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Rationale for the real-time and dynamic cell death assays using propidium iodide

Hong Zhao¹, Jadwiga Oczos², Pawel Janowski², Dominika Trembecka², Jurek Dobrucki², Zbigniew Darzynkiewicz¹, and Donald Wlodkowic^{3,4,*}

¹Brander Cancer Research Institute and Department of Pathology, New York Medical College, Valhalla, NY, USA ²Division of Cell Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland ³The Bioelectronics Research Centre, University of Glasgow, Glasgow, UK ⁴Department of Chemistry and Polymer Electronics Research Centre, University of Auckland, Auckland, New Zealand

Abstract

We have recently reported an innovative approach to use charged fluorochromes such as propidium iodide (PI) in the real-time, dynamic cell viability assays. The present study was designed to provide a mechanistic rationale for the kinetic assays using cell permeability markers. Uptake of PI by live cells, effect on the cell cycle, long term proliferation capacity, DNA damage response and pharmacologic interactions with anticancer drugs were studied using both laser scanning microscopy and laser scanning cytometry. Exposure of human carcinomic alveolar basal epithelial A549 cells in cultures to 1.5 or 7.5 μM of PI for 24 h had minimal effect on cell cycle progression including DNA replication as measured by incorporation of 5'-ethynyl-2-deoxyuridine (EdU) detected by the "click chemistry" approach and measured by laser scanning cytometry. A modest reduction, from 44% to 40% or 33%, in frequency of DNA replicating cells was seen after 48 h at 1.5 or 7.5 μM concentration of PI. There was no evidence of increased phosphorylation of histone γH2AX in cells growing in the presence of 1.5 or 7.5 μM of PI for up to 48 h. Confocal image analysis, of HeLa and NIH 3T3 mouse embryonic fibroblasts growing in the presence of PI showed granular distribution in cell cytoplasm suggesting PI accumulation in endosomes and progressive increase in fluorescence of nucleoli reflecting PI binding to nucleolar RNA. The overall responses of cells to cytotoxic agents were also not affected by the growth in the presence PI. Our data lend further support to the notion that propidium iodide can be effectively used in real-time, kinetic viability assays.

Keywords

Cell viability; cell proliferation; cell cycle; DNA damage response; gammaH2AX; DNA replication; autophagy; endocytosis

INTRODUCTION

Cell viability tests that probe plasma membrane integrity have wide application in various disciplines of biology and medicine to explore cytotoxicity of different pharmacological agents or environmental toxins. Most tests are performed in the "end-point" fashion that only reveals frequency of live versus dead cells at the time of harvesting or at the

*Corresponding author: The Bioelectronics Research Center, University of Glasgow, G12 8LT, Glasgow, United Kingdom, d.wlodkowic@elec.gla.ac.uk.

intermittent time-points of cell culture (1). Since cell death within cell populations is a stochastic process the possibility to identify and quantify individual cells at the time of their demise offers much greater sensitivity and accuracy compared with the end-point assays. We have recently proposed an innovative approach to dynamically trace cell death in real-time using fluorochromes such as propidium iodide (PI), SYTOX Green, SYTOX Red and YO-PRO 1 (1, 2).

These charged probes are generally excluded from live cells and the disruption of plasma membrane integrity leads to their intracellular penetration and strong staining of nucleic acids. Since it is generally assumed that plasma permeability markers are themselves cytotoxic, they are used only as the end-point markers for live versus dead cell discrimination. We have recently observed that growth and proliferation of several cell lines appeared to be unaffected in their presence (2). These initial results indicated that plasma membrane integrity remained preserved and no significant perturbation of the cell cycle progression was apparent in the cells growing in the presence of such probes for extended periods of time (2). As such they can be conveniently applied in the kinetic real-time viability tests with cell death rapidly detected in real-time as a distinct increase in fluorescence signal easily measured by flow cytometry or time-lapse fluorescence microscopy (2). Considering vast time savings and kinetic data acquisition such assays have the potential to be applied in a number of areas including accelerated anti-cancer drug discovery and high-throughput screening routines (2–4).

Our initial results prompted us to look in more detail on the interaction of fluorescent plasma membrane permeability markers with living cells (2). The present study was therefore designed to explore effects of PI on living cells, its intracellular distribution and/or compartmentalization, the effects on cell cycle including DNA replication and possible interaction with genomic DNA that could be detected by the DNA damage response as measured by histone H2AX phosphorylation. The overall responses of cells to cytotoxic during dynamic protocols were also evaluated as described by us previously (2). Combined with our prior reports on the subject, current data provide a rationale for the real-time cell death assays with vast applications in high-throughput pharmacological screening of anti-cancer therapeutics (2).

MATERIALS AND METHODS

Cells, cells treatment

A549 cells were purchased from American Type Culture Collection (ATCC #CCL-185, Manassas, VA). The cells were cultured in Ham's F-12K Nutrient Mixture (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (GIBCO/BRL; Grand Island, NY) in 25 ml FALCON flasks (Becton Dickinson Co., Franklin Lakes, NJ) at 37.5 °C in an atmosphere of 95 % air and 5% CO₂. The cells were maintained in exponential and asynchronous phase of growth by repeated trypsinization and reseeding prior to the culture reaching confluency. The cells were then trypsinized and seeded at low density (about 5×10⁴ cells per chamber) in 2-chambered Falcon CultureSlides (Beckton Dickinson). PI (Molecular Probes, Eugene, OR) was added into cultures 24 h after cells seeding into CultureSlides to obtain final concentration of 1.5 µM or 7.5 µM (1.0 or 5.0 µg/ml). HeLa cells and NIH 3T3 mouse embryonic fibroblasts were cultured using standard methods, and prepared for microscopy as described previously (5, 6). Briefly, cells were grown on round coverslips in Petri dishes, transferred to microincubator, covered with medium without Phenol Red, buffered for contact with air, and imaged.

Cytotoxicity and Phototoxicity assessment

Analysis of cytotoxicity and phototoxicity was performed 6, 12, 24 and 48 h after addition of 0.5 µg/ml PI (Molecular Probes) as described by us previously (1, 2). During phototoxicity experiments cells grown in the presence of propidium iodide were every hour illuminated with a 100 W Mercury Arc lamp for up to 2 s. Cells were then harvested, washed twice with PBS and counterstained with 5 nM of SYTOX Red (Molecular Probes), a spectrally distinct marker of plasma membrane permeability (excitation 640 nm, emission 658 nm). Subsequent flow cytometric analysis was performed using a BD FACSCalibur (Becton Dickinson) analyzer, equipped with a 15 mW Argon-ion and 20 mW red diode lasers. Logarithmic amplification scale using the following configuration was applied: 635 nm laser excitation line, FL4 detection channel (675 nm BP) for collection of SYTOX Red fluorescence signal. Acquisition was performed in 1024 channels resolution scale using CellQuest Pro software (Becton Dickinson). A typical run consisted of a sample with 10,000 cells.

Cell cycle analysis

Analysis of cell cycle profile (DNA content) was performed 12, 24 and 48 h after adding 0.5 µg/ml of PI. It was carried out using standard flow cytometry protocol as described by us elsewhere (1, 2). Briefly, cells were harvested, washed twice with PBS, fixed with 70% ethanol, and incubated with RNase A (Sigma Aldrich, St. Louis, MO, USA) and PI (1 µg/ml; Molecular Probes) for 1h at 37°C. The cell cycle profile was estimated on a BD FACSCalibur analyzer equipped with a 15 mW Argon-ion laser (Becton Dickinson). A linear amplification scale for the 488 nm excitation line and emission collected at 610 nm was used. Acquisition was performed in 1024 channels using CellQuest Pro software (Becton Dickinson). A typical run used a sample with 10,000 cells. Data analysis was performed using open access WinMDI ver.2.8 (<http://facs.scripps.edu/software.html>) software.

Cell proliferation

To examine long-term effects of propidium iodide treatment, cells were seeded at an initial concentration of 1×10^4 cells/ml and treated with 0.5 µg/ml of PI for up to 5 days. After every 24 hours a sample of cells was removed and a viable cell count was microscopically estimated by a standard Trypan Blue (Life Technologies, Grand Island, NY, USA) exclusion assay (1, 2).

Incorporation of 5'-ethynyl-2-deoxyuridine (EdU)

24 or 48 h after addition of PI the EdU (Invitrogen-Molecular Probes) was added into cultures at 10 µM concentration and the cells were cultured for additional 60 min. The cells were then fixed with 1% methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) in PBS for 15 min on ice, followed by treating on slides with 0.1% Triton X-100 (Sigma) in PBS for 15 min. The cells were then washed in PBS and incorporated EdU was detected with the detection solution as specified by the company (Invitrogen-Molecular Probes).

Detection of histone H2AX phosphorylation

Following incubations with PI for 24 or 48 h the cells on slides were rinsed with PBS and then fixed with 1% methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) in PBS for 15 min on ice followed by suspension in 70% ethanol where they were stored at -20° C for 2–24 h. The fixed cells were then washed twice in PBS and treated on slides with 0.1% Triton X-100 (Sigma) in PBS for 15 min, and then in a 1% (w/v) solution of bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress nonspecific antibody (Ab) binding. The cells were then incubated in a 100 µl volume of 1% BSA containing 1:200

dilution of phospho-specific (Ser 139) H2AX mouse monoclonal antibody (Ab) (Biolegend, San Diego, CA) for 1.5h at room temperature or overnight at 4°C. The cells were then incubated with secondary Alexa Fluor 488 tagged Ab (Invitrogen/Molecular Probes, at 1:100 dilution) for 40min. Prior to fluorescence measurements by laser scanning cytometry the cells were counterstained with 2.8 μ M 4,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St Louis, MO) in PBS for 15 min. Other details of cell incubations with the primary and secondary Ab were presented before (7, 8).

Measurement of cell fluorescence by LSC

Cellular green fluorescence representing either incorporation of EdU or phospho-specific γ H2AX Abs immunofluorescence (IF) and the blue emission of DAPI stained DNA was measured using an LSC (iCys; CompuCyt, Cambridge, MA) utilizing standard filter settings; fluorescence was excited with 488-nm argon, and violet (405 nm) lasers. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3,000 cells were measured per sample. The experiments were twice repeated yielding essentially identical results. Other details are given in figure legends.

Confocal microscopy

Confocal images of PI in live cells were recorded using Bio-Rad MRC1024 scanning system, equipped with 100 mW Argon ion laser (ILT, Salt Lake City, UT, USA), Nikon Diaphot (Nikon, Amsterdam, The Netherlands) inverted microscope, a PalnApo 60X NA 1.4 oil immersion lens and a micro-incubator (Life Sci. Resources, Cambridge, UK). Excitation was 488 nm, primary dichroic 510 DCLP, secondary dichroic 565 DRLP, the emission filter 585LP.

RESULTS

Observation of live A549 and HeLa cells growing in the presence of PI by fluorescence and confocal microscopy revealed the presence of red fluorescence essentially in all cells, with distinct differences in fluorescence intensity between individual cells. Fig. 1 shows the representative field of view of the HeLa cells cultured in the presence of 7.5 μ M PI for 5, 24, and 48 h with the red fluorescence predominantly localized in nucleoli. At 5 h granular fluorescence at the cell surface was observable, while the cytoplasm and the nucleus remained either unstained or showed only a very weak red fluorescence. Over time red PI fluorescence appeared in granular structures localized in the cytoplasm and as a diffused cytoplasmic background. The intensity of nucleolar fluorescence was more pronounced after 48 h of cell growth in the presence of PI compared with 24 h. At 48 h cells showed marked signs of damage with strong PI staining. Interestingly, even after such a long time of exposure to PI and despite its presence in the cytoplasm and nucleoli, many cells showed very little or no PI staining in their chromatin. Figure 1 depicts examples that represent both viable and damaged cells following PI exposure. The granular cytoplasmic localization of PI essentially similar to that seen on HeLa cells (Fig. 1) was also observed in A549 cells growing in the presence of PI (Supplementary Fig. 1).

Observation by interference contrast (Nomarski) microscopy of A549 carcinomic cells after 24 h or 48 h of culturing revealed essentially similar cell densities in cultures containing 1.5 or 7.5 μ M PI as compared with control cultures having no PI (not shown). Interestingly, cell growth and proliferation were not visibly affected by the presence of PI. These data support our earlier observations on a panel of hematopoietic and solid tumor cell lines (Table 1) (2) After 24 h of culturing the frequency of cells incorporating EdU was similar in cultures containing 1.5 or 7.5 μ M PI (44% and 42%) as in the cultures having no PI (45% and 44%) (Fig. 2). After 48 h of culturing however, a minor decline (to 40%) in the culture containing

PI at 1.5 μM concentration and more pronounced decrease (to 33%) at 7.5 μM PI were apparent (Fig. 2 E, D, F).

Most notably, cell growth in the presence of 1.5 or 7.5 μM PI for 24 or 48 h did not induce phosphorylation of histone H2AX (Fig. 3). In fact, a minor decrease in phosphorylation levels of H2AX was seen in cultures growing in the presence of 1.5 or 7.5 μM PI for 48 h (Fig. 3, E, F) compared to control (Fig. 3 A, B). The effect of PI on distribution of cells in particular phases of the cell cycle was also negligible (Fig. 4), manifesting as an increase in proportion of cells in G₁ and concomitant decrease in S phase, seen after 48 h of growth in the presence of 7.5 μM PI (Fig. 4 F).

Potential interactions with propidium iodide and agents whose cytotoxicity is being tested can introduce bias in estimating the toxic effects in dynamic real-time assays. To exclude this possibility, we have expanded our initial screening of selected cytotoxic agents on a panel of tumor cell lines including T-cell leukemia (Jurkat), promyelocytic leukemia (HL60) and invasive breast carcinoma (MDA-MB-231) (Fig 5 A, B) (2). Our results indicate that responses of cells to a various cytotoxic agents were not affected by the growth in the continuous presence of propidium iodide (0.5 $\mu\text{g}/\text{ml}$). Excellent agreement (Pearson linear correlation $R^2 = 0.9924$) between results obtained with end-point vs. dynamic protocols confirms lack of any negative interactions between the probe and the evaluated anti-cancer drugs (Fig 5 C).

DISCUSSION

The major popularity of propidium iodide (PI) in cell viability assessment stems from its propensity for being excluded from live cells. This cationic fluorochrome is considered to be impermeant to plasma membrane and therefore commonly used to detect cells with compromised plasma membrane integrity. Following cell entry PI stains DNA by virtue of binding to double-stranded nucleic acids by intercalation which leads to over 30-fold enhancement of its fluorescence (9, 10). The convenient excitation (maximum at 535 nm) and emission (maximum at 617 nm) properties of PI made it the dye of choice for cytometers equipped with a 488 nm laser excitation source. Exposure of cell populations to PI dissolved in culture media or saline identifies dead cells that either died by mode of necrosis, are at late stages of apoptosis, as well as cells with mechanically or chemically damaged plasma membrane that are unable to exclude this probe (11, 12).

The apparent exclusion of PI by live cells has recently prompted us to develop innovative protocols for real-time, dynamic quantification of cell viability (2). The rationale behind development of such assays stems from the fact that cell death within cell populations is inherently a stochastic process. Therefore the possibility to identify and track individual cells up to the point of their demise offers much greater sensitivity and accuracy compared with the end-point assays. Although it is generally assumed that plasma permeability markers are themselves cytotoxic, our preliminary study proved that cell viability, proliferation and responses to selected cytotoxic agents were unaffected by growth in the presence of PI and other cyanine probes such as SYTOX Green, SYTOX Red and YO-PRO 1 (Table 1) (2). While some increase in fluorescence of live cells growing in their presence was seen, the intensity of fluorescence was many-fold enhanced upon cell death. We therefore initially concluded that moderate increase in fluorescence of live cells indicates some intracellular uptake of PI during prolonged exposure (2). Expanding these pilot studies we have observed accumulation of red fluorescence within A549 and HeLa cells exposed in cultures to 1.5 or 7.5 μM of PI. This indicates that PI is not entirely excluded by live cells (Fig. 1 and Supplementary Fig 1). The presence of PI in the cytoplasm was manifested in the form of granules, which were heterogeneous in size (Supplementary Figure 2,

Supplementary time-lapse movie). Their shape and localization was inconsistent, however, with the pattern of cytoplasmic RNA distribution and points to the uptake by endocytosis with subsequent deposition in endocytic vesicles. As mentioned, PI in solution has very low fluorescence intensity and its intercalation into double helical nucleic acids is needed for the fluorescence enhancement. Fluorescence enhancement in endocytic vesicles probably reflects the presence of DNA and RNA fragments internalized during constitutive formation of autophagosomes (13, 14).

Confocal images demonstrated also redistribution of PI fluorescence to the diffused cytoplasmic pattern after prolonged incubation with PI (Fig 1). Exclusion of PI fluorescence from the nucleoplasm, however, indicates that DNA in nuclear chromatin is protected and inaccessible for PI to intercalate in living cells. This is in a stark contrast to strong DNA stainability seen after plasma membrane permeabilization and/or cell fixation. The torsional (topological) stress is likely the major force that prevents the unwinding of DNA, required to accommodate the intercalating dye molecules (10, 15, 16). The presence of polyamines such as spermidine, spermine or putrescine, that protect DNA from binding of intercalating agents can also contribute to the limited accessibility of DNA to PI (17, 18).

Surprisingly, despite the strong binding of PI to nucleoli and concentration in endocytic/endophagocytic vesicles, its effect on cell viability and cell cycle progression was minimal during cell culture in the presence of up to 7.5 μM). Moreover, the responses of cells to selected cytotoxic agents were not affected by the growth in the presence PI.

Taken together our current study lends further support to our previous observations of the unperturbed cell growth in the presence of PI and of the potential utility of this fluorochrome for use in real-time assays of cell viability (2). In such assays, the fluorescence markers, applied supravivally, should have a minimal effect on structure, function and survival of the cells being studied. Findings that PI neither perturbs cell growth properties nor affects DNA integrity to the point of induction of DNA damage form a rationale for dynamic labeling with such viability markers. Lack of any noticeable influence on cell responses to or interaction with cytotoxic agents during growth in the presence of PI allows for a straightforward adaptation for the real-time pharmacological profiling of anti-cancer drugs. Moreover, reduction of sample processing steps achieved with such kinetic protocol is important for the preservation of fragile apoptotic cells. We conclude that the current data provide a foundation for the development of a new generation of assays with vast applications in both flow and imaging cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Fluorescence confocal microscopy of HeLa cells incubated with PI (7.5 μM), showing the subsequent stages of PI entry into live cells and changes of the staining pattern - the initial accumulation of the dye on the plasma membrane (row A, image collected at 5h), appearance of fluorescent granules in cytoplasm (B – 24h, C – 48h), an increasing intensity of staining of nucleoli, nucleoplasm and cytoplasm (B – 24h, C, D – 48h) and cells heavily loaded with PI after 48h (D). Left panels – non-confocal transmitted light images, center panel – fluorescence of PI, right panels – PI fluorescence overlaid with transmitted light image (composite). Scale bar 10 μm , magnification was identical in A, B, C and D.

Figure 2.

Effect of PI exposure on DNA replication in A549 cells. The bivariate distributions showing cellular DNA content vs incorporation of EdU (1 h pulse of labeling) by the cells growing in the absence of PI (A- 24 h; B – 48 h) and presence of 1.5 μ M PI for 24 h (C) and 48 h (E) or 7.5 μ M PI for 24 h (D) and 48 h (F). Shown is the percent of cells incorporating EdU, above the threshold marked by the dashed-line.

Figure 3.

Phosphorylation of histone H2AX in A549 cells growing in the presence of PI. The bivariate distributions (DNA content vs expression of γ H2AX) of cells growing in the absence of PI (A, 24 h; B, 48 h) or presence of 1.5 μ M PI for 24 h (C) and 48 h (E) or 7.5 μ M PI for 24 (D) and 48 h (F). DNA content frequency histograms from the respective cultures are shown in the insets. As a positive control cells were exposed to 50 J/m² of UV B light and then cultured for 15 min (G).

Figure 4.

Distribution of A459 cells growing in the absence or presence of PI in different phases of the cell cycle. DNA content frequency histograms as shown in insets in Fig 3 were deconvoluted to estimate percent of cells in G₁, S and G₂M phases of the cell cycle. A and B, cells growing in the absence of PI for 24 and 48 h, respectively, C and E, cells growing in the presence of 1.5 and 7.5 μ M PI for 24 and 48 h respectively and D and F, cells growing in the presence of 7.5 μ M PI for 24 and 48 h, respectively. The dashed horizontal lines show the percentage level of G₁ and S-phase cells in the cultures containing no PI to better visualize minor changes seen in the cultures containing PI.

Figure 5.

Screening of cytotoxic agents is not affected by the growth in the continuous presence the PI fluorochrome (0.5 μg/ml). Invasive breast carcinoma MBA-MB-231 cells were challenged for 24 hours with increasing doses of Brefeldin A in the presence of propidium iodide (dynamic assay) (A). T-cell leukemia Jurkat cells were challenged for 24 hours with increasing doses of BH3 mimetic HA14-1 also in the presence of propidium iodide (dynamic assay) (B). Cumulative correlation analysis of results from end-point vs. dynamic assays performed on HL60, Jurkat and MBA-MB-231 cell lines challenged with varying doses of Cycloheximide (CHX), Brefeldin A (BFA) and chrome BH3 mimetic (HA14-1). Note excellent agreement between results obtained with end-point vs. dynamic no-wash

protocols that indicates no interactions between the probe and anti-cancer drugs (Pearson linear correlation $R^2 = 0.9924$). Cell viability was assessed using FACS Calibur flow cytometer equipped with 488 nm excitation line and standard optical filter configuration as described previously (2).

Table 1

Effects of continuous PI exposure (24 hours, 0.5 µg/ml) on a panel of selected cancer cell lines.

Cell lines		Influence of continuous culture in the presence of propidium iodide (PI; 0.5 µg/ml) on:				
		Cytotoxicity	Phototoxicity	Cell cycle	DNA damage	Cell proliferation
Hematopoietic	U937	-	-	-	N/A	-
	HL60	-	-	-	N/A	-
	K562	-	-	-	N/A	-
	Jurkat	-	-	-	N/A	-
	MOLT-4	-	-	-	N/A	-
Solid tumors	U2OS	-	-	-	N/A	-
	Saos2	-	-	-	N/A	-
	MDA-MB-231	-	-	-	N/A	-
	MCF-7	-	-	-	N/A	-
	HeLa	-	-	-	-	-
	A549	-	-	-	-	-