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Hydroxyethyl disulfide as an efficient metabolic assay for cell viability in vitro

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Abstract

Cell viability assays have a variety of well known practical and technical limitations. All the available approaches have disadvantages, such as non-linearity, high background and cumbersome protocols. Several commonly used tetrazolium chemicals rely upon generation of a colored formazan product formed by mitochondrial reduction of these compounds via phenazine methosulfate (PMS). However, sensitivity is inherently limited because their reduction relies on mitochondrial bioreduction and cellular transport of PMS, as well as accessibility to tetrazolium chemicals. In this study, we identify hydroxyethyl disulfide (HEDS) as an inexpensive probe that can measure cellular metabolic activity without the need of PMS. In tissue culture medium, HEDS accurately quantitated metabolically active live cells in a linear manner superior to tetrazolium based and other assays. Cell toxicity produced by chemotherapeutics (cisplatin, etoposide), oxidants (hydrogen peroxide, acetaminophen), toxins (Phenyl arsine oxide, arsenite) or ionizing radiation was rapidly determined by the HEDS assay. We found that HEDS was superior to other commonly used assays for cell viability determinations in its solubility, membrane permeability, and intracellular conversion to a metabolic reporter that is readily transported into the extracellular medium. Our findings establish the use of HEDS in a simple, rapid and low cost assay to accurately quantify viable cells.

Keywords

glutathione; glucose metabolism; cytotoxicity; cell proliferation; cisplatin; toxins

INTRODUCTION

The response of cells to chemotherapeutic agents, radiation and toxins is generally measured by clonogenic, cell proliferation, apoptosis, lactate dehydrogenase, ATP, alamarblue and tetrazolium salt based assays. Although one or many of these approaches have been used for more than three decades in biomedical sciences, each one of them has disadvantages ranging

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from non-linearity to high background and complicated cumbersome and time consuming protocols. Clonogenic assay is a measure of colony (50 cells/colony) forming ability of a single cell, which quantifies the cytotoxic effects of drugs or radiation (Franken et al., 2006; Kuwahara et al., 2010; Pomp et al., 1996; Wouters et al., 2010). Most studies for high throughput screening (HTS) of cancer drugs avoid using this assay because of the difficulties in terms of time consuming and complicated protocols combined with low plating efficiency (PE) of the cells at low density (Pomp et al., 1996). Cell proliferation assay by automated Coulter cell counter measures the total number of cells with a better sensitivity, but this assay requires extensive sample preparation (trypsinization and dilution of samples) and limits the ability to do drug HTS (Gantchev et al., 1996; Jiffar et al., 2004; Kurz et al., 2001).

The cell survival assay by alamar blue is not widely used since its bioreduction is affected by various factors in the tissue culture medium (O'Brien et al., 2000; Davis et al., 2011). Further, it requires up to 8 hrs incubation for a better response curve (Voytik-Harbin in et al., 1998). Under certain conditions, it is toxic to certain cell types. High serum or high protein concentrations in the medium also affects the fluorescence metabolite from alamar blue, which may limit its use in certain cell types that require high serum conditions. The response curve obtained with almar blue reaches a plateau at cell concentrations higher than 20,000 cells (Davis et al., 2011).

In cancer research, the most commonly used assay for drug HTS is the 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay, which is limited in sensitivity due to the insolubility of the formazan product produced by mitochondrial reduction of MTT (Lechpammer et al., 2002; Scudiero et al., 1988; Selvakumaran et al., 2003; Twentyman and Luscombe, 1987; Young et al., 2005). Further, the cells can not be reused for other assays. Although MTT assay was replaced by using other tetrazolium salts such as 2,3-bis(2-methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium (WST-1) and 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium (MTS), the sensitivity and reproducibility of these improved assays required an additional compound phenazine methosulfate (PMS) or phenazine ethosulfate (PES), an intermediate electron acceptor (Lechpammer et al., 2002; Scudiero et al., 1988; Selvakumaran et al., 2003; Twentyman and Luscombe, 1987; Young et al., 2005). This assay is complicated since the extracellular reduction of such compounds is dependent on the intracellular mitochondrial reduction and cellular transport (influx and efflux) of PMS/PES, and accessibility to reduce XTT, WST or MTS (Funk et al., 2007; Lechpammer et al., 2002; Scudiero et al., 1988; Selvakumaran et al., 2003; Twentyman and Luscombe, 1987; Young et al., 2005). Further, change in pH of the medium may also affect the reduction of tetrazolium salts by phenazine since it crystalizes at high pH (Vistica et al., 1991). Additionally, phenazine is a known generator of oxygen free radicals and cause potassium loss in cells (Ghosh and Quayle, 1979; Kise et al., 1994; Picker and Fridovich, 1984). Studies had shown that in addition to mitochondrial mediated reduction, other factors such as serum and membrane enzymes may also affect the bioreduction of tetrazolium compounds (Geys et al., 2010; Knight and Dancis, 2006; Kuhn et al., 2003). These factors may be responsible for the higher error reported by other laboratories (Wagener et al., 2008).

A single probe that can measure the metabolically active cells without additional electron acceptor/donor, cell lysis or interference from medium might offer a better cell viability assay. One candidate compound of potential use is hydroxyethyldisulfide (HEDS). Viable cells reduce HEDS to mercaptoethanol (ME), a free sulfhydryl-containing compound, in the absence of any toxic effect on cells suggesting its rapid reduction by intracellular pathways (Ayene et al., 2008; Biaglow et al., 2006). ME produced inside the cells as a result of HEDS

reduction by oxidative pentose phosphate cycle (OPPC) is extruded into the extracellular medium (Ayene et al., 2008; Biaglow et al., 2006). This conversion of HEDS into ME by mammalian cells can be easily measured in the extracellular medium without the need for cell lysis.

Although we have demonstrated HEDS to ME bioconversion in cell culture using HPLC/EC detection and DTNB assays, our studies did not determine whether HEDS could be used in an assay to quantify cell density or cellular responses to radiation, chemotherapeutic agents and toxins (Ayene et al., 2008; Ayene et al., 2000; Ayene et al., 2002; Biaglow et al., 2003; Biaglow et al., 2000; Biaglow et al., 2006). In this report, we define a HEDS assay that can accurately measure metabolically active live cells and quantify cell death after a cytotoxic insult.

MATERIALS AND METHODS

Cells

Human colon (HT29, HCT116) and breast (MCF7 and SKBR3) cancer cell lines were purchased from the American Type Tumor Collection. MCF10A cells were a gift of S.K. Muthuswamy (Cold Spring Harbor Laboratory, USA). Chinese hamster ovary (CHO) cells were provided by Thomas D. Stamato (Lankenau Institute for Medical Research, USA). Cells at different cell densities were plated in 60 mm dishes, 6 well or 96 well plates in 2ml, 1ml and 100 μ l respectively. DMEM containing 15% FCS and 25 mM HEPES were used for HT29, HCT116, MCF7 and SKBR3 cells. In some instances, McCoy 5A containing 15% FCS and 25mM HEPES were used for these cells. MCF10A cells were cultured in DMEM/F12 containing 5% horse serum, EGF (100 μ g/ml), hydrocortisone (1 mg/ml), cholera toxin (1 mg/ml), insulin 10 mg/ml and Penn/Strep (10 μ l/ml) media.

Cell density and Plate formats

For comparative studies (Figure 1 and Table 1) with other commonly used cell survival assays (XTT, WST-1, alamarblue, PrestoBlue and CellTiter Glo), we used 0, 1, 5, 10, 20 and 40 $\times 10^3$ cells in a 96 well plate, which are commonly used to determine the slope and linear regression of the response curves for cell survival assays. To determine the use of HEDS assay to measure the cytotoxic response (Figures 2,3,4,5,6a), we used 5,000 cells/well in a 96 well format since the data in Fig. 1 showed a reasonable OD of 0.8 for 5000 cells for HEDS assay. Additionally, we have also used 1000 cells, which exhibited 0.3 OD, in some of these experiments (Figures 2,3,4,6a) to determine the use of our assay for low density cells since it is also widely used in toxicology studies. We have also determined (Figures 6b, 7, 8) whether HEDS assay can also work for other procedures (>800,000 cells/well in a six well format and replating) commonly used for cell survival assays (Coulter counter and clonogenic assay) after radiation and cytotoxins. Additionally, this procedure also enables a better comparison between HEDS assay and Coulter counter since high cell density and replating procedure in a six well format gives minimum variations in the calculation of surviving fraction from Coulter cell counter and colony assay.

Cisplatin Treatment

HCT116 cells were plated at the desired density (1000 or 5000 cells) in a 96 well plate as described above on the day before treatment with cisplatin (Sigma, USA). Cells were exposed to different doses (0, 5, 10, 20, 40, 50 μ M) of cisplatin (Bedford Laboratories, USA) and incubated for 16 hours at 37 $^{\circ}$ C in a humidified 5% CO₂ incubator. After the desired incubation, cells were washed three times to remove extracellular cisplatin and replenished with the full growth medium. The effects of cisplatin under these conditions

were measured by HEDS, WST-1 and XTT assays at different days (0, 1, 2, 3) after cisplatin treatment.

Hydrogen peroxide and acetaminophen treatment

5,000 HCT116 cells were seeded into a 96 well plate on the day before treatment with hydrogen peroxide or acetaminophen. Cells were incubated with different concentrations of hydrogen peroxide (0, 100, 200, 300, 400, 800 μ M) or acetaminophen (0, 10, 20, 30, 40, 50, 60mM) for 3 and 16 hours respectively at 37 °C in a humidified 5% CO₂ incubator. After the desired incubation, cells were washed three times to remove extracellular hydrogen peroxide and replenished with the full growth medium. The effects of hydrogen peroxide under these conditions were measured by HEDS assay and Coulter cell counter 3 days after treatment. The effects of acetaminophen were measured by HEDS assay and Coulter cell counter immediately after 16 hours treatment.

Phenylarsine oxide and arsenite treatment

For experiments with PAO, HCT116 cells (1000 and 5000) were plated in a 96 well platform on the day before treatment. For experiments with arsenite, 800,000 cells were plated in 6 wells plate on the day before treatment. A stock solution of 35 mM PAO (Sigma, USA) and 35mM arsenite (Sigma, USA) were prepared fresh in DMEM growth medium. Cells were exposed to different doses of PAO (0, 1, 2, 4, 6, 8, 10, 20 μ M) or arsenite (0, 10, 20, 40, 60 μ M) and incubated at 37 °C in a humidified 5% CO₂ incubator. After 2 and 24 hours incubation with PAO and arsenite respectively, cells were washed three times to remove extracellular arsenic and replenished with the full growth medium. The effects of PAO under these conditions were measured by HEDS assay and Coulter cell counter in a 96 well plate after 3 days. To quantify the toxicity, arsenite treated cells were replated at low cell density in fresh full growth medium in duplicates in 6 wells plate and the HEDS assay and cell count by Coulter Counter were carried out simultaneously after 6 days.

Etoposide Treatment

Assays that measure the toxicity are also used widely for assaying anticancer efficacy of etoposide in various human cancer cells. We therefore compared the sensitivity of the HEDS assay to Coulter cell counter in measuring the survival of human breast cancer cells MCF7, SKBR3 and primary breast cells MCF10A. 800,000 cells were plated in a six well plate on the day before treatment with etoposide (Sicor Pharmaceuticals, California, USA). Cells were exposed to different doses (0, 50, 100 μ M) of etoposide and incubated at 37 °C in a humidified 5% CO₂ incubator. After the desired incubation time, cells were washed three times to remove extracellular etoposide and replenished with the full growth medium. To quantify the toxic effects of etoposide, cells were replated at low cell density in fresh full growth medium in duplicates in six wells plate and the HEDS assay and Coulter cell count were carried out after six days.

Hypoxia and Irradiation

800,000 HCT116 cells in full growth medium with 15% FCS and 25 mM HEPES were plated in 60 mm Nunc tissue culture dishes on the day before the experiment. Cells were checked under the microscope to make sure the cells were uniformly distributed and have the same density in all the wells. Hypoxia was carried out by custom made hypoxia chambers as described by Tuttle et al (Tuttle et al., 2007). Briefly, Nunc dishes containing cells were placed in aluminum hypoxic chambers and hypoxia was introduced by repeated removal and reintroduction of nitrogen after every 5 minutes for 30 minutes, a procedure that reduces the oxygen concentration to less than 0.5%. Cells in 60mm dishes placed in an aluminum chamber were irradiated either in air or hypoxia at different doses (dose rate

9.63Gy/min) at room temperature using a J.L. Shepherd Mark I ^{137}Cs irradiator. The effects of radiation under these conditions were measured by HEDS assay six days after irradiation.

Cell growth measured by HEDS assay in a 96 well plate

HEDS assay kit for 96 wells plate was provided by the LIMR Development Inc., Wynnewood, PA, USA. HEDS and 5,5-dithiobis 2-nitrobenzoic acid (DTNB) were prepared as per the manufacturers' instructions in glucose free DMEM growth medium and glutathione buffer respectively and stored at 4 °C. For a 96 wells plate assay, cells in each well of the plate were mixed with 5 μl HEDS reagent and incubated in a humidified CO_2 incubator at 37 °C for 2hrs. The amount of mercaptoethanol (ME) produced by bio-reduction of HEDS was estimated by DTNB at 412 nm in a microplate reader.

Cell growth measured by XTT and WST-1 survival assays

The XTT and WST-1 reagents (Cayman Chemicals Company, Ann Arbor MI) were prepared as per the manufacturers' instructions. Briefly, the XTT or WST-1 reagents were reconstituted with electron mediator solution immediately before use, aliquoted and stored at -20 °C. Ten μl of the reconstituted reagent was added to each well, mixed and then incubated for 2 hours at 37 °C in a CO_2 incubator. The absorbance of each sample was measured using a microplate reader at 450 nm.

Cell growth measured by alamarblue and prestoblue survival assays

Ten μl of alamarblue or prestoblue (Invitrogen, Frederick, MD) reagent was added to each well, mixed and then incubated for 2 hours at 37 °C in a CO_2 incubator. The absorbance of each sample was measured using a microplate reader by absorbance detection at 570nm or fluorescence detection at excitation and emission wavelength of 540–570 and 580–610nm respectively.

Cell growth measured by CellTiter Glo survival assay

The CellTiter Glo reagent (Promega, Madison, WI) was prepared as per the manufacturers' instructions. Briefly, CellTiter Glo buffer was mixed with cellTiter Glo substrate to reconstitute the lyophilized enzyme/substrate mixture. Hundred μl of the reconstituted reagent was added to cells in each well in a special opaque-walled 96 well plate and mixed for 2 minutes on an orbital shaker to induce cell lysis. These plates were then incubated at room temperature for 10 minutes and the luminescence of each sample was measured using a microplate reader as per the manufacturer's instructions.

Simultaneous cell counting by Coulter counter and HEDS assay in a 6 well plate or 60 mm dish

Immediately after treatment, the cells were replenished with fresh growth medium. Each dish containing cells was incubated at 37 °C in a humidified 5% CO_2 incubator. After 18h incubation, the attached cells in these dishes were trypsinized, mixed with medium and counted using a Coulter counter. Approximately 20000 cells from each sample were plated in duplicates in new six wells plate with complete growth medium and incubated in a humidified 5% CO_2 incubator at 37 °C for 5 days before measuring survival. Duplicate dishes were used for counting total number of cells in each dish after trypsinization using a Coulter counter.

The cell growth in the duplicate dishes were measured by 'HEDS assay kit for 60 mm dish' provided by LIMR Development Inc., Wynnewood. Briefly, dishes were incubated with 50 μl of HEDS reagent in a humidified CO_2 incubator at 37 °C for 2 hrs. After this incubation, 0.5 ml of extracellular medium removed from the attached cells was mixed with 0.5 ml of

sulfosalicylic acid (SSA) buffer in microfuge tubes and centrifuged in a microfuge. The supernatant (150 µl) was mixed with 1200 µl of phosphate buffer and 150 µl of DTNB. The optical density of this reaction mixture was measured at 412 nm.

The surviving fraction at a given dose of irradiation was calculated using the following formula:

$$\text{Fold increase in cell number} = \frac{\text{number of cells after 6 days}}{\text{Number of cells plated on day 1}}$$

$$\text{Surviving Fraction} = \frac{\text{Fold increase in cell number or O.D. at 412 nm after radiation}}{\text{Fold increase in cell number or O.D. at 412 nm without radiation}}$$

Statistical Analysis

Values were presented as Mean $\pm$ Standard Error (SE) calculated from three to five independent replicates. The sensitivity and the consistency of the assay were determined from the slope/intercept and the biological variability respectively. The slope and intercept, which represent the sensitivity of the assay, were calculated using linear (Figure 1) or polynomial curve fit (Figures 2, 3, 4). The half maximal inhibitory concentration (IC₅₀) was calculated from the slope and intercept of individual dose response curve. The biological variability i.e. percentage of coefficient of variance (CV) was calculated from the formula $100 \times \text{SD} / \text{mean}$, where SD is the standard deviation of mean of three to five independent replicates. The statistical significance of the differences between the groups was determined by Analysis of Variance (ANOVA) with the p values presented in Table 1 or the legend of Figures 5, 6b and 7.

RESULTS

Comparison of XTT, WST and HEDS assays in measuring the number of cells in a 96 well plate

Figure 1 is a graph of the application of the HEDS cell survival assay compared to other commercially available assays (WST-1 and XTT) used for cell survival in tissue culture medium. Thiol containing compounds can be measured by dithiobis nitrobenzoic acid. In order to utilize HEDS for measuring the cell survival in a 96 well plate, we determined the use of dithiobis nitrobenzoic acid to quantify ME, also a thiol-containing compound, produced from HEDS by its bioreduction in cells. Our results demonstrated that HCT116 cells incubated with HEDS for 2 hrs and then incubated with DTNB for 2 min showed an O.D. of 2.5 – 3.0 with a background O.D. of ~0.3 suggesting the potential to use the HEDS/DTNB combination for cell survival. The results also demonstrated that HEDS incubated for 2 hrs with MCF10A, MCF7, HCT116, SKBR3 and CHO cells at various cell densities (counted by Coulter Counter) showed a better sensitivity (slope) and dynamic range (r^2 values) compared to the WST-1 and XTT-1 assays (Figure 1 and Table 1). However, HEDS, WST-1 and XTT assay had similar slope and r^2 values with cell number in HT29 cells (Table 1). These results demonstrated that HEDS assay worked better than the WST-1 and XTT-1 assays in most cell lines. Comparison with other cell proliferation assays (alamar blue, presto blue, cell titre glo) under similar conditions in representative cell lines (HCT116 and HT29) tested have demonstrated that the r^2 values are better for HEDS assay (Table 1). The biological variability (% CV) of the slopes determined for MCF10A, MCF7, HT29, HCT116, SKBR3, CHO cells are lower for HEDS (2, 7, 9, 11, 5, 5%) as compared to WST-1 (14, 13, 8, 29, 23, 33%) and XTT (10, 5, 7, 8, 10, 16%). Similarly, alamar blue (30,

43% for absorbance; 14, 12% for fluorescence), prestoblue (10, 43% for absorbance; 5, 3% for fluorescence) and cell titer glo (7, 28%) have higher %CV as compared to HEDS assay. These results suggested that HEDS assay worked as good as or better than the other assays.

Comparison of HEDS, WST and XTT assays in measuring the response of cancer cells to cisplatin in a 96 well plate

We determined the application of HEDS assay in comparison with WST-1 and XTT to quantify the toxicity of cisplatin in human cells in a 96 well plate. The cell survival was measured by HEDS, WST-1 or XTT assays at 0 (immediately), 1, 2 and 3 days after overnight incubation of different concentrations of cisplatin (Figures 2–4 and Table 2). The results showed a cisplatin dose-dependent decrease in HEDS conversion measured up to 3 days after overnight incubation of cells with micromolar concentrations of cisplatin. The results not only demonstrated that this novel assay could be used to measure the kinetics of cell survival after cisplatin treatment but also the superior sensitivity of the novel assay with a higher slope and intercept and lower %CV in most cases for the slope and IC₅₀ compared to the other two assays (Table 2).

Comparison of HEDS assay with Coulter counter cell count in measuring the response of cancer cells to hydrogen peroxide and acetaminophen in a 96 well plate

The HCT116 cells in 96 well plates were incubated with different concentrations of hydrogen peroxide (0, 100, 200, 300, 400, or 800 μ M) and acetaminophen (0, 5, 10, 20, 40, 60mM) for 3 and 16 hours respectively (Figure 5). The cell survival was measured by HEDS assay and Coulter cell count at 1 and 3 days after exposure to acetaminophen and hydrogen peroxide respectively. The results demonstrated that a 3 hr incubation of cells with hydrogen peroxide was toxic to cells. The dose dependent decrease in HEDS conversion measured 3 days after incubation with micromolar concentrations of H₂O₂ demonstrated that this assay is sensitive enough to determine the toxicity of H₂O₂. The results demonstrated that HEDS assay exhibited similar IC₅₀ and low %CV ($122 \pm 4 \mu$ M; 3%) as the Coulter counter ($120 \pm 4 \mu$ M; 3%) suggesting that this assay is suitable to measure the toxic effects of hydrogen peroxide. Similarly, the toxicity of acetaminophen, which is known to induce GSH depletion similar to hydrogen peroxide, was effectively measured by HEDS assay with similar IC₅₀ and low %CV (11 ± 0.3 mM; 3%) as the coulter cell counter (10 ± 0.3 mM; 3%).

Comparison of HEDS assay with Coulter counter cell count in measuring the response of cancer cells to environmental toxins PAO (96 well plate) and arsenite (six well plate)

We tested the application of the HEDS assay in quantifying the toxicity of PAO and arsenite in human cells (Figure 6). Our assay quantified the toxic effects of short term exposure (2hrs) of PAO on human colon cells plated at low (1000 cells) and high (5000) cell density in a 96 well plate (Figure 6a). The assay was carried out 3 days after PAO treatment. The assay was extremely sensitive to measure the toxicity of PAO in both low and high density cells at concentrations as low as 1 μ M with IC₅₀ and %CV (1000 cells density: $0.6 \pm 0.08 \mu$ M with 11% CV; 5000 cells density: $3 \pm 0.18 \mu$ M with 6% CV) close to that measured by Coulter counter (1000 cells density: $0.3 \pm 0.01 \mu$ M with 3% CV; 5000 cells density: $2.2 \pm 0.01 \mu$ M with 0.5% CV). Preliminary results indicated that arsenite is not as toxic as PAO. We therefore tested this assay for relatively higher concentrations of arsenite after longer (24 hours) exposure of HCT116 cells to arsenite. This experiment was carried out in a six well plate to compare HEDS assay with the cell count measured by Coulter counter. The cell growth was measured six days after removing the arsenite from the extracellular medium by washing the cells three times and replating them at low cell density in six wells plate. The results in Figure 6b demonstrated an arsenite concentration dependent decrease in the survival of human HCT116 cells incubated with arsenite for 24 hours. Although the slopes and CVs are similar (0.03 ± 0.005 with 15%CV vs. 0.038 ± 0.004 with 10% CV) for both

HEDS assay and coulter counter, the IC₅₀ for arsenite was 2 fold higher ($29 \pm 3 \mu\text{M}$ with 10% CV) measured by HEDS assay as compared to Coulter Counter assay ($13 \pm 1 \mu\text{M}$ with 7% CV). The results demonstrated that HEDS assay exhibited similar slope as the Coulter counter for PAO (Figure 6a) and arsenite (Figure 6b) suggesting that this assay is suitable to measure the toxic effects of environmental pollutants such as arsenicals.

Comparison of HEDS assay with Coulter cell counter in measuring the response of cancer cells to etoposide in a six well plate

The graph in Figure 7 shows the application of the HEDS assay in quantifying the toxicity of etoposide in human cells. This experiment was carried out in a six well plate to compare HEDS assay with the cell count measured by Coulter counter. Cell growth was measured six days after removing the etoposide from the extracellular medium by washing the cells three times and replating them at a low cell density in a six well plate. The results in Figure 7 demonstrated an etoposide concentration-dependent decrease in the survival of human HCT116 cells incubated with etoposide for 24 hours. The results also demonstrated that the HEDS assay exhibited IC₅₀ and low %CV close to that calculated from Coulter counter for MCF7 ($27 \pm 0.6 \mu\text{M}$ with 2.4% CV vs. $36 \pm 5 \mu\text{M}$ with 15% CV), SKBR3 ($18 \pm 0.06 \mu\text{M}$ with 0.3% CV vs. $21 \pm 0.5 \mu\text{M}$ with 2.5% CV) and MCF10A ($41 \pm 3 \mu\text{M}$ with 7% CV vs. $32 \pm 5 \mu\text{M}$ with 14% CV) suggesting that this assay is suitable to measure the toxic effects of etoposide. The biological variation is also lower for HEDS assay as compared to the cell counter assay.

Comparison of HEDS assay with Coulter cell counter in measuring the hypoxic resistance of cancer cells to radiation in 60mm tissue culture dishes

Ionizing radiation is widely used in cancer therapy. It is also a major environmental concern due to the potential use of radioactive dirty bombs by terrorists and nuclear accidents. There is an unmet need for a sensitive cell toxicity assay that could be used for various studies with ionizing radiation. We determined the effectiveness of HEDS assay to measure the toxicity of ionizing radiation in human cells (Figure 8). This assay was carried out in 6 wells plate similar to that used for studies with arsenite and etoposide to simultaneously measure the toxicity by Coulter cell count and HEDS assay. The surviving fraction calculated by Coulter counter after aerobic radiation demonstrated decreased cell survival with increase in the dose of radiation (Figure 8a). A similar trend was also observed when the surviving fraction was calculated using the HEDS assay. We tested the efficiency of this assay to measure the hypoxic resistance (oxygen enhancement ratio) of human colon cancer cells HCT116. An oxygen enhancement ratio (2 ± 0.2 with 10% CV) close to that of coulter counter (1.5 ± 0.1 with 7% CV) indicated that HEDS assay was able to measure the resistance of these cells to radiation under hypoxic condition (Figure 8b).

DISCUSSION

This study sought to develop and validate a novel tissue culture medium-based biomarker of metabolically active live cells that could be used to quantify cell cytotoxicity to various agents and radiation. We had previously demonstrated using a state of the art HPLC electrochemical detector that live rodent cells convert HEDS into ME in vitro (Ayene et al., 2000). Most importantly, we had demonstrated that the HPLC peak for ME was observed in the extracellular medium only after incubation of rodent and human cells with HEDS (Ayene et al., 2000). In the absence of HEDS, the extracellular medium did not have any detectable amount of mercaptoethanol. These studies also demonstrated that the conversion of HEDS into ME is dependent on the activity of the oxidative pentose phosphate cycle (OPPC), suggesting that this assay could be used for all mammalian cells (Ayene et al., 2008; Ayene et al., 2000; Ayene et al., 2002; Biaglow et al., 2003; Biaglow et al., 2000;

Biaglow et al., 2006). There are several biochemical pathways involved in the reduction of HEDS in live mammalian cells, presently below.

Direct reduction of HEDS by GSH and NADPH may produce ME, NADP and GS-adduct (reactions 1 and 2).

Normally, in cells, the generation of NADPH produced by oxidative pentose phosphate cycle (OPPC) recycles GSSG back to GSH (reactions 2 and 3), which is also involved in the reduction of HEDS (Biaglow et al., 2000; Biaglow et al., 2006).

We know from our studies and those of others that the reduction of HEDS is also facilitated by thiol transferase linked reactions (Bjornstedt et al., 1997). However, the thioltransferase enzyme also utilizes GSH in the reduction of HEDS to stimulate the second step of reaction 1 (Bjornstedt et al., 1997). The schematic representation in figure 9 shows that five major metabolic pathways, which ultimately require OPPC for their activity, may be involved in “HEDS conversion into ME” (Figure 9).

Based on the DTNB reaction of the metabolite of HEDS and our previous high pressure liquid chromatography and electrochemical detector (HPLC/EC) data that identified ME as the only DTNB reactive thiols produced by HEDS bioreduction (Ayene et al., 2000), our current findings demonstrated that ME is the main product produced from HEDS in these human cancer cells (HCT116, HT29, MCF7, MCF10A, SKBR3) and CHO cells. Our results demonstrated that the metabolic conversion of HEDS into DTNB reactive product correlates with the cell number measured by Coulter counter. Additionally, the correlation between DTNB reactive product after HEDS incubation and cell number measured by Coulter counter showed a better linearity than currently available (XTT, WST-1, alamar blue, presto blue and cell titer glo) assays (Table 1). Although the results presented in Figures 1–5 and 6a, which were all carried out in 96 well formats, were sufficient to demonstrate the novelty and efficiency of the HEDS assay, we have also presented data for 6 well formats in figures 6b, 7 and 8. We have used six wells format to demonstrate that this assay could also be used as well as the Coulter counter in 6 wells format that is quite commonly used in determining the toxicity of radiation and chemotherapeutic agents.

Cisplatin is one of the most commonly used chemotherapeutic agents in humans (Barabas et al., 2008) and there remains great interest in understanding the mechanisms of cancer cells resistance to platinum compounds. The normal tissue toxicity of platinum compounds is a dose limiting factor in cancer therapy (Barabas et al., 2008). Addressing these issues requires a more efficient cell survival assay that could determine the changes in survival of cancer cells. We therefore tested the sensitivity of this assay in measuring the cell survival of human colon cancer cell HCT116 after cisplatin treatment. The results demonstrated that HEDS assay is suitable to determine the survival of cells after treatment with cisplatin in a 96 well plate.

Hydrogen peroxide (H_2O_2) is used to understand the signaling pathways during oxidative stress and to screen/test the efficacy of antioxidants (Groeger et al., 2009; Muller et al., 2007). Because of the wide use of this oxidant, we determined the application of HEDS assay in quantifying the toxicity of hydrogen peroxide in human cells in a 96 well plate. The results demonstrated that HEDS assay can estimate the survival of cells after treatment with hydrogen peroxide. Although hydrogen peroxide is known to induce glutathione depletion, the HEDS assay worked as well as the Coulter counter suggesting that modest GSH depletion does not affect the HEDS assay. Consistent with this, our data with acetaminophen, which is also known to deplete GSH, demonstrated that acetaminophen induced toxicity measured by HEDS test is comparable to Coulter cell counter analysis suggesting that the GSH depletion, if any, is either not strong enough to affect the HEDS assay or GSH is regenerated by cells at the time of the assay. This is consistent with previous studies that have shown that the GSH depletion by acetaminophen is mild in HUH6 cells and regenerated to the control level after 5 hours in liver (Neuwelt et al., 2009; Henderson et al., 2000). However, strong GSH depletion persisted during the assay may have some influence on the measurement. Under these conditions, the HEDS test can still be equally effective if the time of incubation is increased longer than 2 hours or the assay is done after GSH regeneration.

The results with hydrogen peroxide and acetaminophen demonstrated that HEDS assay has no disadvantages in these cells under normal growth medium. However, it may be affected in zero glucose medium, G6PD deficient cells or cells completely depleted of GSH since glucose and G6PD and to some extent GSH are essential for the bioreduction of HEDS. However, this assay will be certainly useful for chemotherapeutic agents, oxidant, toxins and radiation or other cytotoxins that have mild effects on multiple pathways involved in the metabolism of HEDS. This is further confirmed by the current results that clearly demonstrated the application of HEDS assay for four different classes of cytotoxic agents (DNA damaging agents, oxidants, toxins, radiation).

Environmental pollution is a major concern worldwide. In the U.S., arsenical toxins rank third of all hazardous chemicals in highly polluted superfund sites. In ground water inorganic arsenic commonly exists as arsenate (As^{5+}) and arsenite (As^{3+}) and the reduction of As^{5+} to As^{3+} may increase As^{3+} content in soil (Singh, 2006). In air, the semimetallic form of arsenic oxidizes rapidly and at high temperatures will produce arsenic trioxide, a compound used in humans to treat certain types of cancer (Evens et al., 2004). Phenyl arsenic compounds are the main contaminants in ground water at abandoned sites with a history of arsenic containing chemical warfare agents (Kroening et al., 2008). The beneficial use of arsenic in cancer and potential harmful effects in humans at low exposure prompted us to explore HEDS assay for measuring arsenic-induced cell death. We therefore tested the application of the HEDS assay in quantifying the toxicity of PAO and arsenite in human cells (Figure 6). The results demonstrated that HEDS assay can effectively measure the toxic effects of environmental pollutants in human cells.

Etoposide is a topoisomerase II inhibitor used in cancer therapy (Hande, 1998). Etoposide is known to kill cancer cells by producing DNA double strand breaks (Hande, 1998). Like many other chemotherapeutic agents, etoposide is also toxic to normal cells (Joel et al., 1996; Kobayashi and Ratain, 1994; Massimino et al.). The results demonstrated that HEDS assay is a good measure of the toxic effects of topoisomerase inhibitor in vitro.

The cellular response to radiation is also dependent on the oxygen concentration in the cells (Vaupel and Mayer, 2007). Several previous studies have demonstrated that cells under hypoxic conditions are resistant to radiation as compared to that in aerobic condition (Vaupel and Mayer, 2007). Hypoxic cells are quite common in most solid tumors, which

determine the outcome of radiation therapy. Hypoxic cancer cells are also responsible for tumor vasculature and growth (Vaupel and Mayer, 2007). Although certain drugs are used to target hypoxic cancer cells, there still remains a great need for a fast cytotoxic assay to measure hypoxic resistance that will be useful to screen better hypoxic drugs (Ahn and Brown, 2007). The results demonstrated that HEDS assay can be used to measure the hypoxic resistance of cancer cells to radiation.

Our results suggested that this simple extracellular medium based biochemical assay could be used to determine the survival of cells that produce NADPH. The continuous recycling of NADP to NADPH, which requires a fully functional OPPC and glucose substrate, is essential to convert HEDS into ME. Glucose is converted into glucose-6-phosphate by hexokinase in all living cells. Glucose-6-phosphate is used as a substrate by G6PD/OPPC to produce NADPH. NADPH is used directly or as a cofactor to reduce HEDS into ME, which is released into the tissue culture medium. This conversion is dependent on the glucose level and active metabolic pathway of live cells. Unlike glutathione disulfide, HEDS is a unique non-toxic disulfide that is readily converted into a reduced thiol by glucose-dependent metabolic activity of live cells and transported into the extracellular medium in vitro. Our preliminary results demonstrated that ME produced from HEDS bioreduction is not toxic to cells under the conditions used in this protocol. Although ME is a well known reducing agents for proteins in vitro, it is less effective as compared to DTT in changing the function of protein in intact cells (Valetti and Sitia, 1994). Further, it is unlikely to be toxic since it maintains the integrity of proteins and cells (Janjic and Wollheim, 1992).

Our present results now demonstrate that this metabolic conversion of HEDS can be used to determine cell density and response of cancer cells to radiation, chemotherapeutics, chemical oxidant and environmental toxins. The HEDS conversion directly correlated to cell proliferation measured by Coulter counter suggesting that the conversion of HEDS into ME is a direct measurement of live cells. Dead cells will fail to convert HEDS into ME due to the loss of OPPC activity. This conclusion is further confirmed by our current findings that revealed a dose-dependent decrease in ME released into the tissue culture medium by cells treated with cisplatin, etoposide, hydrogen peroxide, arsenicals and radiation as compared to untreated cells. Our results demonstrated a direct quantification of cell death or loss of survival, since the loss of HEDS conversion correlated with decrease in cell number measured by the Coulter counter. Most importantly, the lack of conversion of HEDS into ME could be easily quantified by dithiobisnitrobenzoic acid, with the simple HEDS+DTNB biochemical assay taking less than 2.5 hours to complete in the laboratory.

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Abbreviations

G6PD	glucose-6-phosphate dehydrogenase
HEDS	hydroxyethyl disulfide
OPPC	oxidative pentose phosphate cycle
ME	mercaptoethanol
DTNB	dithiobisnitrobenzoic acid
HTS	high throughput screening

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Highlights

- Cell viability assays have a variety of practical and technical limitations.
- Our findings establish the use of hydroxyethyl disulfide (HEDS) to quantify cells.
- HEDS quantified metabolically active live cells better than other assays.
- Cell toxicity produced by various agents was rapidly determined by the HEDS assay.

Figure 1. Comparison of HEDS, XTT and WST assays in measuring the number of cells in a 96 wells plate

Cell density dependent conversion of HEDS in colon (HCT116, HT29) and mammary (MCF7) cells was measured by medium based DTNB assay that measures the conversion of HEDS into a non-toxic mercaptoethanol (ME). Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted. Linear regression analysis of the cell number response curve and ANOVA analysis of differences between the HEDS assay and XTT or WST-1 are presented in table 1. The biological variability as determined by % coefficient of variance (%CV) for these results is presented in Table 1. Similar analysis for additional cell lines (MCF10A, SKBR3 and CHO cells) and other assays (almarblue, prestoblue and CellTiter Glo) are presented in table 1.

Figure 2. HEDS assays in measuring the response of human colon cancer cells HCT116 to cisplatin

Cell survival was determined by HEDS assay after overnight incubation with different concentrations (0, 10, 20, 30, 40, 50 μM) of cisplatin in a 96 wells plate at 0 (immediately), 1, 2 and 3 days after treatment as described in Figure 1. The cisplatin dose response curves were presented for 0 and 1 day after treatment in Figure 2. The IC₅₀ calculated from the slope and intercept of regression analysis (polynomial curve fit) of the cisplatin dose response curves and biological variability (%CV) for the slopes and IC₅₀ for HEDS assay measured after 0, 1, 2 and 3 days are presented in table 2. The high slope and intercept for HEDS assay in most cases demonstrated that it is suitable for measuring the cisplatin toxicity in mammalian cells. Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted.

Figure 3. WST assays in measuring the response of human colon cancer cells HCT116 to cisplatin

Cell survival was determined by WST and PMS reagents after overnight incubation with different concentrations (0, 10, 20, 30, 40, 50 μ M) of cisplatin in a 96 wells plate at 0 (immediately), 1, 2 and 3 days after treatment as described in Figure 1. The cisplatin dose response curves were presented for 0 and 1 day after treatment in Figure 3. The IC₅₀ calculated from the slope and intercept of regression analysis (polynomial curve fit) of the cisplatin dose response curves and biological variability (%CV) for the slopes and IC₅₀ for WST-1 assay measured after 0, 1, 2 and 3 days are presented in table 2. The low slope and intercept for WST-1 in most cases demonstrated that it has inherent problems in measuring the cisplatin toxicity in most mammalian cells. Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted.

Figure 4. XTT assays in measuring the response of human colon cancer cells HCT116 to cisplatin

Cell survival was determined by XTT and PMS reagents after overnight incubation with different concentrations (0, 10, 20, 30, 40, 50 μM) of cisplatin in a 96 wells plate at 0 (immediately), 1, 2 and 3 days after treatment as described in Figure 1. The cisplatin dose response curves were presented for 0 and 1 day after treatment in Figure 4. The IC₅₀ calculated from the slope and intercept of regression analysis (polynomial curve fit) of the cisplatin dose response curves and biological variability (%CV) for the slopes and IC₅₀ for XTT assay measured after 0, 1, 2 and 3 days are presented in table 2. The low slope and intercept for XTT in most cases demonstrated that it has inherent problems in measuring the cisplatin toxicity in most mammalian cells. Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted.

Figure 5. HEDS assay in measuring the response of human colon cancer cells HCT116 to hydrogen peroxide and acetaminophen in a 96 wells plate

The effects of hydrogen peroxide under these conditions were measured by HEDS assay (scale on the left Y axis) and Coulter cell counter (scale on the right Y axis) 3 days after treatment. The effects of acetaminophen were measured by HEDS assay (scale on the left Y axis) and Coulter cell counter (scale on the right Y axis) immediately after 16 hours treatment. Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted. HEDS assay exhibited similar IC₅₀ and low %CV as the Coulter counter for H₂O₂ ($122 \pm 4 \mu\text{M}$; 3% vs. $120 \pm 4 \mu\text{M}$; 3%) and acetaminophen ($11 \pm 0.3 \mu\text{M}$; 3% vs. $10 \pm 0.3 \mu\text{M}$; 3%). Statistical (ANOVA analysis) comparisons of the HEDS assay relative to that of Coulter counter was not significantly different for the IC₅₀ calculated after hydrogen peroxide (Figure 6a) or acetaminophen (Figure 6b) exposure.

Figure 6. HEDS assay in measuring the response of human colon cancer cells HCT116 to environmental toxins PAO (96 wells plate) and arsenite (six well plate)

The toxic effects of short term exposure (2 hrs) of PAO was measured by HEDS assay (scale on the left Y axis) and Coulter cell counter (scale on the right Y axis) at low (1000) and high (5000) cell density in a 96 well plate after 3 days. Arsenite toxicity after 24 hrs exposure was determined in a six well plate to compare the HEDS assay (scale on the left Y axis) with Coulter counter (scale on the right Y axis). Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted. HEDS assay exhibited IC₅₀ for PAO close to that of the Coulter counter at 1000 (IC₅₀: 0.6 ± 0.08 μ M with 11% CV vs. 0.3 ± 0.01 μ M with 3% CV) and 5000 (IC₅₀: 3 ± 0.18 μ M with 6% CV vs. 2.2 ± 0.01 μ M with 0.5% CV) cells density. Although the slope is same (0.03 ± 0.005 with 15%CV vs. 0.038 ± 0.004 with 10% CV) for both HEDS assay and coulter counter, the IC₅₀ for arsenite was 2 fold higher (29 ± 3 with 10% CV) measured by HEDS assay as compared to Coulter Counter assay (13 ± 1 with 7% CV). Statistical (ANOVA analysis) comparisons of the HEDS assay relative to that of Coulter counter was not significantly different for the slopes calculated after PAO (Figure 6a) and arsenite (Figure 6b) exposures suggesting that HEDS assay is comparable to Coulter counter in measuring the toxic effects of arsenicals.

Figure 7. Comparison of HEDS assay and Coulter cell counter in measuring the response of breast cancer cells (MCF7, SKBR3) and primary breast cells (MCF10A) to etoposide

This experiment was carried out in a 6 well plate to compare HEDS assay (scale on the left Y axis) with Coulter counter (scale on the right Y axis). The cell growth was measured six days after removing the etoposide from the extracellular medium by washing the cells three times and replating them at low cell density in duplicates in six wells plate. Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted. HEDS assay exhibited IC_{50} close to that of the Coulter counter for MCF7 ($27 \pm 0.6 \mu\text{M}$ with 2.4% CV vs. $36 \pm 5 \mu\text{M}$ with 15% CV), SKBR3 ($18 \pm 0.06 \mu\text{M}$ with 0.3% CV vs. $21 \pm 0.5 \mu\text{M}$ with 2.5% CV) and MCF10A ($41 \pm 3 \mu\text{M}$ with 7% CV vs. $32 \pm 5 \mu\text{M}$ with 14% CV). Statistical (ANOVA analysis) comparisons of HEDS assay relative to that of Coulter counter was not different for the IC_{50} measured after etoposide exposure suggesting that HEDS assay is comparable to Coulter counter in measuring the toxic effects of etoposide.

Figure 8. Comparison of HEDS and cell proliferation assays in measuring the response of normoxic and hypoxic cancer cells (HCT116) to radiation

The effects of radiation were measured by HEDS assay or Coulter counter six days after irradiation. HEDS assay exhibited oxygen enhancement ratio (2 ± 0.2 with 10% CV) close to that of the Coulter counter (1.5 ± 0.1 with 7% CV). Each value was the mean $\pm$ standard error (SE) calculated from three to five independent replicates with SE as shown unless smaller than points plotted. Statistical (ANOVA analysis) comparisons of HEDS assay relative to that of Coulter counter was not different for the oxygen enhancement ratio measured suggesting that HEDS assay is comparable to Coulter counter.

Figure 9. Biochemical pathways involved in bioreduction of HEDS

Schematic representation of the various pathways involved in the cellular interactions of HEDS. HEDS reacts directly with NADPH to produce mercaptoethanol (ME) and NADP (Pathway 1). It may also react with GSH directly and/or in a reaction catalyzed by glutathione-S-transferase (GST) to produce mixed disulfide (MESSG) of glutathione (GSH) and ME (Pathway 2). The mixed disulfide MESSG reacts with GSH and produces ME and oxidized GSH (GSSG) by the catalytic action of glutaredoxin (GRX) (Pathway 3). The glutathione disulfide GSSG reacts with NADPH and produces GSH by the catalytic action of glutathione reductase (GRase) (Pathway 4). The conversion of GSSG to GSH i.e. GSH recycling requires NADPH recycling ($\text{NADP}^+ \rightarrow \text{NADPH}$) by oxidative pentose phosphate cycle (OPPC) (Pathway 5).

Table 1

The mean ± SE and %CV of slope and r² values calculated from individual linear curve fit of cell density dependent response curves from three to five independent replicates.

	r ²																					
	slope								Cell titer Glo		HEDS		WST-1		XTT		Alamarblue		Prestoblue		Cell titer Glo	
Cells	HEDS	WST-1	XTT	Alamarblue		Prestoblue				Lumin.	Abs.	Abs.	Abs.	Abs.	Abs.	Fluor.	Fluor.	Abs.	Abs.	Fluor.	Fluor.	Lumin.
	Abs.	Abs.	Abs.	Abs.	Fluor.	Abs.	Fluor.															
MCF10A	0.041 ± 0.001 (3%)	0.007 ± 0.001 * (p<0.001) (14%)	0.01 ± 0.001 ** (p<0.001) (10%)									0.98 ± 0.003 (0.3%)	0.84 ± 0.055 * (p<0.01) (7%)	0.77 ± 0.02 ** (p<0.01) (3%)								
MCF7	0.065 ± 0.005 (7%)	0.015 ± 0.002 * (p<0.001) (13%)	0.019 ± 0.001 ** (p<0.001) (5%)									0.97 ± 0.01 (1%)	0.73 ± 0.12 * (p<0.01) (16%)	0.74 ± 0.09 ** (p<0.01) (12%)								
HT29	0.021 ± 0.002 (9%)	0.025 ± 0.002 (NS) (8%)	0.015 ± 0.001 (NS) (7%)	0.0033 ± 0.001 # (p<0.001) (30%)	330 ± 47 (14%)	0.001 ± 0.0001 # (p<0.001) (10%)	337 ± 17 (5%)	811 ± 53 (7%)				0.99 ± 0.002 (0.2%)	0.93 ± 0.02 (NS) (2%)	0.98 ± 0.002 (NS) (0.2%)	0.64 ± 0.03 # (p<0.01) (5%)	0.93 ± 0.03 # (p<0.1) (3%)	0.55 ± 0.04 # (p<0.01) (7%)	0.83 ± 0.003 # (p<0.01) (0.4%)				0.89 ± 0.01 # (p<0.01) (1%)
HCT116	0.08 ± 0.009 (11%)	0.017 ± 0.005 * (p<0.001) (29%)	0.025 ± 0.002 ** (p<0.001) (8%)	0.0023 ± 0.001 # (p<0.001) (43%)	1146 ± 147 (13%)	0.0023 ± 0.001 # (p<0.001) (44%)	1039 ± 33 (3%)	359 ± 102 (28%)				0.93 ± 0.012 (1%)	0.37 ± 0.1 * (p<0.01) (27%)	0.72 ± 0.06 ** (p<0.01) (8%)	0.58 ± 0.12 # (p<0.01) (21%)	0.831 ± 0.05 # (p<0.01) (6%)	0.602 ± 0.048 # (p<0.01) (8%)	0.84 ± 0.01 # (p<0.01) (1%)				0.87 ± 0.01 # (p<0.01) (1%)
SKBR3	0.018 ± 0.001 (5%)	0.013 ± 0.003 (NS) (23%)	0.006 ± 0.001 ** (p<0.001) (17%)									0.97 ± 0.011 (1%)	0.71 ± 0.1 * (p<0.01) (14%)	0.88 ± 0.01 ** (p<0.01) (1%)								
CHO	0.019 ± 0.001 (5%)	0.006 ± 0.002 * (p<0.01) (33%)	0.02 ± 0.002 (NS) (10%)									0.96 ± 0.011 (1%)	0.33 ± 0.1 * (p<0.01) (30%)	0.67 ± 0.1 (p<0.1) (15%)								

* ANOVA statistical comparisons of slope and r² for HEDS assay relative to WST-1 were significant (p<0.01) for MCF10A, MCF7, HCT116 and CHO cells. Additionally, the r² for HEDS assay relative to WST-1 was significant (p<0.01) for SKBR3 cells.

** ANOVA statistical comparisons of slope and r² for HEDS assay relative to XTT were significant (p<0.01) for MCF10A, MCF7, HCT116 and SKBR3 cells. Additionally, the r² for HEDS assay relative to WST-1 was significant (p<0.01) for CHO cells.

ANOVA statistical comparisons of slope for HEDS assay relative to alamar and presto for comparable absorbance measurements were significant (p<0.01) for the two cell lines (HCT116 and HT29) tested. Although the slope for the fluorescence measurement for presto and alamar blue are higher, the r² values for these measurements are significantly lower than the HEDS assay.

The % CV of the slopes and r² values are presented within parentheses for each condition.

Table 2

Slope, IC50 and biological variability (%CV) calculated from three to five independent replicates of cisplatin dose response curves shown in Figures 2–4.

Cell Density	Day	HEDS				WST				XTT			
		slope		IC50		slope		IC50		slope		IC50	
		Mean ± SE	%CV	Mean ± SE (μM)	%CV	Mean ± SE	%CV	Mean ± SE (μM)	%CV	Mean ± SE	%CV	Mean ± SE (μM)	%CV
1000	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1000	1	0.18 ± 0.06	33	2.2 ± 0.4	18	ND	ND	ND	ND	ND	ND	ND	ND
1000	2	0.29 ± 0.07	24	1.9 ± 0.37	19	0.15 ± 0.05	33	2.8 ± 0.9	32	0.07 ± 0.02	28	4.1 ± 2.6	62
1000	3	0.66 ± 0.018	3	1.68 ± 0.09	5	0.18 ± 0.11	61	0.53 ± 1.8	336	0.3 ± 0.1	33	1.63 ± 0.22	13
5000	0	0.016 ± 0.006	37	31.5 ± 6	19	ND	ND	ND	ND	ND	ND	ND	ND
5000	1	0.09 ± 0.010	11	12 ± 0.9	8	0.004 ± 0.02	500	6 ± 14	219	0.048 ± 0.01	20	12 ± 1.2	9.5
5000	2	0.5 ± 0.13	26	3.4 ± 0.49	14	0.23 ± 0.13	56	7.6 ± 2.6	34	0.32 ± 0.15	46	7.2 ± 5.2	72
5000	3	0.55 ± 0.04	7	2.95 ± 0.21	7	0.26 ± 0.14	53	50.6 ± 54	106	0.12 ± 0.1	83	25 ± 16	63