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Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes

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ABSTRACT

Technological developments are driving *in vitro* methods towards integrated "omic" strategies. However, there is still an over reliance on classical viability assays for dose range finding. Such assays are not readily suited to the investigation of subtle alterations in cell function and most require termination of the experiment, which makes it difficult to monitor temporal alterations in repeat-dose long term exposure experiments. To this end, we investigated the use of lactate production as a marker of cell stress in long term repeat dose experiments.

We conducted daily exposures to eight compounds at five concentrations for 14 days on human renal proximal tubular cells (RPTEC/TERT1), human hepatoma cells (HepaRG) and mouse fibroblasts (BALB-3T3) cells. Compounds were chosen from a training set used in the 7th EU Framework project Predict-IV and consisted of amiodarone, diclofenac, troglitazone, cadmium chloride, cephaloridine, cidofovir, cyclosporine A and buflomedil. At days 1, 3, 7 and 14, lactate was measured in the supernatant medium. At day 14, cells were assayed for resazurin reduction capability and subsequently lysed in methanol for ATP determination. Compound-induced loss of viability was comparable across all cell lines. For all cell types, when cell viability was compromised at day 14, lactate production was induced during the treatment period. In some situations, lactate also fell below control values, indicating cell death. Thus, temporal alterations in supernatant lactate provides information on the time and concentration of stress induction and the time and concentration where cell death becomes the dominant factor.

Supernatant lactate production is a simple, cheap and non-invasive parameter. Since many molecular pathways converge on the glycolytic pathway, enhanced lactate production may be considered as a global marker of sub-lethal injury and thus an ideal marker for investigating temporal alterations in long term repeat dose testing *in vitro* regimes.

1. Introduction

The use of integrated "omic" technologies in *in vitro* toxicological strategies aims to identify novel stress and dysfunction pathways and to further elucidate existing pathways (Vinken et al., 2008). The ultimate goal is to shift the dependence on classical cell death endpoints such as MTT, lactate dehydrogenase (LDH) release and neutral red uptake, towards the use of more sensitive, mechanistic-based injury markers. However, until such novel cell stress markers are identified and validated, classical cell death endpoints must still be used prior to "omic" strategies for dose ranging experiments. This is a particularly confounding problem for long-term repeat dose toxicity testing *in vitro*, as the majority of cell death assays are either invasive (i.e. may interfere with the experiment) or end-point based (i.e. require experiment termination) and thus are not suited for temporal monitoring. An appropriate marker for repeat dose testing needs to be measured repeatedly, should be non-invasive (non-interfering) and provide information about the time and dose of initiation of stress or toxicity (Prieto et al., 2006).

Lactate production via anaerobic metabolism of glucose is an example of such a potential marker. Practically all animal cells exhibit a basal rate of glycolysis and various stress situations increase glycolysis rates. One of the best characterised inducers of glycolysis is hypoxia. Under hypoxic conditions, where available oxygen is limiting, HIF-1 alpha activation results in transcriptional up-regulation of a number of glycolytic enzymes to maintain sustainable ATP levels via enhanced glycolysis leading to increased lactate pro-

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duction (Semenza, 1998; Signorelli et al., 2010). Additionally, there are many lines of evidence to suggest that proliferating cells favour glycolysis over oxidative metabolism despite the fact that oxidative metabolism is energetically more efficient. Cancerous cells, which are highly proliferative, are known to have very high glycolytic metabolism (Warburg, 1956). Indeed, inhibition of LDH-A decreases glycolysis, increases mitochondrial respiration and reduces cell proliferation of tumour cells (Fantin et al., 2006). However, this phenomenon may be a consequence of enhanced proliferation and not necessarily directly linked to a cancerous phenotype *per se* (Najafov and Alessi, 2010). For example, silencing LDH-A or inhibiting it pharmacologically has been shown to prevent glucose consumption, lactate production and proliferation in primary pulmonary microvascular endothelial cells (Parra-Bonilla et al., 2010).

We have previously demonstrated that human proximal tubular cells increase glucose consumption and concomitantly lactate production in response to cyclosporine A (CsA) and that this occurs also at sub-cytotoxic concentrations (Jennings et al., 2009). However, CsA induced cell stress is also associated with a decreased cell proliferation (Jennings et al., 2007). Since CsA can cause mitochondrial disturbances due to blockage of the mitochondrial transition pore and also causes enhanced H_2O_2 production (Jennings et al., 2007), the increase in lactate production with this compound may be related to mitochondrial dysfunction.

Thus, increased lactate production might be brought about by hypoxia, tissue repair (proliferation), oxidative stress and mitochondrial disturbances. Since lactate measurements can be conducted off-line in the collected supernatant medium using a simple colorimetric assay, we investigated the usefulness of this metabolite for the evaluation of compound induced cell stress and toxicity, using a broad range of pharmaceutical toxins in three different cell types. The results demonstrate that lactate is an excellent temporal marker of compound induced cell stress.

2. Materials and methods

All chemicals unless otherwise stated were purchased from Sigma and were of the highest grade available. Cidofovir was a gift from Gilead Sciences (Foster City, USA). CsA was purchased from Calbiochem (Merck, Darmstadt Germany) and troglitazone from Cayman Europe (Estonia).

2.1. Routine cell culture

All cell types were cultured in 10 cm dishes (Sarstedt) at 37 °C in a 5% CO_2 humidified atmosphere in their respective growth medium. The telomerase immortalised human proximal tubule cells, RPTEC/TERT1 (Wieser et al., 2008) were cultured in hormonally-defined medium (HDM). HDM consisted of a 1 to 1 mixture of Dubelcco's modified Eagle's medium (DMEM, Invitrogen, cat. No. 11966) and Ham's F-12 nutrient mix (Invitrogen, cat. No. 21765) supplemented with 2 mM glutamax (Invitrogen, cat. No. 35050), 5 μg/L insulin, 5 μg/L transferrin and 5 ng/L sodium selenite, 100 U/mL penicillin and 100 μg/mL streptomycin, 10 ng/mL epithelial growth factor and 36 ng/mL hydrocortisone. This medium contains 5 mM glucose final. RPTEC/TERT1 cells were used between passage 78 and 82. The human hepatoma cell line HepaRG (obtained from Biopredic International, Rennes, France) (Guillouzo et al., 2007) was cultured in William's E medium (Invitrogen, cat No. 22551) supplemented with 2 mM glutamax, 5 μg/L insulin, 5 μg/L transferrin, 5 ng/L sodium selenite, 100 U/mL penicillin and 100 μg/mL streptomycin, 9% FCS (Biochrom AG), 200 ng/mL hydrocortisone. HepaRG cells were used between passage 22 and 25. The mouse embryonic fibroblast cell line BALB-3T3 (clone A31) (purchased from ATCC, CCL-163) (Aaronson and Todaro, 1968) was cultured in DMEM (Sigma, cat. No. D 5030), 7% FCS, 5 mM glucose, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. 3T3 cells were used between passage 70 and 75. Under routine conditions, cells were fed three times a week and sub-cultured by trypsinisation when at or near confluence.

2.2. Long-term repeat dose experiments

To allow a better comparison of the response of the three cell lines to the compounds, all cell lines were cultured in the RPTEC/TERT1 HDM, as described above. Preliminary experiments demonstrated that 3T3 and HepaRG cells could be maintained without problems in this medium. However, in the absence of FCS, HepaRG cells required to be cultured on human collagen IV coated plastic (8.8 μg/cm^2). All cells were cultured to confluence in 96 well cell culture plates (Greiner Bio One). RPTEC/TERT1 and HepaRG cells were seeded 2 weeks prior to treatment in order to establish a fully quiescent state. 3T3 cells were treated 3 days after seeding.

The compounds chosen were selected from a panel of compounds used as training chemicals in the 7th EU Framework project Predict-IV (http://www.predict-iv.toxi.uni-wuerzburg.de/). Cadmium chloride ($CdCl_2$), cephaloridine, cidofovir, diclofenac and buflomedil were dissolved in medium. Cyclosporine A (CsA) and troglitazone were dissolved in dimethylsulfoxide (DMSO) and then diluted in medium to a maximum level of DMSO of 0.1% (used as respective control). Amiodarone was dissolved in methanol and then diluted in medium to a maximum level of methanol of 0.125% (used as respective control). Before the start of the treatment, each compound was diluted in HDM to the five concentrations to be tested and aliquots for every feeding day were frozen at −20 °C.

Cells were exposed repeatedly to fresh compounds every 24 h for 14 days. Supernatants were collected on days 1, 3, 7 and 14 for lactate measurement. At the end of the 14 day exposure, cell viability was assessed using two parameters. Redox potential was measured with a resazurin reduction assay in live cells and ATP levels were measured in methanol homogenates. Experiments were conducted three times in duplicate.

2.3. Resazurin reduction assay

After treatment, each well was washed once in 100 μl 44 μM resazurin HDM and incubated with a further 100 μl of resazurin solution for 1 h at 37 °C. The fluorescent product resorufin was detected at 540 nm excitation and 590 nm emission using a Tecan GENios plus plate reader.

2.4. ATP assay

ATP levels in methanol cellular extracts were measured using the ATPlite kit from PerkinElmer (6016731) according to the manufacturer's instructions.

2.5. Lactate assay

Lactate was measured in supernatant medium using a colorimetric assay as originally described by Babson (Babson and Phillips, 1965). The lactate assay is based on the conversion of lactate to pyruvate by lactate dehydrogenase (LDH) activity reducing NAD to NADH. NADH reduces N-methylphenazonium methyl sulphate (PMS) to PMSH which reduces p-iodonitrotetrazolium violet (INT) to INTH. In detail, 10 μl of supernatant medium was incubated with 90 μl of assay solution consisting of 86 mM triethanolamine HCl, 8.6 mM EDTA.Na, 34 mM $MgCl_2.6H_20$, 326 nM PMS, 790 nM INT, 7% ethanol, 0.4% Triton-X-100, 3.3 mM β-NAD and 4 U/mL lactate dehydrogenase. After approx. 7 min, the