glycolysis to generate ATP and synthetic intermediates for biomass production is a hallmark of many cancers.[1-4] The tumor microenvironment is highly heterogeneous in nature, poorly vascularized at the center, and exists in nutrient-poor conditions with limited glucose and oxygen availability. Interestingly, the mitochondrial electron transport chain is able to operate with low oxygen levels, and also plays important energetic and biosynthetic roles in sustaining cancer cell growth under nutritionally challenged environments.[5-11] In this regard, cancer cells exhibit high levels of mitochondrial biogenesis and generate a greater mitochondrial mass to fuel cancer growth.[5-11] Further, aerated cancer cells also utilize mitochondrial oxidative phosphorylation (OxPhos) to proliferate by utilizing the end product of glycolysis, pyruvate, to sustain TCA cycle processes. Hence, aerobic cancer cells establish a symbiotic metabolic plasticity with glycolytic cancer cells to sustain proliferation.[12,13]

The mitochondrial outer- and inner membranes act as rigid barriers for passive diffusion of several small molecule xenobiotics. Lipophilic phosphonium cations generated from triphenylphosphine (TPP) have been conjugated with a wide variety of small molecules with reported potent and selective mitochondria targeting capabilities for cancer and other biomedical applications.[14-17] TPP cations have a large ionic radius with a hydrophobic surface and efficiently enter the mitochondrial matrix due to the negative membrane potential inside the matrix. These cations do not require any specific transporters for mitochondrial translocation and many studies have shown that they accumulate several hundred-fold inside the mitochondria compared to cytoplasm.[14-17] In this regard, extensive literature reported structure activity studies have revealed that increased lipophilicity of TPP-conjugate enables enhanced mitochondrial targeting.[18] In fact, accumulation of simple alkyl-TPP conjugates was found to be directly proportional to hydrocarbon chain length (methyl < decyl, etc.).[19,20] In contrast, highly polar templates, including peptides, have been conjugated to TPP and were found to have limited mitochondrial accumulation.[21] Accordingly, the capacity of TPP-based drugs to target and accumulate in the mitochondria can be fine-tuned by altering lipophilicity.

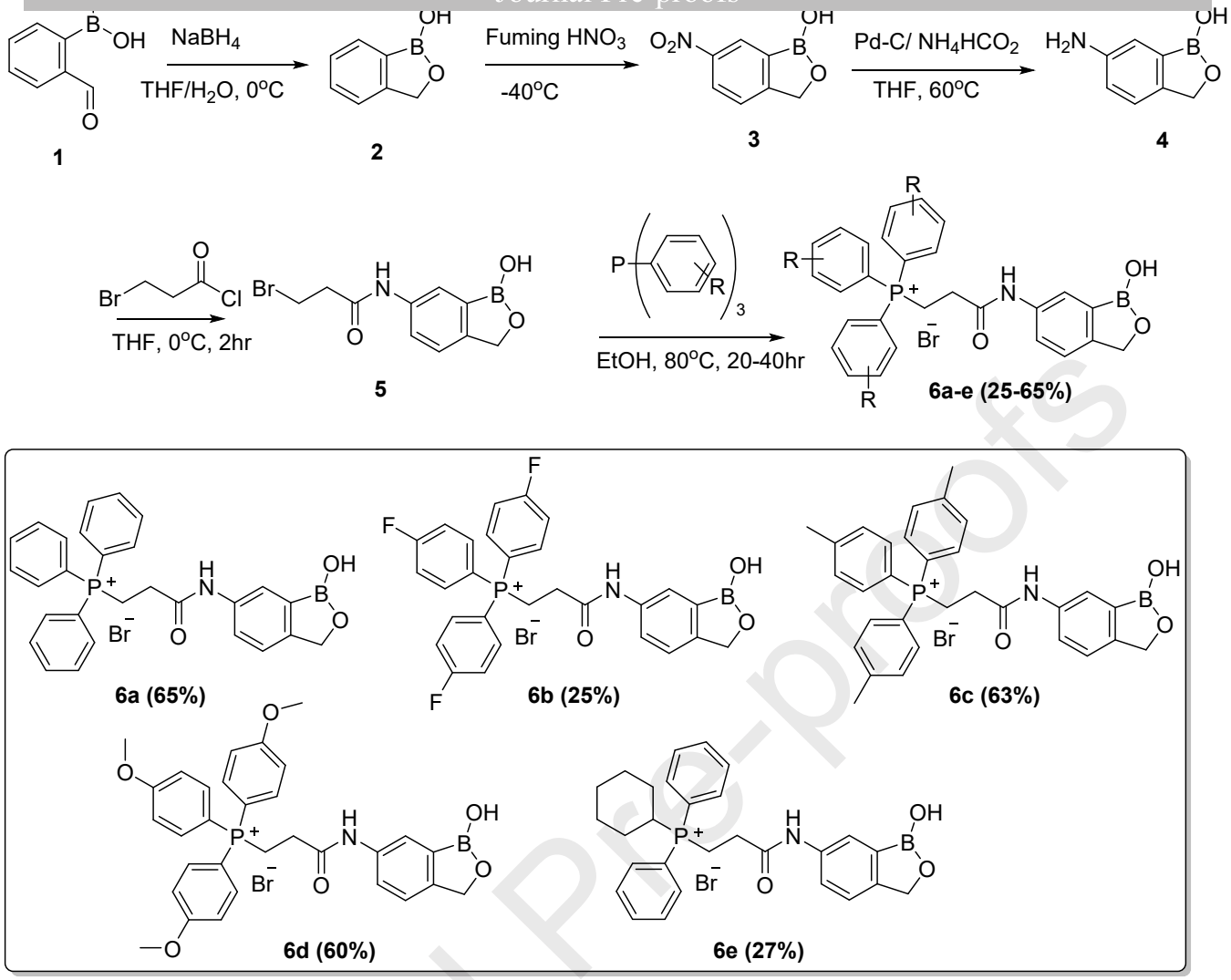
However, many lipophilic TPP appended compounds suffer from low therapeutic index to be suitable as targeted anticancer agents. There are several known pharmacological OxPhos inhibitors including oligomycin (ATP synthase), rotenone (complex I), and antimycin (complex III) which are utilized to study mitochondrial function, but are not suitable for clinical

use. the index with high levels of off-target toxicities, and rotenone causes Parkinson's-like side effects.[22,23] Additionally, clinically used antidiabetic drug metformin has a high therapeutic index and is widely studied as an OxPhos inhibitor for cancer treatment.[24] However, its lack of potency limits its utility as an anticancer agent in an advanced setting. Similarly, some antibiotic drugs have been shown to target mitochondria with potential anticancer applications.[25,26]

Phenyl group appended benzoxaboroles are cyclic boronic acids which have attracted a lot of attention in recent years by pharmaceutical industry due to their metabolic stability and several interesting pharmacological properties.[27-35] We also reported on the synthesis of functionalized benzoxaboroles as potential medicinal agents.[36-40] In this regard, we envisioned that benzoxaboroles conjugated to TPP cations could be selectively delivered to mitochondria to develop them as potential anticancer agents. The idea behind design of these conjugates is to utilize the generally non-toxic nature of benzoxaborole structural unit to deliver them to mitochondria with high therapeutic index. Molecules that exhibit cytotoxicity at low micromolar concentration may be difficult to achieve high therapeutic index to target mitochondria, and molecules that have no cytotoxicity even at high concentrations (> 100 micromolar) may not be useful as anticancer agents. Our goal in this project is to identify a molecule that is moderately cytotoxic with IC_{50} values in the range of 10 to 50 micromolar concentration where a compromise between cytotoxicity and therapeutic index can be potentially achieved. With this idea in mind, we hypothesized that 6-aminobenzoxaborole would be a good starting material that can be readily converted in to phosphonium conjugates via its bromoalkylamides. Further, it has been extensively illustrated that TPP-based cations have increased uptake into cancer cells when compared to normal cells, as the plasma-membrane potential of potential selectivity and non-toxic nature of these candidate compounds.

The synthesis of aminobenzoxaborole was accomplished starting from 2-formylphenylboronic acid **1**. Sodium borohydride reduction of the aldehyde **1** resulted in benzoxaborole **2** which was nitrated with fuming nitric acid at low temperature to obtain 6-nitrobenzoxaborole **3**.[41] Pd-C catalyzed reduction of nitro group in **3** provided the corresponding aminobenzoxaborole **4**.[42] The amine group in **4** was acylated with 3-bromopropionyl chloride to obtain bromoamide **5**. Treatment of **5** with arylphosphine in refluxing ethanol afforded the arylphosphonium-benzoxaborole conjugates (Scheme 1). Using this procedure with bromide **5**, five different phosphonium-benzoxaboroles conjugates (**6a-6e**) with different stereoelectronics were synthesized (Scheme 1). The reactions with unsubstituted and electron donating phosphines were relatively facile compared to the electron withdrawing tri-fluorophenylphosphine, and sterically hindered cyclohexyl triphenylphosphine. These two examples gave lower yields with substantial recovery of starting materials. Protracted and elevated heating did not improve the reaction yields.

All synthesized compounds were evaluated for their effects on cell viability using MTT assay.[44] Murine metastatic breast cancer 4T1, human breast cancer MCF7, human colorectal adenocarcinoma WiDr, and human pancreatic MIAPaCa-2 cancer cells were utilized for this assay. The compounds **6a**, **6b**, **6d** and **6e** did not exhibit effects on cell viability in all these cell lines up to 100 μM concentration, whereas compound **6c** exhibited IC_{50} value in the range of 24-50 μM (Table 1) across the tested cell lines. Based on these studies, **6c** was selected as the lead compound for further *in vitro* evaluation of its effect on mitochondrial and glycolysis parameters.



Scheme 1: Synthesis of arylphosphonium-benzoxaborole conjugates **6a-6e.** [36]

Table 1. MTT IC_{50}* (μM) values of arylphosphonium-benzoxaborole conjugates in 4T1, MCF-7, MIA PaCa-2, and WiDr cell lines

Compound	4T1	MCF-7	MIA PaCa-2	WiDr
5	>100	>100	>100	>100
6a	>100	>100	>100	>100
6b	>100	>100	>100	>100
6c	50±13	46±2	44±5	24±7
6d	>100	>100	>100	>100
6e	>100	>100	>100	>100

*The average±SEM values of at least three independent experimental values.

The metabolic profiles of WiDr and 4T1 cells treated with compound **6c** were evaluated utilizing standard Seahorse XFe96 based mitochondrial and glycolytic stress tests (MST and GST) according to the manufacturer's protocols.[45-47] In both MST and GST, cells were treated with the test compound **6c** at 100 and 50 μM concentrations, and the corresponding oxygen consumption rates (OCR) following the addition of specific mitochondrial perturbants enabled quantification of mitochondrial damage. In MST, mitochondrial ATP production was calculated by observing the acute change in OCR following the addition of ATP synthase inhibitor oligomycin. Simultaneously, mitochondrial proton leak was calculated, and is defined as the basal OCR unrelated to ATP synthesis. Mitochondria require maintenance of a proton gradient across the inner membrane for ATP synthase to function, and damage by drugs or xenobiotics can perturb the membrane integrity leading to proton leak across the membrane. Hence, an increase in proton leak is usually linked with a decrease in ATP production. In the presence of compound **6c**, a significant decrease in ATP production was observed at 100 and 50 μM in WiDr cells. In 4T1 cells, ATP production was decreased significantly only at 100 μM concentration (Figure 1A). In both the cell lines, compound **6c** significantly increased proton leak at both concentrations, indicating that it may be damaging the mitochondrial membrane and thus, allowing protons to "leak" down their gradient (Figure 1B). Maximum mitochondrial respiration and spare respiratory capacity in control cultures can

be i[illegible] east cell line, a decreased maximal respiration suggested that the cells were unable to reach their maximum OCR compared to the control (Figure 1C). These results indicate that compound **6c** inhibits the cells ability to oxidize mitochondrial respiratory substrates including sugars, fats, amino acids, etc. - possibly by inhibiting the electron transport chain. Surprisingly, compound **6c** did not affect maximal respiration in 4T1 cell line even at 100µM concentration cancer cell line with rapid proliferation dynamics. This cell line exhibits high metabolic plasticity between glycolysis and mitochondrial OxPhos[9] and due to the aggressive metabolic nature of these cells, inhibition of respiration may only be accomplished at higher concentrations of **6c**. Compound **6c** did not affect spare respiratory capacity in either WiDr and 4T1 cell lines (Figure 1D).

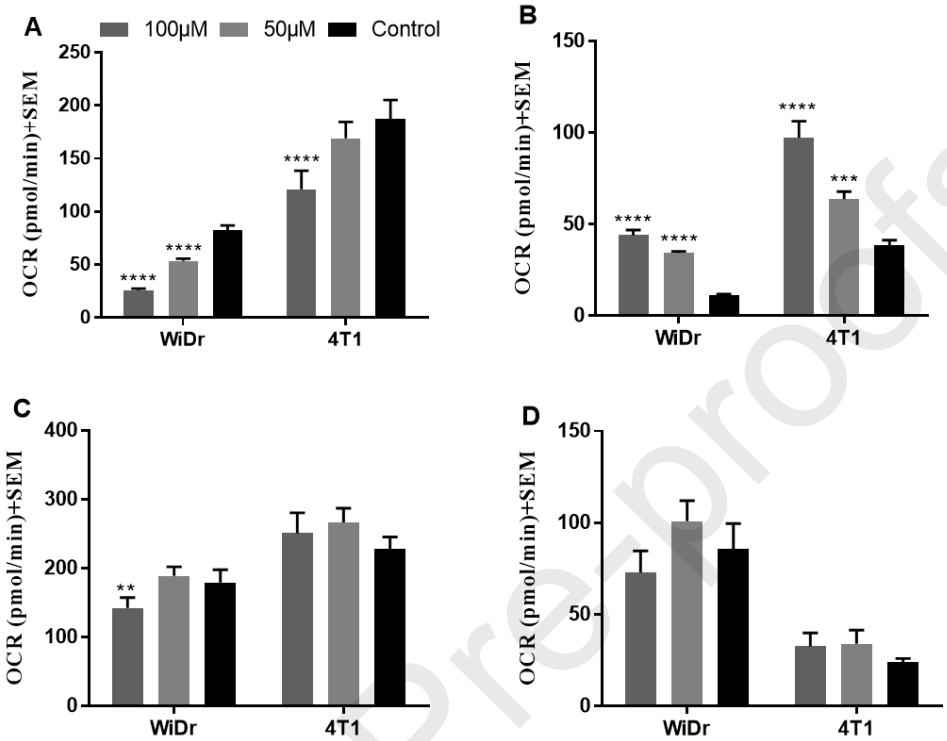


Figure 1: Mitochondrial stress test of compound **6c** in WiDr and 4T1 cell lines. The graphs represent mitochondrial parameters (**A**) ATP production (**B**) proton leak (**C**) maximal respiration, and (**D**) spare respiratory capacity. The average+SEM values of at least three independent experimental values were calculated. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$, indicating statistically significant changes in OCR when comparted to control (vehicle, DMSO).

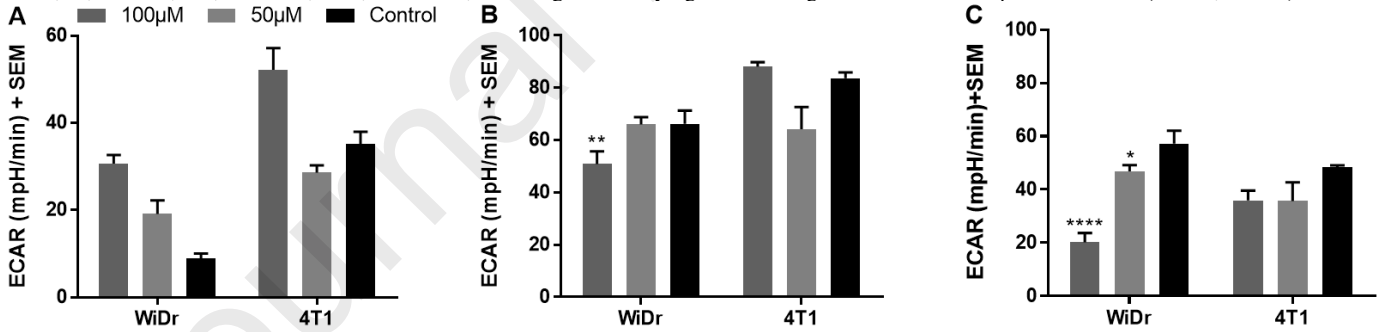


Figure 2: Glycolysis stress test of compound **6c** in WiDr and 4T1 cell lines. The graphs represent glycolytic parameters (**A**) glycolysis (**B**) glycolytic capacity and (**C**) glycolytic reserve. The average+SEM values of at least three independent experimental values were calculated. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$, indicating statistically significant changes in OCR when comparted to control (vehicle, DMSO).

In GST, glycolysis is proportional to the rate at which glucose is metabolized to pyruvate and exported as lactate and H^+. Hence, rates of glycolysis can be directly associated with extracellular acidification rate (ECAR), which was the reporting measurement of glycolysis in these experiments. Compound **6c** increased glycolysis compared to the control in both WiDr and 4T1 cell lines at 100 and 50 µM concentrations (Figure 2A). Increased glycolysis in this regard is consistent with **6c**-induced mitochondrial dysfunction as the cells must exhibit a marked shift in metabolism to maintain energetic homeostasis. We then evaluated compound effects on glycolytic capacity which is defined as cells capability to undergo maximum theoretical glycolysis when mitochondrial OxPhos is inhibited by oligomycin. Interestingly, glycolytic capacity was significantly decreased by candidate compound **6c** at 100 µM compared to the control in WiDr cell line, whereas in 4T1 cell line, compound **6c** did not have any effect on glycolytic capacity (Figure 2B). Severe mitochondrial damage induced in WiDr cells may result in metabolite accumulation limiting the ability of these cells to heighten compensatory glycolytic rates. Glycolytic reserve is the capacity of the cell to increase its glycolytic rate (above basal glycolysis) in response to an energetic demand driven by OxPhos inhibitor oligomycin. Here, it was observed that glycolytic reserve was decreased at both tested concentrations as evidenced by decreased compensatory ECAR in the presence of oligomycin and **6c** in WiDr and 4T1 cell lines (Figure 2C).

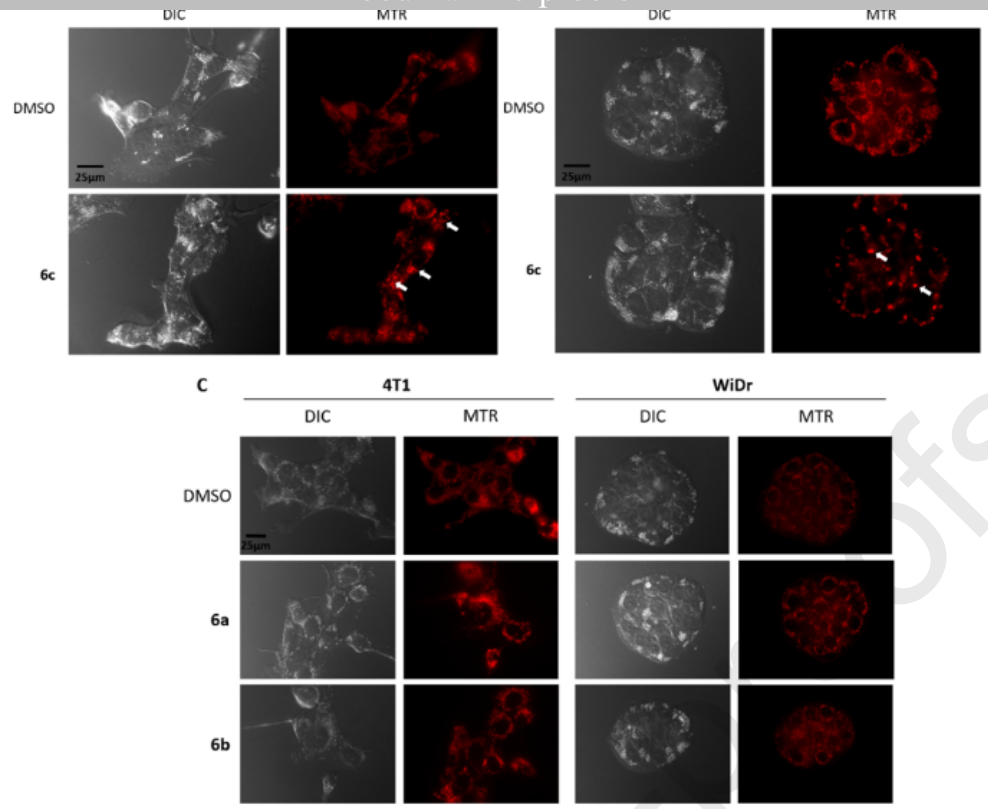


Figure 3: Epifluorescent microscopy experiments in (**A**) 4T1 and (**B**) WiDr cells indicate **6c**-treated cultures exhibit substantial fragmentation of mitochondrial networks when compared to control cultures. (**C**) Non-toxic All images were captured using the same magnification (see scale bar, 25µm), and are representative of overall culture appearances (3-5 fields of view) across three independent biological replicates. Arrows indicate regions of mitochondrial fragmentation.

To further explore the effects of candidate compounds on mitochondrial vitality, epifluorescent microscopy experiments were employed.[49] In this regard, 4T1 and WiDr cells were seeded in MatTek glass-bottom dishes and were exposed to test compounds (**6a**-**6c**, 100µM) or vehicle (DMSO) for 24 hours. Unsubstituted compound **6a** and electron withdrawing substituent fluoro-substituted **6b** were chosen as non-cytotoxic analogs to compare with the cytotoxic lead tolyl substituted analog **6c** to compare mitochondrial morphological effects with compound treatment. Following exposure, compounds were removed and cells were then exposed to Mitotracker Red-CMXROS (MTR), a mitochondrial targeting fluorescent probe that accumulates and fluoresces as a function of membrane potential. This probe provides information on compound effects on mitochondrial morphology and vitality. These experiments revealed that candidate compound **6c** led to drastic effects on mitochondrial morphology, with a more substantial affect in WiDr cells (Figure 3A&B). Heightened sensitivity of WiDr mitochondria toward **6c** treatment interestingly correlated with enhanced effects of WiDr cell viability when compared to the 4T1 cell line. Further, it was observed that treatment with the non-toxic **6a** and **6b** examples led to very minor effects on mitochondrial morphology (Figure 3C), lending evidence that **6c**-induced mitochondrial damage may be responsible increased effects on cell viability. Observed fragmentation of mitochondria in **6c** treated cultures is consistent with increased mitochondrial fission observed during apoptosis, further suggesting a mitochondrial mediated mechanism of cell death.[50,51] However, the mechanism of action of the candidate compounds described in this manuscript are likely acting via pleiotropic means to elicit effects on cancer cell viability, and could be a combination of cytostatic and/or toxic depending on tissue type and cellular context.

In conclusion, we have synthesized arylphosphonium-benzoxaborole conjugates as potential agents for selective mitochondria targeted anticancer agents. These compounds were evaluated for their *in vitro* anticancer properties and compound **6c** exhibited effects on cell viability in 4T1, MCF-7, MIA PaCa2 and WiDr cell lines. Based on its *in vitro* cytotoxicity, compound **6c** was selected as the lead candidate compound and was evaluated for its metabolic profile using Seahorse XFe96 mitochondrial and glycolytic stress tests. Candidate **6c** exhibited significant disruption of maximal respiration and ATP production as determined from MST in WiDr and 4T1 cell lines. As anticipated, candidate **6c** did not inhibit many of the glycolytic parameters as evidenced by GST in the same cell lines. These studies also showed that compound **6c** induced glycolysis in cancer cells most likely due to inhibition of mitochondrial respiratory function. Finally, epifluorescent microscopy experiments revealed that **6c** led to substantial perturbations in mitochondrial morphology in WiDr and 4T1 cells when compared to non-toxic analogs, indicating that enhanced mitochondrial targeting capacity of this derivative may be responsible for the observed effects on cell viability. Our results also provide opportunities for combination therapy of **6c** with various glycolytic inhibitors for even more potent therapeutic protocol targeting metabolic plasticity, which is very common in many tumors.

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43. *Materials:* 2-formyl phenylboronic acid (AKSci), sodium borohydride (AKSci), fuming nitric acid (Alfa-Aesar), 10% Pd-C (Sigma-Aldrich), ammonium formate (AKSci), aryl phosphoniums (AKSci), were purchased from commercial sources. All other chemicals were of reagent grade quality and purchased from Sigma-Aldrich. The ^{1}H- and ^{13}C-NMR spectra were plotted on a Varian Oxford-500 spectrometer. High-resolution mass spectra (HRMS) were recorded using a Bruker BioTOF II ESI mass spectrometer. Elemental analysis (CHN) results were obtained from Atlantic Microlab services.
44. *Cell culture conditions and cytotoxicity:* 4T1 cells (ATCC): RPMI-1640 supplemented with 10% FBS and penicillin-streptomycin (50U/ml, 50µg/ml). MCF7 cells (ATCC): α-MEM (500 mL) supplemented with EGF (6.25 µg), hydrocortisone (0.5 mg), non-essential amino acids (1%), insulin (0.5 mg), HEPES (10 mM), sodium pyruvate (1 mM), and 30 mL FBS. MIA PaCa-2 cells (ATCC): DMEM supplemented with 10% FBS, 2.5% horse serum, and penicillin-streptomycin (50U/ml, 50µg/ml). WiDr cells (ATCC): MEM supplemented with 10% FBS and penicillin-streptomycin (50U/ml, 50µg/ml). Cancer cells were seeded in 96-well plate at a density of $5x10^3$ cells/well and incubated for 18-24 hours. Test compounds were added into the wells at various concentrations and incubated for further 72 hours. 10 µL of MTT (5 mg in 1 mL of 1X PBS) was added into the wells and incubated for 4 hours followed by the addition of 100 µL of 10% SDS in 0.01N HCl was added to quench the reaction. The plates were further incubated for 4 hours and the absorbance was recorded at 570 nm using BioTek Synergy 2 SLFA microplate reader. IC_{50} was calculated using Graphpad Prism software by plotting absorbance on y-axis and log[C] on x-axis.
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47. *Seahorse XFe96® based mitochondrial and glycolysis stress tests:* 20,000 cells per well were seeded in a Seahorse XF 96-well microplate and incubated 18-24 hours at 37ºC at 5% CO_2. For MST, cells were treated with test compounds, followed by the addition of oligomycin (ATP synthase inhibitor), FCCP (proton uncoupler), and rotenone+antimycin A (mitochondrial complex I and III inhibitors, respectively), at regular time intervals. For GST, the cells were treated with test compounds, followed by the addition of glucose (glycolysis initiator), oligomycin (ATP synthase inhibitor) and 2-deoxyglucose (glycolysis inhibitor) at regular time intervals. OCR and ECAR were recorded in real-time for MST and GST, respectively, using a Seahorse XFe96®analyzer (Agilent). Glycolytic and mitochondrial parameters were calculated using the Wave 2.4.0 software (Agilent). *Statistical Analysis:* Statistics were computed using GraphPad Prism version 7.0. Repeated measures

untreated groups for all Seahorse studies. A *P*-value of <0.05 was considered significant.

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49. *Epifluorescent microscopy experiments of mitochondrial moprphology:* 4T1 and WiDr cells (100,000cells/dish) were plated in glass bottom 35mm MatTek dishes (MatTek Corp, #P35G010C) and were incubated at 37ºC and 5% CO_2 for 48 hours. Cells were then exposed to compound 6a-6c (100µM) or vehicle (DMSO, 0.1% w/v) for 24 hours. Cells were then exposed to Mitotracker- and 5% CO_2. Media was then aspirated and replaced with PBS supplemented with 5% FBS for imaging. Cells were imaged using a Nikon TE2000 epifluorescent microscope and a Photometrics Dyno CCD camera.
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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: