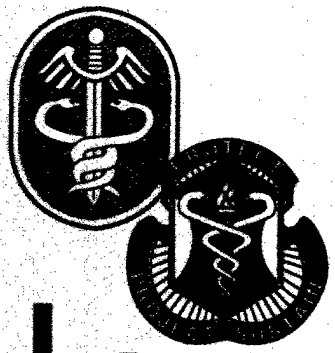

# U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

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# Comparison of Three Fluorescence Microplate Assays for Assessment of HD Cytotoxicity

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September 2002





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Abstract

The cytotoxic effect of sulfur mustard (HD) was examined in cultured human epidermal keratinocytes (HEK, Clonetics # 2971, of neonatal origin). Three different cytotoxicity assays of adherent cells in 96-well culture plates were compared, i.e., procedures employing alamar blue, calcein-AM or ethidium homodimer (EthD-1) as the fluorescent probe. HD was administered at 100 μM increments over the range of 100-500 μM. Cytotoxicity was assessed at 24, 48 and 96 hr post-exposure. HD-induced cytotoxicity was readily detectable with all three dyes. All three procedures indicated that cell death occurs over an extended period of time, with some cells dying both before and after 24 hr post-exposure to HD. Essentially linear concentration-dependent declines in alamar blue and calcein-AM fluorescence (indicating cell death) were observed at 24 hr. Further, the cytotoxic response measured using both alamar blue and calcein-AM increased sequentially with time post-exposure. The severity of the cytotoxic response was greater with alamar blue than with calcein-AM at all three time periods examined. EthD-1 fluorescence (enhanced with cell death) was increased between 0 and 24 hr and between 24 and 48 hr, but was not further enhanced at 96 hr after HD. EthD-1 had a smaller dynamic range (difference in fluorescence between dead [HD exposed] and live [non-exposed] cells) and was thus a less sensitive indicator of HD cytotoxicity than the other two fluorescent dyes. Differences in the cytotoxic response measured by these three procedures undoubtedly arise because of differences in the molecular reactions and the particular aspects of cellular function that the individual dyes monitor. The overall data indicate that HD cytotoxicity in adherent HEK can be assessed using either alamar blue or calcein-AM. Alamar blue is more sensitive than calcein-AM and is therefore recommended for general use. Alamar blue also offers the additional advantage that it is non-toxic and freely diffuses from cells in both oxidized and reduced forms. It can therefore be used to obtain replicate analyses on the same cell preparations (following washout from the medium). The assessment of HD cytotoxicity using adherent HEK in multiwell plates is rapid, does not require sophisticated instrumentation, and is free of cellular damage produced by detachment procedures required for flow cytometry analyses. Difficulties associated with the multi-well plate technique for the assay of HD cytotoxicity are relatively minor and involve the use of suboptimal controls.

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## Introduction

Sulfur mustard (HD) has been used as a chemical warfare agent because of the highly debilitating effect it has on exposed soldiers. Direct contact with skin, eyes and respiratory passageways produces painful inflammatory and necrotic lesions requiring weeks of intensive medical care (Smith and Dunn, 1991). Cell death appears to be a critically important component of lesion formation *in vivo*. Actively growing keratinocytes of the basal epidermal layer are considered to be the primary cellular targets of HD (Smith et al., 1992). HD is also cytotoxic to a variety of cell types *in vitro* (Smith et al., 1992; Dabrowska et al., 1996; Meier and Millard, 1998; Sawyer, 1999). An area of active research interest at the MRICD has been the development of *in vitro* screening procedures to evaluate potential antagonists of HD-induced cytotoxicity. Flow cytometric procedures have been routinely employed (Smith et al., 1991, 1992,1999; Martens, 1997). These procedures are adequate for non-adherent cells, such as blood lymphocytes. However, a difficulty has been observed in conducting these analyses on keratinocytes (Guzman et al., 2000), which require attachment to the culture vessel for growth. Adherent cells must be detached by trypsinization, which is believed to result in cellular damage. Because of this, a number of multi-well plate assays have been considered as alternatives for adherent cells (Guzman et al., 2000). The current investigation was conducted to further evaluate several multi-well plate approaches for monitoring HD cytotoxicity. The specific procedures involved the use of alamar blue, calcein-AM or EthD-1 as cytotoxicity indicators. The molecular reactions involved with each of these procedures are briefly described below. Procedures for all three probes are established and generally accepted cytotoxicity assays. The author has played no role in the development of these assays or in their validation. For this, the reader is referred to the papers cited below for each cytotoxicity indicator. This study was conducted merely to examine the use of these three procedures for the assay of HD-induced cytotoxicity.

Alamar blue is a recently developed tetrazolium dye specifically introduced as a cell viability indicator (Page et al., 1993). This dye detects cellular redox activity, i.e., the patency of cellular energy generating capabilities (Andrews et al., 1997), which are lost early during the process of cell death (Hirsch et al., 1998; Pettitt and Cawley, 2000). The blue dye is reduced by cellular enzymes (primarily mitochondrial) to a pink product, which also has markedly different fluorescence properties from the oxidized form. Reaction product (the reduced form) can therefore be quantified either spectrophotometrically or fluorimetrically. Both oxidized and reduced forms are freely permeable to cell membranes, and are non-toxic (Ahmed et al., 1994). This allows the repeated administration of alamar blue for repeated assay of the same cell preparations (Page et al., 1993).

Calcein-AM (the acetoxymethyl ester of calcein) is an indicator of the functional activity of cellular esterases. The calcein-AM ester readily enters both live and dead cells. Active esterases of living cells hydrolyze the dye into the free calcein form, which is both impermeable to cell membranes and of markedly greater fluorescence than the ester form (Weston and Parish, 1990; Papadopoulus et al., 1994). Living cells thereby accumulate

fluorescent free calcein, whereas the relatively non-fluorescent ester form is maintained at the medium concentration (at equilibrium) in both live and dead cells. Calcein-AM is cytotoxic to a variety of cultured cell lines (Liminga et al., 1999) and therefore cannot be used for repetitive cytotoxicity determinations on the same cell preparations.

The mechanism of the EthD-1 cytotoxicity reaction is identical to that of propidium iodide, used for viability/cytotoxicity determinations for many years. The homodimer of ethidium is now available, thereby eliminating potential health hazards associated with handling of the bromide salt. EthD-1 is impermeable to the intact membranes of living cells, but freely permeable to cells with damaged membranes. EthD-1 binds to nucleic acids, resulting in markedly enhanced fluorescence of the molecule (Papadopoulus et al., 1994). The basis of the assay is quantification of highly fluorescent nucleic acid-bound EthD-1. Thus, the EthD-1 procedure differs from alamar blue and calcein-AM procedures in that cell death results in an increase in the measured fluorescence.

## Methods

Primary HEK of neonatal origin (batch #2971, Clonetics, San Diego, CA) were cultured in keratinocyte growth medium (KGM, Clonetics). Tertiary cultures were seeded into 96-well culture plates at a density producing ca. 95 % confluence at the time of HD exposures (the next morning). Cultures were exposed to HD (100-500 μM, at 100 μM increments) in a toxic fume hood. HD (of approximately 98 % purity, Edgewood Chemical Biological Center, APG, MD) was dissolved in ethanol and stored at -70° C. At the time of exposures, the HD-ethanol stock was diluted in ice-cold KGM and added to the cells as rapidly as possible to prevent spontaneous hydrolysis of HD (each HD concentration was prepared separately from the ethanol stock). Final concentrations of ethanol ranged from 0.03 to 0.15 %. The standard operating procedure required that the HD-exposed cells remained in the toxic fume hood (at room temperature) for 60 min after exposure. They were transferred into the culture incubator. In plates used for 96-hr measurements, the medium was carefully aspirated (to avoid detaching cells damaged by HD) at about 48 hours and replaced with fresh KGM. No evidence that the cells the non-HD control wells were growing on top of each other or up the sides of the wells was obtained by microscopic examination.

Cytotoxicity determinations were conducted at 24, 48 and 96 hr after HD exposure. One hour prior to the designated time period, alamar blue, calcein-AM and EthD-1 were added to the cultures. Alamar blue was assayed according to directions provided by the supplier (Biosource International, Camarillo, CA). Alamar blue was diluted in KGM prior to addition to the plates (the final concentration was 5%, vol/vol). Any non-specific fluorescence attributable to the cells or KGM was corrected for by subtracting 0 time measurements (obtained immediately upon addition of the dye to each plate). Calcein-AM and EthD-1 were assayed simultaneously and in the same cells using a Molecular Probes (Eugene, OR) Live/Dead Cytotoxicity Assay Kit (#L3224). As recommended by Molecular Probes, the medium was replaced with phosphate-buffered saline (PBS) to

remove potentially interfering factors. Final concentrations of calcein-AM and EthD-1 were 8 and 4 μM, respectively. Calcein-AM and EthD-1 assays included freshly killed control wells prepared by a 10-min pretreatment with 0.1 % saponin in PBS. For all assays, fluorescence reaction product was measured using a Cytofluor 4000 multi-well plate reader (Perceptive Biosystems, Foster City, CA). Filter combinations employed were in agreement with specifications of Biosource and Molecular Probes. Excitation and emission filter combinations (peak nm/bandwidth nm) employed were as follows: for alamar blue, 530/25, 580/50; for calcein-AM, 485/20, 530/25; and for EthD-1, 530/25, 620/40. Fluorescence measurements were made on calcein-AM and EthD-1 plates at 0, 30, 60 and 90 min after addition of the fluorescent probes. The 90-min values were used for statistical analysis. Similarly, alamar fluorescence determinations were made at 0, 1, 2 and 3 hr following addition to the plates, with the 3-hr values analyzed statistically. Assays were conducted such that each column (consisting of 8 wells) of each plate was used to obtain one data point. Results of 8 different assays (duplicate assays from each of 4 different exposures) were combined for statistical analysis, i.e., each data point expressed below represents the overall mean value of 64 wells (8 wells in each of 8 replicate assays).

Statistical analyses were conducted on the raw fluorescence intensity data expressed as a percentage of the respective 100% control value. However, 100% control values were calculated differently for the individual assays. For alamar blue and calcein-AM the non-HD-treated control wells were assigned a value of 100%. This was done primarily to eliminate inter-assay variability (evident in variability in fluorescence intensity of non-HD-treated controls), which appeared to be related to day-to-day variations in the metabolic reactivity of the cultures. EthD-1 results were expressed in terms of a percentage of the saponin-killed control wells, as recommended by Molecular Probes. Multiple statistical assays were performed, as follows. Initially, two-way analysis of variance (ANOVA) was used to examine the effects of time and concentration for each procedure. With all three procedures, significant interaction was present. However, in attempting to follow up with one-way ANOVAs and multiple comparison procedures, a problem became evident. Because the data from HD-treatment groups were expressed as a percentage of the appropriate control values, the data failed initial variance and normality tests. This necessitated the use of Kruskal-Wallis ANOVA on ranks and multiple comparisons of the ranked means using Dunn's test. The results of these analyses differed markedly from inferences drawn by simple visual inspection of the data. A particular difficulty was encountered when the individual data points were compared with their respective 100% control value. Therefore, these analyses were discontinued and alternate procedures sought. The following series of analyses were adopted, results of which are presented with the data below. The EthD-1 data were transformed (100 – X) to allow direct comparisons among results obtained with the assays. Each data point was compared with its respective control value by a one-sample t-test. For comparison of the three different assay procedures and for comparison of the 24-, 48- and 96-hr time points, 3-way mixed model ANOVA was used (the 100 % controls were not considered for these analyses). Significant 3-way and 2-way interaction was evident. One-way ANOVA was then used to compare the assays at each individual time and concentration point. Also,

one-way ANOVA was used to compare time differences with each assay at each concentration point. Follow-up multiple comparisons of the 3 assay procedures or the 3 time points at each HD concentration were made using Tukey's tests. Two-way ANOVA was also used to obtain statistical evaluation of overall differences among the assays and also among the time points. However, these analyses also included an interaction term (representing time and concentration or assay and concentration, respectively). In both instances, the interactions were statistically significant. Since this indicates that relationships among assays and among the time periods vary with concentration, further testing would have only yielded results that are difficult to interpret, and these analyses were therefore halted (results of these overall effect analyses are not presented). Statistical significance in all tests was defined as $p < .05$.

## Results

Results obtained using each assay procedure are graphed separately in Fig. 1, allowing comparisons of the time-dependent effects of HD with each procedure. These same data are graphed according to time periods in Fig. 2, to facilitate comparisons among the assays at each time period examined. In addition, the means are presented in Table 1 with results of t-tests comparing each data point with its respective HD control value. Table 2 depicts results of the multiple comparison analyses, showing differences among the time points and among the assays at each HD concentration.

From Fig. 1 it is apparent that a major increase in cytotoxicity occurred between 100 and 200 μM HD and that cytotoxicity is sequentially increased with time post-exposure. Very similar response patterns were evident with alamar blue (Fig, 1, upper graph) and calcein-AM (Figure 1, middle graph). Both alamar blue and calcein-AM fluorescence decreased linearly over the 100-500 μM range at 24 hr after HD. Further declines in fluorescence were evident with both probes at 48 and again at 96 hr after HD. At both 48 and 96 hr, essentially maximal declines in alamar blue and calcein-AM fluorescence occurred with 200 μM HD, higher concentrations failing to produce more severe loss of reactivity.

EthD-1 exhibited a markedly different pattern of responses from alamar blue or calcein-AM (Fig. 1, bottom graph). This was only in part due to cell death resulting in increased EthD-1 fluorescence. The same enhancement in cytotoxicity between 100 and 200 μM HD observed with alamar blue and calcein was also evident with EthD-1. This effect was apparent with EthD-1 as early as 24 hr and was markedly enhanced at 48 hr, as indicated by differences in fluorescence between the 200 μM HD group relative to the 100 μM HD treatment group (as well as the non-HD treated controls, which are expressed in the figure as 0 time post-exposure group). At both 24 and 48 hr, 300-500 μM HD failed to produce further elevations in fluorescence. At 96 hr post-exposure, however, this elevation in fluorescence occurring with 200 μM HD (relative to 100 μM HD) was not evident. Rather, a gradual increase in measured fluorescence occurred from 100-500 μM HD. Unlike alamar blue and calcein, EthD-1 did not indicate additional cell death occurring between 48 and 96 hr (see Discussion). It is also apparent from Fig. 1 that the dynamic

range of the EthD-1 procedure, i.e., the difference in fluorescence between live (e.g., the non-HD control groups) and dead (e.g., the 48- or 96-hr 500 μM HD groups) cells is markedly smaller (from a minimum of about 20 % to a maximum of about 50 % of the saponin-killed control values) than with either alamar blue (from the 100 % control value to about 0 %) or calcein-AM (from the 100 % control value to about 10 %).

Additional information can be derived when results from the assay procedures are graphed according to the individual time periods examined (Fig. 2). Here it can be seen that alamar blue showed a more severe loss of fluorescence than calcein-AM at 24 (upper graph), 48 (middle graph) and 96 hr (lower graph) post-exposure. This manner of presentation also reveals several similarities in results obtained using the 3 fluorescence probes. For example, at 24 hr, only small differences in fluorescence are apparent among the various concentrations of HD with all three probes (indicating that relatively little cell death has occurred by 24 hr). At 48 hr after HD (middle graph), all 3 probes showed marked concentration-dependent effects, indicating greater cell death at the higher HD concentrations. This was evident as severe declines in alamar blue and calcein-AM fluorescence with 200-500 μM HD, whereas EthD-1 fluorescence was approximately doubled relative to the 100 μM HD value. At 96 hr after HD, there was virtually a complete elimination of alamar blue fluorescence at 200 μM and higher concentrations, indicating that these HD concentrations eventually kill nearly all of the exposed cells.

The data are also presented in Table 1, with results of t-tests comparing each data point (time and HD concentration) to the respective control value. The 100 % control values differed among the assays. Alamar blue and calcein-AM fluorescence data are expressed as a percentage of non-HD control values. EthD-1 fluorescence values are expressed in terms of the freshly killed saponin controls. All alamar blue and calcein-AM fluorescence values from HD treatment groups were significantly different from the respective controls. All but 3 EthD-1 values (24 hr, 100 μM HD; 96 hr, 100 μM HD; and 96 hr, 300 μM) also differed from the controls. However, the reader is cautioned that the analyses conducted (repetitive t-tests) are associated with a relatively high probability of error, increasing the chance of incorrectly concluding that individual values are significantly different from their control.

Results of multiple comparison analyses (Tukey's tests) of the individual data points are given in Table 2. The EthD-1 data were transformed (100 – X) to facilitate comparisons among the assay procedures (so that cytotoxicity produced a decline with all assays). Table 2 is divided into 2 sections. The upper section shows comparisons of the time points with each assay and HD concentration. An example is given to facilitate understanding. Results of time comparisons at 100 μM HD with alamar blue indicate that 24,48 > 96. Referring back to Table 1 for the actual fluorescence values, this is interpreted as 65.3 (the 24-hr mean) is not different from 59.6 (the 48-hr mean), but both 65.3 and 59.6 are greater than 34.9 (the 96-hr mean). The major observations to be derived from this portion of Table 2 are that, with few exceptions, 1) alamar blue and calcein-AM values are significantly reduced from 24 to 48 hr and again from 48 to 96 hr, 2) EthD-1 fluorescence values are increased from 24 to 48 hr (the 100 – X values are

significantly lower at 24 than 48 hr), and 3) EthD-1 values are significantly reduced between 48 and 96 hr (the transformed values are significantly higher at 96 than 48 hr). The lower section of Table 2 shows comparisons among the assay procedures at each time point and concentration. Considering the 24 hr, 100 μM time point as an example, C > E > A. Once again referring back to Table 1, this is interpreted as 92.6 (the calcein-AM mean) is greater than 80.9 (100 – the EthD-1 mean), and 80.9 is greater than 65.3 (the alamar blue mean). Similar comparisons can be made at all other data points. The lower section of Table 2 can be summarized as follows. With few exceptions, alamar blue values are significantly lower than corresponding calcein-AM values. In addition, calcein-AM values are generally significantly lower than the transformed EthD-1 values. These results give statistical confirmation for several observations described above, namely that alamar blue measurements are more severely depressed than calcein-AM after HD exposure and that both alamar blue and calcein-AM show more marked alterations in fluorescence than does EthD-1.

## Discussion

At the USAMRICD, flow cytometric methods have been routinely employed for the assessment of HD-induced cytotoxicity (Smith et al., 1991, 1992, 1999; Martens, 1997). However, these procedures appear to be inadequate for cytotoxicity determinations on cells that require adherence to a culture vessel for growth. Namely, detaching the cells requires relatively harsh procedures (i.e., trypsinization with calcium removal) that may cause cell damage (Guzman et al., 2000). A number of microplate procedures were therefore considered for cytotoxicity assays of adherent cells, including the alamar blue and calcein-AM assays employed herein (Guzman et al., 2000). The major conclusion of this study was that microplate analyses could be used only for the qualitative assessment of HD-induced cytotoxicity, because reaction product quantification did not yield linear concentration-dependent responses. In contrast, the current results demonstrate that both alamar blue and calcein-AM show essentially linear concentration-dependent responses at 24 hr. Linear concentration-dependent responses were not obvious at subsequent time periods because essentially maximal cytotoxicity was present with only 200 μM HD. There was no evidence of any problem whatsoever with the quantitative assessment of HD-induced cytotoxicity with either alamar blue or calcein-AM. These findings appear to directly contradict those of Guzman et al. (2000) and indicate that both procedures are suitable for the quantitative assessment of HD-induced cytotoxicity. Reasons for these discrepant findings are apparent with a closer inspection of the HD concentrations and post-exposure time periods employed in the two studies. In the Guzman et al. study, only one time-point (24 hr) and 3 HD concentrations (50, 100 and 300 μM) were used. The current study, however, was more extensive with measurements made after 100-500 μM HD and as late as 96 hr post-exposure. From the results presented herein, 100 μM HD produced relatively little cytotoxicity (Fig 2, Table 3), and higher concentrations resulted in cell death occurring primarily later than 24 hr post-exposure (Fig 1, Table 3). Thus, the absence of suitable concentration-dependent responses in the earlier study appears to be due to the HD concentrations and single assay time employed. It is noteworthy that

the delayed cell death in HD-exposed HEK reported here is not a novel finding. Substantial HEK death occurring between 48 and 72 hr after HD exposure has been observed previously (Smith et al., 1999).

As indicated above, both alamar blue and calcein-AM procedures appear to be suitable for the screening of potential antagonists of HD cytotoxicity in adherent cells. Alamar blue exhibited a more rapid and complete cytotoxic response than calcein-AM at all time periods employed (Fig 2). Alamar blue is therefore the recommended method for assay of HD cytotoxicity in adherent cells. Alamar blue also offers the additional advantage that it is non-toxic (Ahmed et al., 1994), thus enabling the repeated administration of dye for repeated assay of the same cells (Page et al., 1993). The assessment of HD cytotoxicity in adherent HEK in multi-well plates is rapid, without requirements of sophisticated or expensive instrumentation, and free of cellular damage produced by detachment procedures required for flow cytometric assays of cells that require adherence to a culture vessel for growth.

EthD-1 appears to be less acceptable for the quantitative assessment of HD cytotoxicity. EthD-1 has a smaller dynamic range than alamar blue or calcein-AM (Fig. 1). In addition, a major difference in EthD-1 results relative to those obtained using alamar blue and calcein-AM was detected. The latter two showed continuing cell death between 48 and 96 hr; this was not apparent with EthD-1 (Fig. 1). The absence of a further increase in EthD-1 fluorescence at 96 hr is most likely due to a loss in ethidium-nucleic acid binding with prolonged cell degeneration. Support for this conclusion is provided by the observation that EthD-1 fluorescence with HD never exceeded approximately 50 % of the saponin-control value. It therefore appears that assay timing may be extremely critical using EthD-1 for cytotoxicity determinations; i.e., for a period of time (several days using HEK and the conditions described herein) there is a progressive increase followed by a decline in EthD-1 reactivity. If the time-course of the cytotoxicity reaction is not carefully analyzed with each particular cell type and assay conditions, it is entirely possible that a prominent cytotoxic effect could be largely missed using EthD-1. This problem is not present with alamar blue or calcein-AM.

Difficulties associated with the use of multi-well plate cytotoxicity determinations are relatively minor and involve the use of suboptimal assay controls. This stems from an HD-induced inhibition of cell proliferation (Smith et al., 1999) resulting in a difference in cell numbers between control and HD-exposed wells. Inhibition of proliferation occurs at lower concentrations than cytotoxicity and therefore is the predominant effect at low HD concentrations (this effect was evident at the lowest HD concentration examined, i.e., 13 μM by Smith et al., 1999). How HD-induced inhibition of proliferation influences the results of the multi-well plate cytotoxicity assays can be more clearly understood by considering the following. At the time of the HD exposures, an equal number of cells are present in all wells. However, non-exposed control cells continue to proliferate, whereas those exposed to HD do not. Thus, control wells will contain more cells than HD-exposed wells at the time of assays (and this difference in cell number may be greater with increasing time post-exposure). One may envision the current fluorescence

measurements, comparing HD-treated non-proliferating cells to actively proliferating controls, as actually representing a combination of the effects of HD on proliferation and viability. However, it is apparent that the degree of confluence of the cells at the time of HD exposure will be a major determinant of the magnitude of this effect. Cell division in the non-HD controls will also be markedly curtailed as confluence is approached. The keratinocytes used in this study were approximately 95 % confluent at the time of exposures, thus tending to minimize this effect. Exactly how differences in cell number between HD-exposed and control wells have altered the results of the alamar blue, calcein-AM and EthD-1 assays used herein is given in the Appendix, for the interested reader with a strong mental constitution. The issue of unequal cell counts in control vs. HD-exposed wells has been considered for biochemical measurements taken at prolonged times post-exposure (Smith et al., 1999). Smith et al. investigated the use of cell protein contents to standardize measurements and account for differences in cell number between control and HD-exposed wells. Unfortunately, cell protein contents were altered by HD treatment, and this correction was found to be unsuitable. This author is unaware of an appropriate means to correct for this problem. Although the addition of a proliferation-inhibiting anti-metabolite to control wells at the time of HD exposures is an obvious possibility, it must be demonstrated that any potential compound is devoid of any additional effects on cellular metabolism.

It should be emphasized that results from these multi-well plate assays cannot be used to determine an actual percentage of live or dead cells. Reasons for this are as follows. HD may influence mitochondrial function, esterase activity or ethidium-nucleic acid binding (or even membrane permeability to these probes) in cells that remain alive. In addition, HD causes keratinocyte lysis at high concentrations (Moser and Meier, 1998; Moser et al., 1999). The released mitochondrial enzymes, esterases or nucleic acids may still react with alamar blue, calcein-AM or EthD-1, respectively (although this has not been demonstrated). To calculate accurate numbers of live or dead cells, it must be established that these factors do not alter the assay results. However, this was not the objective of the current study. The objective was merely to evaluate these procedures as rapid assays for screening potential antagonists of HD-induced cytotoxicity. This objective was attained, without expression of the results in terms of an exact percentage of live or dead cells.

Postscript

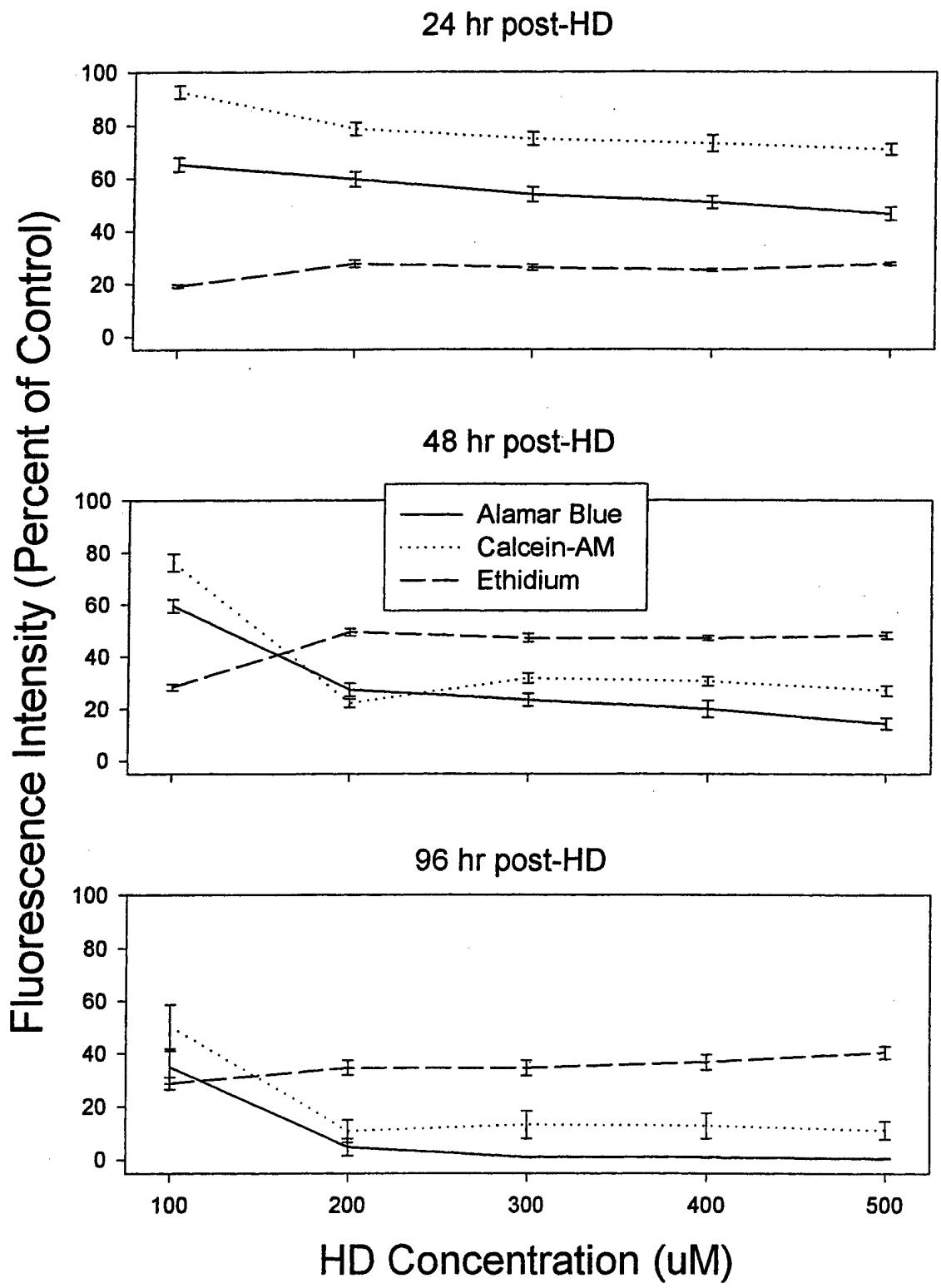
At a division review of this work on 4 April 2002, an objection was raised to the use of ethanol as a solvent for HD (by Drs. William Smith and Henry Meier). The reviewers cited a Stall or Shall working with poly (ADP-ribose) polymerase, and indicated that ethanol enhanced DNA damage produced by HD. The author has not been able to obtain the references. However, studies were conducted to determine whether the findings reported herein are obtained without the use of ethanol (data not shown). Available HEK were from an adult donor (Clonetics, #2426). There are kinetic differences between adult and neonatal derived cells, as witnessed during these supplemental studies and in previous work (unpublished). Neonatal HEK exhibit a greater cytotoxicity at 100 μM HD but greater residual cell survival at all higher HD concentrations (at 48 hr post-exposure) than those of adult origin. In addition, the adult cells respond to saponin differently from the neonatal cells. (0.1 % saponin does not kill all cells, since there is still appreciable calcein-AM accumulation in the cells. Almost all cells are killed with 0.2 % saponin, but there was a progressive loss in EthD-1 reactivity during the 90-min incubations. Finally, 0.15 % saponin was found to kill most of the cells, although some ability to hydrolyze calcein-AM was retained. This concentration was used for subsequent comparisons.) Despite these differences, many of the results obtained with ethanol as a solvent (in HEK of neonatal origin) were confirmed in the absence of ethanol (in HEK of adult origin, data not shown). Both alamar blue and calcein revealed concentration-dependent cytotoxicity that increased with time post-exposure. Alamar blue showed a more rapid and severe cytotoxic response than calcein-AM. EthD-1 also showed generally similar responses with and without ethanol as a solvent. EthD-1 reactivity was enhanced between 24 and 48 hr, and not further enhanced at 96 hr. However, the EthD-1 fluorescence of HD treatment groups was slightly higher than observed in neonatal HEK (values were generally 60-70 % of the 0.15 % saponin controls) and did not appear to be diminished from 48 to 96 hr. However, the number of analyses conducted was not sufficient to analyze statistically.

Figure Legends

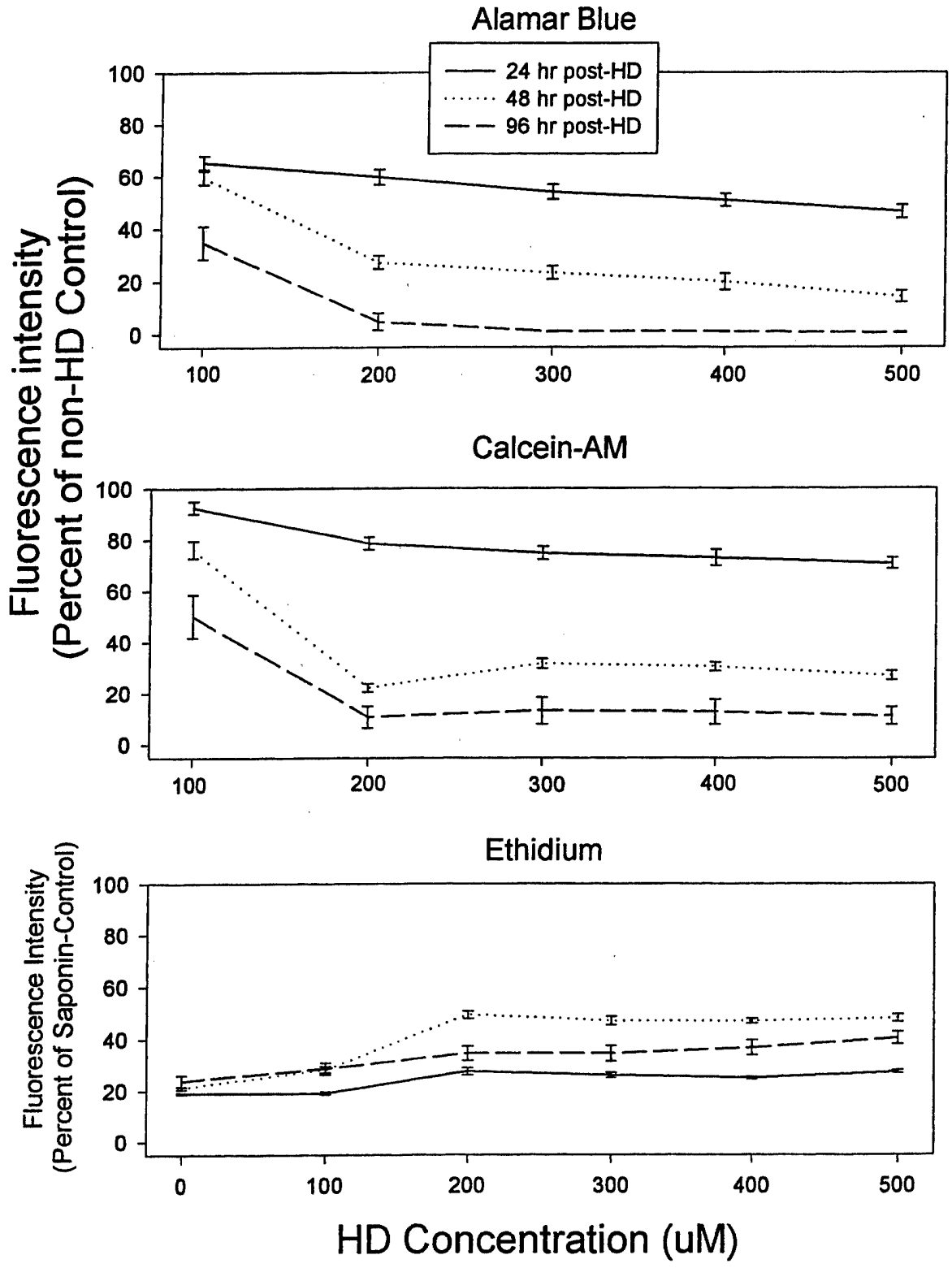
Fig. 1. Comparison of alamar blue, calcein-AM and ethidium results at individual time periods. The results are graphed by time of assay to facilitate comparisons among the three assay procedures. Methods and additional details are given in the text. Alamar blue fluorescence is more severely depressed than calcein-AM fluorescence at almost all time points. Ethidium fluorescence is enhanced in association with cytotoxicity.

Figure 2. Time-dependent cytotoxic responses evident with alamar blue, calcein-AM or ethidium. The results are graphed by assay procedures to facilitate comparisons among the time periods. Sequential reductions in both alamar blue and calcein-AM fluorescence are evident from 24 to 48 and from 48 to 96 hr. It should be noted that the error bars with 300-500 μM HD are not apparent on the 96-hr alamar blue curve, because the errors are not greater than the width of the line used to connect the data points (symbols were not used in preparation of these figures so that the error bars would not be obscured by them). Ethidium showed a large increase in fluorescence (indicating cytotoxicity) from 24 to 48 hr, but a reduction from 48 to 96 hr (see text).

# Comparison of Alamar Blue, Calcein-AM and Ethidium at Each Time Period Post-HD



# Time-Dependent Responses of Alamar Blue, Calcein-AM and Ethidium



## Table 1. Comparison of Alamar Blue, Calcein-AM and Ethidium Procedures for Assessing HD Cytotoxicity

| | 100 μM | 200 μM | 300 μM | 400 μM | 500 μM | Control |
|---|---|---|---|---|---|---|
| Alamar Blue | | | | | | |
| 24 hr | 65.3 ± 2.6* | 59.7 ± 2.9* | 53.9 ± 2.8* | 50.9 ± 2.4* | 46.5 ± 2.5* | 100 |
| 48 hr | 59.6 ± 2.6* | 27.3 ± 2.5* | 23.4 ± 2.5* | 20.0 ± 3.1* | 14.3 ± 2.2* | 100 |
| 96 hr | 34.9 ± 6.2* | 4.8 ± 3.2* | 1.0 ± 0.2* | 1.0 ± 0.2* | 0.5 ± 0.2* | 100 |
| Calcein-AM | | | | | | |
| 24 hr | 92.6 ± 2.4* | 78.6 ± 2.4* | 74.8 ± 2.5* | 73.0 ± 3.1* | 70.8 ± 2.1* | 100 |
| 48 hr | 76.2 ± 3.3* | 22.2 ± 1.6* | 31.7 ± 1.9* | 30.5 ± 1.7* | 27.0 ± 1.9* | 100 |
| 96 hr | 50.3 ± 8.4* | 10.8 ± 4.2* | 13.2 ± 5.2* | 12.8 ± 4.8* | 11.1 ± 3.4* | 100 |
| Ethidium | | | | | | |
| 24 hr | 19.1 ± 0.6 | 27.6 ± 1.4* | 26.1 ± 1.0* | 25.1 ± 0.5* | 27.5 ± 0.6* | 19.0 ± 0.3 |
| 48 hr | 28.3 ± 1.2* | 49.5 ± 1.4* | 47.2 ± 1.6* | 47.1 ± 0.9* | 48.2 ± 1.3* | 21.1 ± 0.6 |
| 96 hr | 28.7 ± 2.3 | 34.6 ± 2.8* | 34.5 ± 3.0 | 36.8 ± 2.9* | 40.5 ± 2.4* | 23.8 ± 2.3 |

Cytotoxicity data obtained using alamar blue, calcein-AM or ethidium. Fluorescence intensity values (mean ± standard error of the mean) are expressed in terms of non-HD controls (alamar blue and calcein-AM) or of saponin-killed controls (ethidium). Results of one-sample t-tests comparing each data point with the respective non-HD control value are also shown. Values denoted by (*) are significantly different from the control, $p < .05$.

Table 2. Results of Statistical Comparisons of Assay Procedures and Time Differences

Comparison of Times for each Assay and HD Concentration

| | 100 μM | 200 μM | 300 μM | 400 μM | 500 μM |
|---|---|---|---|---|---|
| Alamar Blue | 24,48>96 [a] | 24>48>96 [a] | 24>48>96 [a] | 24>48>96 [a] | 24,48>96 [a] |
| Calcein-AM | 24,48>96 [a] | 24>48>96 [b] | 24>48>96 [a] | 24>48>96 [a] | 24>48>96 [a] |
| Ethidium | 24>48,96 [a] | 24,96>48 [a] | 24>96>48 [b] | 24>96>48 [a] | 24>96>48 [b] |

Comparison of Assay Procedures at each Time Point and HD Concentration

| | 100 μM | 200 μM | 300 μM | 400 μM | 500 μM |
|---|---|---|---|---|---|
| 24 hr | C>E>A [a] | E,C>A [a] | E,C>A [a] | E,C>A [a] | C,E>A [a] |
| 48 hr | E,C>A [a] | E>A,C [a] | E>C>A [b] | E>C>A [a] | E>C>A [a] |
| 96 hr | E>A [a,c] | E>C,A [a] | E>C,A [a] | E>C>A [b] | E>C>A [b] |

A, Alamar blue. C, Calcein-Am. E, EthD-1. ( [a] ), $p<.05$. ( [b] ), $p<.01$. ( [c] ), C=E and C=A. The text provides examples to facilitate interpretation of the results.

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Appendix. Effects of unequal cell counts in HD-exposed vs. control wells on the alamar blue, calcein-AM and EthD-1 assays described herein.

As described in the Discussion section, the inhibition of proliferation by HD will result in higher cell counts in non-HD control wells relative to HD-exposed wells. This will produce artefactual changes in the results, which occur in a predictable pattern. The magnitude of this effect is unknown, but is believed to be highly dependent on the density of the cultures at the time of HD exposure. Cultures used in the current study were approximately 95 % confluent at the time of HD exposures, thus tending to minimize the significance of differences in cell counts between HD-exposed and control wells. Exactly how the results will be affected will differ for each of the three assay procedures, primarily because results of the assays were analyzed differently. How results for each assay are influenced by differences in cell number between HD-exposed and non-HD control wells is as follows. With alamar blue, the data were expressed as a percentage of the non-HD control wells. Since the non-HD control wells contain more cells than HD-exposed wells, the non-HD control fluorescence value is too high. This results in an artefactual enhancement in the measured cytotoxicity. Since HD virtually completely blocks proliferation at all concentrations employed, the difference in cell number between HD-exposed and control wells will be equivalent at all HD concentrations (with the caveat that cell lysis may be greater with increasing concentrations of HD (see Discussion). This same effect will also be evident with calcein-AM assays, but here an additional factor must be considered. As recommended by Molecular Probes, analysis of calcein-AM data involved the subtraction of fluorescence readings obtained on freshly killed (with saponin) control wells. Because these control wells contain more cells than the HD-treated wells, this results in a further inappropriate exaggeration of the severity of cytotoxicity (the subtracted saponin-killed control value will be too high). It is possible that this control subtraction may result in negative numbers when few live cells remain in exposed wells. With EthD-1, higher cell counts in both saponin-killed and in non-HD control wells relative to the HD-exposed wells will both reduce the measured cytotoxicity. Values from HD-exposed groups will be artefactually reduced (thereby reducing measured fluorescence and apparent cytotoxicity) because they are expressed in terms of a percentage from the saponin-killed control fluorescence (which is too high because proliferation has continued in these wells until the time of assay). The increase in fluorescence induced by HD will also be artefactually reduced because the HD-exposed values are compared with the non-HD control (non-HD control fluorescence will be too high since more cells are present in these wells).