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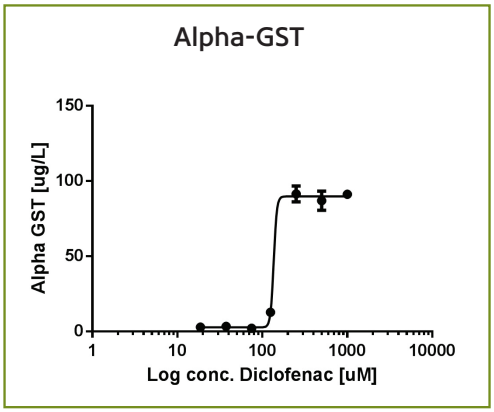

Technical Application for
Alpha-GST ELISA - Catalogue No. TE1051

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Alpha-GST Release as a Predictive Marker of Drug Induced Hepatotoxicity in 3D Liver Models

Introduction and Background

Drug induced liver injury (DILI) is a major obstacle in the development of new pharmaceutical compounds. Evaluation of potential DILI effects of novel compounds during pre-clinical phases is, therefore, a prerequisite for their safe entry into clinical trials. Hepatocytes, the major cell type in the liver, contain high amounts of metabolic enzymes, which are released upon cellular injury into cell culture supernatant. Besides the commonly used markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alpha-GST is increasingly being used as a liver injury marker. Over 150 articles have been published on its use in hepatotoxicity and in a wide range of clinical conditions. Alpha-GST provides advantages as a biomarker for hepatotoxicity due to its high cytosolic concentration in hepatocytes and its rapid release upon their injury[1]. Detection of alpha-GST release is therefore a specific and early indicator of hepatocyte injury.

Key Features

- Highly sensitive detection of released alpha-GST from 3D Liver Spheroids
- Detect hepatotoxicity in rat and human liver spheroids with as little as 1 x 10^3 cells per spheroid

Mitochondrial injury can also be an indicator of hepatocyte damage and it leads to decreases in ATP production, making the intracellular ATP assay a sensitive test for this pathological process. By combining the intracellular ATP and the alpha-GST assays, one can potentially detect both hepatotoxicity and obtain information as to the potential toxic mechanism.

Most 3D liver models are co-cultured spheroids that mimic liver physiology by providing a native cell structure with dense inter-hepatocyte contacts. These cultures typically do not require artificial extracellular matrix components[2]. The 3D liver spheroid in the 96-well format is viable for several weeks, making long-term toxicity testing possible. Since only 1 x 10^3 hepatocytes per spheroid are required, a large number of 3D hepatocytes can be produced from a single vial of primary cells. However, the low number of hepatocytes also means that very sensitive assays for monitoring changes in cellular status are required. In this study, the alpha-GST release assay, using alpha-GST ELISA kits (TECOmedical, Gewerbestrasse, Switzerland) and the CellTiter-Glo® Luminescent Cell ATPassay (Promega, Madison, USA) were compared as tests for hepatotoxicity using 3D liver spheroids. As test substances, two compounds with known hepatotoxic effects were chosen: Amiodarone[3] and Diclofenac[5].

Materials and Methods

Assays, 3D Spheroids, Media and Compounds

- Human Alpha-GST EIA (TECOmedical Group, Cat: TE1051)
- Rat Alpha-GST EIA (EKF Diagnostic, Cat: BIO64RT)
- CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Cat: G7572)
- 3D human liver spheroid culture
- 3D rat liver spheroid culture
- Maintenance media for 3D human liver spheroids
- Maintenance media for 3D rat liver spheroids
- Diclofenac sodium salt
- Amiodarone hydrochloride
- Dimethly sulfoxide (DMSO)

Cell Culture and Test Set-up

3D rat liver spheriods were treated with Amiodarone at the following concentrations: 500 µM; 250 µM; 125 µM; 62.5 µM; 31.25 µM; 15.63 µM; 7.81 µM. 3D human liver spheroids were treated with Diclofenac at the following concentrations: 1000 µM; 500 µM; 250 µM; 25 µM; 62.5 µM; 31.25 µM; 15.63 µM. Control cells were treated with DMSO in 70 µl maintenance medium per well. After 5 days incubation, the release of alpha-GST into the culture medium of the spheroids was quantified. Measurement of viability was performed with CellTiter-Glo®.

Alpha-GST EIA

After incubation with compounds, cell culture supernatants from at least 3 independent liver spheroids were frozen at -20°C until alpha-GST assessment. The alpha-GST assays were performed according to the manufacturer's instructions whereby the cell culture supernatants were diluted 1:1 with sample diluent (50µl + 50µl before incubation in the ELISA plates. The same procedure was applied for rat and human samples. EIAs were read with the Tecan Infinite M200Pro (Teccan Group Ltd., Männedorf, Switzerland with measurement and reference wavelengths of 450 nm and 630 nm, respectively.

ATP Assay

Measurement of ATP content in liver spheroids was performed with CellTiter-Glo® Cell Viability assay. The assay was performed according to the manufacturer's protocol and with an increased incubation time of 20 min minutes. Luminescence was quantified the Tecan Infinte M200Pro.

Results and Discussion

Rat Liver Microtissues and Amiodarone

Amiodarone is known to cause hepatotoxicity by affecting mitochondrial function[3]. Cell toxicity was studied by simultaneously measuring the decrease of ATP

concentration and the release of the cytosolic protein alpha-GST. As is evident in Figure 1, the treatment of rat liver spheroids with Amiodarone led to a dose-dependent decrease in ATP production and a concomitant release of alpha-GST. The IC_{50} generated using both biomarkers corresponded very closely (ATP: 55.26 µM alpha-GST: 52.36 µM) and were in agreement with previously published studies on cultures of rat hepatocytes in 2D-culture (IC_{50} of 38.3 µM)[4]. In comparison with 2D cultivated HepG2 cells (IC_{50} of 78.9 µM)[4] the rat liver microtissues showed increased sensitivity towards this drug.

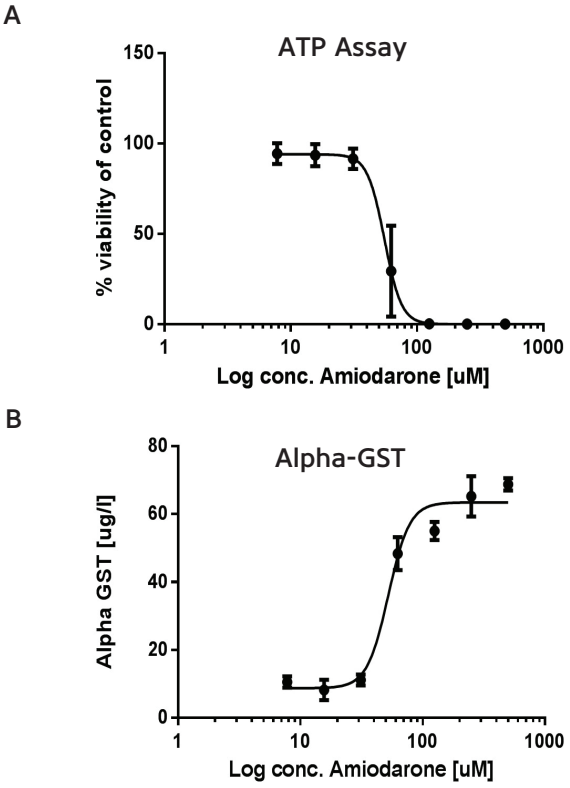

Figure 1. Dose response curves of rat liver microtissues treated over 5 days with Amiodarone. Measurement of intracellular ATP-content (A) and released alpha-GST (B) from the same tissues (n=4).

Human Liver Microtissues and Diclofenac

Diclofenac is known to cause hepatotoxicity via mechanisms that involve mitochondrial injury[5]. As shown in Figure 2, it produces a concentration-dependent cytotoxic effect on human liver spheroids, as assessed by CellTiter-Glo® (ATP production) and alpha-GST release, with IC_{50} values of 91.3 µM (ATP) and 136.2 µM (alpha-GST).

Compared with published data on 2D cultures (IC_{50} of primary human hepatocytes 331 µM, primary rat hepatocytes 392 µM and HepG2 399 µM)[4,5] the 3D human liver spheroids were more sensitive than conventional standard liver *in vitro* models. The toxicity of Diclofenac requires active phase I+II enzyme metabolism. These enzyme systems are highly expressed in 3D liver models, but are severely down-regulated in other model systems. In this case, the observed difference between the calculated IC_{50} from ATP or alpha-GST assay may be an indication that decreases in ATP production and the release of alpha-GST are reflecting different aspects of the pathological process. Possibly, the cells could survive a certain level of mitochondrial injury with a decrease in ATP production and retain viable, but when a threshold is exceeded the cells die and alpha-GST is released.

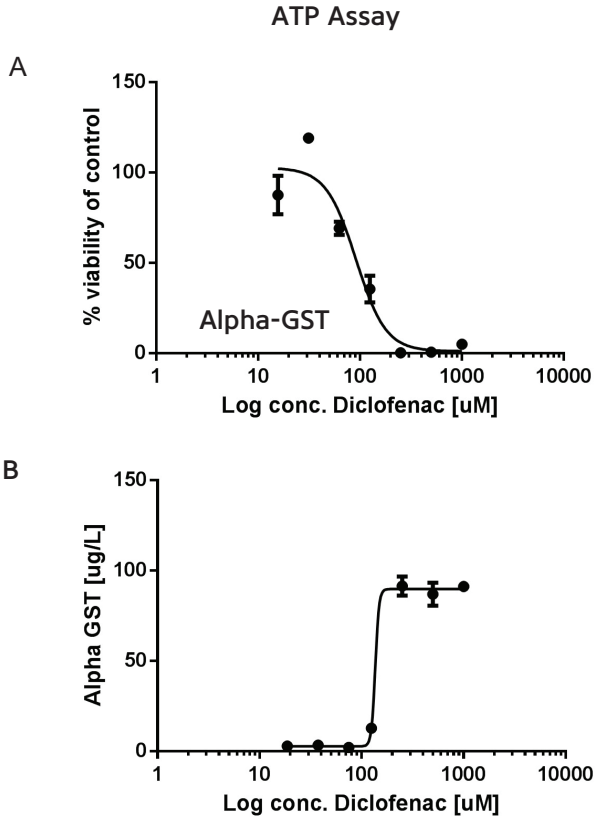

Figure 2. Dose response curves of human liver microtissues treated over 5 days with Diclofenac. Measurement of intracellular ATP-content (A) and released alpha-GST (B) from the same tissues (n=3).

Conclusion

In summary, alpha-GST release assessed with the ELISA assays was shown to be a highly reliable and sensitive biomarker to detect hepatotoxicity utilizing single liver spheroids. Alpha-GST release showed similar sensitivity as the reduction in ATP production. However, since the reduction in ATP production and the release of alpha-GST reflect different pathological processes, the assays in combination provide complementary information. This was demonstrated in this study where both Amaridone and Diclofenac were known to affect mitochondrial function.

Since destruction of the spheroids is not required for assessment of alpha-GST in the supernatant, the assay conveniently enables monitoring of spheroid culture viability over time. This can provide more detailed toxico-kinetics and reduce the number of liver cultures required. Thus, the combination of the alpha-GST ELISA with 3D liver spheroids is a valuable combination that is well-suited for the *in vitro* iassessment of drug-induced hepatotoxicity.

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inSphero Biomimetic Microtissue Technology

Application note published via work of InSphero AG,
(Schlieren, Switzerland; Brunswick, ME; Waldshut, Germany).
Visit www.insphero.com for more information on 3D liver spheroid technology.

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