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Effects of Selected Inhibitors of Protein Kinases and Phosphatases on Cellular Respiration: An *In Vitro* Study

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Abstract

Inhibitors of protein kinases/phosphatases are known to alter cellular metabolism. Effects of these rapidly identified small molecules on cellular respiration (mitochondrial O_2 consumption) have not been adequately investigated, especially in healthy organs. This in vitro study measured cellular respiration in tissues from C57BL/6 mice with and without GSK2126458 (PI3K/mTOR inhibitor), BEZ235 (PI3K/mTOR inhibitor), GDC0980 (PI3K/mTOR inhibitor), GSK1120212 (trametinib, MEK inhibitor), sorafenib, regorafenib (multikinase inhibitors), and cyclosporine (calcineurin inhibitor). Cellular respiration was measured by the phosphorescence oxygen analyzer, aided by the O_2 probe Pd(II)-meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin. Cyanide inhibited O_2 consumption, confirming the oxidation occurred in the respiratory chain. Renal cellular respiration decreased 26-34% in the presence of 10 μM GSK2126458 ($p<0.001$), 10 μM BEZ235 ($p<0.001$), or 1.0 μM GDC0980 ($p<0.001$). Liver cellular respiration decreased 20-32% with 10 μM GSK2126458 ($p=0.048$), 0.1 μM BEZ235 ($p=0.028$), or 0.1 μM GDC0980 ($p=0.016$). Heart cellular respiration decreased 19-27% with 10 μM GSK2126458 ($p=0.078$), 10 μM BEZ235 ($p=0.040$), or 10 μM GDC0980 ($p=0.036$). GSK1120212, sorafenib, regorafenib, and cyclosporine had no effects on cellular respiration. Thus, cellular bioenergetics (the biochemical processes involved in energy conversion) is interconnected with PI3K/PTEN/Akt/mTOR; and inhibitors of this cascade impair cellular respiration. This biomarker (cellular respiration) senses the activity/toxicity of this class of molecularly targeted agents.

Keywords: Cellular respiration; PI3K inhibitor; mTOR inhibitor; MEK-1/2 inhibitor; Multikinase inhibitor; Calcineurin inhibitor

Abbreviations

PI3K: Phosphoinositide 3-kinase; Mtor: Mammalian Ttarget of Rapamycin; MEK: Mitogen-activated Protein/extracellular Signal-regulated Kinase; PBS: Phosphate-buffered Saline; Pd phosphor: Pd(II) Complex of Meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin; $1/\tau$: Phosphorescence Decay Rate; k: Rate of Cellular Respiration (in μM O_2 min^{-1}); kc: Corrected Rate of Cellular Respiration (in μM O_2 min^{-1} mg^{-1}).

Introduction

Intracellular kinase-dependent signals regulate vital cellular functions including nutrient transport, metabolic reactions, proliferation, and response to toxins [1]. Inhibitors of some of these enzymes (protein kinases and phosphatases) have been developed for treatment of various human diseases (e.g., cancer and aberrant immune responses) [2,3]. Many of these small molecules are in clinical use as monotherapy or with conventional cytotoxic agents. This therapeutic approach promotes apoptosis by targeting critical processes, such as survival pathways (e.g., PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK) and cellular bioenergetics (metabolic reactions involving cellular respiration and production of adenosine triphosphate (ATP)) [4,5].

Studies addressing the effects of these agents targeting biomolecules on organ functions are limited, as drug development studies focus on derangements involving target organs. Agents that target regulators of the metabolic pathways are in clinical development and many (e.g., inhibitors of MEK, PI3K, mTOR, and Akt) are in phase 3 clinical trials [4] and one is recently approved (the PI3K inhibitor idelalisib). Inhibiting these signals is expected to disturb the metabolic processes of normal tissue. Blocking PI3K, for example, causes multifaceted alternations in glucose transport/ metabolism including feedback activation of insulin signals. Inhibition of mTOR activates AMP-activated protein kinase, resulting in improved cellular bioenergetics, including increased substrate-level phosphorylation and ↑oxidative phosphorylation [4].

This study investigated the effects of selected inhibitors of PI3K, mTOR, MEK, sorafenib family multikinases, and calcineurin (a calcium-dependent serine-threonine phosphatase) on renal, cardiac, and hepatic cellular respiration.

Methods

Reagents and solutions

GSK2126458 (PI3K inhibitor, cat. #HY-10297), BEZ235 (PI3K/mTOR inhibitor, cat. #HY-15174), GDC0980 (PI3K/mTOR inhibitor, cat. #HY-13246), and GSK1120212 (MEK inhibitor, cat. #HY-10999) were purchased from MedChem Express, LLC (Princeton, NJ). Sorafenib (multikinase inhibitor) and regorafenib (multikinase inhibitor) were purchased from Selleck Chemicals (Houston, TX, USA). All compounds were dissolved in DMSO (5 mg/mL) and stored at -20°C. The immunosuppressant cyclosporine (calcineurin inhibitor, m.w. 1202.61; dissolved in DMSO at 50 mg/dL and stored at -20°C) was purchased from MedChem Express, LLC (Princeton, NJ). Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Pd phosphor (2.5 mg/mL=2 mM) and Na cyanide (1.0 M) were stored at -20°C. Roswell Park Memorial Institute medium (RPMI) 1640 and remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

C57BL/6 (9-10 weeks old) mice were housed at the animal facility in rooms maintained at 22°C, 60% humidity and 12-h light-dark cycles. The mice had ad libitum access to standard rodent chow and filtered water. The study received approval from the Animal Ethics Committee-College of Medicine and Health Sciences (A29-13; in vitro assessment of the effects of nephrotoxic drugs and toxins on renal cellular respiration in mice).

Tissue collection and processing

Urethane (25% w/v, 100 µL per 10 g) was used to anesthetize the mice. Tissue fragments (10 to 20 mg each) were cut manually with sterile scalpels (Swann-Morton, Sheffield, England) and immediately processed for measuring cellular respiration in the presence and absence of designated concentrations of the drugs [6,7]. Alternately, specimens were incubated at 37°C in 50 mL RPMI or phosphate-buffered saline (gassed with 95% O_2: 5% CO_2) with and without the drugs for up to 6 h; at designated times, samples were removed from the incubation solution and processed for measuring cellular respiration [6,7].

Cellular respiration

The phosphorescence O_2 analyzer was used to measure O_2 consumption [6-9]. Briefly, samples were exposed to 600 per min pulsed flashes. O_2 concentration was measured with the Pd phosphor, 625 nm absorption/800 nm emission. The phosphorescence was detected by the Hamamatsu photomultiplier tube (#928). The phosphorescence decay rate ($1/\tau$) was exponential; $1/\tau$ was linear with O_2 concentration: $1/\tau=1/\tau_o+ k_q[O_2]$, $1/\tau$=phosphorescence decay rate with O_2, $1/\tau_o$=phosphorescence decay rate without O_2, and k_q=second-order O_2 quenching rate constant ($s^{-1}\bullet\mu M^{-1}$) [8]. Rate of respiration (k, $\mu M\ O_2\ min^{-1}$) was the negative of the slope $d[O_2]/dt$. The value of k was divided by specimen weight (k_c, $\mu M\ O_2\ min^{-1}\ mg^{-1}$). A program was developed using Microsoft Visual Basic 6, Microsoft Access Database 2007, and Universal Library components (Universal Library for Measurements Computing Devices), which allowed direct reading from the PCI-DAS 4020/12 I/O Board (PCI-DAS 4020/12 I/O Board) [9]. O_2 measurements were performed at 37°C in sealed glass vials. Respiratory substrates were endogenous metabolic fuels and nutrients (e.g., glucose) in RPMI. $[O_2]$ decreased linearly with time. This zero-order process was inhibited by cyanide, confirming O_2 consumption occurring due to the process of respiration [6,7].

Statistical analysis

Data were analyzed on SPSS statistical package (version 19), using the nonparametric (2 independent samples) Mann-Whitney test.

Results

Figure 1A shows representative runs of renal cellular O_2 consumption in RPMI with and without the PI3K/mTOR inhibitor GSK2126458. Each run represented a specimen that was collected from a mouse and processed for measuring cellular respiration immediately after collection. A summary of the results is shown in Figure 1C. The rate of respiration (k_c in $\mu M\ O_2\ min^{-1}\ mg^{-}$ mean ± SD) without addition (control) was 0.99 ± 0.24 (n=11 mice) and with the addition of 10 µM GSK2126458 was 0.74 ± 0.18 (n=11 mice, p=0.023), Figure 1C. Thus, GSK2126458 significantly decreased renal cellular respiration by 25%.

The same experiments were repeated in phosphate-buffered saline (PBS). Representative runs are shown in Figure 1B and a summary of the results in Figure 1D. The value of k_c without addition was 0.90 ± 0.06 $\mu M\ O_2\ min^{-1}\ mg^{-1}$ (n=7 mice) and with the addition of 10 µM GSK2126458 was 0.68 ± 0.11 $\mu M\ O_2\ min^{-1}\ mg^{-1}$ (24% lower, n=6 mice, p=0.004), Figure 1D. Thus, the inhibition of renal cellular respiration by GSK2126458 was independent of nutrients (e.g., glucose) present in RPMI.

Figure 2A shows representative runs of renal cellular mitochondrial O_2 consumption in samples incubated *in vitro* with and without GSK2126458 (PI3K/mTOR inhibitor), GSK1120212 (MEK inhibitor), or both agents. Briefly, several renal specimens were collected from one mouse and incubated at 37°C in RPMI with and without the compounds. At designated times; specimens were removed from incubation solutions, rinsed with RPMI, and processed for measuring O_2 consumption at 37°C. A summary of the results is shown in figure 2B (five separate experiments, five mice, 8-15 runs per condition). The rate of respiration (k_c in $\mu M\ O_2\ min^{-1}\ mg^{-1}$) without addition was 0.53 ± 0.14 (n=15 mice), with 10 µM GSK2126458 was 0.39 ± 0.09 (26% lower, p=0.031), with 10 µM GSK1120212 was 0.47 ± 0.08 (11% lower, p=0.397), and with 10 µM of both compounds was 0.35 ± 0.07 (34% lower, p=0.004). Thus, renal cellular respiration was significantly decreased in the presence of the PI3K/mTOR inhibitor GSK2126458, but not the MEK inhibitor GSK1120212. The degree of inhibition was similar to that observed over the shorter incubation discussed above.

It is worth noting, however, that the values of kc in untreated specimens incubated for ≤6 h (Figure 2B) were significantly lower than untreated specimens without incubation (Figure 1C), p<0.001. This finding reflects deterioration of the renal tissue *in vitro*. More pronounced deteriorations were observed in heart and liver specimens (data not shown). Therefore, the remaining experiments were performed as described for Figures 1B and 1C).

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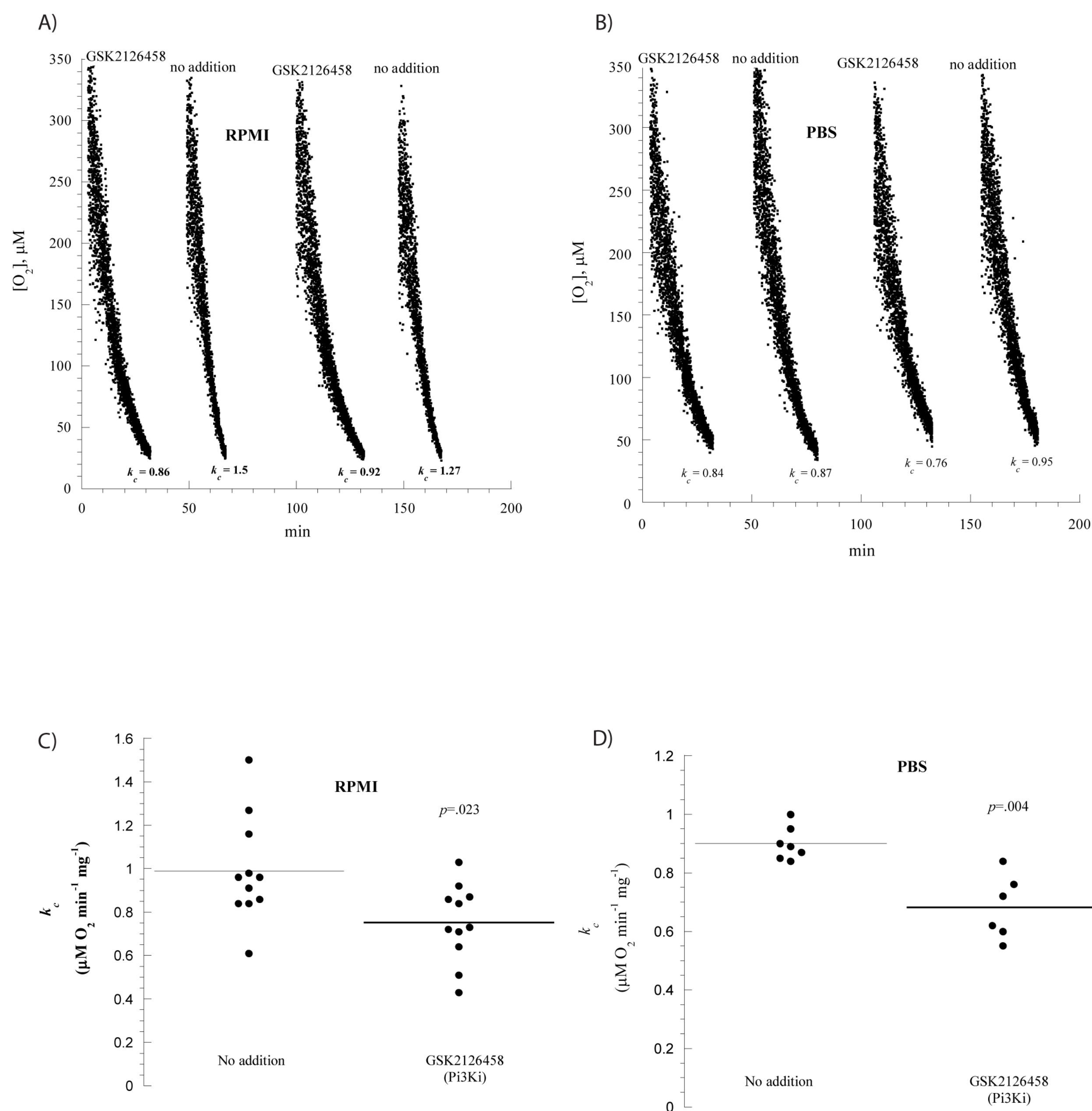

Figure 1: Effects of the PI3K inhibitor GSK2126458 on renal cellular respiration. Panels A-B: Each run represented a renal specimen that was collected from a C57BL/6 mouse and processed immediately for measuring cellular respiration in RPMI (A) or PBS (B) with and without the addition of 10 µM GSK2126458. Rate of respiration (k, µM O_2 min^{-1}) was the negative of the slope of $[O_2]$ vs. t. The values of kc (µM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run. Panels C-D are summaries of all measurements in RPMI (C; 11 separate experiments, 11 mice per condition) and PBS (D; six separate experiments, 7 mice for untreated condition and 6 mice for treated condition). The lines are means.

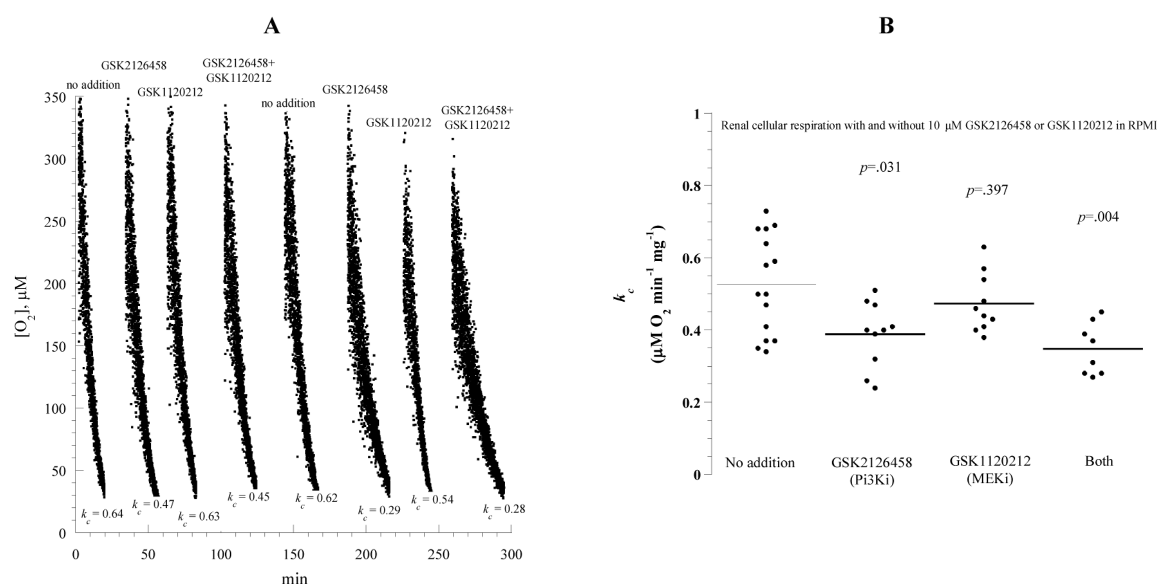

Figure 2: Effects of GSK2126458 (PI3K inhibitor) and GSK1120212 (MEK inhibitor) on renal cellular respiration. Panel A: Representative runs of renal cellular respiration. Multiple renal specimens were collected from a C57BL/6 mouse and incubated at 37°C in RMPI with and without the addition of 10 μM GSK2126458 alone, 10 μM GSK1120212 alone or combination of both compounds. At designated periods, specimens were removed from the incubation solution, rinsed with RPMI, and then placed in the O_2 vial for measuring cellular respiration. Rate of respiration (k, μM O_2 min^{-1}) was the negative of the slope of $[O_2]$ vs. t. The values of k_c (μM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run. Panel B is a summary of all measurements in RPMI (five separate experiments, one mouse per experiment, 8-15 runs per condition); the lines are means.

	Drug Concentration	**kc (μM O_2 min^{-1} mg^{-1})**	**Inhibition (%)**	**P**
GSK2126458 (PI3K/mTOR inhibitor)	0	1.12 ± 0.27 (22)	-	-
	0.1 μM	1.36 ± 0.23 (4)	0	0.150
	1.0 μM	1.02 ± 0.19 (9)	9	0.453
	10 μM	0.74 ± 0.18 (11)	34	<0.001
BEZ235 (PI3K/mTOR inhibitor)	0	0.72 ± 0.18 (18)	-	-
	0.1 μM	0.75 ± 0.20 (8)	0	0.724
	1.0 μM	0.62 ± 0.15 (16)	16	0.126
	10 μM	0.40 ± 0.13 (10)	31	<0.001
GDC0980 (PI3K/mTOR inhibitor)	0	0.93 ± 0.20 (12)	-	-
	0.1 μM	0.98 ± 0.21 (7)	0	0.400
	1.0 μM	0.62 ± 0.07 (8)	27	<0.001
	10 μM	0.64 ± 0.10 (12)	26	0.003
GSK1120212 (MEK inhibitor)	0	1.30 ± 0.32 (4)	-	-
	10 μM	1.03 ± 0.18 (7)	21	0.164
Cyclosporine (calcineurin inhibitor)	0	1.07 ± 0.17 (4)	-	-

	10 µM	1.00 ± 0.23 (8)	7	0.461
Sorafenib (multikinase inhibitor)	0	0.96 ± 0.18 (6)	-	-
	10 µM	0.90 ± 0.22 (12)	6	0.494
Regorafenib (multikinase inhibitor)	0	1.02 ± 0.28 (4)	-	-
	10 µM	1.01 ± 0.20 (8)	0	1.00

Table 1: Effects of selected inhibitors of protein kinases and phosphatases on renal cellular respiration. For each measurement, a renal fragment was collected from C57BL/6 mouse and processed immediately for measuring cellular respiration in RPMI with and without designated concentrations of the drugs. The values of k_c are mean ± SD (n); n=number of animals. Each measurement is a separate experiment.

Tables 1 and 2 summarize the results of the studied compounds in renal, cardiac, and hepatic tissues. All measurements were determined in RPMI with and without designated drugs exactly as described for Figures 1B and 1C).

As shown, the PI3K/mTOR inhibitors GSK2126458, BEZ235, and GDC0980 significantly inhibited cellular respiration in the three studied organs (Tables 1 and 2). The inhibition was concentration-dependent; 100 nM BEZ235 or GDC0980 significantly inhibited hepatic cellular respiration (Table 2). GSK1120212 (MEK inhibitor), cyclosporine (calcineurin inhibitor), sorafenib (multikinase inhibitor), and regorafenib (multikinase inhibitor), on the other hand, had no significant effect on cellular respiration (Table 1).

		Drug Concentration	**k_c (µM O_2 min^{-1} mg^{-1})**	**Inhibition (%)**	**P**
Heart	**GSK2126458**	0	0.48 ± 0.09 (6)	-	-
		10 µM	0.39 ± 0.12 (11)	19	0.078
	BEZ235	0	0.33 ± 0.08 (8)	-	-
		0.1 µM	0.26 ± 0.07 (6)	21	0.142
		10 µM	0.24 ± 0.12 (7)	27	0.040
	GDC0980	0	0.37 ± 0.11 (8)	-	-
		10 µM	0.29 ± 0.08 (16)	22	0.038
Liver	**GSK2126458**	0	0.93 ± 0.20 (6)	-	-
		10 µM	0.73 ± 0.18 (11)	22	0.048
	BEZ235	0	0.87 ± 0.16 (4)	-	-
		0.1 µM	0.60 ± 0.12 (8)	20	0.028
	GDC0980	0	1.06 ± 0.24 (4)	-	-
		0.1 µM	0.72 ± 0.16 (8)	32	0.016

Table 2: Effects of the PI3K/mTOR inhibitors on heart and liver cellular respiration. For each measurement, a tissue fragment was collected from C57BL/6 mouse and processed immediately for measuring cellular respiration in RPMI with and without designated concentrations of the drugs. The values of k_c are mean ± SD (n); n=number of animals. Each measurement is a separate experiment.

Discussion

The adverse impact of disrupting PI3K signaling on cellular respiration is demonstrated here using highly selective PI3K/mTOR inhibitors (GSK2126458, BEZ235, and GDC0980) with three vital organs (the heart, liver, and kidney), Tables 1 and 2. These results are consistent with the well-known roles of PI3K/PTEN/Akt/mTOR pathway in cell metabolism, including insulin and insulin signaling [4].

GSK1120212 (trametinib, an oral MEK1/2 inhibitor) has no effect on renal cellular respiration (Table 1), even when the incubation is extended to about 6 h (Figures 2A and 2B). The combination of GSK1120212 and GSK2126458, however, is more potent than GSK2126458 alone (apparently additive, Figure 2B). GSK1120212 is effective in melanoma and colorectal cancer (inducing cell cycle arrest). The drug is typically given with other targeted therapies [10,11].

The sorafenib family multikinase inhibitors (sorafenib and regorafenib) and cyclosporine have no effect on cellular respiration (Table 1). The results are consistent with a recent finding that the multikinase inhibitors are much more effective when combined with

PI3K inhibitors [12]. The results are also consistent with a previous study showing cyclosporine has no effect on cell line respiration [13].

It is important to explain the rational for using relatively high drug concentrations (≥100 nM, Tables 1 and 2) to elicit the cellular response to blocking the PI3K signals. First, drug exposure time is relatively brief (≤1 h). Second, distribution of the drug in *ex vivo* tissue fragments is relatively slow. Due to these experimental limitations, it is unclear whether concentrations <100 nM are inhibitory over a longer incubation time.

Signal transduction controls vital cellular processes including: metabolism, growth, and survival [1-5]. Consistently, derangements in cellular bioenergetics are expected in therapies that alter cell signaling [14]. The PI3K/PTEN/Akt/mTOR pathway regulates cell glucose uptake [15]. Consistently, insulin resistant hyperglycemia is a common dose-limiting toxicity of PI3K inhibitors [16]. This adverse bioenergetic event, however, has not been adequately investigated in off-target organs, such as the heart, liver, and kidney. The results in Tables 1 and 2 show the studied PI3K/ mTOR inhibitors impair cellular respiration in these vital body organs. This finding suggests monitoring therapies that block PI3K/mTOR signaling should include biomarkers of cellular bioenergetics.

The activities of BEZ235 and GSK2126458 were compared in tamoxifen-resistant breast cancer cell lines. Both drugs inhibited AKT signaling, but BEZ235 was a more potent inhibitor of p70S6K and rpS6 [17].

The rapamycin derivatives temsirolimus and everolimus (mTOR-targeted therapies) are approved treatments for renal cell carcinoma [18]. Exposure to these drugs leads to feedback activation of PI3K-PTEN-AKT, which overcomes the effects of inhibiting mTOR [19]. BEZ235, on the other hand, targets both mTOR and PI3K, preventing the undesirable feedback activation of PI3K [20].

Signals through PI3K/PTEN/Akt/mTOR integrate critical processes between the plasma membrane and cellular compartments. Aberrant pathways have been implicated in numerous cancers [2,3]. Consistently, increased phosphatidylinositol 3,4,5-trisphosphate, generated by the activity of PI3K, has been associated with tumor survival; and agents that block PI3K have been shown to induce rapid apoptosis [4,5]. Activities of these rapidly identified small molecules have been linked to suppressing cellular metabolism [21]. Some of these drugs (sirolimus, cyclosporine, and tacrolimus) are potent immunosuppressants, and monitoring their cytotoxicities requires novel systems, such as the one described here.

Mitochondria use energy derived from oxidations in the respiratory chain to generate adenosine 5'-triphoshate (oxidative phosphorylation). These vital organelles respond to apoptotic signals by releasing pro-apoptotic molecules that trigger the caspase (*cys*teine-dependent *asp*artate-directed prote*ase*) cascade. Caspase activation leads to mitochondrial perturbation, which involves opening mitochondrial permeability transition and collapsing the electrochemical potential. Thus, induction of apoptosis and mitochondrial dysfunction are coupled processes. These facts emphasize the need for using mitochondrial biomarkers for monitoring adverse events of molecularly targeted therapies.

Caspase activation is more likely to result in cell death in the presence of mitochondrial dysfunction (impaired cellular bioenergetics). This cellular dependency on aerobic metabolism does not necessarily apply to cancer cells, which are more capable of surviving on anaerobic metabolism ("aerobic glycolysis" or Warburg effect) [22]. It is worth noting that other biomarkers of mitochondrial function include modulators of thioredoxin/thioredoxin-interacting protein [23] and death-associated protein kinase interactome [24]. These important reactions are involved in mitochondria respiration, permeability, and electrochemical potential, generation of reactive O_2 species, induction of caspases, and regulation of calcium homeostasis. O_2 consumption, however, is the easiest method of monitoring drug-induced cellular toxicities.

In summary, inhibitors of PI3K/ mTOR invoke potent inhibitory effects on the cellular respiration of vital organs (Figures 1 and 2, Tables 1 and 2). Inhibitors of MEK, sorafenib family multikinases, and calcium-dependent serine-threonine phosphatase have no effects on cellular respiration (Table 1). Cellular respiration is a useful tool for assessing compounds that inhibit PI3K/mTOR signaling.

Author Contribution

Saeeda Almarzooqi, Alia Albawardi, Ali Alfazari, Robert Mallon, and Abdul-Kader Souid designed the study, carried out the analysis, interpreted the data, and drafted the manuscript. Sami Shaban programed the oxygen analyzer and performed the data analysis. Dhanya Saraswathiamma and Hidaya Mohammed Abdul-Kader performed the oxygen measurements. All authors read, edited, and approved the final manuscript.

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