Investigating Dihydroorotate Dehydrogenase Inhibitor Mediated Mitochondrial Dysfunction in Hepatic *in vitro* Models

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Abstract

Inhibition of dihydroorotate dehydrogenase (DHODH), the rate-limiting enzymatic step in *de novo* pyrimidine synthesis, has broad immunosuppressive effects *in vivo* and shows promise as a therapeutic target for the treatment of malignancies, viral infections and auto-immune diseases. Whilst there are numerous DHODH inhibitors under development, leflunomide and teriflunomide are the only FDA approved compounds on the market, each of which have been issued with black-box warnings for hepatotoxicity. Mitochondrial dysfunction is a putative mechanism by which teriflunomide and leflunomide elicit their hepatotoxic effects, however it is as yet unclear whether this is shared by other nascent DHODH inhibitors. The present study aimed to evaluate the propensity for DHODH inhibitors to mediate mitochondrial dysfunction in two hepatic *in vitro* models. Initial comparisons of cytotoxicity and ATP content in HepaRG® cells primed for oxidative metabolism, in tandem with mechanistic evaluations by extracellular flux analysis identified multifactorial toxicity and moderate indications of respiratory chain dysfunction or uncoupling. Further investigations using HepG2 cells, a hepatic line with limited capability for phase I xenobiotic metabolism, identified leflunomide and brequinar as positive mitochondrial toxicants. Taken together, biotransformation of some DHODH inhibitor species may play a role in mediating or masking hepatic mitochondrial liabilities.

Keywords: Dihydroorotate dehydrogenase, mitochondria, dysfunction, DILI, HepaRG®, HepG2

Introduction:

Human dihydroorotate dehydrogenase (DHODH) is a ubiquitous flavin mononucleotide (FMN) protein localised to the inner mitochondrial membrane (IMM). DHODH catalyses the fourth and rate-limiting enzymatic step, the ubiquinone-mediated oxidation of dihydroorotate to orotate and the concomitant reduction of FMN to dihydroflavin mononucleotide ($FMNH_2$), in *de novo* pyrimidine biosynthesis[1]. DHODH uses the quinone pool as its electron acceptor, thus contributing to the generation of the electrochemical gradient through the activities of ubiquinol-cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV)[2]. Therefore, DHODH provides a functional link between the pyrimidine biosynthesis pathway and the mitochondrial electron transport chain (ETC)[3,4].

The inhibition of DHODH has broad immunosuppressive effects *in vivo*, including cytostatic effects upon B and T lymphocyte proliferation following activation. Rapidly dividing cells have an especially high demand for pyrimidine nucleotides in order to execute nucleic acid synthesis[5]. Therefore targeting DHODH activity, as evidenced by a number of studies, shows promise for the treatment of conditions characterised by aberrant activations of the immune system, viral infection or various cancers (i.e. auto-immune diseases, myeloid malignancies and transplant rejections)[1,6–9].

Currently there are two U.S. Food and Drug Administration (FDA) approved DHODH inhibitors on the market; leflunomide, an isoxazole derivative used for the management of rheumatoid arthritis and its active analogue teriflunomide, an agent used for the management of relapsing-remitting multiple sclerosis[9]. Once administered, leflunomide is thought to undergo enzymatic conversion to teriflunomide facilitated by the cytochrome P450 (CYP450) isozyme family[10–12]. In addition, several other inhibitors of DHODH have been described and are currently under various stages of development for a variety of disease indications (Table 1)[1].

Recently there has been renewed interest in the class for the treatment of myeloid malignancies due to encouraging pre-clinical evidence of anti-tumour activity across several of the compounds listed[13]. Furthermore, in the advent of the global SARs-CoV-2 pandemic several pre-print studies have indicated that DHODH inhibitors may be effective host-targeting antivirals (HTAs)[14–16]. Given the high unmet clinical need for both acute myeloid leukaemia (AML) and SARS-CoV-2 treatments, the development of potent and selective DHODH inhibitors or the repurposing of existing ones is currently of great interest to the wider medical community[13].

Table 1 An update on the status of approved/experimental dihydroorotate dehydrogenase inhibitors in 2020[1].

Compound	Sponsor	Disease	Status
AG-636	Agios Pharmaceuticals, Inc	• Lymphoma	Phase I
ASLAN003	ASLAN Pharmaceuticals	• Acute Myeloid Leukaemia	Phase II
BAY2402234	Bayer	• Myeloid Malignancies	Phase I
Brequinar Sodium	Clear Creek Bio	• Acute Myeloid Leukaemia	Phase I/II
Brequinar Sodium	Clear Creek Bio	• SARS-CoV-2 Infection	Phase I/II
Leflunomide (Arava®)	Sanofi	• Rheumatoid Arthritis • Active Psoriatic Arthritis	FDA Approved
Leflunomide (Arava®)	Sanofi	• Polymyalgia Rheumatica • Multiple Myeloma	Phase III

		• Triple Negative Breast Cancer	Phase I/II	
			Phase I/II	
Leflunomide (Arava®)	Sanofi	• SARS-CoV-2 Infection	Phase I	
Manitimus (FK778)	Astellas Pharma Europe Ltd. (Sanofi-Aventis)	• Immunosuppressive Therapy for Transplantations	Phase II	
PP-001	Panoptes Pharma Ges.m.b.H	• Non-Infectious Uveitis • Keratoconjunctivitis	Phase I/II	
PTC299	PTC Therapeutics	• Acute Myeloid Leukaemia • Metastatic Breast Cancer • Brain and Central Nervous System Tumours	Phase I	
PTC299	PTC Therapeutics	• Pneumonia • SARS-CoV-2 Infection	Phase II/III	
Teriflunomide (Aubagio®)	Sanofi	• Relapsing-Remitting Multiple Sclerosis	FDA Approved	
Vidofludimus calcium (IMU-838)	Immunic Therapeutics	• Relapsing-Remitting Multiple Sclerosis • Ulcerative colitis	Phase II	
Vidofludimus calucim (IMU-838)	Immunic Therapeutics	• SARS-CoV-2 Infection	Phase II/III	

Abbreviations: FDA, U.S. Food and Drug Administration. Notes: Information regarding stage of development and disease association were derived from https://clinicaltrials.gov.

However, safety concerns have arisen due to post-marketing reports of severe liver injury from patients receiving leflunomide between August 2002 and May 2009[17]. This has resulted in the FDA issuing a black-box warning for hepatic injury in 2010 following a review that identified 49 cases of severe liver injury, 14 of which were fatal[18]. Consequently teriflunomide, as the primary metabolite of leflunomide, was issued with a similar warning in the prescribing information due to comparable steady-state plasma concentrations achieved via both direct and indirect (leflunomide) administration[19]. Furthermore, under the recommendation of the FDA, trials concerning the use of PTC299 for the treatment of refractory or recurrent central nervous system tumours in adults were ceased due to two cases of hepatotoxicity, one of which was fatal[20]. It has yet to be determined whether other DHODH inhibitors under development pose a similar risk of adverse hepatotoxic reactions in recipients.

This issue is particularly pertinent as drug-induced liver injury (DILI) is a major driver of both pre- and post-market drug attrition thus hampering the development and repurposing of therapeutically applicable compounds[21]. The liver is extremely vulnerable to toxic insults, with DILI accounting for > 50 % of acute liver failure cases in the clinic[22,23]. In addition, the nature of DILI is often idiosyncratic, characterised by complex dose-response relationships and heavily influenced by inter-individual variation in patient susceptibility factors. Therefore, predicting the potential clinical risk for hepatocellular injury within a pre-clinical setting is often notoriously difficult[24].

Whilst the precise nature of DHODH inhibitor associated hepatotoxicity has yet to be resolved, previous studies using hepatic cell lines have implicated mitochondrial dysfunction as a putative mechanism by which leflunomide and teriflunomide elicit their adverse effects *in vitro*[10,25].Mitochondria are essential intracellular organelle that are innately linked to energetic homeostasis and cellular signalling. Mitochondria are also integral regulators of cell death, therefore perturbations can often result in the initiation of death signalling cascades[26]. It is as yet unclear if other DHODH inhibitors have a similar impact upon mitochondrial respiration.

This study aimed to evaluate the mitotoxic, and by extension, the hepatotoxic potential of a panel of DHODH inhibitors in two readily available hepatic *in vitro* models, HepG2 cells and their more physiologically relevant counterpart, HepaRG® cells[27,28]. Initial end-point comparisons of cytotoxicity and ATP content were performed in HepaRG® cells acutely conditioned to either glucose or galactose media[29–31], whilst comparative experiments were conducted in HepG2 cells. Subsequently, compounds of interest were carried forward for mechanistic investigation via extracellular flux analysis to examine mitochondrial oxygen consumption rate and substrate driven respiration.

Materials and Methods

Materials:

All forms of Dulbecco's modified eagle medium (DMEM), foetal bovine serum (FBS), phosphate buffered saline (PBS) and type I rat tail collagen were purchased from Life Technologies (Paisley, UK). All extracellular flux analyser (XF^{e}96) consumables were purchased from Seahorse Bioscience or Agilent Technologies (North Billerica, USA and Santa Clara, USA respectively). HepaRG® cells, basal medium, growth and differentiation supplements were acquired from Biopredic International (Saint Grégoire, France). William's E medium powder (with L-glutamine, without glucose) was manufactured by United States Biological. HepG2 cells were sourced from the European Collection of Cell Cultures (Salisbury, UK). Lactate dehydrogenase cytotoxicity detection kit was purchased from Roche Diagnostics Ltd (West Sussex). ASLAN003 and BAY2402234 were kindly donated by ASLAN Pharmaceuticals Ltd. All other materials and compounds were purchased from Sigma Aldrich (Dorset, UK) unless otherwise specified.

Cell Culture:

HepG2 cells were routinely maintained in high glucose (25 mM) DMEM (41965039, Gibco) supplemented with L-glutamine (4 mM) (CAS: 56-85-9), 10 % (v/v) FBS, sodium pyruvate (1 mM) (CAS: 113-24-6) and HEPES (1 mM) (CAS: 7365-45-9). All cells were incubated in a humidified environment at 37 °C with 5 % (v/v) CO_2. Cell populations were used between passages 2-20 as per vendor instructions.

Undifferentiated HepaRG® cells were supplied at passage 12 and cultured as specified by Biopredic International (Saint Grégoire, France). Briefly, cells were thawed and allowed to propagate in HepaRG® growth medium (basal medium plus growth supplements) for two weeks, with twice weekly media changes. Cells were collected via trypsinisation (0.05 % trypsin-EDTA) and seeded into appropriate culture vessels using seeding densities recommended by the vendor. Cells were maintained in growth media for a further two weeks followed by two weeks in differentiation media (basal medium plus differentiation supplements). Media changes were performed twice weekly, fully differentiated cells were used within a period of four weeks. All experiments were performed using cells at a passage number < 20.

Acute Metabolic Modification Assays:

Acute metabolic modification assays were performed in HepG2 and differentiated HepaRG® cells as previously described. Briefly, HepG2 (1×10^5/well) and undifferentiated HepaRG® (9×10^3/well) cells were seeded into 96-well, collagen coated plates (50 µg/mL in 0.02 M acetic acid)[29,32].

HepaRG® assay medium was prepared from glucose-free William's E medium (W1105-05), supplemented with insulin (5 µg/mL) (CAS: 11061-68-0), L-glutamine (2 mM), hydrocortisone (50 µM) (CAS: 50-23-7) and sodium bicarbonate (3.7 mg/mL) (CAS: 144-55-8). To this, either D-glucose (11 mM) or D-galactose (10 mM) was added as stipulated by Biopredic International (Saint Grégoire, France). HepG2 assay medium was prepared from serum- and glucose- free DMEM (11966025), supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) and HEPES (1 mM). To this, either D-glucose (25 mM) (CAS: 492-62-6) or D-galactose (10 mM) (CAS: 59-23-4) was added.

On the day of assay, HepG2 cells and differentiated HepaRG® cells were washed and acutely conditioned (2 h) with their respective glucose or galactose media formulations. Following pre-conditioning, serial dilutions of compound were dispensed into the culture plates and incubated with the cells for either 2 or 24 hours (5 % (v/v) CO_2, 37 °C). The final solvent concentration for all experiments was 0.5 % (v/v) DMSO (CAS: 67-68-5).

Endpoint measures of total ATP content, lactate dehydrogenase activity (lysates and supernatants) and total protein were made for each well. All values are reported as a percentage of the corresponding vehicle control (0.5 % (v/v) DMSO).

Determination of IC_{50} Values:

IC_{50} values, the concentration at which total ATP content and/or LDH retention levels reach 50 % of the vehicle control, were calculated by non-linear regression using GraphPad Prism® 7 software (GraphPad Software Inc, CA, USA). Direct mitochondrial dysfunction was defined as a significant difference between the ATP IC_{50} values in glucose and galactose media with a ratio ≥ 2 (IC_{50}-ATP_{glu}/IC_{50}-$ATP_{gal} \geq 2$). Induction of mitochondrial dysfunction prior to the onset of cell death was defined as (IC_{50}-LDH_{gal}/ IC_{50}-$ATP_{gal} \geq 2$)[29–31].

Respirometry by Extracellular Flux Analysis:

Undifferentiated HepaRG® cells were seeded into collagen coated (50 µg/mL in 0.02 M acetic acid) XF^e96 cell culture microplates at a density of 5×10^3 cells/ well and differentiated as previously described[29,32].

Mitochondrial stress test:

Following compound pre-treatment (24 h) or prior to acute compound injection, culture medium was replaced with 175 µL unbuffered XF assay medium supplemented with glucose (25 mM), L-glutamine (2 mM) and sodium pyruvate (1mM) and adjusted to pH 7.4. Cells were incubated for 1 hour in a CO_2 free incubator (37 °C) prior to the start of the assay.

A mitochondrial stress test was conducted, consisting of sequential injections of 1 µM oligomycin (ATP synthase inhibitor) (CAS: 579-13-5), 0.75 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (ionophore) (CAS: 370-86-5) and 1 µM rotenone/antimycin A (complex I/III inhibitors respectively) (CAS: 83-79-4/CAS: 1397-94-0), with each injection followed by three or five measurement cycles. For acute exposure studies, the mitochondrial stress test was preceded by the injection of DHODH inhibitors at the specified concentrations. Oxygen consumption rates (OCR) were normalised to total protein content of the well and expressed as pmol/min/µg.

OCR values were used to calculate the following respiratory parameters: non-mitochondrial respiration (NMR) = lowest OCR value after the injection of rotenone/antimycin A, basal respiration (BR) = last measurement before oligomycin – NMR, proton leak (PL) = lowest OCR

value after oligomycin – NMR, ATP-linked respiration (ALR) = BR - PL, maximal respiratory capacity (MRC) = highest OCR measurement after injection of FCCP – NMR and spare respiratory capacity (SRC) = MR – BR.

Substrate Driven Respiration at Complexes I and III:

Differentiated HepaRG® cells were pre-treated with compounds for 24 hours. Following treatment, culture medium was replaced with 175 µL mitochondrial assay solution (MAS) buffer (5mM $MgCl_2$ (CAS: 7786-30), 220 mM mannitol (CAS: 69-65-8), 70 mM sucrose (CAS:57-50-1), 10 mM KH_2PO_4 (CAS: 7778-77-0), 2 mM HEPES (CAS: 7365-45-9), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (CAS: 13368-13-3) and 0.4 % (w/v) fatty acid free bovine serum albumin (BSA) (CAS: 9048-46-8), pH 7.2), supplemented with constituents to stimulate oxygen consumption via complex I (4.6 mM ADP (CAS: 20398-34-9), 30 mM malic acid (CAS: 636-61-3), 22 mM glutamic acid (CAS: 6893-26-1), 0.2 % (w/v) BSA and 1 nM recombinant perfringolysin O (rPFO) (102504-100, Agilent Technologies)) or complex III (4.6 mM ADP, 500 µM duroquinol (CAS: 527-18-4), 1 µM rotenone, 40 µM malonic acid (CAS: 141-82-2), 0.2 % (w/v) BSA and 1 nM rPFO).

Each run included a pre-programmed calibration and 3 cycles of mix/measure/wait (30 secs/2 mins/30 secs) to establish a baseline OCR prior to the injection of any compounds. Following this, a mitochondrial stress test was conducted as previously described. Individual complex activities were normalised to the respective vehicle control. Raw OCR values were normalised to total protein content of the well and expressed as pmol/min/µg.

Statistical Analysis:

Data are representative of at least three independent experiments (n=3) and all values are expressed as mean ± standard error (S.E.M) as appropriate. Statistical analyses were preformed using GraphPad Prism® 7 software (GraphPad Software Inc, CA, USA). Data were tested for Gaussian distribution using the Shapiro-Wilk normality test before statistical significance was determined using an unpaired t-test with Welch's correction or one-way Analysis of Variance (ANOVA) with Dunnett's correction for multiple comparisons.. A p-value ≤ 0.05 was accepted as the significance threshold.

Results

Exposure to DHODH inhibitors induces time-dependent multifactorial toxicity in HepaRG® cells:

The potential for DHODH inhibitors to induce mitochondrial toxicity in HepaRG® cells was assessed using the acute metabolic modification assay over a 2- and 24-hour period. Baseline HepaRG® metabolism was manipulated by substituting 11 mM glucose with 10 mM galactose and 2 mM L-glutamine in the assay medium. Carbohydrate substitution forces the cells to initiate glycolysis via the oxidation of galactose to pyruvate, thus rendering net ATP gain from glycolysis negligible and resulting in an increased reliance upon oxidative phosphorylation for ATP synthesis[33]. Ultimately this allowed for the detection of compounds that perturb respiratory function in the absence of compensatory glycolytic activity. Comprehensive validation of the method with HepaRG® cells has been performed previously by Kamalian *et al.*, using positive mitochondrial toxicants with well characterised modes of action[29,33].
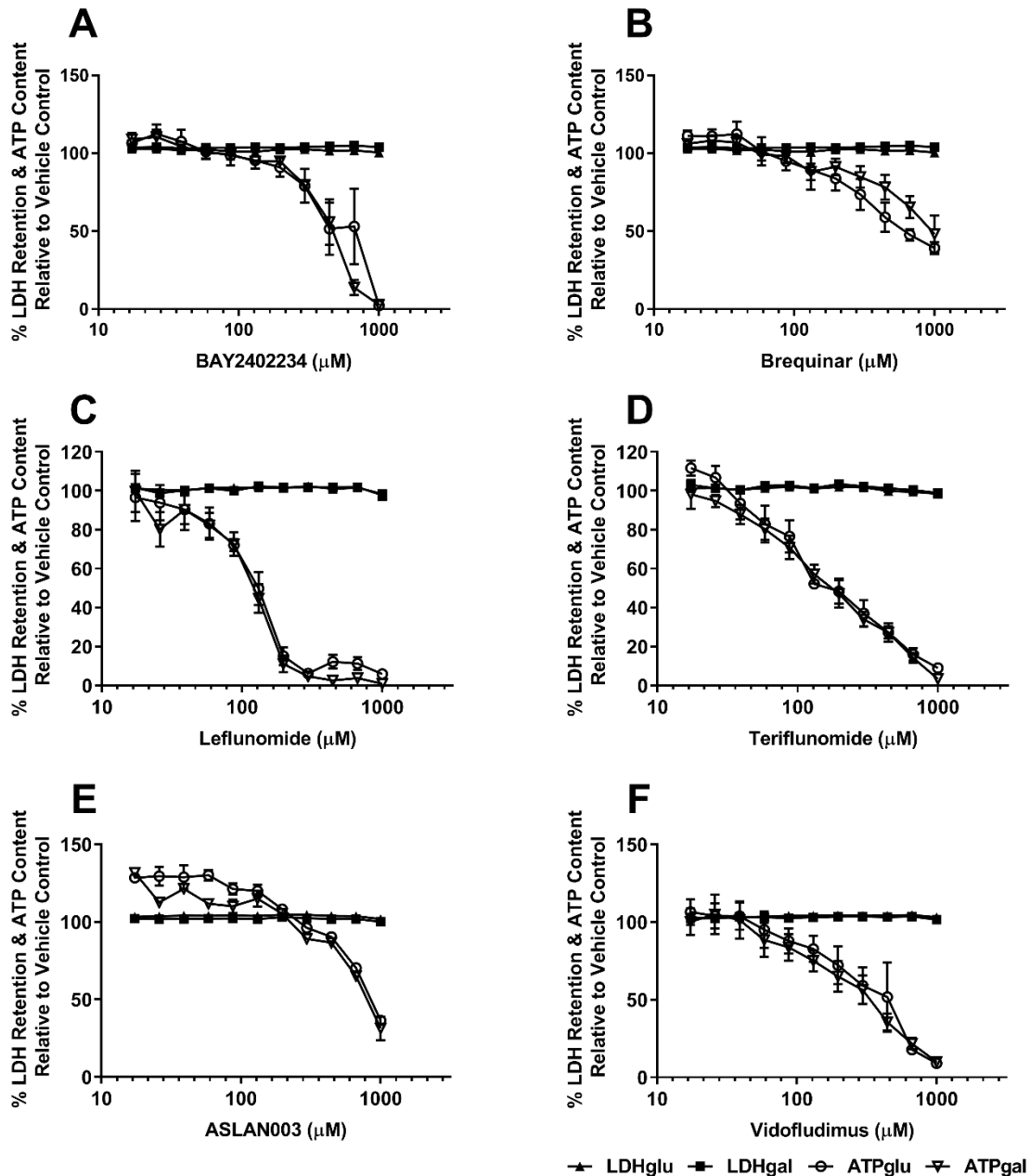
Figure 1 The effect of acute (2 h) DHODH inhibitor application (0-1000 µM) upon ATP content and LDH retention in differentiated HepaRG® cells conditioned to glucose and galactose media. Results are expressed as percentage of the corresponding vehicle control and graphical values are displayed as mean ± S.E.M (n=3).

As depicted in Figure 1, each of the DHODH tested reduced cellular ATP content with no appreciable separation between the glucose and galactose media conditions over 2 hours. Leflunomide and teriflunomide were the most potent of the compounds tested (IC$_{50}$ ATP$_{gal}$ 108.4 ± 15.2 and 182.6 ± 33.2 respectively), however there was no evidence of cell membrane rupture (loss of LDH retention) at equimolar concentrations. After an extended 24 hour incubation period, a similar trend was observed (Figure 2). Leflunomide and teriflunomide reduced cellular ATP content the most potently (IC$_{50}$ ATP$_{gal}$ 144.2 ± 5.9 and 143.7 ± 35.2 respectively), followed closely by vidofludimus (IC$_{50}$ ATP$_{gal}$ 197.8 ± 26.7). However, reductions in ATP content were accompanied by marked losses in cell membrane integrity in response to BAY2402234 and leflunomide treatment. ASLAN003 reduced cellular ATP content the least

potently of the compounds tested (IC$_{50}$ ATP$_{gal}$ 542.4 ± 65.6) and was characterised by increased ATP$_{glu}$ and ATP$_{gal}$ content compared to vehicle at the lower end of the concentration range. Increased intracellular ATP content, particularly in cancer cells may be indicative of compensatory metabolic adaptations to cytotoxic compounds. Such changes have been linked to various mechanisms including apoptotic cell death, cellular stress pathways and chemoresistance[34,35]. The rank order of toxicity at 24 hours (ATP content) was as follows: leflunomide ≡ teriflunomide > vidofludimus > brequinar ≡ BAY2402234 > ASLAN003.
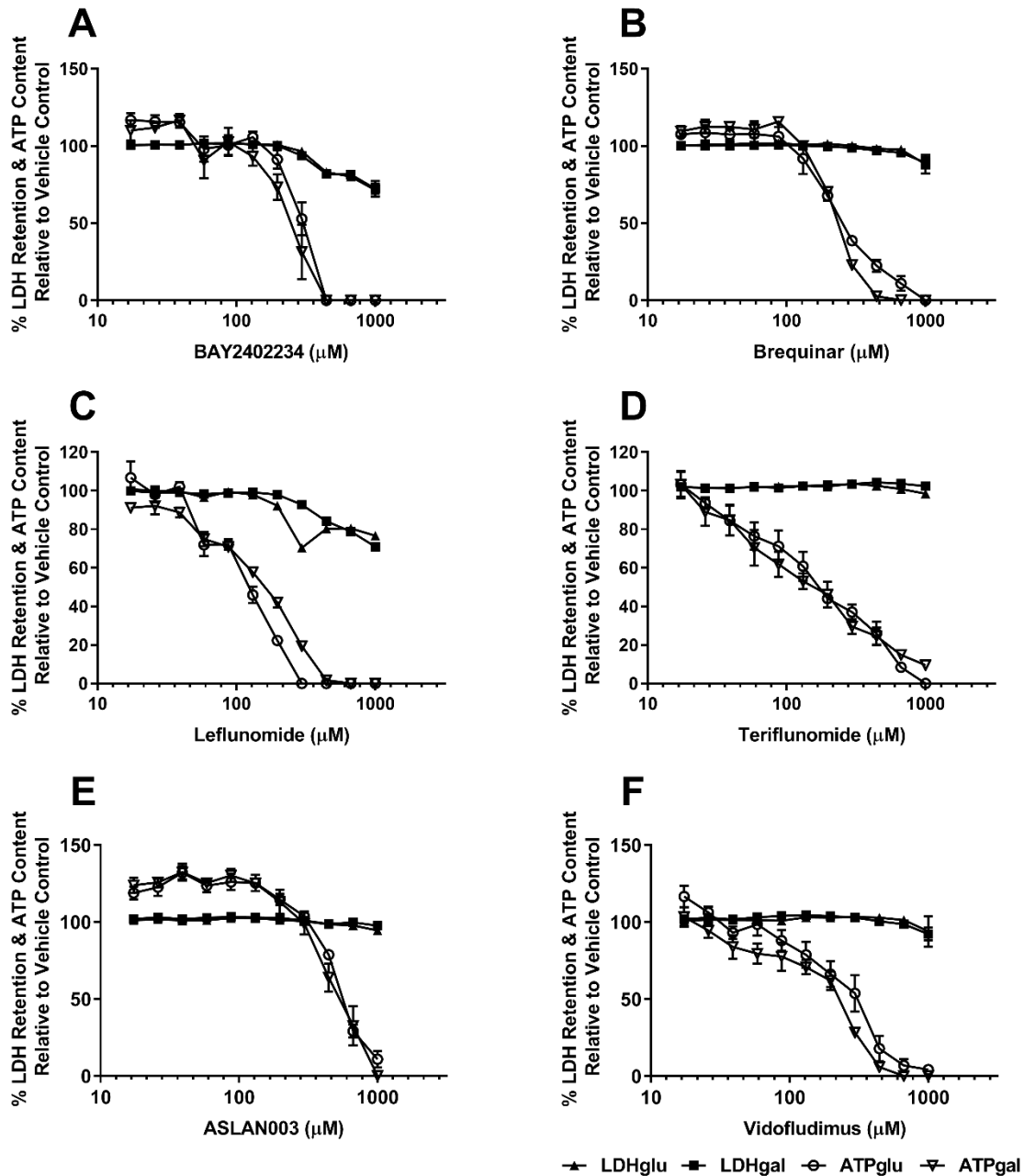

Figure 2 The effect of extended (24 h) DHODH inhibitor application (0-1000 µM) upon ATP content and LDH retention in differentiated HepaRG® cells conditioned to glucose and galactose media. Results are expressed as percentage of the corresponding vehicle control and graphical values are displayed as mean ± S.E.M (n=3).

Mitochondrial toxicity parameters were defined by calculating the ratio between the IC$_{50}$ values for ATP$_{glu}$ versus ATP$_{gal}$, whereby a ratio ≥ 2 indicated that the test compound was a direct

respiratory toxicant, thus had a more pronounced effect in galactose media. An IC_{50} ratio ≥ 2 for LDH_{gal} versus ATP_{gal} indicated that mitochondrial dysfunction was preceding cell death, in line with previous definitions. As indicated in Table 2, for each compound and time point with the exception of brequinar (2 h) and ASLAN003 (2/24 h), mitochondrial dysfunction could be defined as preceding cytotoxicity[29–31]. The greatest separation between ATP_{glu}/ATP_{gal} was observed in response to vidofludimus (24 h).

Table 2 Summary of accompanying IC_{50} values for each compound in HepaRG® cells as determined by non-linear regression. Results are displayed as mean ± S.E.M (n=3). Statistical significance was determined by unpaired t-test with Welch's correction. *p-value < 0.05, **p-value <0.01, ***p-value < 0.001.

Compound	Hours	LDH IC_{50} (µM) ± S.E.M		ATP IC_{50} (µM) ± S.E.M		IC_{50} ATP_{glu}/ ATP_{gal} (p-value)	IC_{50} LDH_{gal}/ ATP_{gal} (p-value)
		Glucose	Galactose	Glucose	Galactose		
BAY2402234	2	> 1000	> 1000	554 ± 119	484 ± 39	1.1 (n/s)	> 3 (n/d)
	24	> 1000	> 1000	308 ± 4.1	253 ± 22	1.2 (n/s)	> 4 (n/d)
Brequinar	2	> 1000	> 1000	614 ± 137	874 ± 125	0.7 (n/s)	> 1.1 (n/d)
	24	> 1000	> 1000	294 ± 10	261 ± 1.6	1.1 (n/s)	> 4 (n/d)
Leflunomide	2	> 1000	> 1000	118 ± 19	108 ± 15	1.1 (n/s)	> 9 (n/d)
	24	> 1000	> 1000	112 ± 4.7	144 ± 5.9	0.8 (*)	> 7 (n/d)
Teriflunomide	2	> 1000	> 1000	178 ± 32	182 ± 33	1.0 (n/s)	> 6 (n/d)
	24	> 1000	> 1000	179 ± 32	143 ± 35	1.3 (n/s)	> 7 (n/d)
ASLAN003	2	> 1000	> 1000	863 ± 6.7	818 ± 47	1.05 (n/s)	> 1.22 (n/d)
	24	> 1000	> 1000	561 ± 14	542 ± 65	1.04 (n/s)	> 1.84 (n/d)
Vidofludimus	2	> 1000	> 1000	372 ± 109	310 ± 58	1.2 (n/s)	> 3 (n/d)
	24	> 1000	> 1000	267 ± 56	197 ± 26	1.4 (n/s)	> 5 (n/d)

Abbreviations: n/d, value could not be determined; n/s, value not statistically significant.

Lack of appreciable differential toxicity between media conditions (IC_{50} $ATP_{glu/gal}$ ratios < 2), in any case, was consistent with multifactorial toxicity rather than mitochondrial dysfunction alone. Though it should be acknowledged that there are several examples of compounds (e.g. troglitazone, chlorpromazine and sertraline) which have known mitochondrial liabilities yet fall under the umbrella of multifactorial toxicity based upon the defined screening thresholds[31,36,37]. In the case of leflunomide, previous studies have demonstrated that *in vitro* cytotoxicity may be linked to not only mitochondrial dysfunction, but also endoplasmic reticulum (ER) stress and the activation of MAPK (JNK and ERK1/2) signalling pathways[25].

However, it is important to recognise the limitations of the assay as a first line screening tool. Whilst total ATP content is often used as a surrogate marker for mitochondrial function, it provides no deeper mechanistic insight as to the nature of the perturbations taking place. Furthermore, it is limited in its ability to detect all forms of mitochondrial dysfunction and does not take into account depletions of ATP reserves due to the activation of defensive and/or compensatory mechanisms[38].

Functional assessment of HepaRG® bioenergetics reveals the differential impacts of DHODH inhibitors upon the electron transport chain:

Extracellular flux analyser technology (XF^{e}96) was used in conjunction with HepaRG® cells to monitor the effects of DHODH inhibitor exposure (24 h) on cellular bioenergetics. Monitoring changes to OCR in real-time is known to be a more sensitive measure of mitochondrial function than measuring total ATP content[39,40]. Test concentrations were selected based upon the IC_{50} ATP_{glu} values (24 h) for each compound in addition to the extrapolated IC_{25} and IC_{75} values and not on DHODH inhibitory potency (Table 5). Using the respiratory toxicant analytical framework set out by Kamalian *et al.*, the compounds were subsequently categorised based upon their effects on the respiratory chain, specifically basal respiration, ATP-linked respiration and spare respiratory capacity[29]. Results are summarised in Table 3 and displayed fully in Figure 3, representative mitochondrial stress test profiles are displayed in Supplementary Figure 1. **Table 3** Summary of outcomes from the functional assessment of HepaRG® mitochondrial respiration in the presence of DHODH inhibitors.

Compound	Concentrations (µM)	Bioenergetic Profile	Interpretation
BAY2402234	150, 300, 450	BR ↑ ALR → SRC ↓	Possible uncoupling properties at highest test concentration.
Brequinar	150, 300, 450	BR ↓ ALR ↓ SRC ↓	Profile associated with inhibition of ETC activity.
Leflunomide	50, 100, 150	BR ↓ ALR ↓ SRC →	Reduced basal and ATP-linked respiration. Possible mild ETC dysfunction.
Teriflunomide	100, 200, 300	BR ↓ ALR ↓ SRC ↓	Profile associated with inhibition of ETC activity.
ASLAN003	250, 500, 750	BR ↑ ALR → SRC ↓	Possible uncoupling properties at highest test concentration.
Vidofludimus	125, 250, 375	BR ↑ ALR → SRC ↓	Possible uncoupling properties at highest test concentration.

Abbreviations: BR, basal respiration; ALR, ATP-linked respiration; SRC, spare respiratory capacity; ETC, electron transport chain

A reduction in cellular SRC is often seen as a primary indicator of direct electron chain dysfunction. A decrease in SRC in the presence of compounds may be regarded as a 'warning signal' for impending mitochondrial toxicity, defined as a reduction in ATP-linked respiration[29,41]. All compounds, with the exception of leflunomide reduced spare respiratory capacity in a dose dependent manner, with reductions associated with BAY2402234, brequinar, teriflunomide and ASLAN003 reaching statistical significance (Figure 3**A-B**, **D-E**).

Interestingly, vidofludimus, BAY2402234 and ASLAN003 increased basal respiration at their IC_{75} values (375, 450 and 750 µM respectively), indicating hallmarks of mild mitochondrial uncoupling (Figure 3**A, E-F**). Uncoupling of oxidative phosphorylation increases basal respiration as the rate limiting step of ATP synthesis is no longer coupled to oxygen consumption, enabling OCR to increase[42].
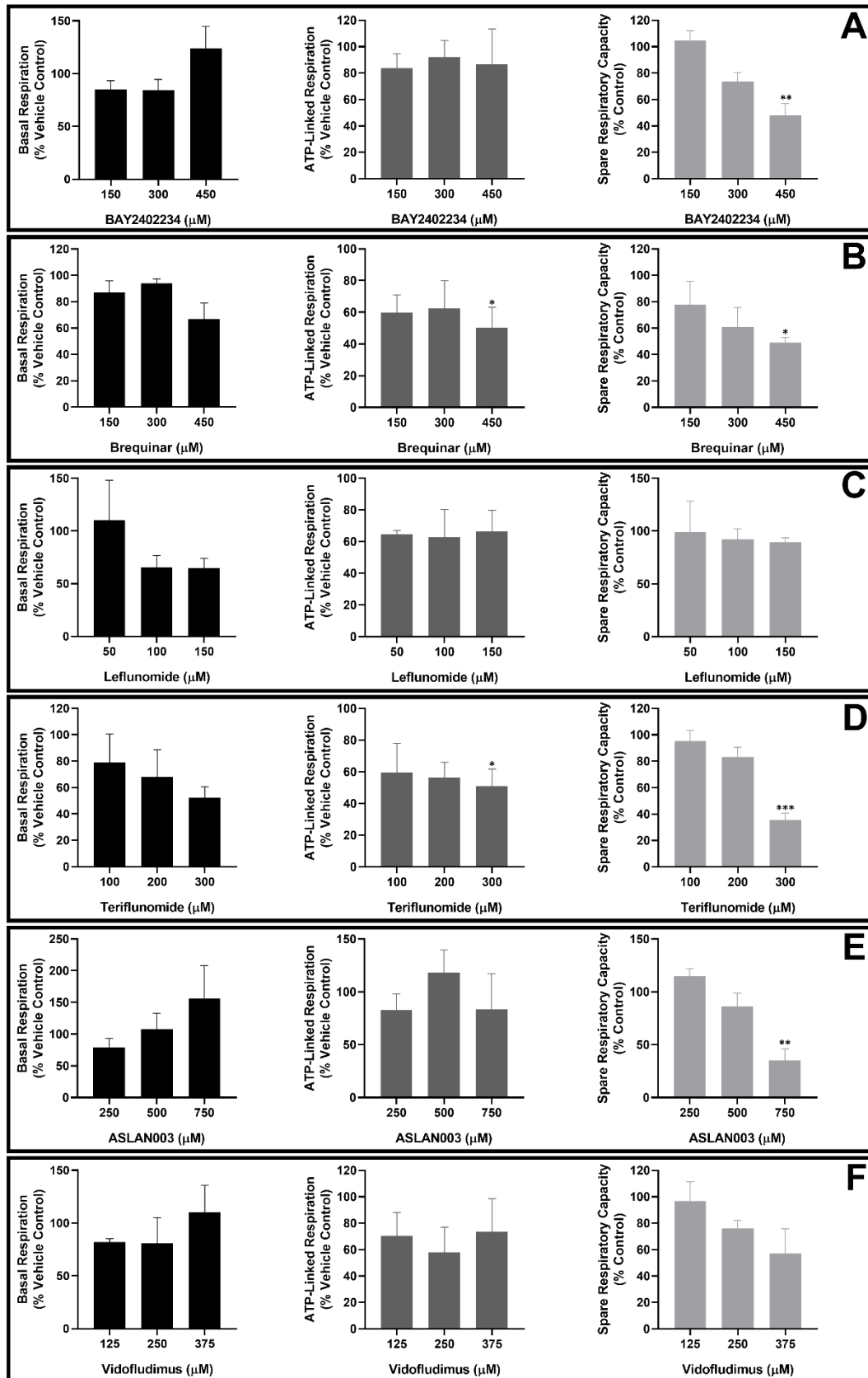
Figure 3 Examining the effects of DHODH inhibitors (24 h) upon bioenergetic parameters in HepaRG® cells compared to vehicle control (0.5 % (v/v) DMSO). **(A)** BAY2402234, **(B)** brequinar, **(C)** leflunomide, **(D)** teriflunomide, **(E)** ASLAN003 and **(F)** vidofludimus.Graphical values are displayed as mean ± S.E.M (n=3) and were normalised to µg protein per well. Statistical significance was determined by one-way ANOVA with Dunnett's correction for multiple comparisons. *p value < 0.05, **p value < 0.01, ***p value < 0.001.

Furthermore, to the best of our knowledge, respiratory uncoupling by these compounds has not been reported previously, thus warrants further investigation.

In contrast, brequinar and teriflunomide exhibited profiles which were consistent with direct, albeit mild, ETC inhibition. At their IC_{75} values, both compounds reduced basal respiration and significantly decreased SRC and ATP-linked respiration. However, in line with the acute metabolic modification testing, these effects were not particularly pronounced in the HepaRG® cell line and were not supportive of direct perturbations of mitochondrial function leading to cytotoxicity. Furthermore, the proportion of OCR dedicated to ATP-linked respiration was only significantly decreased by brequinar and teriflunomide at their IC_{75} values, despite profound effects on total ATP content as measured previously (Figure 3**B, D**).

Leflunomide displayed a profile which could be attributed to mild ETC dysfunction at the concentrations tested (Figure 3**C**). However, the data were atypical in that there was no clear decease in SRC despite reductions in basal and ATP-linked respiration. Investigations performed by Eakins *et al.*, 2016 have reported that this type of respiratory profile is more typical of ATP synthase inhibitors[43]. Prior research has identified leflunomide as a mitochondrial toxicant in HepG2 cells and sub-mitochondrial fractions, with a greater potency than that of its metabolite teriflunomide. Although, it must be acknowledged that these tests were not conducted using high-resolution respirometry platforms[10]. Furthermore, low concentrations (~ 50 µM) of leflunomide have also been shown to promote mitochondrial elongation, upregulate mitofusin (MFN1/2) expression and confer stress resistance across different species and cell types[44].

However, it may be that the bioenergetic profile derived from leflunomide-treated HepaRG® cells could be better explained by examining the *in vitro* model itself. HepaRG® cells are favoured for their phenotypic similarities to fresh human hepatocytes, specifically the expression of CYP450 and bile acid transport enzymes and their ability to form bile canaliculi-like structures[32,45–49]. By the very virtue of their metabolic competency it is likely that HepaRG® cells rapidly convert leflunomide to its active metabolite due to the expression of CYPs that are involved with the biotransformation of the parent compound (e.g. CYP1A2, CYP3A4). It has been previously reported that CYP3A4 expression in differentiated HepaRG® cells is ~2.5 fold higher than in fresh primary human hepatocytes (PHH)[28,45,50].

It must be noted that these investigations are intended to provide mechanistic insight as to the potential effects of DHODH inhibitors upon respiratory chain functionality. Specifically, caution should be exercised if comparing results between compounds as the concentrations were selected based upon observed effect on ATP concentration and not upon concentrations required for pharmacological effect, *in vitro* or clinical. As an illustration, in the case of BAY2402234, brequinar or ASLAN003 the test concentrations employed in this study are substantially greater than those required for pharmacological effect (Table 5), compared with leflunomide for which the concentrations tested more closely match clinical concentrations [10,25].

Acute brequinar and leflunomide exposure induces mitochondrial toxicity in HepG2 cells:

In order to substantiate the biotransformation hypothesis, HepG2 cells, which are reported to either totally lack or express negligible levels of CYP450 isozymes[45], were used to screen for DHODH inhibitor-induced mitochondrial dysfunction using the acute metabolic modification assay (Figure 4).

As depicted in Figures 4**B** and 4**C**, there was a distinct separation between the dose curves for ATP_{glu} and ATP_{gal} in response to acute (2 h) brequinar and leflunomide administration in HepG2 cells, a trend not replicated in the presence of the other compound panel members (Figure 4**A, D** and **F**) and not recapitulated in HepaRG® cells (Figure 1 and Figure 2). To a lesser extent, ASLAN003 showed greater toxicity under galactose conditions (Figure 4**E**). Interestingly, in stark contrast to the HepaRG® cells, BAY2402234 did not reduced cellular ATP content in HepG2 cells. In fact, total ATP levels increased compared to the vehicle control for all concentrations except 1000 µM ATP_{gal} (Figure 4**A**).
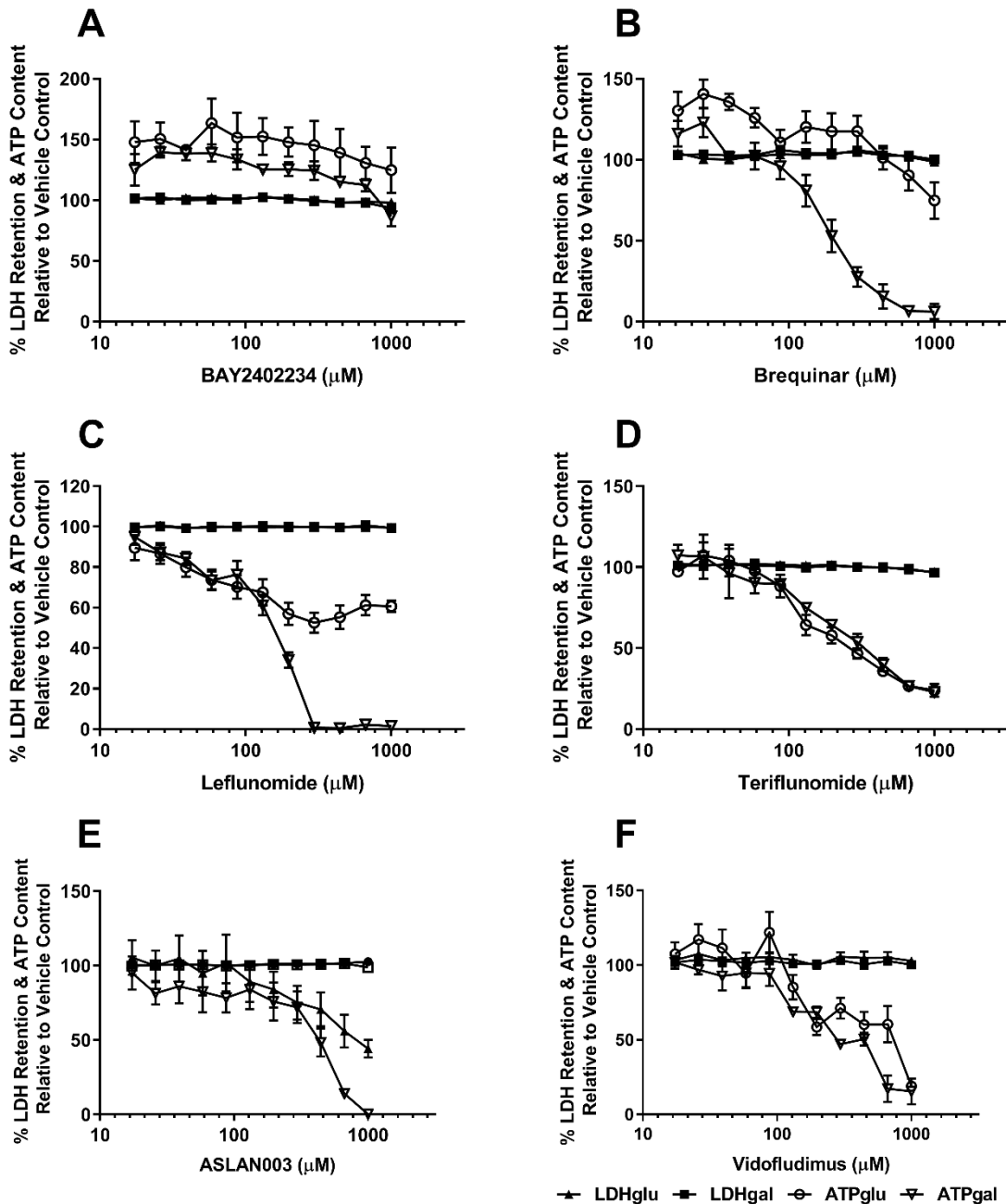

Figure 4 The effects of 2 h **(A)** BAY2402234, **(B)** brequinar, **(C)** leflunomide, **(D)** teriflunomide, **(E)** ASLAN003 and **(F)** vidofludimus upon ATP content and LDH retention in HepG2 cells conditioned to glucose and galactose media. Results are expressed as percentage of the corresponding vehicle control and graphical values are displayed as mean ± S.E.M (n=3).

A summary of the accompanying IC_{50} values are displayed in Table 4. In accordance with the outlined definitions for a positive mitochondrial toxicant, brequinar and leflunomide (2 h) had IC_{50} ATP_{glu}/ATP_{gal} ratios of > 4.80 and 6.34 respectively, surpassing the threshold of $\geq$ 2. In addition, IC_{50} LDH_{gal}/ATP_{gal} ratios of > 4.80 and > 7.75 indicated that in both cases mitochondrial dysfunction preceded cell death. ASLAN003 failed to breach the threshold of $\geq$ 2 for a positive mitochondrial toxicant (IC_{50} ATP_{glu}/ATP_{gal} ratio 1.77), however enhanced potency under galactose conditions was evident at higher concentrations. In contrast, teriflunomide and vidofludimus displayed hallmarks of multifactorial toxicity with ATP_{glu}/ATP_{gal} ratios of 0.87 and 1.68 respectively. Taken together, these data would suggest that leflunomide and brequinar are potent mitochondrial toxicants under acute conditions and prior to their biotransformation *in vitro*. Conversely, BAY2402234 did not reduce cellular ATP content in the HepG2 model and thus it could be questioned whether differences in pharmacokinetics of the compound in HepaRG® cells, for example metabolic by-products or cellular distribution may play a role in the onset of toxicity. To fully understand this, further work would be required.

Carrying forward the aforementioned compounds of interest (leflunomide, BAY2402234, Brequinar and ASLAN003), supplementary mechanistic analyses were performed in HepaRG® cells using an extracellular flux analyser instrument. Rather than adopting a drug pre-incubation strategy as employed previously, the selected DHODH inhibitors were acutely injected during the assay (Supplementary Figure 2, Supplementary Figure 3, Supplementary Table 1). Test concentrations were selected based upon the IC_{25} ATP_{glu} values (2 h) rather than the IC_{50} ATP_{glu} (2 h) due to solubility concerns when preparing high concentration stocks for the XF injection ports.

The results garnered from these investigations largely mimicked the trends observed in the HepG2 metabolic modification assays. Acute application of BAY2402234 in HepaRG® cells had no significant effects upon respiratory parameters whilst brequinar, leflunomide and ASLAN003 displayed varying degrees of mitochondrial dysfunction.

These points exemplify the need, from a first line screening perspective, to carefully consider *in vitro* model selection. The toxic effects of compounds may be missed or severely understated if an inappropriate cell model or time point is selected[22,29,45]. In the case of leflunomide in particular, a combination of biotransformation in HepaRG® cells over 24 hours and a lower starting concentration range (50–150 µM leflunomide versus 100-300 µM teriflunomide), could have potentially resulted in the toxic effects of leflunomide upon mitochondrial function being missed entirely (Figure 3**C**).

Table 4 Summary of accompanying IC_{50} values for each compound in HepG2 cells as determined by non-linear regression. Results are displayed as mean ± S.E.M (n=3). Statistical significance was determined by unpaired t-test with Welch's correction. *p-value < 0.05, **p-value <0.01, ***p-value < 0.001.

Compound	Hours	LDH IC_{50} (µM) ± S.E.M		ATP IC_{50} (µM) ± S.E.M		IC_{50} ATP$_{glu}$/ ATP$_{gal}$ (p-value)	IC_{50} LDH$_{gal}$/ ATP$_{gal}$ (p-value)
		Glucose	Galactose	Glucose	Galactose		
BAY2402234	2	> 1000	> 1000	> 1000	> 1000	~1 (n/d)	~1 (n/d)
Brequinar	2	> 1000	> 1000	> 1000	209 ± 32	> 4.80 (n/d)	> 4.80 (n/d)
Leflunomide	2	> 1000	> 1000	818 ± 182	129 ± 14	6.34 (**)	> 7.75 (n/d)
Teriflunomide	2	> 1000	> 1000	278 ± 40	320 ± 21	0.87 (ns)	> 3.13 (n/d)
ASLAN003	2	> 1000	> 1000	698 ± 185	394 ± 86	1.77 (ns)	> 2.54 (n/d)
Vidofludiumus	2	> 1000	> 1000	599 ± 114	333 ± 19	1.68 (ns)	> 3.00 (n/d)

Abbreviations: n/d, value could not be determined; n/s, value not statistically significant.

Further examining the impacts of leflunomide, teriflunomide and ASLAN003 exposure upon mitochondrial function in HepaRG® cells:

In order to further delineate the impacts of leflunomide, teriflunomide and ASLAN003 on mitochondrial function, assessment of ETC complex (I/III) driven respiration was performed in permeabilised HepaRG® cells by supplying substrate-inhibitor cocktails specific to the complex of interest. Cells were treated 24 hours prior to the assay, after which a mitochondrial stress test was performed. The effects of leflunomide, teriflunomide and ASLAN003 upon state 3$_{(uncoupled)}$ respiration for complexes I and III are presented in Figure 5.

In agreement with studies examining the effects of teri/leflunomide[3,10] and experimental DHODH inhibitors upon ETC complex function in sub-mitochondrial fragments, extracellular flux analysis identified both leflunomide and teriflunomide as inhibitors of complex III function.Teriflunomide showed significant inhibition of complex I driven respiration, however this was not observed in response to leflunomide administration. Conversely, ASLAN003 inhibited complex I more potently than complex III. It has been reported that some DHODH inhibitors act as ubiquinone binding site inhibitors, therefore mammalian respiratory chain enzymes that bind ubiquinone or ubiquinol could potentially be sites of drug interaction[3,51–53]. Whilst not addressed in this study, teri/leflunomide have also been shown to inhibit F_1F_0 ATP synthase (complex V) and adenine nucleotide translocator (ANT) activity, thus hindering both

ATP synthesis and the translocation of mitochondrial ATP with cytosolic ADP[10]. This may partially account for the rapid ATP depletion observed across hepatic models.

The role of DHODH inhibition in the onset of ATP depletion was evaluated by supplementing the culture medium with orotate (1 mM) and/or uridine (1 mM) in order to salvage the pyrimidine pathway downstream of DHODH (Figure 6). The addition of orotate alone did not reverse the ATP depletion induced by ASLAN003 (500 µM), leflunomide (100 µM) or teriflunomide (200 µM). However, the addition of uridine in combination with orotate significantly reduced the effects of ASLAN003 and leflunomide on HepaRG® cells. It is important to note that although supplementation with uridine/orotate can circumvent the effect
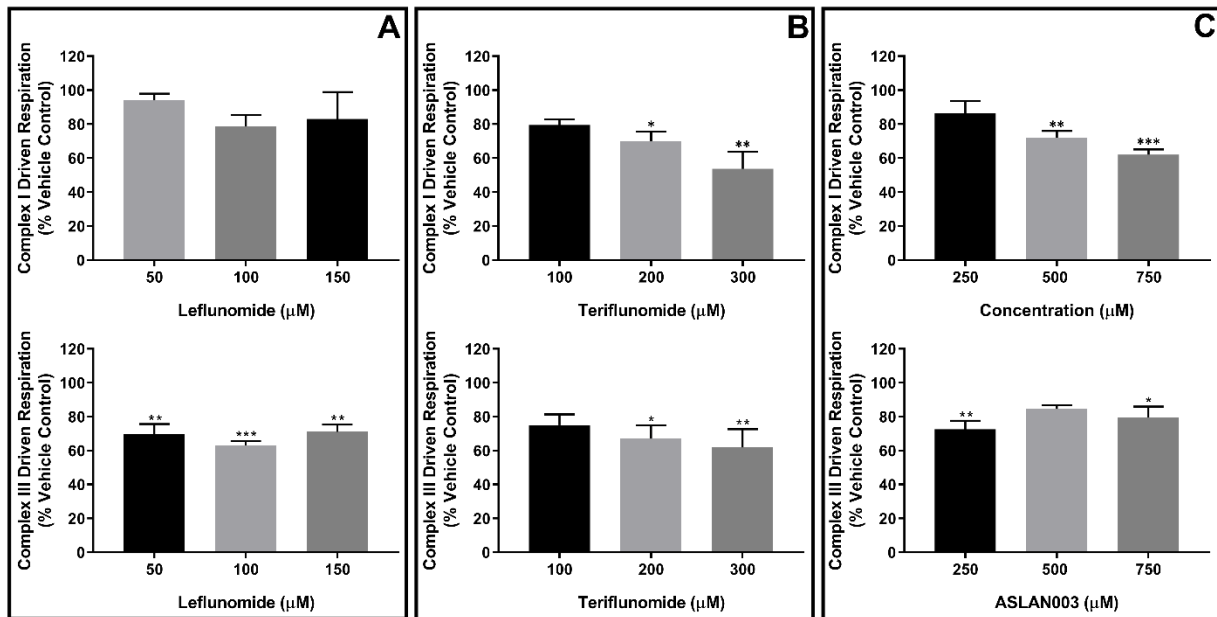

Figure 5 The effect of leflunomide **(A)**, teriflunomide **(B)** and ASLAN003 **(C)** upon ETC complex (I & III) driven respiration. HepaRG® cells were pre-treated with DHODH inhibitors 24 hours prior to cell permeabilisation and delivery of complex specific substrates using an extracellular flux analyser (XF^{e}96) instrument. Complex driven respiration was defined as state 3$_{(uncoupled)}$ respiration, normalised to the vehicle control (0.5 % (v/v) DMSO). Graphical values are displayed as mean ± S.E.M. (n=3) and results were normalised to µg protein per well. Statistical significance compared to the vehicle control was determined by one-way ANOVA with Dunnett's correction for multiple comparisons *p value < 0.05, **p value < 0.01, ***p value < 0.001.

of DHODH inhibition upon the pyrimidine pathway, it could not reduce any effect of DHODH inhibition on mitochondrial respiration or upon direct insult of the ETC. It could therefore be inferred from these data that a proportion of the toxic effects of DHODH inhibitors on cellular ATP content in HepaRG® cells are independent of their effects on the pyrimidine pathway, though further mechanistic investigations would need to be conducted in order to confirm this.
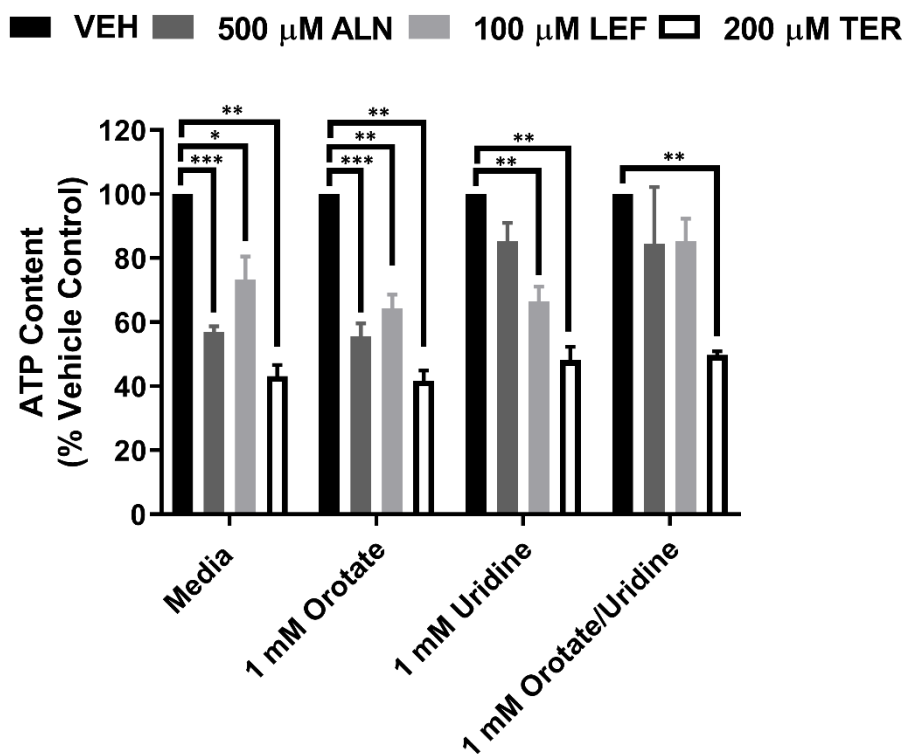.

Figure 6 The effects of orotate (1 mM) and uridine (1 mM) supplementation on ATP depletion induced by DHODH inhibitors in HepaRG® cells (24hrs). Test concentrations were selected based upon 24 hrs IC_{50} ATP_{glu} values. Results are expressed as percentage of the corresponding vehicle control (0.5 % (v/v) DMSO) and graphical values are displayed as mean ± S.E.M. Statistical significance was determined by one-way ANOVA with Dunnet's test for multiple comparisons. *p-value < 0.05, **p-value <0.01, ***p-value < 0.001. Abbreviations: ALN, ASLAN003; LEF, leflunomide; TER, teriflunomide.

Examining the therapeutic efficacy of dihydroorotate dehydrogenase inhibitors in relation to their *in vitro* toxicity:

As summarised in Table 5 the majority of DHODH inhibitors tested, with the exception of leflunomide and teriflunomide, have an IC_{50} value for human DHODH (hDHODH) inhibition in the low nanomolar range. The three compounds with notable therapeutic efficacy include BAY2402234, brequinar and ASLAN003. Conversely, the equivalent IC_{50} ATP_{glu} values in HepaRG® cells (24 h) were far greater, in the low to mid micromolar range. This was particularly true of the more potent DHODH inhibitors, as denoted by the exceptionally high IC_{50} ATP_{glu}/ IC_{50} hDHODH ratios, a surrogate measure of *in vitro* toxicity in relation to efficacy. In contrast, the less potent DHODH inhibitors, particularly leflunomide, had IC_{50} ATP_{glu} values which aligned more closely with their reported IC_{50} hDHODH or plasma C_{max} values [10,25].

It is therefore clear that the compound concentrations used throughout the present study, excluding leflunomide, were more than sufficient to fully inhibit hDHODH *in vitro*. When the inhibitory potency of each compound is taken into account, it is apparent that their associated toxicities are not consistent mechanistically and do not appear to show a dependency upon DHODH inhibition in hepatocarcinoma cells.

Table 5 Summary of IC_{50} ATP_{glu} values in HepaRG® cells (24 h) and IC_{50} values for human dihydroorotate dehydrogenase (hDHODH) inhibition derived from literature review.

Compound	IC_{50} ATP_{glu} (µM)	IC_{50} hDHODH (µM)	Ratio IC_{50} ATP_{glu}/ IC_{50} hDHODH	References
BAY2402234	308	0.0012	256,667	[13]
Brequinar	294	0.010	29,400	[54,55]
Leflunomide	112	98	1.14	[54]
Teriflunomide	179	1.3	137.6	[56]
ASLAN003	542	0.035	15,485	[57]
Vidofludiumus	197	0.16	1,231	[58]

Conclusions

Due to the sustained and widespread use of leflunomide and teriflunomide in the clinic, investigations into the pathomechanistic basis of DHODH inhibitor related liver injuries are a necessity for both the repurposing and safer use of the drug class going forward. It is also important to establish whether nascent pharmaceuticals, with related modes of action, harbour the same or similar potential for hepatic damage. For example ASLAN003, a second generation DHODH inhibitor, is structurally distinct from leflunomide and teriflunomide and exhibits a favourable hepatic safety profile under trial conditions in patients with acute myeloid leukaemia[57]. Indeed, this was confirmed in the present study whereby ASLAN003 depleted cellular ATP and LDH content less potently than leflunomide and teriflunomide. It is therefore pivotal to understand whether a DILI concern will be shared across a family of compounds due to inextricably linked pharmacological and/or toxicological mechanisms.

As previously mentioned, respiratory chain dysfunction has been recognised as a putative mechanism by which leflunomide, and its active analogue teriflunomide, elicit their hepatotoxic effects *in vitro*[10,25]. Therefore, it is necessary to determine whether there is a shared risk of mitochondrial dysfunction across other DHODH inhibitors, not least due to the innate pharmacological targeting of mitochondrial pathways through their intended mode of action[1]. Importantly, mitochondrial dysfunction is increasingly implicated as a major mechanism of DILI. Impaired mitochondrial respiration disrupts energetic homeostasis, which can result in cellular dysfunction or death depending on the severity of the deficit. Whilst moderate impairments may only result in dysfunction at a localised cellular level, severe or sustained perturbations can result in organ damage[39,59]. This is particularly pertinent for patients with pre-existing liver diseases or elevated liver enzymes receiving medications which are potentially hepatotoxic.

The present study has demonstrated that amongst the DHODH inhibitors tested, both cytotoxic potential and propensity to perturb mitochondrial function in HepaRG® cells varied between compounds. At 24 hours BAY2402234, ASLAN003 and vidofludimus exhibited profiles which were consistent with mild mitochondrial uncoupling whilst teriflunomide, brequinar and to a lesser extent leflunomide behaved similarly to traditional ETC inhibitors. However, although HepaRG® cells are considered to be a more physiologically relevant hepatic model compared to HepG2 cells, benefitting from phenotypic similarity to fresh human hepatocytes (i.e. CYP450 expression) with enhanced culture longevity, they are not the most appropriate model for examining hepatotoxicity associated with parent compounds or pro-drugs[29,45].

This point was exemplified when examining leflunomide-associated mitochondrial toxicity in the HepaRG® model. Despite prior evidence of adverse mitochondrial events associated with leflunomide exposure being present in the literature, the same effect was not recapitulated in HepaRG® cells via preliminary mitochondrial toxicity screening at 2 and 24 hours or at 24 hours using an extracellular flux analyser[10,25]. However, significant differential toxicity was apparent after 2 hours of leflunomide exposure in metabolically modified HepG2 cells and was also evident when using an acute injection strategy on the XF^{e}96 instrument with HepaRG® cells. This has provided a line of evidence to suggest that leflunomide, prior to its biotransformation *in vitro*, is a potent mitochondrial toxicant. Indeed, this is in agreement with research suggesting that leflunomide-induced hepatotoxicity is exacerbated by CYP450 inhibitors *in vitro* and can result in fatal hepatitis in the clinic[50,60].

Further work in HepG2 cells also examined the possibility that the remaining DHODH inhibitors may be undergoing biotransformation, thus understating their mitotoxic potential in the HepaRG® model. It was subsequently demonstrated that brequinar exposure generated an $ATP_{glu/gal}$ ratio that was indicative of acute mitochondrial dysfunction. Conversely, BAY2402234 did not elicit reductions in ATP content in HepG2 cells, indicating that energetic disruptions may be mediated by a metabolite in HepaRG® cells and not necessarily the parent compound itself. Again, these points were further substantiated when examining acute DHODH inhibitor exposure in HepaRG® cells on an XFe96 analyser, thus reinforcing the need to carefully consider the metabolic competencies of the *in vitro* model selected for mitochondrial toxicity screening.

Furthermore, from a bioenergetic standpoint, side-by-side comparisons of the effect of leflunomide treatment upon total ATP content across HepG2, HepaRG® and fresh human hepatocytes showed that HepaRG® cells were more resistant to ATP depletion than their counterparts[25]. This may be owing, in part, to a greater proportion of their maximal respiratory capacity being dedicated to spare respiratory capacity at baseline. This is in stark contrast to HepG2 cells which, due to their proliferative nature, have less reserve capacity and dedicate a greater proportion of their maximal OCR to ATP-linked respiration[29]. Therefore, considerations should also be made in regards to the inherent susceptibility of the chosen model to mitochondrial insults.

Finally, it is important to assess the role that inter-individual variation may play in dictating patient susceptibility to adverse events as often these factors are not addressed when using homogeneous populations of cells or pre-clinical species during screening. For example, patients with subclinical mitochondrial insufficiencies i.e. mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) variants that alter respiratory chain functionality, may potentially be more susceptible to DHODH inhibitor mediated mitochondrial dysfunction[61,62], particularly at complexes I and III[3,10]. Whilst the effect of DHODH inhibitors upon mitochondrial respiratory parameters were demonstrably mild in the HepaRG® model, this does not detract from the potential amplification of drug-induced mitochondrial dysfunction amongst some recipients.

Conflicts of Interest

Funding

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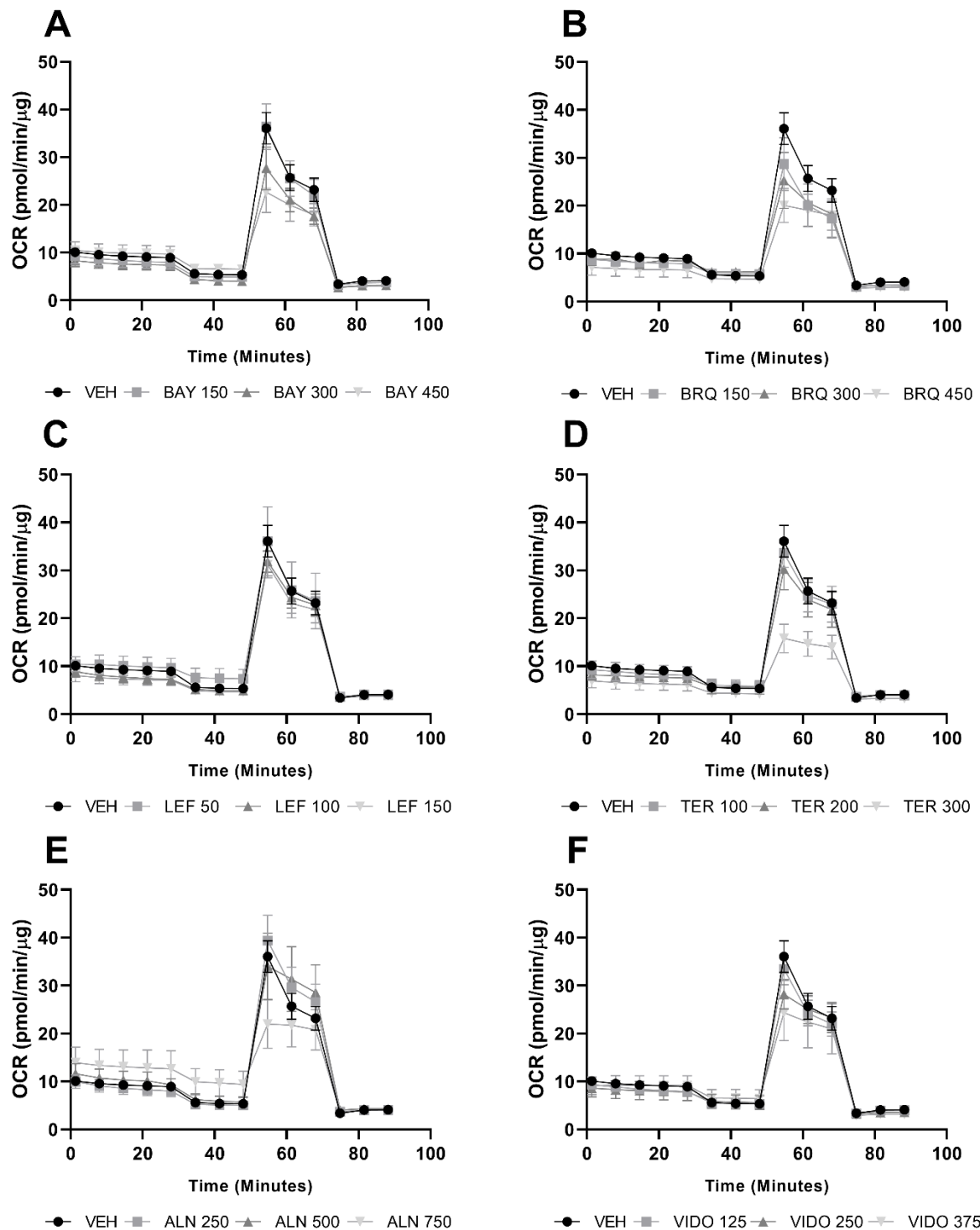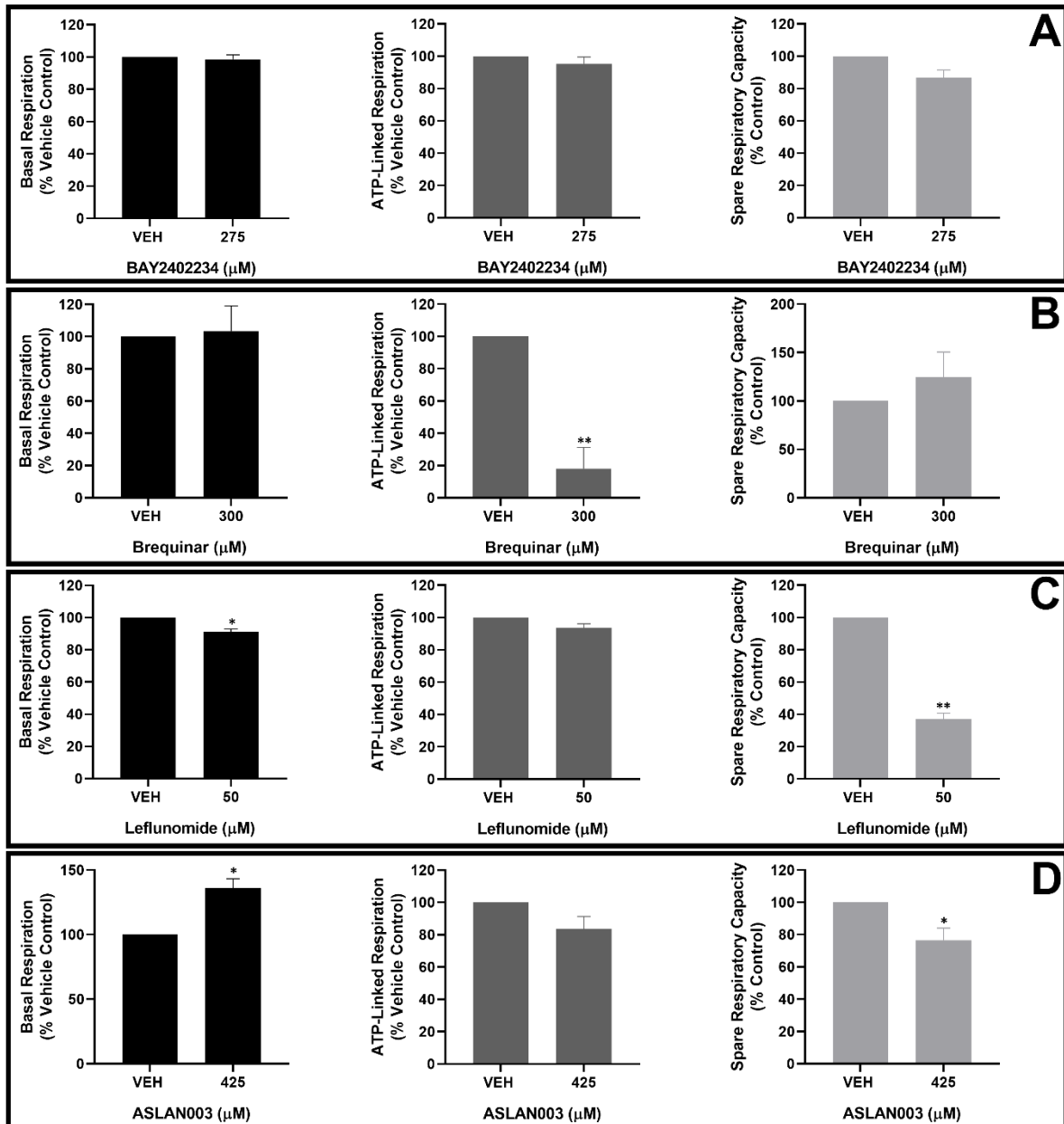
Supplementary Figure 1 Representative XF mitochondrial stress test profiles examining the effects of DHODH inhibitors (24 h) upon bioenergetic parameters in HepaRG® cells compared to vehicle control (0.5 % (v/v) DMSO). **(A)** BAY2402234, **(B)** brequinar, **(C)** leflunomide, **(D)** teriflunomide, **(E)** ASLAN003 and **(F)** vidofludimus.Graphical values are displayed as mean ± S.E.M (n=3) and were normalised to µg protein per well
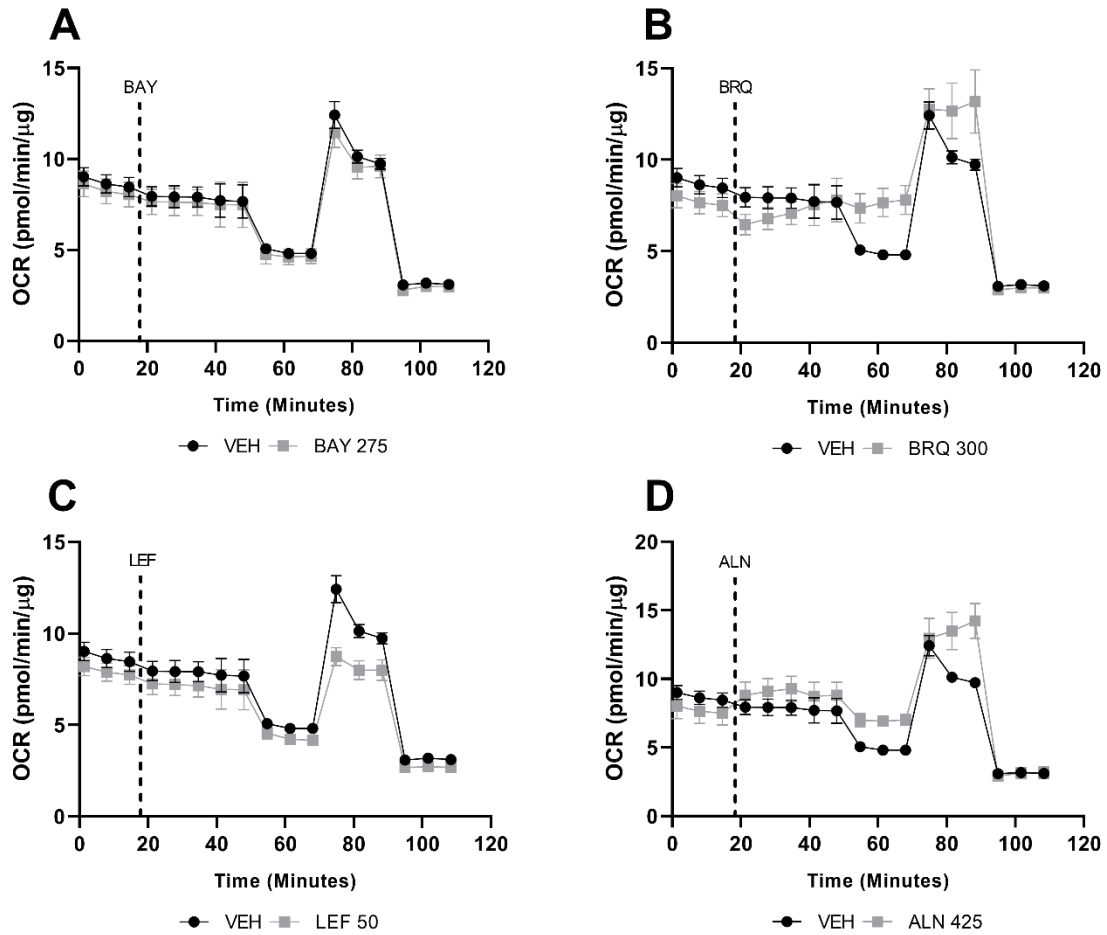
Supplementary Figure 2 Representative XF mitochondrial stress test profiles examining the acute effects of DHODH inhibitors upon bioenergetic parameters in HepaRG® cells compared to vehicle control (0.5 % (v/v) DMSO). **(A)** BAY2402234, **(B)** brequinar, **(C)** leflunomide **(D)** ASLAN003. Dashed lines represent the point at which the test compound was injected into the XF well plate. Graphical values are displayed as mean ± S.E.M (n=3) and were normalised to µg protein per well.

Supplementary Figure 3 Examining the acute effects of DHODH inhibitors upon bioenergetic parameters in HepaRG® cells compared to vehicle control (0.5 % (v/v) DMSO). **(A)** BAY2402234, **(B)** brequinar, **(C)** leflunomide, **(D)** ASLAN003.Graphical values are displayed as mean ± S.E.M (n=3) and were normalised to µg protein per well. Statistical significance was determined by un-paired t-test with Welch's correction. *p value < 0.05, **p value < 0.01, ***p value < 0.001.

Supplementary Table 3 Summary of outcomes from the functional assessment of HepaRG® mitochondrial respiration in response to acute DHODH inhibitor exposure.

Compound	Concentrations (µM)	Bioenergetic Profile	Interpretation
BAY2402234	275	BR → ALR → SRC ↓	No significant effects observed
Brequinar	300	BR → ALR ↓ SRC ↑	Profile associated with inhibition of ETC activity.
Leflunomide	50	BR ↓ ALR → SRC ↓	Profile associated with inhibition of ETC activity.
ASLAN003	425	BR ↑ ALR ↓ SRC ↓	Possible uncoupling properties at test concentration.