Nur Salwani Bakar[1, 3,*] Farhad Kamali[1], Colin D. A. Brown[2]

[1]Institute of Cellular Medicine, 4th Floor William Leech Building, Medical School, Newcastle University NE2 4HH, UK.

[2]Institute for Cell and Molecular Biosciences, 1st Floor Cookson Building, Medical School, Newcastle University, NE2 4HH, UK.

[3]Present address: School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.

Effect of Statins on Functional Expression of Membrane Transporters in L6 Rat Skeletal Muscle Cells

Abstract— Background: Statins reduce LDL-cholesterol and the risk of atherosclerosis. They are generally safe, although statin-induced myopathy is relatively common. Membrane transporters play a crucial role in determining statin side effects. Little is known regarding the interaction of drug transporters in muscle cells with statins. Study aims: The present study aimed to determine the effect of statins on functional expression of monocarboxylate transporters (MCTs) and multidrug resistance-associated proteins (MRPs) in L6 rat skeletal myotube cells. Methods: Relative gene expression at mRNA level was confirmed by RT^2 Profiler^{TM} Rat Drug Transporter PCR array. The uptake of ^{3}H-labelled DL-lactate (1 µCi/ml) was measured to functionally expressed MCT function. The inhibition of [^{3}H]-DL-lactate uptake was assessed in the presence or absence of statins and compared to that of the MCT inhibitors, phloretin and CHC. Transporter-mediated dye efflux was used as functional assay for the MRP efflux transporters. Results: In L6 rat skeletal myotubes, relatively high mRNA expression level was observed for Mct1and Mrp1for uptake and efflux transporters, respectively. The [^{3}H]-DL-lactate uptake was shown to be a concentration-, pH-dependent and Na^+-independent manner with Michaelis-Menten constant (K_m) value of 16.17 ± 2.4 mM vs 15.63 ± 3.0 mM in the presence and absence of Na^+, respectively. The maximum velocity of substrate binding (V_{max}) of the DL-lactate uptake inhibition by lipophilic statins; simvastatin and atorvastatin, were in the same order as phloretin and CHC, while no significant inhibitory magnitude with hydrophilic statins; pravastatin and rosuvastatin. However, the L6 rat skeletal myotubes did not exhibit lactate efflux function. Among four of statins used, only simvastatin showed an affinity inhibition of MRP function in L6 cells. Conclusions: This study has shown that lipophilic statins significantly inhibit functional expression of MCTs, even though they have not shown relatively high inhibition impact on MRPs.

***Keywords*— Lactate, L6 cells, Monocarboxylate transporter, Multidrug resistance-associated protein, Statins**

Received 19 Oct 2016.
Revised 21 Nov 2016.
Accepted 25 Nov 2016.
Published Online 13 Dec 2016

*Corresponding Author:
Nur Salwani Bakar
Email: nursalwani@usm.my

1 INTRODUCTION

3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, known as statins is, a class of drugs to lower LDL-cholesterol and the risk of atherosclerosis. Despite its proven long-term safety and efficacy by many large scale studies [1, 2], statins-related myotoxicity (SRM), the most commonly reported side effect, have been reported among statin users [3].

In light of tremendous advances in pharmacogenetic field in the last decade, it is becoming extremely difficult to ignore the contribution of single nucleotide polymorphism (SNP) in solute carrier organic transporter 1B1 (SLCO1B1) gene had in determining statin response and side effects. The SLCO1B1 gene encodes organic anion–transporting polypeptide 1B1 (OATP1B1), a hepatic transporter which mediates the uptake of statins from portal blood. SNPs in this gene have been shown to affect statin transport function and thereby have profound effects on statin response and side effects. Undeniably, a SNP in the SLCO1B1 for example rs4363657, which is located within chromosome 12, has been shown to play a crucial role in determining statin plasma concentrations and subsequent risk in developing myopathy [4]. The SLCO1B1 polymorphism markedly increased plasma-concentrations of active simvastatin acid and resulted in higher risk of simvastatin-induced myopathy [5]. Although it

has been recognised that elevated plasma statin concentration increases the risk of muscle toxicity [6-8], the aetiology of SRM is not well understood. Since many patients develop SRM such as muscle aches and pain, it is very important to determine the factors which control local skeletal muscle statin concentrations. In fact, little is known regarding the molecular determinants of statin distribution into skeletal muscle and its relevance to toxicity.

There are several key studies that highlighted muscle symptoms, such as fatigue and cramp, may be due to lactate build-up in muscles. These lactate accumulations may be inducing dysfunctional monocarboxylate transporters (MCTs) possibly MCT1 and MCT4, two of which highly expressed lactate transporters in striated muscle [9, 10] and transcribed by SLC16A1 and SLC16A3 gene, respectively [10]. Other studies, has shown that exposure to monocarboxylate lactate, was found to be associated with the generation of reactive oxygen species (ROS) and the up-regulation of genes related to mitochondrial lactate oxidation complex in both *in vitro* and *in vivo* study using rat skeletal muscle cells [11, 12].

Physiologically, the role of MCTs in skeletal muscle is undeniable since the organ is the major site of lactate production and removal in the body. So far, there is no study has been carried out to investigate the association of the membrane transporter with the risk of SRM i.e., myotoxicity happens as a result of inhibition to the MCT function by a statin and subsequently increases muscular lactate levels for which it might be perceived as muscle pain or cramp. Moreover, substrates for MCTs have not been limited to endogenous metabolites but also xenobiotics such as statins, gamma-hydroxybutyrate and valproic acid [13-16] which thus suggest their potential role in predicting SRM.

Our group has previously shown that HK-2 cells (a proximal tubule cell line originated from human kidney) express MCT1 at both mRNA and functional levels [17]. A number of studies also have characterised MCT functions in L6 skeletal muscle cells [12, 18, 19], by which MCT1 in particular mediated lactate transport in both mitochondrial and sarcolemma membrane of a striated muscle fibre. However, limited evidence is available to explain how statins affect the function of MCT1 in muscle cells. There are evidences indicating that MCT4, but not MCT1, being the statin target and upregulated during statin-induced cytotoxicity [20, 21]. In terms of blood lactate transport and removal, MCT1 and MCT4 were both considered important mediators for blood lactate removal [22, 23] thus implicating their vital role in maintaining muscle lactate concentration. Therefore in this study, we determined whether SRM could be promoted by the inhibition of MCT1 function by statins primarily looking at their ability to inhibit lactate uptake into the L6 cells. It is possible that the inhibition of MCT1 by statins could advocate myotoxicity due to affected lactate transport.

Apart from the above mentioned SLC transcribing membrane transporters, the interplay role by efflux transporters typically ATP-binding cassette (ABC), in muscle such as multi-drug resistance associated protein (MRPs), may also modulate statin local exposure in muscle and eventually intensify muscle toxicity in the event of function inhibition of the transporters. Moreover, our group had also shown that 5-(3-92-(7-chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid (MK571) could be used as a high affinity MRP inhibitor in an MRP-mediated CMFDA efflux assay using HK-2 cells [17]. This dual dye assay was developed to evaluate the impact of two inhibitors, MK571 (MRP inhibitor) and CSA (MDR1 inhibitor); whereby the accumulation of dye provides an indirect measure of efflux inhibition of the ABC transporters. Therefore, the aim of the second part of this study was to evaluate the impact of statins on functional expression of efflux transporters. In order to assess the relative affinity of different statins to the MRPs function, the magnitude of the dye efflux inhibition was compared to MK571.

2 MATERIALS AND METHODS

2.1 Materials and reagents

Number RT^2 Profiler^{TM} Rat Drug Transporter PCR array (Catalogue no: PARN-070Z) and reagents were purchased from Qiagen Ltd (Crawley, UK). Simvastatin, atorvastatin, pravastatin, and rosuvastatin were gifts from AstraZeneca (Alderley Park, Cheshire, UK). [$^{2\text{-}3}$H]-DL-lactate (at activity of 20 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). SV Total RNA Isolation System was purchased from Promega (Southampton, UK). SYBR Green Dye Master Mix for real-time polymerase chain reaction was purchased from Roche Applied Sciences (Burgess Hill, UK). Reagents for dye assay; 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Invitrogen

(Paisley, UK). Unless otherwise stated, all other reagents including phloretin (well-defined MCT1 inhibitor) and α-cyano-4-hydroxycinnamate, CHC (a typical MCT1, 2 and 4 inhibitors) were purchased from Sigma-Aldrich (Dorset, UK).

2.2 L6 rat muscle cell line

The L6 rat muscle cell line (ATCC® number: CRL-1458™), supplied at myoblast stage, was kindly provided by Dr Audrey Brown of Newcastle University. L6 exists as myoblasts when cultured in maintenance culture medium, which comprises high glucose DMEM supplemented with 10 % foetal calf serum, 200 units/ml penicillin and 200 µg/ml streptomycin, until 70-80 % confluency. The cells were then introduced to differentiation medium (high glucose DMEM supplemented with 2 % horse serum, 200 units/ml penicillin and 200 µg/ml streptomycin), upon which the cells differentiate into myotube until approximately 80 to 90 % confluent. L6 cells used in this study were within passage numbers 11 through 30.

For the uptake and efflux experiments, L6 cells were seeded at 50,000 cells/well and 20,000 cells/well onto 24-well plates and 96-well plates (Coster, Corning Incorporated Corning, UK), respectively, for 2 days or until the indicated confluence. Cells were then differentiated into myotube formation by incubating them with differentiation media. The cell monolayers were fed a fresh differentiation media every 2 days and were used for uptake experiments on day 7.

2.3 Isolation of total RNA and qPCR array for rat membrane transporters

On day 7 of differentiation, total RNA was isolated from L6 cells at myotube stage to determine the expression of a series of uptake (both SLC and SLCO sub-groups) and efflux (ABC group) transporters by qPCR array plate. In order to assess whether statins affect gene expression of the uptake transporters in L6 myotubes, the cells were pre-treated on day 5 with simvastatin (2 µM) for 48 hrs prior to RNA extraction and compared with untreated control cells (contained 0.02 % methanol only). The protocols for RNA extraction and qPCR array were carried out as previously described [17].

2.4 Functional assay of monocarboxylate transporters (MCTs)

2.4.1 ^{3}H-DL-lactate uptake assay

Lactate uptake experiments by L6 cells seeded on 24-well plates in the presence of statins were carried out according to the previously described protocols [17].

2.4.2 ^{3}H-DL-lactate efflux assay

The efflux of radiolabelled substrate was used to functionally measure the expression of MCT. Briefly, cells were pre-incubated with 300 µl of Krebs containing radiolabelled DL-lactate (50 µM) for 60-90 minutes at pH 6.0, 37 °C to ensure cells were loaded with radiolabelled substrate. After incubation, cells were then washed with ice-cold Krebs at pH 7.4 three times. To initiate lactate efflux, cell monolayers were incubated with 300 µl pre-warmed ^{3}H-DL-lactate-free Krebs buffer at pH 6.0 with and without 500 µM inhibitor. After several time intervals, the cell monolayers were solubilised in 0.5 mL of 0.05 % SDS and transferred to scintillation vials.

The radioactivity was measured as described earlier [17]. Fractional efflux rate (% of radiolabelled lactate efflux every 2 min intervals) was calculated according to (**1)** and the magnitude of MCT-mediated ^{3}H-DL-Lactate efflux in Krebs-inhibitor solution was compared to that of control with Krebs buffer only (refer *Supplementary document* for an example of calculation).

$$\text{Fractional efflux rate} = \frac{\text{Amount of effluxed lactate}}{\sum \text{Remains of intracellular radioactivity}} \times 100\ \% \quad (1)$$

2.5 Functional assay of Mrp efflux transporters

The L6 cells were seeded at a density 20,000 cells/well and cultured for 24 hours before induction of differentiation into myotube formation for 6 or 7 days. Retention of MRP-mediated fluorescent product of CMFDA (i.e. glutathione methylfluorescein, GSMF) was used to determine MRPs function in L6 cells and has been previously described [17].

2.6 Statistical analysis

All statistical analysis on data was performed using GraphPad Prism software version 4 (GraphPad Software Inc. San Diego, CA, USA). For kinetic analysis, the Michaelis-Menten constant (K_m) and maximum velocity of substrate binding (V_{max}) of DL-lactate uptake were analysed from a fitted nonlinear regression analysis.

Statistical difference between group means was tested using Student's unpaired t-test (also known as independent- samples t-test) or a One-Way ANOVA with Dunnett's post-hoc test as appropriate. Paired t-test was used when the mean of continuous variables between groups were related in some way. A value of $p< 0.05$ was considered statistically significant. Unless otherwise stated, results are expressed as the mean ± SEM from at least three separate experiments performed on separate days.

3 RESULTS

3.1 Relative mRNA expression of key uptake and efflux transporters in L6 myotubes

Among the SLC and SLCO genes transcribing uptake transporters, mRNA for Mct1 (transcribed by Slc16a1), Mct8 (Slc16a2) and Oatp3a1 (Slco3a1) were found to be highly expressed in L6 cells in contrast to Mct4 (Slc16a3) expression (**Figure 1A**).

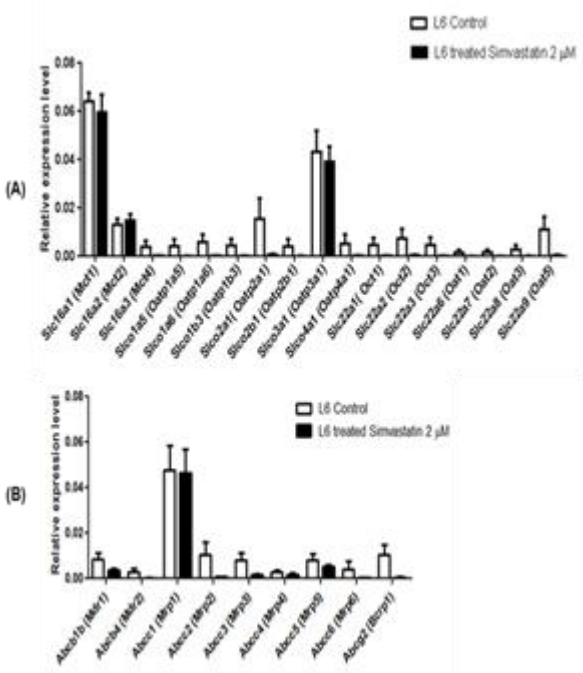

Figure. 1 A selection of key (A) uptake and (B) efflux transporters in cDNA of L6 myotubes analysed by drug transporters qPCR array. The relative expression level was analysed by standard qPCR Array and data analysis from SABiosciences website (http://www.sabiosciences.com/pcrarraydataanalysis.php) and calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to the geometric mean of five housekeeping genes provided by the array. Error bars represent the mean ± SEM (n=3).

Among ABC efflux transporters, mRNA of Mrp1 (Abcc1) was expressed at highest level compared to Mrp2 (Abcc2), Mrp3 (Abcc3), Mrp4 (Abcc4), Mrp5 (Abcc5) and Mrp6 (Abcc6). The mRNA expression of other efflux transporters such as Bcrp1 (Abcg2) and Mdr1 (Abcb1) were also expressed at low level (**Figure 1B**). Among the highly mRNA-expressed uptake (i.e., Mct1 and Oatp3a1) and efflux (i.e., Mrp1) transporters in the L6 myotubes, pre-treatment with simvastatin (2 µM for 48 hours) did not significantly reduce the level of expression in compared to the control.

3.2 The expression of MCT1 function in L6 cells

The uptake of ^{3}H-DL-lactate (50 µM) was used to assess MCT function in L6 cells. The time course for lactate uptake into L6 cells at pH 6.0 (at 37°C) is shown in **Figure 2A**. The ^{3}H-DL-lactate uptake was linear up to 5 minutes. Therefore a 2 minutes incubation time was chosen for subsequent uptake experiments. The effect of extracellular pH and sodium ion concentration on ^{3}H-DL-lactate uptake was examined over the pH range of 5.5 to 7.4. ^{3}H-DL-lactate uptake was significantly higher ($p<0.001$) at lower extracellular pH (pH 5.5 vs pH 7.4) in the presence and absence of Na^+. The uptake at pH 5.5 was found not to be affected by Na^+ concentration (**Figure 2B**). The uptake experiments were subsequently performed at pH 6.0 in the presence of Na^+ to mimic physiological conditions.

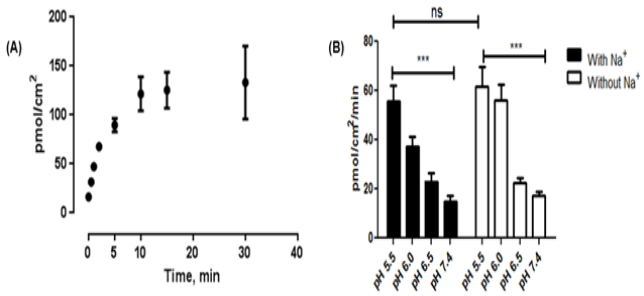

Figure. 2 Time course and pH-dependency uptake of ^{3}H-DL-Lactate in myotubes of L6 rat muscle cells. (A) The ^{3}H-DL-Lactic acid (50 µM) uptake by L6 (at pH 6.0 and 37°C) was linear up to 5 minutes and 2 minutes incubation time was chosen for subsequent experiments. (B) Effect of pH and Na+ on the uptake of DL-lactate (50 µM) by L6 cells. Data are presented as mean ± SEM (n= 12) from three independent determinations. *** $p< 0.001$; Na^+, sodium ion.

The kinetics of ^{3}H-DL-lactate uptake by the L6 cells is shown in **Figure 3A** and **Figure 3B**. The uptake of DL-lactate (1 µCi/mL) was shown to be concentration-dependent (0.1 mM to 20 mM). A nonlinear regression analysis with a simple

Michaelis-Menten equation gave a K_m value of 16.17 ± 2.4 mM (95% CI = 11.47- 20.87) in the presence of Na^+ and the K_m value of 15.63 ± 3.0 mM (95% CI= 9.75 – 21.51) in the absence of Na^+. The K_m values in both conditions were found not to be significantly different from each other (**Figure 3C**) suggesting that the presence of Na^+ has no impact upon the kinetics of the transporter in the cells.

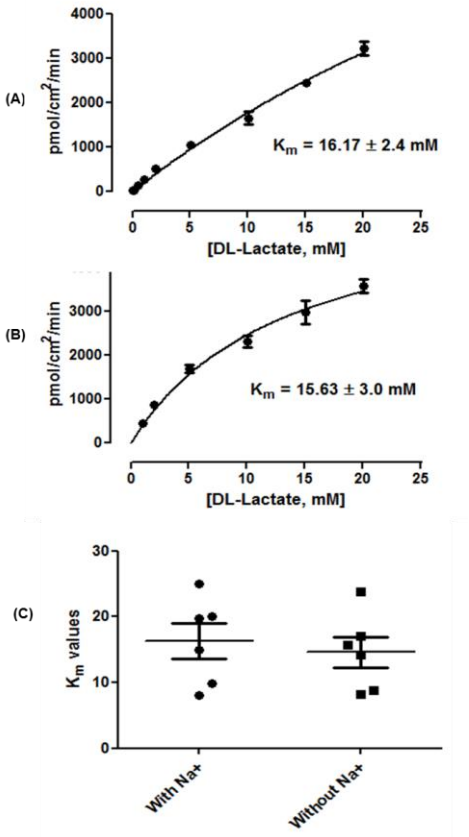

Figure. 3 Concentration-dependence of DL-lactate uptake by L6 myotubes. ^{3}H-DL-Lactate uptake at 0.1 – 20 mM final concentration (at pH 6.0 and 37 °C) was determined over 2 minutes in (A) the presence of extracellular Na+ and (B) the absence of extracellular Na^+. The K_m values were 16.17 ± 2.4 mM (95% CI: 11.47 - 20.87) and 15.63 ± 3.0 mM (95% CI: 9.75 – 21.51) in the presence and absence of Na^+, respectively. Data are presented as mean ± SEM from three independent determinations. (C) The mean K_m value in the presence of Na^+ (16.17 ± 2.4 mM) was not significantly different (paired t-test) compared to that without Na^+ (15.63 ± 3.0 mM). Data are presented as mean ± SEM (n = 6) from six parallel determinations. Na+, sodium ion.

3.3 The impact of statin on the MCT-mediated ^{3}H-labelled DL-lactate uptake

Inhibition assay was performed to determine whether statins affect functional expression of Mct1 in particular. L6 cells were incubated with ^{3}H-DL-lactate in the absence and the presence of statins (i.e. simvastatin, atorvastatin, pravastatin and rosuvastatin). To confirm that statin was a substrate for Mct1, the magnitude inhibition of DL-lactate uptake by all tested statins were compared to phloretin (a well-defined Mct1 inhibitor) and CHC (a typical Mct1, 2 and 4 inhibitors). **Figure 4** summarises the degree of DL-lactate uptake inhibition. Simvastatin and atorvastatin significantly ($p<0.001$) inhibited DL-lactate uptake to the same degree as phloretin and CHC with IC_{50} values of 10.7 ± 1.2 µM and 7.4 ± 0.9 µM, respectively (**Table 1**). In contrast, the inhibitory effects of pravastatin and rosuvastatin were not significant even up to 1 mM.

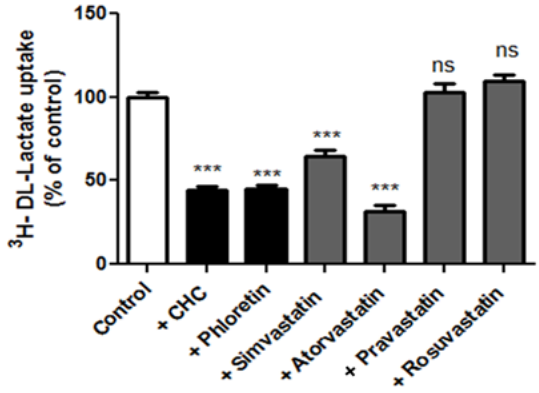

Figure. 4 The DL-lactate uptake (50 µM) in the presence of statins, CHC and phloretin (all at 1 mM). Data are mean ±S.E.M. n = 12, each data point was derived from a triplicate of experiments and from four independent determinations. Data were analysed using One-Way ANOVA with Dunnett's post-test and compared to that of control without the presence of inhibitor. CHC, α-Cyano-4 hydroxycinnamic acid *N*-ethyl-*N*,*N*-diisopropylammonium salt;*** p< 0.001; ns, non-significant.

TABLE I
IC_{50} AND V_{max} VALUES FOR THE ABILITY OF THE AGENTS TO INHIBIT ^{3}H-DL-LACTATE (50 µM) UPTAKE INTO L6 MYOTUBES.

Inhibitors	IC_{50} values (95% CI)	V_{max} (mean ± SEM)
Phloretin	8.8 ± 0.7 µM (7.43 – 10.17)	72.07 ± 0.9 %
Simvastatin	10.7 ± 1.2 µM (8.35 – 13.05)	61.30 ± 1.1 %
Atorvastatin	7.4 ± 0.9 µM (5.64 – 9.16)	69.60 ± 0.8 %

The fractional efflux rate of ^{3}H-DL-Lactate (50 µM) at each time interval was low and the values were consistently at the level below than 10% from the baseline reading **(Figure 5)**. Furthermore, the fractional efflux rates for DL-lactate were similar when co-incubated with Mct1 inhibitors, phloretin (a typical MCT1 inhibitor),

CHC (non-specific MCT inhibitor), simvastatin (a representative of lipophilic statin) and pravastatin (a representative of hydrophilic statin), at a concentration of 500 µM of each inhibitor to that of control (substrate only). In contrast to the uptake function of Mct1 (**section 3.2**), this finding suggests that Mct1 in the L6 muscle cells does not exhibit efflux function for DL-lactate.

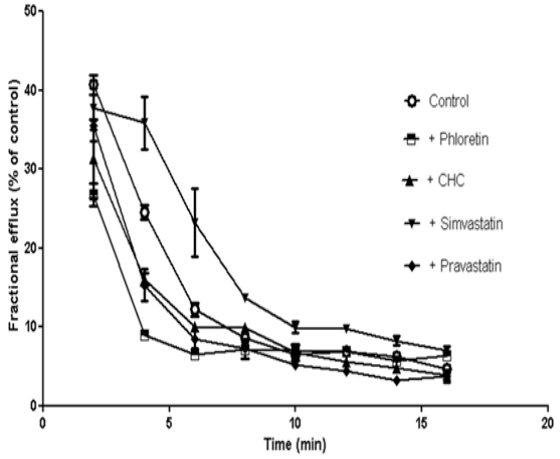

Figure. 5 Efflux assay for determination of MCT1-mediated ^{3}H-DL-Lactate (50 µM) efflux in L6 cells. There was no significant inhibition of DL-lactate (50 µM) efflux. Efflux rates were approximately 10 % for all conditions. All inhibitors were at 500 µM and each point represented as mean ±SEM (n =9) from three independent experiments. MCT, monocarboxylate transporter; CHC, α-Cyano-4 hydroxycinnamic acid *N*-ethyl-*N*,*N*-diisopropylammonium salt.

3.4 The impact of statins on the MRP-mediated CMFDA efflux

Our group previously published that MK571 could be used a specific inhibitor of MRP-mediated GSMF (a fluorescent component of CMFDA), as an assay to functionally characterised the functional expression of MRP transporter using HK-2 cell line. Consistent with the previous findings in the HK-2 cells, GSMF retention was significantly higher when L6 cells were treated with MK571 compared to that of untreated control with CMFDA dye only (**Figure 6A**) ($p < 0.0001$). Pre-treatment of L6 myotubes with (**Figure 6B1**) and without (**Figure 6B2**) simvastatin (2 µM for 48 hours) did not result in different MK571 IC_{50} values (0.90 ± 0.2 µM vs 1.03 ± 0.2 µM) which thus suggested that simvastatin did not modify Mrp function in the cells.

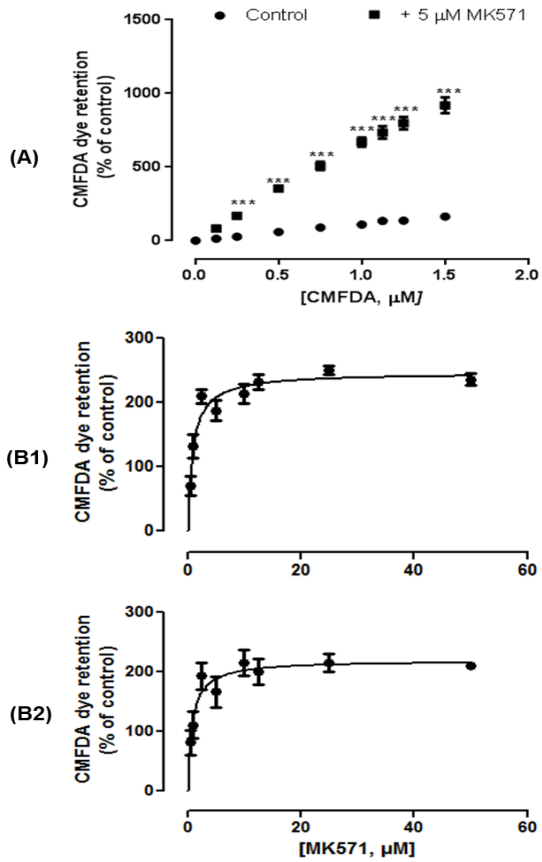

Figure. 6 Inhibition of MRP-mediated CMFDA efflux by MK571 in L6 cells. (A) Addition of MK571 at 5 µM resulted in a significant increase in CMFDA dye retentioncompared to that of control wells with K_m value of 6.66 ± 3.6 µM. ***P<0.0001 demonstrates the level of significance compared to the control without MK571 pre-treatment (paired T-test). (B) Dose-response curve for MRP-mediated CMFDA (1 µM) efflux inhibition by MK571 in L6 cells. L6 myotubes were exposed with simvastatin (B1) and without simvastatin (B2) pre-treatment for 48 hrs and resulted in MK571 IC50 values of 0.90 ± 0.2 µM and 1.03 ± 0.2 µM, respectively. Dye retention was measured after treatment with a range of MK571 concentrations after 40 minutes. Each point represents mean + SEM (n = 18) from 3 independent experiments.

Among four statins used in this experiment, only simvastatin was significantly inhibited ($p<0.05$) MRP-mediated GSMF efflux, however the magnitude of the inhibition was more than five-fold lower to that seen with MK571 (**Figure 7)**.

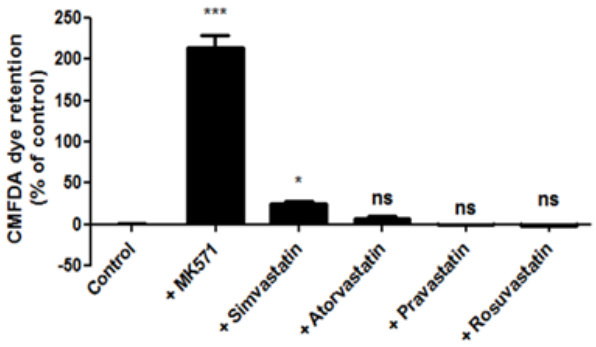

Figure. 7 The inhibition by statins of the MRP-mediated CMFDA dye efflux in L6 cells. The concentration of all inhibitors used were 10 μM. Data are presented as mean ± SD (n=18) from 3 independent experiments. Data were analyzed using one-way ANOVA and Dunnett's post-hoc test;***P<0.0001, *P<0.05 and ns (non-significant) versus control.

4 DISCUSSION

We have, previously, shown both mRNA and functional expression of monocarboxylate transporter 1 (MCT1) in HK-2 cells, using DL-lactate as substrate [17]. In the study, the high MCT-expressed HK-2 cells were found to be transported by mechanism obeying Michaelis-Menten kinetics (K_m). The DL-lactate uptake was likely mediated through the MCT1 transporter although the Na^+ dependence uptake may have suggested the involvement of SMCT1 (sodium-coupled MCT1), but this was not confirmed further [17].

We then extended the functional study of MCT using a model of rat skeletal muscle i.e. L6 cells, since the statin related side effects commonly seen in muscle. In the present study, the K_m value for the DL-lactate uptake in the L6 cells was higher compared to that seen in the previously studied HK-2 cells. Relatively, the mRNA expression level for MCT1 in the HK-2 cells was higher than L6 rat skeletal cells, suggesting its lower affinity to DL-Lactate in the muscle model. Likewise, it could be possible that HK-2 cells extensively transport or eliminate lactate as metabolite substrate and/or product of the endogenous production, thus resulting in lower K_m values (high driving forces) than that of muscle cells, since HK-2 cells were found to express more MCTs; MCT1, MCT2 and MCT4, at both mRNA and protein levels [24].

In contrast to the findings in HK-2 cells [17], we found that the DL-lactate uptake in L6 cells was Na+-independent. Since the absence of SMCT1 in the HK-2 cells was verified by qPCR, it was suggested that the inhibition of Na^+/H^+ exchange in the HK-2 cells resulted in higher lactate uptake in the presence of Na^+ [17]. Therefore, one possible explanation of this discrepancy could be the result of high regulation of lactate/H^+ exchange since it was found as major pH regulator in muscle cell compared to other mechanisms such as Na^+/H^+ exchange and bicarbonate/H^+ [25]. It seems that only the presence of excess proton (H^+) intensifies the lactate transport, in accordance with other previous findings [10, 26] and thus suggests that muscle symptoms among lipophilic statin users in particular, is associated with the disturbance of pH regulation in muscle and eventually lactic acidosis which might lead to apoptosis and toxicity.

Lipophilic statins, simvastatin and atorvastatin, have a significant impact on MCTs, presumably MCT1 since this particular membrane transporter is found to be present in almost all tissues including skeletal muscles, with specific location within the tissues [10]. MCT1 is also localized in sarcolemmal membrane of mitochondrion [27-29], therefore, it is possible that statins cause muscle toxicity by interfering with mitochondrial function. Indeed this has been demonstrated by both *in vitro* and *in vivo* regarding the mode of action of simvastatin [30-32], suggesting that lipophilic statins are better at targeting MCT1 to induce mitochondrial toxicity than hydrophilic statins. Further work in patient cohort is thus needed to determine whether altered function of MCT1 due to genetic mutation would exacerbate statin myotoxicity and/or mitochondrial toxicity.

In contrast to the effect on MCTs, lipophilic statins did not inhibit cellular MRP efflux transporters. The functional expression of efflux transporter in this study is likely to have been attributed to by Mrp1 owing to its relatively high expression level in the L6 myotubes. MRP1 is expressed ubiquitously, and is localised to the basolateral, rather than apical, membrane of epithelial cells. As with MRP2, MRP1 primarily effluxes a wide range of substrates [33, 34], and may acts as the most important efflux transporter for the extrusion of toxins or metabolites from cellular metabolism as suggested by Mueller and colleagues [35]. Among MRPs, although less evidence is available on the capacity of MRP1 for statin efflux than that demonstrated by MRP2

[36], it has been demonstrated that polymorphism in both ABCC1 (MRP1) and ABCC2 (MRP2) genes are equally involved in the incidence of statin resistance and response i.e. patients fail to achieve adequate reduction of LDL-C level [37, 38]. Since MRP2, the 190 kDa membrane glycoprotein, is highly expressed in human apical hepatocytes [39, 40], the mutation and/or inhibition of MRP2 may become a major determinant of biliary excretion of statins resulting in statin plasma elevation, a risk factor for statin-related myotoxicity. Nevertheless, at local exposure in skeletal muscle cells, it has been shown that both atorvastatin and rosuvastatin accumulation is reduced due to MRP1 over-expression [41] suggesting that the efflux of both statins is also attributed to the MRP1 transporter which is consistent with that found by Dorajoo *et al.* in 2008 [42].

5 CONCLUSION

We were able to demonstrate that simvastatin had higher affinity to MRPs than MCT1 and other statins, based upon the inhibition of MRP-mediated CMFDA efflux in the L6 cells. This observation warrants further evaluations possibly by direct transport study using radiolabelled simvastatin possibly in skeletal mitochondrion model.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

FUNDING

This study was supported by a PhD studentship grant (from Ministry of Higher Education Malaysia and Universiti Sains Malaysia) to Professor Farhad Kamali.

ACKNOWLEDGEMENT

N.S.B would like to thank Dr Audrey Brown, Newcastle University, for the generous gift of the L6 cell line and Dr Git Weng Chung (from the Institute for Cell and Molecular Biosciences, Newcastle University) for his technical help and training.

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Supplementary document

Calculation of fractional ^{3}H-DL-lactate efflux

	Reading 1	**Reading 2**	**Reading 3**
Radioactivity collected on the 1st 2 min	4381	4032	3945
Radioactivity collected on the 2nd 2 min	1493	1390	1529
Radioactivity collected on the 3rd 2 min	567	587	506
Radioactivity collected on the 4th 2 min	348	316	366
Radioactivity collected on the 5th2 min	229	233	245
Radioactivity collected on the 6th 2 min	231	238	236
Radioactivity collected on the 7th 2 min	195	197	197
Radioactivity collected on the 8th 2 min	140	137	138
Final radioactivity remained in cells	2846	2821	2804
Total radioactivity	10430	9951	9966

Thus, fractional efflux rate (%) was gained as follow;

	Reading 1	**Reading 2**	**Reading 3**	**MEAN**
1	42.00384	40.51854	39.58459	40.70232
2	24.68177	23.4837	25.39445	24.51997
3	12.44513	12.96092	11.26447	12.22351
4	8.723991	8.016235	9.182137	8.640788
5	6.289481	6.425814	6.767956	6.494417
6	6.770223	7.014441	6.992593	6.925752
7	6.130148	6.244057	6.275884	6.216696
8	4.688547	4.631508	4.690687	4.670247

An example for the values in **Reading 1** from the table above were derived as follow;

42.00384 = 4381/10430*100
24.68177 = (1493/ (10430-4381)*100
12.44513 = (567/ (10430-(1493+4381))*100
8.723991 = (348/ (10430 – (4381+1493+567)*100
6.289481 = (229/ (10430 – (4381+1493+567+348))*100
6.770223 = (231/ (10430 – (4381+1493+567+348+229))*100
6.130148 = (195/ (10430 – (4381+1493+567+348+229+231))*100
4.688547 = (140/(10430 – (4381+1493+567+348+229+231+195))*100