Real-time viability assay based on $^{51}$Cr retention in adherent cells

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The chromium ($^{51}$Cr) release assay has been widely used for viability measurements, even though it has major disadvantages such as high manual workload and poor time resolution. By the use of LigandTracer, $^{51}$Cr release viability measurements on adherent cells can be significantly simplified and improved. LigandTracer enables a time-resolved detection of $^{51}$Cr in target cells, with the result that the effect of toxic material is updated continuously throughout the experiment. Here we explain the principle behind this novel real-time viability assay and show viability curves for known toxic compounds on A431 and U343MGaCl2:6 cell lines.

INTRODUCTION

Assessing toxicology in vitro based on cells as a model organism is of interest both to the industry and the regulatory authorities. Toxicity is still a major reason for failure in drug development (1) and as of 1 June 2007, the European Union launched a new law on chemicals; registration, evaluation, authorization, and restriction of chemicals (REACH) (2). This new law requires all chemicals of large volumes manufactured or imported into Europe to be tested and registered. The massive task of toxicity testing has created a concern for a possible increase of in vivo studies. Therefore, further refinement of the methods capable of measuring cell viability in vitro is needed.

Current methods to decide cell viability usually involve staining a cell line to count viable and dead cells, evaluating morphology of cells, or analyzing metabolic activity (1). One common method relies on chromium ($^{51}$Cr) release (3), which is based on the theory that there is a close relationship between $^{51}$Cr release and cytotoxicity (4).

Although the $^{51}$Cr release assay has been widely used, it has major disadvantages (5). The use of radioactivity and the intense manual workload are some of the negative aspects. Advantages using $^{51}$Cr release include easy uptake of $^{51}$Cr by cells, low spontaneous release, possibility to parallelize in multiwell microplates, and sensitive and simple detection (6). Additionally, $^{51}$Cr can label replicating as well as nonreplicating cells with little harm to the cells (7).

Detailed cytotoxicity studies relying on $^{51}$Cr can be significantly simplified by using LigandTracer (Ridgeview Instruments AB, Uppsala, Sweden), an instrument originally developed for protein-cell interactions in vitro (8). By enabling a time-resolved detection of viability markers in target cells, the effect of toxic material can be detected as it appears. When using manual protocols, information on the viability status can only be determined at the time of cell harvesting. In contrast, when using LigandTracer, information on the viability status is continuously updated in real time throughout the experiment. However, when using LigandTracer, it is not possible to parallelize the viability assay, meaning that it is not suited for screening.

Here, viability measurements have been performed with known toxic compounds and $^{51}$Cr (GE Healthcare, AB, Upplands Vasby, Sweden) as a viability marker.

MATERIALS AND METHODS

LigandTracer Technology

As previously described by Björke and Andersson (8), the adherent cells were seeded in a limited area of a Nunclon cell dish (size 100 × 20; NUNC A/S, Roskilde, Denmark), leaving an empty area for reference purposes (see Figure 1). The cell dish was put on an inclined slowly rotating support, and cell culture medium containing $^{51}$Cr was added. The target cells passed by a detector positioned at the upper part of the dish, and internalized $^{51}$Cr resulted in increased intensity. Subsequent to incubation with $^{51}$Cr, the cell dish was washed once, and a toxic substance was added. If the toxin affected the cells, a release of $^{51}$Cr was seen in real time. Note that LigandTracer measures the $^{51}$Cr still associated with the cells, while manual protocols follow the release of $^{51}$Cr. All measurements were performed using LigandTracer Yellow.

Cell Lines and Reagents

The target cell area consisted of a 1- to 2-mL aliquot of the human squamous carcinoma cell line A431 or the human glioma cell line U343MGaCl2:6 (from now on denoted U343) (both from ATCC, Rockville, MD, USA) in suspension, containing approximately 10$^6$ cells. The cell dishes were kept tilted for a few hours in order for the cells to attach to the surface, prior to addition of 10 mL HAMS-F10 cell culture medium (Biochrom, Berlin, Germany), 10% containing fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Biochrom), and 100 U/mL penicillin and 100 μg/mL streptomycin (PEST; Biochrom), and stored in an incubator with humidified air at 37°C and 5% CO$_2$ for at least 24 h before use.

Viability Measurement Procedure

A cell dish prepared for use in LigandTracer was placed in the instrument, and 1.2 MBq $^{51}$Cr (37 MBq/mL) in 3 mL fresh cell culture
medium were added to the dish. The amount of chromium used for one assay corresponds to 1/800 of the Annual Limit of Intake (ALI) by ingestion. Thus, with proper use of radiation protection devices, such as gloves, safety glasses, and lead containers for waste, the risk for the operator is extremely small. Following 1 h of incubation in LigandTracer, the cells were washed once with cell culture medium, and 3 mL were again added to the dish. Several measurements without toxins were performed to study the repeatability of 51Cr uptake and retention. For dose-response measurements, 1%–0.003% Triton X-100 (Pharmacia Biotech, Uppsala, Sweden) or 0.3–3 mM cupric sulfate (Sigma-Aldrich) were added to the cells. A concentration of 0.1% Triton X-100 has previously been used as an indicator of maximum release of chromium (6) and in lower concentrations for assessing cytotoxicity (9). Throughout all measurements, LigandTracer was placed inside an incubator with humidified air at 37°C and 5% CO2. The instrument was set to detect radioactivity at 10 positions/round (equally distributed along the rim of the cell dish), 4 s/position (activity measurement), and averaged 20 such rounds to generate one data point. Thus, the data points appeared every 16.5 min.

RESULTS AND DISCUSSION

The initial repeatability study proved that uptake and retention of 51Cr without addition of toxin differed typically <10%, provided that the detected 51Cr levels were scaled prior to comparison (data not shown). Moreover, the stable retention indicated that the cells kept the 51Cr when no toxins were added. The dose-response curve of cupric sulfate on U343 cells and Triton X-100 on A431 cells are shown in Figure 2, wherein the curves were scaled with respect to the 51Cr uptake. Following wash, the toxin was added at time 0 in Figure 2.

The data presented in Figure 2A corresponds to retained 51Cr in U343 cells at different cupric sulfate concentrations. Increasing concentrations of cupric sulfate causes the cells to release the 51Cr sooner; the effect of copper ions at 3 mM has an immediate effect on cell viability, whereas the effect of lower concentrations (0.3–1 mM) is visible after 12–14 h.

The data presented in Figure 2B corresponds to retained 51Cr in A431 cells at different Triton X-100 concentrations. Triton X-100 had no toxic effect on the cells during the allotted time period for concentrations 0.003% and 0.006%. A slow release of 51Cr was observed at Triton X-100 concentrations of 0.05% and higher.

The A431 cells had individual morphological appearances at the end of the runs with different concentrations. At Triton X-100 concentrations of 0.1% and higher (where 51Cr release occurred immediately), the cells had collapsed into small, dense bodies. At Triton X-100 concentrations resulting in slow but clear 51Cr release (i.e., 0.01%–0.03%), the cells seemed inflated when compared with healthy cells. This indicates that the cells do not have to be completely dead prior to release of 51Cr. Furthermore, the A431 cells were more likely to detach from the surface when the cell death occurred at a slow pace (i.e., at Triton X-100 concentrations 0.01%–0.03%). Detached cells are also a sign of poor viability, but it is difficult to address the actual cause of a declining 51Cr level in LigandTracer when the cells detach from the plastic surface. All cell dishes were visually inspected under microscope to determine the degree of detached cells after the runs with Triton X-100, and in all cases presented here, the loss of cells was insignificant. Cell detachment is cell-line dependent, and U343 used together with cupric sulfate never showed signs of detachment. The experience in our laboratory is that A431 cells adhere less strongly to cell dishes than U343. Our hypothesis is that in combination with a toxic detergent like Triton X-100, A431 cells become sensitive for mechanical impact and may detach due to unknown reasons. A thorough investigation of adhesive properties of different cell lines under the influence of toxins and other chemicals will be conducted in our laboratory in the near future.

The benefit of 51Cr release curves generated by LigandTracer is clearly seen in Figure 2A. When using a manual 51Cr assay with cell harvesting after 10 h, 0.3 and 1 mM cupric sulfate
would have been judged nontoxic, whereas after harvesting after 15 h it would be judged highly toxic. The release curves also reveal that the toxic action can be quite different. When treated with cupric sulfate, the cells collapse in a similar manner, but with a time delay related to the concentrations. For Triton X-100, the cells degrade immediately, over the course of several hours for lower concentrations and more rapidly at increasing concentrations. Collecting the same information with manual protocols would be impractical and costly.

The results presented in Figure 2 have comparable sensitivity as the standard $^{51}$Cr release assay. Langhans et al. (5) reported that the baseline release of $^{51}$Cr after 4 h was 6%–9%, and the maximum release was 65%–66%. In Figure 2A, the three negative controls decreased to 95% ± 2% after 4 h, which corresponds to a baseline release of ~5%. The residual signal after 4 h in Figure 2B for the three highest concentrations of Triton X-100 was 10% ± 2%, which corresponds to a maximum release of ~90%. Advantages previously mentioned about the manual chromium release method are still valid when using LigandTracer. Furthermore, the measurements in LigandTracer run essentially unattended, which reduces labor time for detailed cytotoxicity measurements. Additionally, a lower level of radioactivity can be used, which improves the labor safety and is beneficial for the environment.

The ability to see how fast the cells take up and release $^{51}$Cr in real time is another important advantage, and the time-resolved data are also a good measurement of data quality. The user will not only see if noise occurs during the run, but also when it happens. Furthermore, a failed experiment can be discarded at an early stage and time can be saved.

Although LigandTracer provides many advantages to viability measurements, it does have some negative aspects. LigandTracer is not suitable for the screening of a large number of toxins, since only one cell dish at
a time is handled. It is not possible to have a lid on the cell dish during measurement, which results in the evaporation of cell culture medium during the run (8). Even though the measurement chamber is sealed, evaporation can affect the results during longer runs (>48 h). For the cells to be in a humid and CO₂ environment, LigandTracer must be placed in an incubator, which might be a space and contamination problem. The radiation detector in LigandTracer Yellow has also shown some sensitivity to humidity and heat when exposed for longer periods of time.

A potential difficulty can be the seeding of the cells, since they have to be in a limited area (Figure 1). If they migrate to the reference area, which is supposed to be empty, the dish is unusable for experiments in LigandTracer. Additionally, the cells need to be adherent and not detach during the run.

In conclusion, a real-time viability assay based on chromium retention has been developed and tested, and high-resolution viability data was provided using a minimum of labor time and reagents. In the light of increased demands for toxicity testing, this method offers a complement to expensive in vivo viability models.

COMPETING INTERESTS STATEMENT

L.V. has been employed by Ridgeview Instruments AB, C.B., H.B., and K.A. are currently employed by Ridgeview Instruments AB, and K.A. is a shareholder of Ridgeview Instruments AB.

REFERENCES
