Cancer Chemother Pharmacol (2011) 68:795–803
DOI 10.1007/s00280-010-1549-9

ORIGINAL ARTICLE

The slow cell death response when screening chemotherapeutic agents

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Received: 19 July 2010 / Accepted: 6 December 2010 / Published online: 31 December 2010

Abstract
Purpose To examine the correlation between cell death and a common surrogate of death used in screening assays, we compared cell death responses to those obtained with the sulforhodamine B (SRB) cell protein–based "cytotoxicity" assay.
Method With the SRB assay, the Hill equation was used to obtain an IC50 and final cell mass, or cell mass present at infinite agent concentrations, with eight adherent cell lines and four agents (32 agent/cell combinations). Cells were treated with high agent concentrations (well above the SRB IC50) and the death response determined as the time-dependent decrease in cells failing to bind both annexin V and vital fluorochromes by flow cytometry.
Results Death kinetics were categorized as fast (5/32) (similar to the reference nonadherent Jurkat line), slow (17/32), or none (10/32), despite positive responses in the SRB assay in all cases. With slow cell death, a single exposure to a chemotherapeutic agent caused a slow, progressive increase in dead (necrotic) and dying (apoptotic) cells for at least 72 h.
Conclusions Cell death (defined by annexin and/or fluorochrome binding) did not correlate with the standard SRB "cytotoxicity" assay. With the slow cell death response, a single exposure to an agent caused a slow conversion from vital to apoptotic and necrotic cells over at least 72 h (the longest time point examined). Here, increasing the time of exposure to agent concentrations modestly above the SRB IC50 provides a method of maximizing cell kill. If tumors respond similarly, sustained low doses of chemotherapeutic agents, rather than a log-kill, maximum tolerated dose strategy may be an optimal strategy of maximizing tumor cell death.

Keywords Apoptosis · Annexin V · Chemotherapy · Cell death · Screening · Cytotoxicity

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Introduction

Although cell death is the endpoint sought in cancer therapy, surrogate endpoints of death are widely employed in cancer drug development and in analyzing the response of tumors to treatment. In vitro screening for chemotherapeutic agents often relies on biochemical metrics of cell function, including the sulforhodamine B (SRB) assay, which measures cell protein [1, 2], the MTT assay, which measures mitochondrial reductase activity [1, 3], and ATP assays, which measure cellular ATP [4, 5]. Clinically, the response to chemotherapy can be determined by imaging fluorodeoxyglucose uptake or tumor volume, or analyzed through the levels of tumor-related serum markers, though none of these methods provides direct information on tumor cell death or tumor cell survival.

Recently, however, a variety of probes have been developed that selectively bind to apoptotic cells (cells about to die) or necrotic cells (dead cells) [6–9]. Two types

of these probes, those based on annexin V [10] and those based on vital fluorochromes [11], are widely used in fluorescence-based in vitro research and can be used to image cell death in vivo by attaching radioactive or paramagnetic reporters. In addition, the use of annexin V and vital dye binding as measurements of cell death, and consequently a lack of binding as survival, is consistent with recent consensus statements on methods of determining cell death [12, 13]. Thus, the chemotherapy-induced death response might be examined in vitro using fluorescent annexin V and vital fluorochromes, and hypotheses formed about the death response evaluated using similar probes in animal tumor models or humans.

Based on these technical developments, we posed two questions: (1) How does the in vitro cell death response compare with a popular in vitro surrogate of cell death, the SRB "cytotoxicity" assay? (2) How do the rates of cell death vary when cell lines are exposed to chemotherapeutic agents?

We therefore examined the cell death response of eight adherent cell lines originating from a variety of tissues (lung, breast, prostate, etc.) to four chemotherapeutic agents with different targets (microtubules, topoisomerase I, etc.) (The selection of cell lines and agents is described below). The death response was the time-dependent decrease in the survival fraction (SF), or fraction of cells that did not bind either annexin V or a vital fluorochrome by dual wavelength flow cytometry after an exposure to an agent. As a reference, we also measured the death response of suspended Jurkat T cells studied previously [14]. Agent/adherent cell line combinations were also characterized with the SRB assay [1, 2, 15].

We found that the cell death response was highly variable and did not correlate with the uniformly positive responses obtained using a common surrogate of cell death, the decrease in cell protein measured by the SRB assay. Measurements of the cell death response were therefore essential to interpret responses to chemotherapeutic agents. In addition, we describe the slow cell death response, an intermediate between frank resistance (no cell death) and susceptibility (fast cell death). With the slow death response, maximizing the duration of exposure to a modest but effective concentration of agent (a time-kill strategy), rather than a maximizing the concentrations present for short durations (a log-kill strategy), can be an optimal strategy for causing cancer cell death.

Materials and methods

The four agents used were vinblastine (VBN), camptothecin (CPT), 5-fluorouracil (5-FU) and paclitaxel (PXL). Agents were from Sigma–Aldrich and dissolved in DMSO to obtain 10 mM stock solutions. Our eight adherent cell lines (A549, MDA-MB-231, PC3, U87 mg, HT29, MCF-7, 786-0, and HeLa) and the suspended Jurkat T-cell line were from the ATCC and cultured by their instructions. Cell lines were selected for their widespread usage and to represent a variety of tissues of origin. Allophycocyanin-labeled annexin V (Annexin-APC) and Sytox Green were from Invitrogen. Propidium iodide was from BD Pharmingen. *SRB cell mass assay:* Approximately 5,000 cells were plated in a 96-well format. Cells were incubated (48 h) with or without agents. Cell masses were determined using based on the SRB cell protein stain [16]. Cells were fixed (10% trichloroacetic acid, 4°C, 1 h), water washed, dried, stained (100 µl 0.4% SRB in 1% acetic acid, 30 min at RT), and then washed four times (1% acetic acid). Dissolved SRB (10 mM Tris, pH 10) was quantified (564 nm, BioTek ELx-800 reader). Cell mass absorbances were fit to the Hill equation (Fig. 1b) with Prism 4.0 (GraphPad Software). *Cell death response:* Cells (80K cells/24 well plate) were exposed to a single dose of agent for the indicated times. Cells were detached (200 µl of 0.05% Trypsin with 0.53 mM EDTA, 10 min @ 37°C), pelleted (400 g, 5 min) and resuspended in 300 µl of Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+}, Mg^{2+} and 1% fetal calf serum. Cells were stained (1 µl of 1 µg/ml propidium iodide, 1 µl of annexin V-APC, both Molecular Probes^{TM}) for 15 min at RT. One ml of DPBS was added, and cells were pelleted as before, resuspended in 300 µl of DPBS, and analyzed with a FACSCalibur flow cytometer. Alternatively, Sytox Green replaced propidium iodide. Dot plot quadrant statistics used FlowJo v8.2.2 software. Concentrations were 10 µM CPT, 50 nM VBN, 100 nM PXL, and 10 µM 5-FU.

Results

To study the kinetics of cell death in response to chemotherapeutic agents, we selected eight adherent cell lines from variety of organs of origin. They were 786-0 (renal cell carcinoma), HeLa (cervical carcinoma), A549 (lung carcinoma), HT-29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), MDA-MB-231 (epithelial breast carcinoma), PC-3 (prostatic adenocarcinoma bone metastasis), and U87 mg (glioblastoma astrocytoma). We then selected four agents to represent a variety of molecular targets; they were camptothecin ("CPT", noncycle-specific, topoisomerase inhibitor), paclitaxel ("PXL", cell cycle-specific, microtubule polymerizer), vinblastine ("VBN" cell cycle-specific, microtubule polymerization inhibitor), and 5-fluorouracil ("5FU", cell cycle-specific inhibitor of DNA and RNA synthesis).

Protocols for obtaining the cell death response and SRB assay allowed cells to proliferate under identical

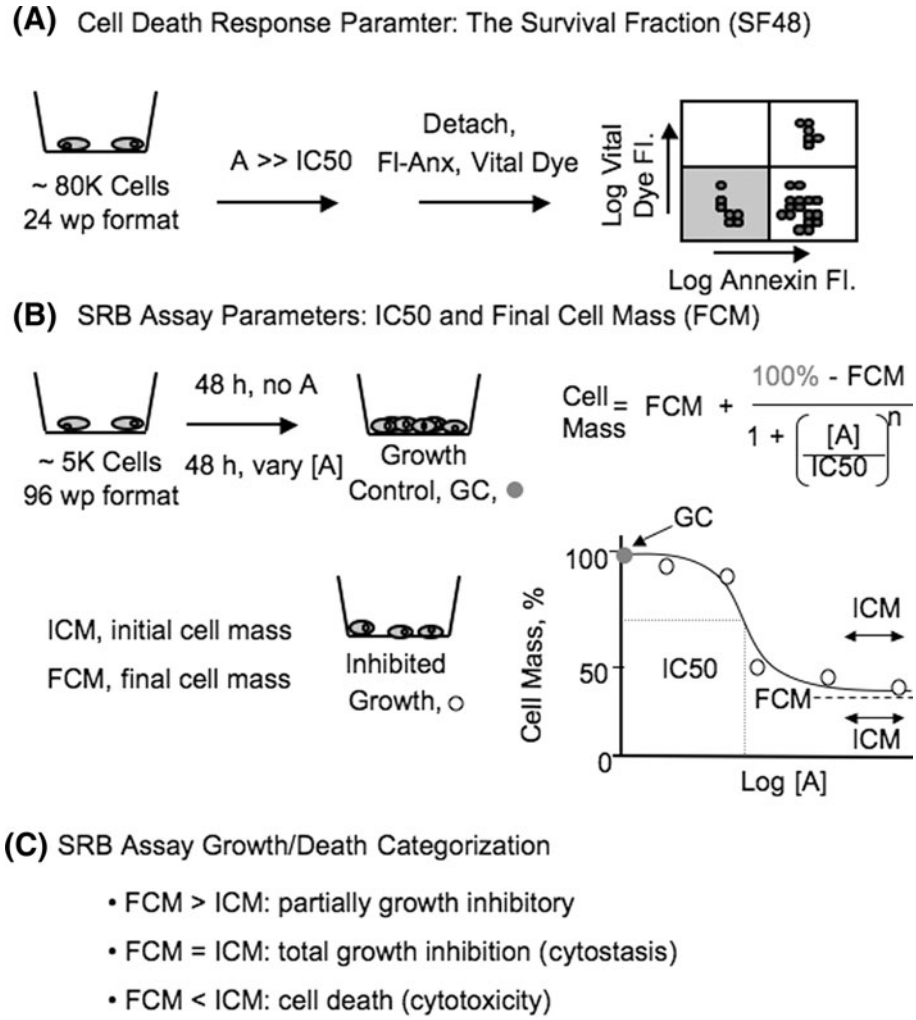

Fig. 1 The cell death response and the SRB cell mass assay. **a** To obtain the death response, cells were plated at 80,000 cells/well in a 24-well plate format and treated with agent concentrations above the IC50 as determined by the SRB assay. After 48 h, cells were detached, incubated with a fluorescent annexin and a vital dye, and analyzed by dual wavelength flow cytometry. The survival fraction at 48 h (SF48) was the fraction of cells negative for annexin and vital dye (represented by the *gray quadrant*). **b** For the SRB assay, cells were plated at 5,000 cells/well in a 96-well format and allowed to proliferate for 48 h. With no agent (No A), the growth control (GC) was the 100% value (*solid gray circle*). Increasing molar concentrations of agent [A] yielded data points (*open circles*) analyzed with the Hill equation to obtain a value of n (slope), an IC50, and a final cell mass (FCM). **c** ICM is not used in curve fitting or data reduction but can be greater than equal to or less than the FCM, yielding a categorization system cell death based on the SRB assay

conditions, though more cells were needed to measure the death response (Fig. 1).

To determine the death response (Fig. 1a), cells were exposed to a chemotherapeutic agent (A), and the binding annexin V and a vital dye were determined by dual channel flow cytometry. The survival fraction (SF) was defined as the proportion of annexin-negative and vital dye-negative cells obtained by dual wavelength flow cytometry (single gray box, lower left quadrant). The SF can be measured as a function of time (Fig. 3a) or after a standard 48-h exposure to agent (SF48, Figs. 2, 3), which is the incubation time used with the SRB assay. The survival fraction (SF) is an in vitro assay parameter that quantifies the inability of a chemotherapeutic agent to produce complete cell death, a sad but common response to chemotherapy seen clinically.

The SRB assay (Fig. 1b, c) measured the decrease in cell mass as cell protein with increasing concentrations of agent. As shown in Fig. 1b, a growth control (GC) is obtained where cells proliferate in the absence of agent. Since nonzero cell mass plateaus were seen over wide concentrations ranges above the IC50 (see Fig. 2), a four parameter Hill equation was employed to analyze data. The GC was set at maximum of 100%, so the three-parameter Hill equation now yielded an IC50, a value of n (steepness), and a final cell mass (FCM). The IC50 was the concentration of the cell mass at the curve midpoint or (GC-FCM)/2. The Hill equation also provided a final cell mass (FCM), which is a computer-generated value for the cell mass at the high concentration plateau of an agent. The quantity GC/ICM (growth control or uninhibited growth cell mass/initial cell mass) is a measure of cell growth and was used to obtain the doubling times of cell lines, see Fig. 4. When cell lines are exposed to high agent concentrations (well above the IC50), FCM/ICM value of the SRB assay can be used to categorize the response of agent/cell line combinations as reviewed in Fig. 1c. Thus, there can be partial inhibition of cell growth (FCM/ICM > 1), total growth inhibition (FCM/ICM $= 1$), or "cytotoxicity" from a reduction below the initial cell mass (FCM/ICM < 1). See for example box 1 of [15].

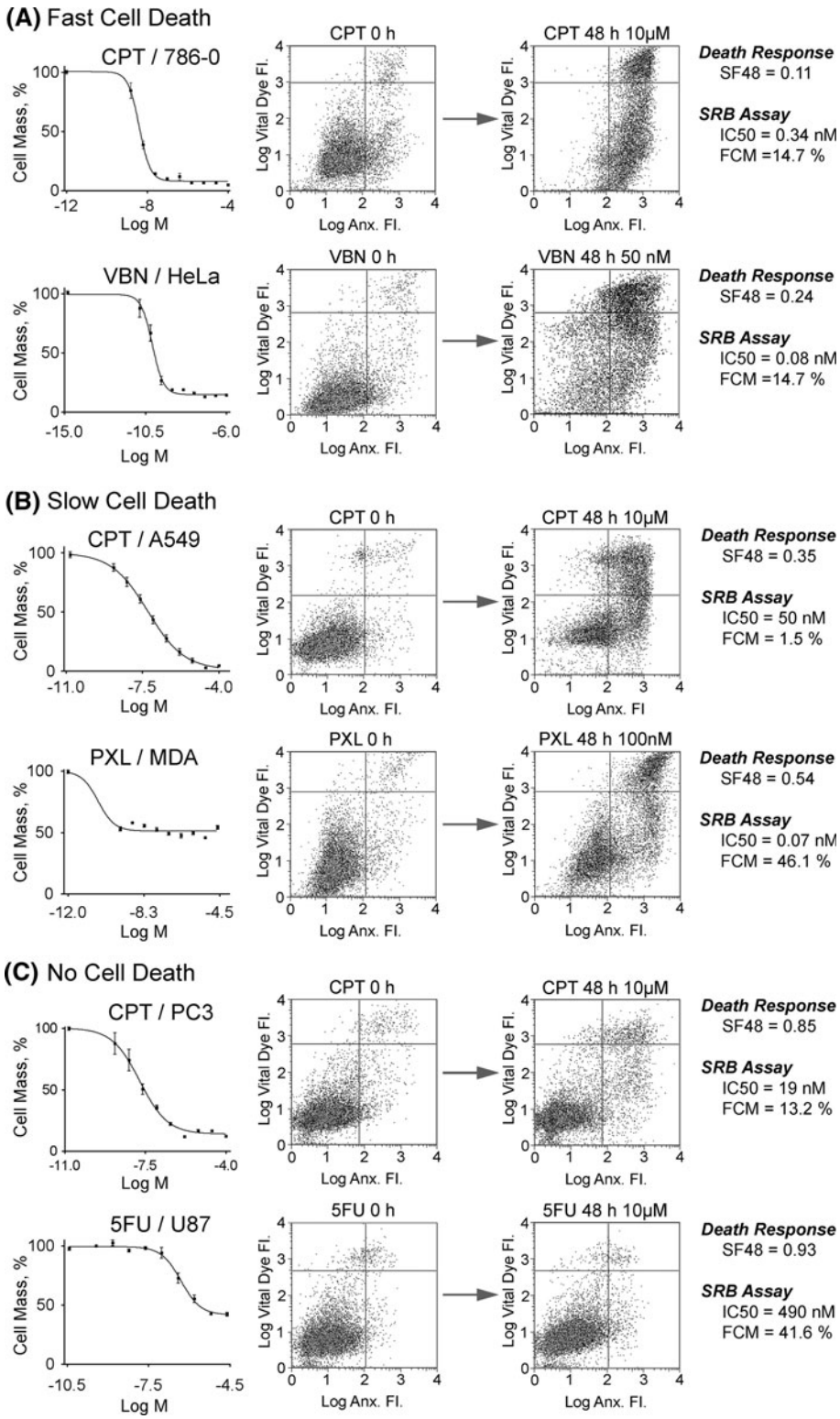

Fig. 2 Comparison of cell death responses with responses shown in SRB assays. **a** Fast cell death responses of the CPT/786-0 and VBN/HeLa combinations. From *left* to *right*: *linear/log* plots of the SRB data fit to the Hill equation, flow cytometry *dot* plots of untreated cells, *dot* plots of treated cells, and a summary of SF48's and SRB assay values. CPT and VBN caused a nearly complete conversion of viable (*lower left*) quadrant cells to annexin and/or vital dye positive populations. **b** Slow death responses with the CPT/A549 and PXL/MDA-MB231 combinations. Agents caused a partial conversion of viable cells to annexin and vital dye binding cells. **c** No death response with the CPT/PC-3 and 5-FU/U87 mg combinations. Viable cells did not convert to annexin or vital dye positive cells

A comparison of the 48 h SRB assay with the flow cytometry dot plots used to obtain SF48 values is shown for eight agent/cell line combinations in Fig. 2. From left to right are (1) the SRB data as linear/log plots fit to the Hill equation, (2) dot plots of untreated cells, (3) dot plots of cells treated for 48 h, and (4) a summary of SRB and cell death data values.

The CPT/786-0 and VBN/HeLa agent/cell line combinations that exhibited the fast death response are shown in Fig. 2a. With the SRB assay, large decreases in cell mass

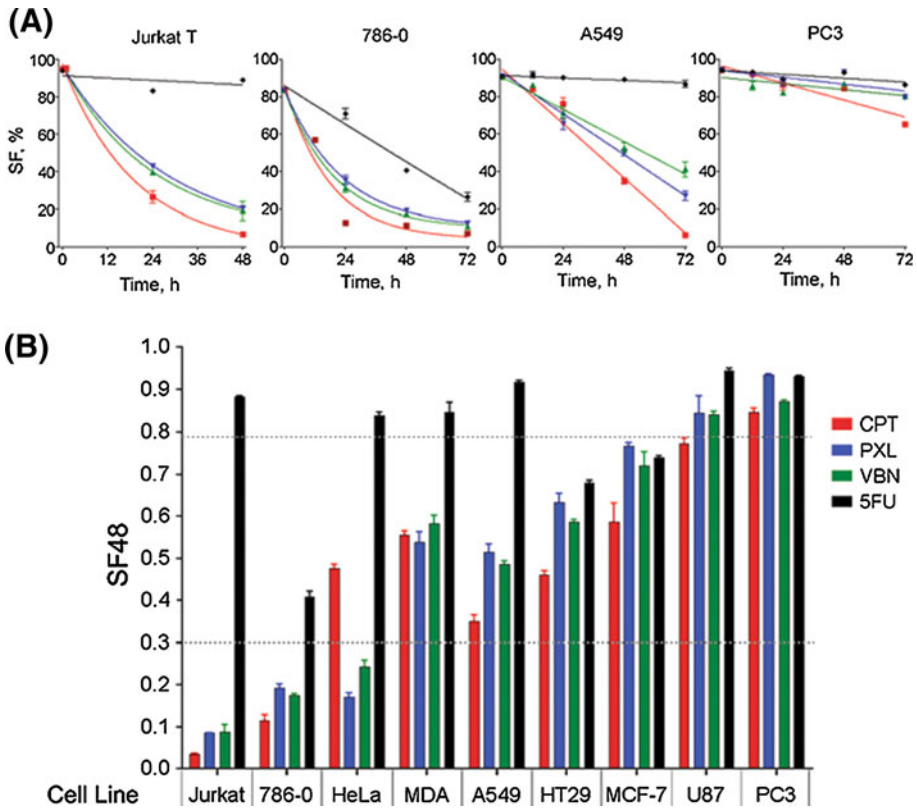

Fig. 3 Cell line-dependent and time-dependent decreases in the SF cell death response parameter. **a** The SF decrease for the suspended Jurkat and adherent 786-0, A549, and PC-3 cell lines. *Lines* were obtained by fits to a linear equation or a simple exponential decay equation. **b** SF48's as a function of cell line and agent for adherent cell lines and suspended Jurkat cells. *Dotted lines* are the cutoffs between fast and slow death responses, and between slow and no cell death responses

(>50%) and well-defined IC50's were obtained, with values given (far right) and listed in Table 1. Coefficients of correlation for the Hill analyses were at least greater than 0.89 and averaged 0.948 ± 0.0345. Because values of n were close to 1, they are not provided. An advantage of using the Hill equation is that it allows others to regenerate SRB response curves over the entire concentration range from the values provided (Table 1, FCM, IC50).

Untreated 786-0 and HeLa cells exhibited the expected preponderance of annexin-negative/vital dye-negative, viable cells (lower left quadrant) that converted to apoptotic and necrotic cells (two right quadrants) after exposure to CPT or VBN. Post-treatment SF48's of 0.11 (CPT/786-0) and 0.24 (VBN/HeLa) indicated a high degree of conversion of viable to apoptotic and/or necrotic cells.

Figure 2b shows the results for two agent/cell line combinations (CPT/A549, PXL/MDA-MB231) that gave a slow death response. Again, the SRB assays showed apparent activity, with decreases in cell mass of at least 50% and well-defined IC50's. However, SF48's of 0.35 (CPT/A549) and 0.54 (PXL/MDA-MB231) indicated only a partial conversion of viable to apoptotic and necrotic cells.

Finally, the results for agent/cell line combinations (CPT/PC3, 5-FU/U87) that exhibited the no death response are shown in Fig. 2c. Although a clear response was seen with the SRB assay, SF48's of 0.85 (CPT/PC3) and 0.93 (5-FU/U87) were obtained, indicating a lack of conversion of viable to apoptotic and/or necrotic cells. SF48's and SRB assay results from Figs. 2 and 3, along with the other adherent cell line/agent combinations studied, are summarized in Table 1. SF48's are also provided for the suspended Jurkat cell line, which were not analyzed by the SRB assay due to the risk of cell loss.

After agent treatment, vital dye-positive, annexin-negative cells (upper left quadrant of dual wavelength flow cytometry, Fig. 1a) were a minor population, as illustrated by the six dual wavelength dot plots shown in Fig. 2. (A similar observation was made in all cases, data not shown). In addition, the fraction of annexin-positive apoptotic and necrotic cells was always greater than the fraction of vital dye-positive necrotic cells (data not shown). Therefore, a cell death model where agents induced the conversion of viable to apoptotic and then to necrotic cells appeared to hold [14].

The time-dependent cell death responses of four cell lines were further examined in Fig. 3a. For Jurkats, the fast response to CPT, and lack of response to 5-FU (Fig. 3a), has been described[14]. The nonlinear decrease in SF seen with VBN, PXL and CPT and Jurkats or 786-0's placed those combinations in our fast cell death category. Linear slow cell death responses were seen with VBN, PXL, CPT and A549's or the 5-FU/786-0 combinations. The no cell death response (see criteria below) was seen with all agents

Table 1 Effects of chemotherapeutic agents by cell death responses and SRB assays

Cell line	Jurkat	786-0	HeLa	MDA-MB-231	A549	HT-29	MCF 7	U87	PC3
Cell death response @ 10 μM camptothecin (CPT)									
SF48	0.039	0.11	0.48	0.56	0.35	0.46	0.59	0.77	0.85
Cell death response	Fast	Fast	Slow	Slow	Slow	Slow	Slow	Slow	None
SRB Assay									
IC50 (molar)	*	3.40 E-10	3.14 E-08	8.98 E-07	5.02 E-08	1.44 E-07	3.97 E-07	6.70 E-08	1.89 E-08
FCM (%)		14.7	3.0	30.3	1.5	12.0	3.3	15.1	13.1
Cell death response @ 50 nM vinblastine (VBN)									
SF48	0.091	0.18	0.24	0.58	0.49	0.59	0.72	0.84	0.87
Cell death response	Fast	Fast	Fast	Slow	Slow	Slow	Slow	None	None
SRB assay									
IC50	*	2.45 E-10	7.67 E-11	6.26 E-12	3.71 E-10	8.80 E-11	3.98 E-10	1.14 E-11	1.67 E-11
FCM (%)		19.4	14.7	46.4	14.5	31.7	24.4	46.8	45.7
Cell death response @ 100 nM paclitaxel (PXL)									
SF48	0.09	0.19	0.17	0.54	0.52	0.63	0.77	0.84	0.93
Cell death response	Fast	Fast	Fast	Slow	Slow	Slow	Slow	None	None
SRB assay									
IC50	*	6.98 E-09	3.24 E-12	6.75 E-11	5.47 E-12	5.86 E-13	1.15 E-08	1.70 E-13	3.67 E-11
FCM (%)		17.6	26.8	46.1	19.5	25.1	28.2	35.9	35.4
Cell death response @ 10 μM fluorouracil (5-FU)									
SF48	0.88	0.41	0.84	0.84	0.92	0.68	0.74	0.93	0.93
Cell death response	None	Slow	None	None	None	Slow	Slow	None	None
SRB assay									
IC50	*	3.90 E-06	5.18 E-07	1.42 E-06	8.45 E-08	3.68 E-07	2.15 E-06	4.90 E-07	1.13 E-06
FCM (%)		23.6	33	49.9	34.7	42.7	14.8	41.6	36.3

ND not determined

* Nonadherent Jurkat cells were not run in SRB assay due to cell loss

and PC-3 cells. SF48's of our eight adherent cell lines and Jurkats are further compared in Fig. 3b. The mean SF48 for untreated cells was 0.95 ± 0.09 (mean $\pm$ 1SD, $n = 8$), providing a 2 SD cutoff between slow cell death and no death response at an SF48 of 0.79. A cutoff between slow and fast cell death responses was set at an SF48 of 0.30, which corresponded to a cutoff between linear and non-linear time-dependent increases in SF48's (Fig. 3a). Using these cutoffs, agent/cell line combinations were scored as fast cell death (5/32), slow cell death (17/32), or no cell death (10/32). The fast death response of adherent 786-0's and suspended Jurkats (CPT, PXL, VBN) indicated matrix attachment was not an all-determining factor in slowing the cell death response. Because cell death responses were a function of both the agent and cell line employed (Fig. 3b), we refer to responses as being a function of agent/cell line combinations. The fast death response we obtained for Jurkats and 786-0's has been seen by others, both with Jurkats and HBS2 lymphoma cells [14]. However, the fast cell death response was atypical, being seen in only 5 of 32 agent/cell line combinations.

It appeared that the nature of the cell line rather than the agent used was an important determinant of the cell death response (Fig. 3). With three agents (VBN, PXL, and CPT), the SF48 showed a similar cell line dependence: Jurkat $\sim$ 786-0 $<$ HeLa $<$ MDA-MB231 $<$ A549 $<$ HT29 $<$ MCF-7 $<$ U87 $<$ PC3. The strong cell dependence of the cell death response can be illustrated with CPT, which caused fast death (Fig. 2a, CPT/786-0), slow death (Fig. 2b, CPT/A549), or no cell death (Fig. 2c, CPT/PC-3). 5-FU was notably ineffective at inducing cell death in all cell lines except 786-0's where a slow death response was obtained.

Although cell line doubling time can be an important consideration in the response to chemotherapy, with faster

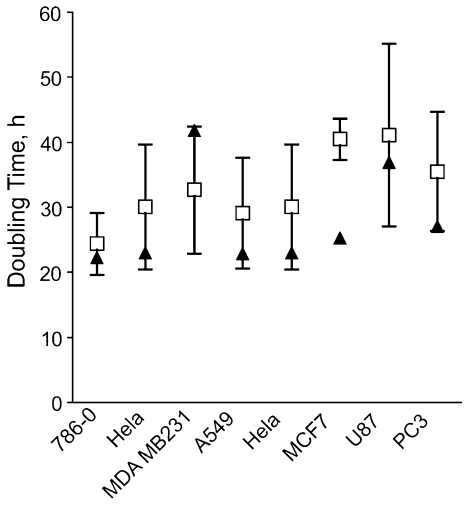

Fig. 4 Cell line doubling times. *Squares* are means and *error bars* are the *standard errors* of at least 6 values. Cell doubling times were from growth controls (Fig. 1b, GC's) and initial cell masses (ICM's) where $k = [\ln (GC/ICM)]/48$ h]. *Triangles* are values from http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html except for HeLa and U87 cell lines which were from [20] and [21], respectively

proliferating lines (shorter doubling times) being more susceptible than slowly proliferating lines to some types of agents [17]. However, our cell lines had a narrow spectrum of doubling times, ranging from as short as 22.4 ± 4.7 h for 786-0's to as long as 41.2 ± 14.0 h for U87's. Doubling times for our cell lines are given in Fig. 4 and compared with literature values.

Discussion

Unfortunately, the SRB assay is widely described as a "cytotoxicity assay" [2, 15, 18, 19] which, based on the lack annexin and vital fluorochrome binding seen with the no cell death response, can be erroneous. Beyond the terminological issue, SRB assay curves can be interpreted as defining cytotoxicity when high concentrations of an agent produce a final cell mass which is below the initial cell mass (see Fig. 1c, FCM < ICM). Our depiction of the categorization of SRB assay curves as indicating partial growth inhibition, cytostasis, or cytotoxicity is that (except for notational changes) used by the NCI for their publically available database. For a recent discussion of the NCI terminology, see Box 1 of [14]. It might be argued that a closer correspondence between the SRB and SF assays could be obtained if the incubation time of the SRB was extended beyond 48 h. However, as noted above, these assays measure different types of responses and not similar responses with different kinetics.

Some 10 of 32 agent/cell line combinations produced a no cell death response (lack of statistically significant effect on SF48 values), though there were large reductions in cell protein and well-defined IC50's with the SRB assay. In other instances, the cell death SF48 assay indicated cell death was occurring though the SRB assay indicated a noncytotoxic, cytostatic response. One of the most striking cases of this was PXL/MDA-MB-231 combination. PXL produced a high-dose plateau (FCM = 46.1%, see Fig. 2b), which is characteristic of a noncytotoxic, cytostatic response as judged by the SRB assay, see Fig. 1c. Nevertheless, the slow death response was occurring. Implications of this are discussed further below.

Thus, the lack of correspondence between the SRB assay and SF values resulted from the fundamentally different nature of the two measurements. The decrease in cell protein mass reflected in the SRB IC50 likely results from a combination of antiproliferative effects and alterations in cell biochemistry such as a decrease in the amount of protein per living cell. The SRB might be more appropriately termed as "cell toxicity," assay since cell death (i.e. "true cytotoxicity") cannot be assessed by this assay. The SF48 overcomes a significant weakness of the SRB assay and potentially other cell toxicity assays as well, by providing the frequency of survivors after cells have been exposed to high agent concentrations. In this view, SRB IC50's provide information on the concentration dependence of a cell line's response, a valuable parameter for characterizing the responses of cell lines to agents, but SF measurements are essential to see whether cell death has occurred at high agent concentrations.

When cells exhibit a slow death response, a protocol for maximizing cell kill (with a single agent) is one which maximizes the time of exposure to an effective agent concentration. The response of A549 cells to 10 μM CPT (Fig. 2b), evident with an exposure at 199 times the SRB IC50 of 50.2 nM, resulted in slow cell death evident as progressive decreases in the survival fraction (SF24 = 0.75, SF48 = 0.35, SF72 = 0.11), see Fig. 3a. Thus, at the termination of the 48-h SRB assay, some 35% of the cells remaining were survivors, but cell survival was reduced to only 11% by continuing the exposure to CPT for an additional 24 h. Another example of slow cell death was the PXL/MDA-MB-231 combination (Fig. 2b). Here, an SRB IC50 of 67 pM and a final cell mass (FCM) of 46.1% were obtained, which were typical of the types of responses to PXL seen for the cell lines we examined (Table 1). Since the value of the FCM (46.1%) was greater than the ICM (ICM = 36.7%), by the SRB assay, PXL produced only partial growth inhibition, or a decidedly noncytotoxic response. See [15]. Yet an exposure to 100 nM PXL, some 1480 times the IC50, produced a steady decrease in survival fraction (SF24 = 0.76, SF48 = 0.54, SF72 = 0.34).

We predict a high cell kill could be obtained if MDA-MB-231 cells were maintained in the presence of 100 nM PXL for a sufficient duration (e.g. greater than 72 h).

Our data summarized in Table 1 demonstrate the broad continuum of cell death kinetics for our 32 agent/cell line combinations. An expansion of Table 1 to include more cell lines and agents may permit the testing of hypotheses regarding the relationship between cell death kinetics and types of agents used (cell cycle-specific, nonspecific, alkylating agents, and antibiotics), between cell death kinetics and site of primary tumor (lung, liver, and pancreas), or between cell death kinetics and the oncogenes driving cell proliferation. The frequency of the no death response suggests that many agent/cell line combinations in the publicly available SRB database are describing the responses of cells to agents that stress them, but which are responses without cell death, see http://dtp.nci.nih.gov/docs/dtp_search.html.

There are two general ways an understanding of the cell death response might yield improved chemotherapeutic protocols. (1) With a slow tumor death response (17 of 32 agent/cell line combinations), chemotherapy protocols may fail because the fraction of surviving cells reflects slow tumor cell death kinetics and not the maximum concentration of agent achieved. However, extending the durations of the current protocols involving maximum tolerated doses by infusing still more agent would be impractical, since doses are already at the limit of toleration. As noted above, it might be possible to determine tumor cell death rates in a patient-specific manner using one of the many probes used to image apoptosis or necrosis. The recognition of slow cell death by probe accumulation would indicate optimal cell kill protocols for those individuals would be those achieving agent concentrations sufficient to drive slow cell death, e.g. 100–1,000 times an SRB IC50, for the longest possible duration. (2) Although we have employed single agents to develop methodology and describe the variability of SF values, many chemotherapeutic protocols are multi-agent in nature. Current methods of selecting combinations of agents often rely on picking agents with different dose-limiting toxicities and different mechanisms of action. Alternatively, a procedure derived from of "SF minimization" might be used to develop new combinations of agents for tumor treatment. SF minimization would involve incubating cell lines with combinations of two or more agents (at concentrations 100–1,000 times the SRB IC50 and sufficient to produce a maximal response), and selecting combinations that minimize survivors (SF values). SF minimization may yield new approaches to developing combination therapies.

Acknowledgments Financial support: NIH R01's EB004472 and EB009691 and by NIH P50CA086355.

Conflict of interest The authors declare no conflict of interest.

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