

# Optimization of Resazurin-based Assay for Cytotoxicity Test in Cholangiocarcinoma Cells

## การปรับปรุงการวิเคราะห์ด้วยรีซ่าซูรินเพื่อวิเคราะห์การตายของเซลล์ในเซลล์มะเร็งท่อน้ำดี

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### ABSTRACT

The viability of cell cultures is usually assessed from the metabolic capability of cells in converting some chemicals to color dyes which can be conveniently measured by multi-well plate reader. However, the inconsistency between the metabolic assays and direct microscopic examination is usually observed and raised some concerns over the validity of the metabolic assay. Resazurin test, one of the metabolic assays similar to the tetrazolium MTT assay, was evaluated against a fluorescent staining microscopic counting methods. The study was performed in cholangiocarcinoma (CCA) cell line KKU-100 and cells were treated with gemcitabine, a highly potent chemotherapeutic agent. Results were revealed that cell density, types of incubation medium and incubation time of resazurin affect the consistency of the metabolic assay as compared with a microscopic counting. Optimization of resazurin-based assay is necessary to provide resazurin test as a simple, rapid and high sensitive cytotoxicity test for the anticancer drugs for cholangiocarcinoma cells.

### บทคัดย่อ

การอุ่รอดของเซลล์เพาะเลี้ยงมักทำการประเมินด้วยการวัดความสามารถในการเมแทบิลิซึมของเซลล์โดยการเปลี่ยนสารเคมีเป็นสีที่สามารถตรวจวัดได้อย่างสะดวกโดยเครื่องอ่านเพลท อย่างไรก็ตามมักมีรายงานกล่าวถึงความไม่เท่าเทียมกันของการประเมินผลระหว่างวิธีการวัดเมแทบิลิซึมของเซลล์กับ

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การสังเกตโดยตรงจากกล้องจุลทรรศน์ ทำให้เกิดความเป็นห่วงถึงความถูกต้องของผลการอ่านจากอ่านสีโดยวิธีต่างๆ การวิเคราะห์ด้วย resazurin ซึ่งจัดเป็นการวัดสีจากการเมแทโนลิซึมของเซลล์ เช่นเดียวกับวิธีอื่น เช่น tetrazolium MTT ได้นำมาศึกษาเบรย์นเทียนโดยวัดการอญ่ารอดของเซลล์มะเร็งท่อน้ำดี KKU-100 ที่ได้รับยาเคมีบำบัด gemcitabine โดยวัดวิธีการย้อมเซลล์ด้วยสีฟลูออเรสเซนท์และนับเซลล์ภายใต้กล้องจุลทรรศน์ กับวิธี resazurin การศึกษาพบว่าจำนวนเซลล์ ชนิดของน้ำยาทำปฏิกิริยา และเวลาของปฏิกิริยา ของ resazurin มีผลต่อความแม่นยำของการวิเคราะห์ เมื่อได้ปรับปรุงวิธีการวิเคราะห์ ทั้งสองวิธีมีประสิทธิภาพใกล้เคียงกัน ดังนั้น วิธีรีชาซูรินจึงเป็นวิธีที่ง่าย รวดเร็ว และมีความไวสูง และสามารถใช้เพื่อทดสอบความไว หรือการตอบสนองต่อยาต้านมะเร็งของเซลล์มะเร็งท่อน้ำดี

**Key Words :** Cholangiocarcinoma cells, Cytotoxicity assay, Resazurin

**คำสำคัญ :** เซลล์มะเร็งท่อน้ำดี การวัดการตายของเซลล์ รีชาซูริน

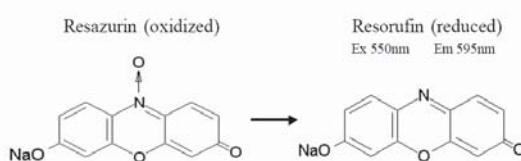
## Introduction

A simple, rapid, sensitive, reliable, safe and cost-effective measurement of cells viability is the ideal test for *in vitro* cell proliferation and cytotoxicity. Apparently, it should not interfere with the compound to be tested. The one step resazurin reduction assay involves the addition of fluorogenic redox indicator, resazurin, to growing cultured cells and the measurement of the fluorescent product resorufin. The resazurin reduction test has been used since the 1950s to assess bacterial or yeast contamination in biological fluids. It is also used to measure the semen quality (Wang et al. 1998; Zrimsek et al. 2004), corneal viability (Perrot et al. 2003), mitochondrial function (Zhang et al. 2004), quantity/proliferation of living organism (Gabrielson et al. 2002; Guerin et al. 2001; Mann and Markham 1998; Martin et al. 2003; Palomino et al. 2002) and cytotoxicity in different types of cells (Batchelor and Zhou 2004; Benavides et al. 2004; Evans et al. 2001; Ó'Brien et al. 2000; Putnam et al. 2002). Resazurin is taken into cells,

the dye becomes reduced to pink and highly fluorescent resorufin (Figure 1) by several mitochondrial enzymes such as flavin mononucleotide dehydrogenase, flavin adenine dinucleotide dehydrogenase, nicotinamide adenine dinucleotide dehydrogenase, nicotinamide adenine phosphate dehydrogenase and cytochromes (Ó'Brien et al. 2000). The extent of this conversion, which is reflected its metabolic capability has presumably been employed as cell viability test. However, the inconsistency of cell viability tests between a morphometric analysis of the cells by fluorescent microscopy and this type of metabolic assay, i.e. resazurin assay and the same kind such as MTT or MTS assay have been observed. This raises the concern over the validity of the metabolic assay in spite of its simple platform.

It has been suggested that each cells has unique metabolic properties and must be individually characterized to determine the experimental parameters (e.g., resazurin concentration, cell density, incubation time and

diluents of resazurin) for optimal conversion from resazurin to reduced form, resorufin. The aim of present study is to optimize the experimental parameters for assessing the cell viability of CCA cells by resazurin assay when cells were treated with gemcitabine, a cytotoxic drug. We validated the resazurin assay with the standard microscopic counting method by staining the cells with acridine orange and ethidium bromide (AO/EB).



**Figure 1** Chemical structures of resazurin and reduction product, resorufin.

## Materials and Methods

### Human CCA Cell Lines and Culture

The human biliary epithelial cell line KKU-100, derived from intrahepatic cholangiocarcinoma, was established in our institute (Sripa et al. 2005). Cells were cultured in Ham's F12 containing 4 mM L-glutamine, 1 mM Na-pyruvate, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin and 10% fetal bovine serum (FBS) and maintained under an atmosphere of 5% CO<sub>2</sub> at 37°C. The media was renewed every 3 days. Confluent KKU-100 cells were harvested by trypsinization with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) and seeded onto 96-well cultured plates at indicated density. Then cells were allowed to attach for 24 h before further experiment.

### Resazurin Reduction Test

The resazurin reduction test was carried out as follows. Briefly, the media were removed and rinsed with phosphate-buffered saline (PBS, pH 7.4). Then 200  $\mu$ l of 0.001% resazurin in medium was added to each well. Plates were incubated for indicated times at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The fluorescence intensity was measured on a scanning microplate spectrofluorometer (Gemini XPS, Molecular Devices, CA, USA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

### Microscopic Counting Method (acridine orange/ethidium bromide (AO/EB) staining)

Briefly, cultured media was removed and KKU-100 cells were washed once with PBS. After addition of 100  $\mu$ l of PBS into each well, 4  $\mu$ l of AO/EB dye mixture (at concentration of 100  $\mu$ g/ml of each dye) (Renvoize et al. 1998) was added. Then cells were examined and counted using a Nikon eclipse TS100 inverted microscope at 200x magnification with excitation filter 480/30 nm; dichloromeric mirror cut-on 505 nm LP; and barrier filter 535/40 nM (Melville, NY, USA). Pictures were taken with a Nikon COLPIX digital camera. Tests were done in triplicate, counting a minimum of 100 total cells each by ImagePro-Plus (version 2.0).

### Cytotoxicity Assay

To compare resazurin reduction assay with the standard microscopic counting method (AO/EB staining) for assessing cytotoxicity of the well-known anticancer drug, gemcitabine.

KKU-100 cells were seeded onto 96-well cultured plates at density of  $5.0 \times 10^3$  cells/well. Gemcitabine diluted in culture medium was added to each well at a constant ethanol concentration of 0.5% (v/v) which had no effect on cell growth. Three drug concentrations (final concentration of 1, 10 and 100 nM) were tested versus controls. After 24 h, the cytotoxicity was determined and compared by using both methods as described above.

#### Statistical Analysis

Data are expressed as the mean  $\pm$  S.E. of the duplicate assays from three independent experiments. Student's *t*-test was used to determine the significant differences between each experimental group. The level of significance was preset at  $p < 0.05$ .

## Results

### Optimization of resazurin reduction test

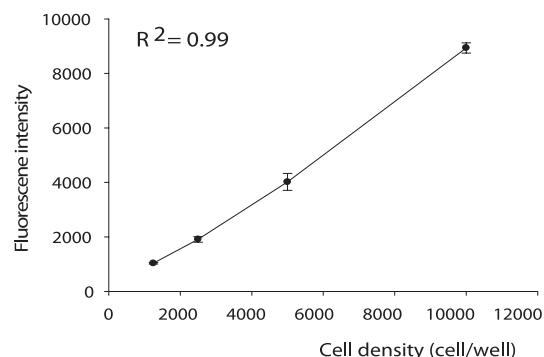
It has been noted that the reduction rate of resazurin is depend on the cellular metabolism and varied among different types of cell lines (Nakayama et al. 1997). KKU-100, a CCA cell line, was seeded onto 96-well cultured plates at varying cell density ( $1.25 \times 10^3$ ,  $2.5 \times 10^3$ ,  $5.0 \times 10^3$ ,  $1.0 \times 10^4$  cells/well) and incubated in the presence of 0.001% resazurin in PBS buffer for 30 min. Figure 2 showed that there was a very good correlation ( $r^2 = 0.99$ ,  $p < 0.05$ ) between the fluorescence intensity of resorufin and cell density (cell number).

We next examine the incubation medium for resazurin reduction assay, three different medium including 0% FBS-media (plain cultured media), 10% FBS-media (full media) and PBS were used to prepare resazurin solution. Figure 3A

shows that the highest reduction rate (30 min incubation time) was observed when using PBS as incubation medium as compared with other two medium. Moreover, resazurin in PBS gave a linear curve over a wide range of cell concentrations (range  $1.25 \times 10^3$  to  $1.0 \times 10^4$  cell/well).

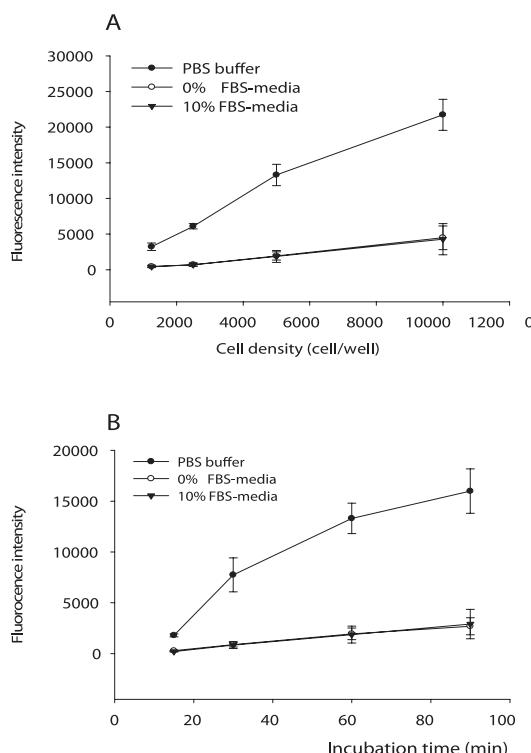
The last parameter to determine was the reduction or incubation time of resazurin. KKU-100 cells at a density of  $5.0 \times 10^3$  cells/well were incubated with resazurin in PBS at indicated times (15, 30, 60, 90 min). Figure 3B showed that a linear increase of the fluorescence intensity of resorufin with incubation time, where the fluorescent signal reached plateau level within 30 min after incubation in PBS buffer, but not the other medium.

These results indicated that, under optimized conditions, the resazurin reduction assay has the potential for evaluation cell viability of CCA cells *in vitro*.



**Figure 2** Resazurin reduction as a function of cell number.

KKU-100 cells with varying cell concentrations were cultured over night and metabolic capacity was assessed by incubating with 0.001% resazurin for 30 min as described in *Materials and Methods*



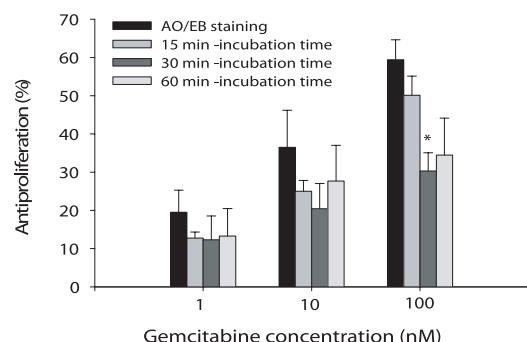
**Figure 3** Effect of incubation mediums in assessing resazurin reduction.

The relationship between three incubation medium and fluorescent signals with regards to. (A) Cell density and (B) Resazurin incubation time. In Figure 3A, KKU-100 cells were seeded in 96-well cultured plate at indicated cell density and then incubated for 30 min with resazurin in different media: PBS buffer (●), 0% FBS-media (○), and 10% FBS-media (▼). In Figure 3B, cells were seeded at a density of  $5.0 \times 10^3$  cells/well before incubation with resazurin in PBS buffer at indicated time.

#### Use of resazurin in drug sensitivity assays

To verify resazurin reduction assay as a tool for assessing cytotoxicity of anticancer drugs, KKU-100 cells were treated with gemcitabine for 24 h and then the cytotoxicity was determined by

resazurin reduction assay, and a standard microscopic counting method (AO/EB staining). Figure 4 shows that the resazurin assay incubated for 15–60 min gave similar pattern of antiproliferation (cytotoxicity). The correlation between both assays was considered high at every incubation time ( $r^2 > 0.9$ ). However, we found that, condition of 15-min incubation time of resazurin gave the highest consistency of antiproliferation as assessed by resazurin assay and microscopic examination. This result indicated that the resazurin reduction assay has the potential for screening cytotoxicity of anticancer drugs in CCA cells *in vitro*.



**Figure 4** Validation of the resazurin reduction assay for assessing the cytotoxicity of gemcitabine in KKU-100 cells.

KKU-100 cells were incubated with three different concentrations of gemcitabine for 24 h before cytotoxicity was assessed by two methods as described in Materials and Methods. Black bar represent antiproliferation by AO/EB staining and gray bars represent antiproliferation by resazurin (incubation time)

## Discussion

The study demonstrated that: (1) optimal conditions to perform resazurin assay as a test for viability include cell density, incubation medium of the reaction and reduction or incubation time, and (2) the resazurin reduction assay had the potential tool for screening in vitro cytotoxicity of anticancer drugs in CCA cells.

We observed a good linearity of the fluorescence intensity of resorufin (product of resazurin reduction) over a wide range of cell concentrations (cell number was in the range  $1.25 \times 10^3$ – $1.0 \times 10^4$  cells/wells, which represent a cell density of  $6.25 \times 10^3$ – $5 \times 10^4$  cells/ml). Interestingly, fluorescence resorufin formation from resazurin in cultured media consisting of FBS or no FBS was relative much lower than that in PBS buffer. It is, therefore, the use of PBS buffer provides an advantage in that it needs short incubation time and more sensitive outcome than the cultured media. There was possible that some components interfere with the resazurin reduction. However, this problem is not yet addressed in this study. It is recognized that reduction of resazurin is a rate kinetics. An incubation time of resazurin is, then, one of major factors affecting the amount of resorufin formation. Incubation time has to be optimized in that the rate of resorufin formation has to be linear with time.

We compared the resazurin-based assay with the standard microscopic counting method (AO/EB) staining for assessing cytotoxicity of gemcitabine. AO/EB staining is a straightforward method for evaluation cell viability since cell

number and morphology will be clearly observed under fluorescence microscope. The DNA binding dyes, AO, permeates all cells and makes the nuclei appear green whereas EB is only taken up by cells with lost of cytoplasmic membrane integrity and stains the nucleus as red color. Thus cells will be classified as (i) live cells will have a normal green nucleus; (ii) apoptotic cells have bright green nucleus with condensed or fragmented chromatin; and (iii) necrotic cells will have major structural changes and orange nucleus (Renvoize et al. 1998). The good linear correlation between both assays was very high ( $r^2 > 0.9$ ) and resazurin assay gave comparable sensitivity to AO/EB staining. It is therefore suggested that optimization of the assay is essential to provide a reliable outcome of cytotoxicity test. We showed that resazurin reduction assay has the potential useful for screening cytotoxicity of anticancer drugs in CCA cells.

In conclusion, a resazurin reduction test is an extremely simple, rapid and sensitive procedure to evaluate cell proliferation. It is also inexpensive and non-toxic. Since this assay is a one step procedure, it can be useful for drug sensitivity test in cell culture systems.

## Acknowledgement

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