

Original Article

The mTOR inhibitor sirolimus suppresses renal, hepatic, and cardiac tissue cellular respiration

Alia Albawardi¹, Saeeda Almarzooqi¹, Dhanya Saraswathiamma¹, Hidaya Mohammed Abdul-Kader², Abdul-Kader Souid³, Ali S Alfazari²

Departments of ¹Pathology, ²Medicine, ³Pediatrics, UAE University, Al-Ain 17666, Abu Dhabi, United Arab Emirates

Received November 23, 2014; Accepted March 2, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: The purpose of this *in vitro* study was to develop a useful biomarker (e.g., cellular respiration, or mitochondrial O₂ consumption) for measuring activities of mTOR inhibitors. It measured the effects of commonly used immunosuppressants (sirolimus-rapamycin, tacrolimus, and cyclosporine) on cellular respiration in target tissues (kidney, liver, and heart) from C57BL/6 mice. The mammalian target of rapamycin (mTOR), a serine/ threonine kinase that supports nutrient-dependent cell growth and survival, is known to control energy conversion processes within the mitochondria. Consistently, inhibitors of mTOR (e.g., rapamycin, also known as sirolimus or Rapamune®) have been shown to impair mitochondrial function. Inhibitors of the calcium-dependent serine/threonine phosphatase calcineurin (e.g., tacrolimus and cyclosporine), on the other hand, strictly prevent lymphokine production leading to a reduced T-cell function. Sirolimus (10 µM) inhibited renal (22%, *P* = 0.002), hepatic (39%, *P* < 0.001), and cardiac (42%, *P* = 0.005) cellular respiration. Tacrolimus and cyclosporine had no or minimum effects on cellular respiration in these tissues. Thus, these results clearly demonstrate that impaired cellular respiration (bioenergetics) is a sensitive biomarker of the immunosuppressants that target mTOR.

Keywords: mTOR, rapamycin, sirolimus, tacrolimus, cyclosporine, O₂ consumption, oxidative phosphorylation

Introduction

The mTOR (a serine/threonine kinase) pathway is known to regulate cellular bioenergetics (the metabolic reactions involved in biotransformation including cellular respiration and accompanying ATP synthesis) [1-3]. Repressing these signals disturbs the metabolism of normal tissue (e.g., alternations in nutrient transport/metabolism) including feedback activation of growth (e.g., insulin) signals [4, 5]. Inhibitors of mTOR (e.g., sirolimus) have been developed for treatment of various human diseases including immune disorders (inducing suppressive effect on the immune system) and cancer (promoting apoptosis) [6-8]. For example, the rapamycin derivatives temsirolimus and everolimus are approved drugs for treatment of renal cell carcinoma [9].

Inhibitors of calcineurin (a calcium-dependent serine/threonine phosphatase), such as tacrolimus (FK-506) and cyclosporine are also commonly used immunosuppressants to prevent graft rejection and to treat a variety of immune

conditions. These potent drugs block critical signaling pathways involved in T cell-mediated adaptive immune responses as well as myeloid cell-mediated innate immune responses [10]. Their effects on cellular respiration (the process of delivering nutrients and O₂ to the mitochondria, oxidation of reduced metabolic fuels, passage of electrons to O₂, and synthesis of ATP) in cell lines and murine kidney have been shown to be insignificant [11, 12].

This study investigated the effects of sirolimus, tacrolimus, and cyclosporine on renal, hepatic, and cardiac cellular respiration using murine tissue. Its main purpose was to use cellular respiration as a surrogate biomarker to differentiate mTOR inhibitors from other molecularly targeted immunosuppressants.

Methods

Reagents and solutions

Sirolimus (rapamycin, mTOR inhibitor, m.w. 914.172; dissolved in DMSO at 5 mg/mL) and

Sirolimus impairs tissue cellular bioenergetics

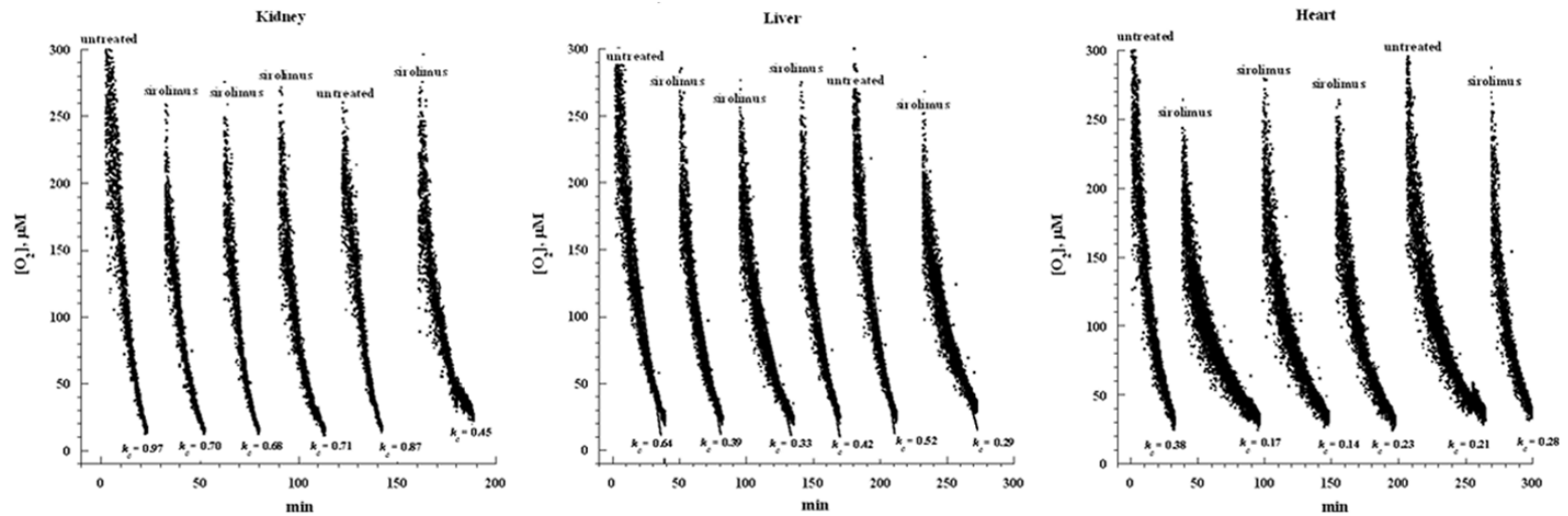


Figure 1. Effects of the mTOR inhibitor sirolimus on renal, hepatic, and cardiac cellular respiration. Representative runs are shown. Each run represented a specimen that was collected from a C57BL/6 mouse and processed immediately for measuring cellular respiration with and without the addition of 10 µM sirolimus. 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. The lines are linear fit.

Sirolimus impairs tissue cellular bioenergetics

Table 1. Effects of the mTOR inhibitor sirolimus on cellular respiration

	Drug Concentration	k_c ($\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$)	Inhibition (%)	<i>P</i>
Kidney	0	0.86 ± 0.11 (8)	-	-
	1.0 μM	0.80 ± 0.07 (8)	7	0.195
	10 μM	0.67 ± 0.09 (8)	22	0.002
Liver	0	0.59 ± 0.08 (8)	-	-
	1.0 μM	0.51 ± 0.07 (8)	14	0.065
	10 μM	0.34 ± 0.05 (7)	39	< 0.001
Heart	0	0.31 ± 0.06 (6)	-	-
	1.0 μM	0.31 ± 0.10 (4)	0	1.0
	10 μM	0.19 ± 0.05 (8)	42	0.005

Tissue fragments were collected from C57BL/6 mice and immediately processed for measuring cellular respiration in RPMI with and without the designated drugs. The values of k_c are mean \pm SD (n).

tacrolimus (FK-506, fujimycin, calcineurin inhibitor, m.w. 804.018; dissolved in DMSO at 5 mg/mL) were purchased from MedChem Express, LLC (Princeton, NJ). Cyclosporine (calcineurin inhibitor, m.w. 1202.61; 50 mg/mL) was purchased from Perrigo® (Minneapolis, MN). All compounds were stored at -20°C .

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, made in dH_2O) and Na cyanide (1.0 M) solutions were stored at -20°C . RPMI 1640 medium and remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

C57BL/6 (10 weeks old) mice were housed at 22°C , 60% humidity, and 12-h light-dark cycles. They had *ad libitum* access to standard rodent chow and filtered water. The study was approved 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 as anesthetic agent. Tissue fragments (10-20 mg) were quickly cut with a sterile scalpel (Swann-Morton, Sheffield, England) and immediately placed in the oxygen vial for measuring cellular respiration at 37°C as described below. The vial contained 1.0 mL RPMI, 3 μM Pd phosphor, 0.5% fat-free albumin, and designated

concentration of the drugs (treated conditions) or DMSO (untreated conditions) [13].

Cellular respiration

The Pd phosphor (625 nm absorption and 800 nm emission) was used for O_2 detection [14]. The phosphorescence was detected by Hamamatsu photomultiplier tube. Samples were exposed to pulsed flashes (600/min). The phosphorescence decay rate ($1/\tau$) was exponential; $1/\tau$ was linear with O_2 concentration: $1/\tau = 1/\tau^\circ +$

$k_q[\text{O}_2]$, $1/\tau =$ phosphorescence decay rate with O_2 , $1/\tau^\circ =$ phosphorescence decay rate without O_2 , and $k_q =$ second-order O_2 quenching rate constant ($\text{s}^{-1} \mu\text{M}^{-1}$) [16]. The rate of respiration (k , $\mu\text{M O}_2 \text{ min}^{-1}$) was the negative of the slope $d[\text{O}_2]/dt$.

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) [17].

O_2 measurements were performed at 37°C in 1-mL sealed glass vials containing 1.0 mL RPMI, 3 μM Pd phosphor solution, 0.5% fat-free albumin, and studied drug or its vehicle. The respiratory substrates were endogenous metabolic fuels and nutrients in RPMI (e.g., glucose). $[\text{O}_2]$ decreased linearly with time; this zero-order process was inhibited by cyanide, confirming O_2 consumption occurred in the respiratory chain [12-15].

Statistical analysis

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

Results

Figure 1 shows representative runs of cellular mitochondrial O_2 consumption with and without the mTOR inhibitor sirolimus. Each run represented a specimen that was collected from the

Sirolimus impairs tissue cellular bioenergetics

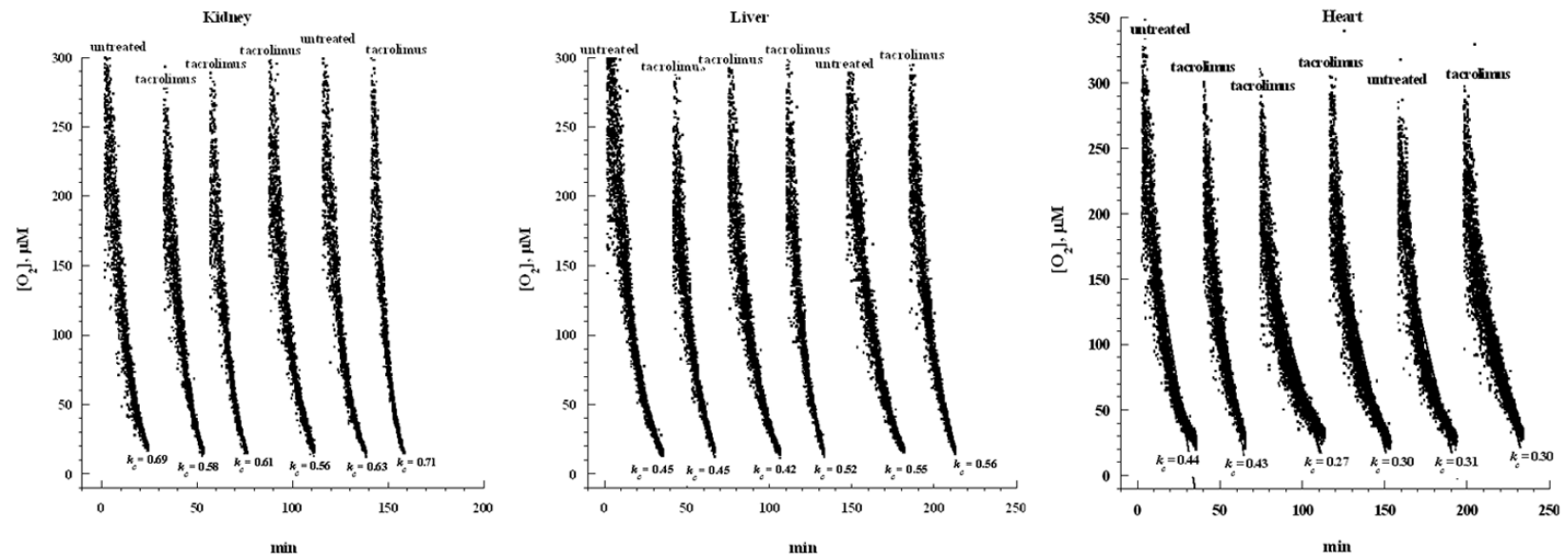


Figure 2. Effects of the calcineurin inhibitor tacrolimus on renal, hepatic, and cardiac cellular respiration. Representative runs are shown. Each run represented a specimen that was collected from a C57BL/6 mouse and processed immediately for measuring cellular respiration with and without the addition of $10 \mu\text{M}$ tacrolimus. Rate of respiration (k_c , $\mu\text{M O}_2 \text{ min}^{-1}$) was the negative of the slope of $[O_2]$ vs. t . The values of k_c ($\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$) are shown at the bottom of each run. The lines are linear fit.

Sirolimus impairs tissue cellular bioenergetics

Table 2. Effects of the calcineurin inhibitor tacrolimus on cellular respiration

	Drug Concentration	k_c ($\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$)	Inhibition (%)	<i>P</i>
Kidney	0	0.71 ± 0.08 (6)	-	-
	1.0 μM	0.73 ± 0.09 (4)	0	0.914
	10 μM	0.58 ± 0.12 (8)	18	0.043
Liver	0	0.53 ± 0.05 (4)	-	-
	10 μM	0.54 ± 0.10 (8)	0	0.933
Heart	0	0.33 ± 0.08 (4)	-	-
	10 μM	0.32 ± 0.05 (8)	3	0.927

Tissue fragments were collected from C57BL/6 mice and immediately processed for measuring cellular respiration in RPMI with and without the designated drugs. The values of k_c are mean ± SD (n).

kidney, liver, or heart and immediately placed in the vial for measuring cellular respiration at 37°C in RPMI with and without the addition of sirolimus. A summary of all results is shown in **Table 1**. The rate of renal cellular respiration (k_c in $\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$, mean ± SD) without addition was 0.86 ± 0.11 (n = 8 mice), with the addition of 1.0 μM sirolimus was 0.80 ± 0.07 (n = 8 mice, $P = 0.195$), and with the addition of 10 μM sirolimus was 0.67 ± 0.09 (n = 8 mice, $P = 0.002$). Thus, sirolimus (10 μM) significantly decreased renal cellular respiration (22%). Consistently, sirolimus (10 μM) significantly decreased hepatic (39%, $P < 0.001$) and cardiac (42%, $P = 0.005$) cellular respiration (**Table 1**).

Figure 2 shows representative runs of cellular mitochondrial O_2 consumption with and without the calcineurin inhibitor tacrolimus. The experiments were performed exactly as described above. A summary of all results is shown in **Table 2**. Tacrolimus (10 μM) slightly decreased renal cellular respiration ($P = 0.043$). Otherwise, the drug had no effects on hepatic ($P = 0.933$) or cardiac ($P = 0.927$) cellular respiration (**Table 2**).

Figure 3 shows representative runs of cellular mitochondrial O_2 consumption with and without the calcineurin inhibitor cyclosporine. The experiments were performed as described above. A summary of all results is shown in **Table 3**. Cyclosporine (10 μM) had no effects on renal ($P = 0.841$), hepatic ($P = 0.933$), or cardiac ($P = 0.109$) cellular respiration (**Table 3**).

Discussion

The deleterious effects of disrupting mTOR signaling on cellular respiration are demonstrated

here in three vital organs (the kidney, liver, and heart), using the highly selective kinase inhibitor sirolimus (rapamycin), **Table 1** and **Figure 1**. These results are consistent with the known role of mTOR in regulating energetic metabolic processes including: cellular respiration (mitochondrial O_2 consumption), nutrient transport, lipogenesis, lipolysis, and protein synthesis [1, 2]. Consistently, sirolimus has been shown to impair murine myo-

cyte respiration [3]. Inhibition of mTOR also has been shown to activate AMP-activated protein kinase (catalyzes the reaction: $\text{AMP} + \text{ATP} \rightarrow 2\text{ADP}$), resulting in improved cellular bioenergetics (\uparrow substrate-level phosphorylation and \uparrow oxidative phosphorylation) [4]. The results here demonstrate that inhibition of cellular respiration dominates the sirolimus biologic effects, perhaps due to the high drug concentration (10 μM) used in this study. It is worth noting, however, that the 10 μM dose was employed in order to elicit the cellular response at a relatively brief exposure (about one hour). Due to this experimental limitations, it is unclear whether concentrations < 10 μM are inhibitory over a longer incubation time. Inhibitors of calcineurin (tacrolimus and cyclosporine), on the other hand, show no or minimum effects on cellular respiration (**Tables 2, 3; Figures 2, 3**) [11, 12].

Activities of the rapidly emerging small molecules that inhibit cellular signaling have been linked to suppressing the metabolism [2-4, 18]. Some of these drugs are potent immunosuppressants, and monitoring their cytotoxicities requires novel systems, such as the one described here.

Suppressing effects of the mTOR inhibitor sirolimus (**Table 1**), and the previously described dual PI3K/mTOR inhibitors GSK2126458, BEZ235, and GDC0980 [12], support the use of cellular respiration as a surrogate biomarker for monitoring drugs targeting mTOR. In contrast, other molecularly targeted agents, such as GSK1120212 (MEK inhibitor), sorafenib/regorafenib (multikinase inhibitors) [12], and tacrolimus/cyclosporine (calcineurin inhibitor; **Tables 1, 2**) have no or minimum effects on cel-

Sirolimus impairs tissue cellular bioenergetics

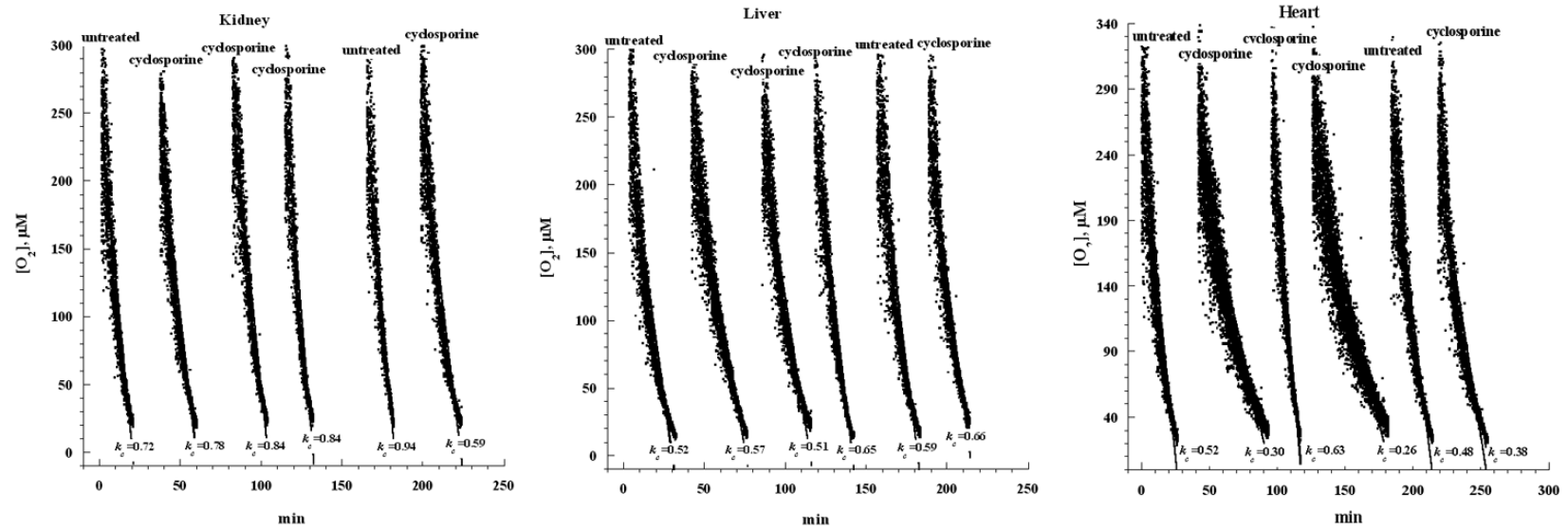


Figure 3. Effects of the calcineurin inhibitor cyclosporine on renal, hepatic, and cardiac cellular respiration. Representative runs are shown. Each run represented a specimen that was collected from a C57BL/6 mouse and processed immediately for measuring cellular respiration with and without the addition of 10 μM cyclosporine. Rate of respiration (k , $\mu\text{M O}_2 \text{ min}^{-1}$) was the negative of the slope of $[\text{O}_2]$ vs. t . The values of k_c ($\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$) are shown at the bottom of each run. The lines are linear fit.

Table 3. Effects of the calcineurin inhibitor cyclosporine on cellular respiration

	Drug Concentration	k_c ($\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$)	Inhibition (%)	<i>P</i>
Kidney	0	0.77 ± 0.13 (5)	-	-
	10 μM	0.80 ± 0.06 (4)	0	0.841
Liver	0	0.64 ± 0.12 (4)	-	-
	10 μM	0.60 ± 0.13 (8)	0	0.933
Heart	0	0.48 ± 0.09 (4)	-	-
	10 μM	0.35 ± 0.14 (8)	27	0.109

Tissue fragments were collected from C57BL/6 mice and immediately processed for measuring cellular respiration in RPMI with and without the designated drugs. The values of k_c are mean \pm SD (n).

lular respiration. Thus, cellular respiration is shown in this study to differentiate between distinct molecularly targeted classes (mTOR inhibition \rightarrow \downarrow cellular respiration).

The mitochondria use energy derived from oxidations in the respiratory chain to produce ATP (oxidative phosphorylation). These vital organelles also are responsible for releasing proapoptotic molecules that trigger the caspase cascade [19]. Caspase activation causes mitochondrial dysfunction [19].

In conclusion, the results show the mTOR inhibitor sirolimus impairs cellular respiration in vital organs (**Table 1**) [12]. Inhibitors of the calcium-dependent serine-threonine phosphatase (tacrolimus and cyclosporine) have no or minimum effects on cellular respiration (**Tables 2, 3**). Cellular bioenergetics is a useful biomarker for compounds that target mTOR signals [20].

Acknowledgements

This research was supported by a grant from the UAE University, NRF (31M096).

Disclosure of conflict of interest

None.

Abbreviations

mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; Pd phosphor, Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetraabenzoporphyrin; $1/\tau$, phosphorescence decay rate; k , rate of cellular respiration ($\mu\text{M O}_2 \text{ min}^{-1}$); k_c , corrected rate of cellular respiration ($\mu\text{M O}_2 \text{ min}^{-1} \text{ mg}^{-1}$).

Address correspondence to: Ali S Alfazari, Department of Medicine, UAE University, Al-Ain 17666, Abu Dhabi, United Arab Emirates. E-mail: a.almelaih@uaeu.ac.ae

References

- [1] Laplante M, Sabatini DM. mTOR signaling at a glance. *J Cell Sci* 2011; 122: 3589-94.
- [2] Schieke SM, Phillips D, McCoy JP Jr, Aponte AM, Shen RF, Balaban RS, Finkel T. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem* 2006; 281: 27643-52.
- [3] Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1 α transcriptional complex. *Nature* 2007; 450: 736-740.
- [4] Tennant DA, Durán RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. *Nat Rev* 2010; 10: 267-277.
- [5] Chen J, Zhao KN, Li R, Shao R, Chen C. Activation of PI3K/Akt/MTOR pathway and dual inhibitors of PI3K and MTOR in endometrial cancer. *Curr Med Chem* 2014; 21: 3070-80.
- [6] Berridge MJ. Signaling defects and disease. *Cell Signaling Biology* 2009; 12.1-12.66.
- [7] Markman B, Dienstmann R, Tabernero J. Targeting the PI3K/Akt/mTOR pathway-beyond rapalogs. *Oncotarget* 2010; 1: 530-543.
- [8] Dang CV. Links between metabolism and cancer. *Genes Dev* 2012; 26: 877-890.
- [9] Ravaud A, Bernhard JC, Gross-Goupil M, Digue L, Ferriere JM. mTOR inhibitors: temsirolimus and everolimus in the treatment of renal cell carcinoma. *Bull Cancer* 2010; 97: 45-51.
- [10] Vandewalle A, Tourneur E, Bens M, Chassin C, Werts C. Calcineurin/NFAT signaling and innate host defence: a role for NOD1-mediated phagocytic functions. *Cell Commun Signal* 2014; 12: 8.
- [11] Tao Z, Jones E, Goodisman J, Souid AK. Quantitative measure of cytotoxicity of anticancer drugs and other agents. *Anal Biochem* 2008; 381: 43-52.
- [12] Almarzooqi S, Albawardi A, Alfazari AS, Sarawathiamma D, Abdul-Kader HM, Shaban S, Mallon R, Souid AK. Effects of inhibiting various protein kinases and phosphatases on cellular respiration. *Journal of Clinical Toxicology*.
- [13] Alfazari AS, Al-Dabbagh B, Almarzooqi S, Albawardi A, Souid AK. A preparation of murine

Sirolimus impairs tissue cellular bioenergetics

- liver fragments for in vitro studies. *BMC Res Notes* 2013; 6: 70.
- [14] Alfazari AS, Al-Dabbagh B, Almarzooqi S, Albawardi A, Souid AK. Bioenergetic study on murine hepatic tissue treated in vitro with atorvastatin. *BMC Pharmacol Toxicol* 2013; 14: 15.
- [15] Alfazari AS, Almarzooqi S, Albawardi A, Sami Shaban, Al-Dabbagh B, Saraswathamma D, Tariq S, Souid AK. Ex vivo study on the effects of sorafenib and regorafenib on murine hepatocytes. *Journal of Clinical Toxicology* 2014; 4: 207.
- [16] Lo LW, Koch CJ, Wilson DF. Calibration of oxygen-dependent quenching of the phosphorescence of Pd-meso-tetra (4-carboxyphenyl) porphine: A phosphor with general application for measuring oxygen concentration in biological systems. *Anal Biochem* 1996; 236: 153-160.
- [17] Shaban S, Marzouqi F, Almansouri A, Penefsky HS, Souid AK. Oxygen measurements via phosphorescence. *Computer Meth Programs Biomed* 2010; 100: 265-268.
- [18] Dehnhardt CM, Venkatesan AM, Chen Z, Delos-Santos E, Ayril-Kaloustian S, Brooijmans N, Yu K, Hollander I, Feldberg L, Lucas J, Mallon R. Identification of 2-oxatriazines as highly potent pan-PI3K/mTOR dual inhibitors. *Bioorg Med Chem Lett* 2011; 21: 4773-4778.
- [19] Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; 305: 626-629.
- [20] Dykens JA, Will Y. The significance of mitochondrial toxicity testing in drug development. *Drug Discovery Today* 2007; 12: 777-785.