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A Reliable Tool to Determine Cell Viability in Complex 3-D Culture: The Acid Phosphatase Assay

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Cell-based assays are more complex than cell-free test systems but still reflect a highly artificial cellular environment. Incorporation of organotypic 3-dimensional (3-D) culture systems into mainstream drug development processes is increasingly discussed but severely limited by complex methodological requirements. The objective of this study was to explore a panel of standard assays to provide an easy-handling, standardized protocol for rapid routine analysis of cell survival in multicellular tumor spheroid-based antitumor drug testing. Spheroids of 2 colon carcinoma cell lines were characterized for evaluation. One of the assay systems tested could reliably be used to determine cell viability in spheroids. The authors verified that the acid phosphatase assay (APH) is applicable for single spheroids in 96-well plates, does not require spheroid dissociation, and is linear and highly sensitive for HT29 and HCT-116 spheroids up to diameters of 650 µm and 900 µm, consisting of 40,000 and 80,000 cells, respectively. Treatment of HT29 and HCT-116 cells with 5-fluorouracil, Irinotecan, and C-1311 revealed critically reduced drug efficacies in 3-D versus monolayer culture, which is discussed in light of literature data. The experimental protocol presented herein is a small but substantial contribution to the establishment of sophisticated 3-D in vitro systems in the antitumor drug screening scenario. (*Journal of Biomolecular Screening* 2007:925-937)

Key words: cell-based assay, 3-D culture, spheroid, antitumor drug testing, cell viability

INTRODUCTION

CELL-BASED ASSAYS have essentially contributed to the reduction of animal test systems in the drug discovery and screening process. However, most cell-based assays, although being more complex than cell-free biochemical test systems, still reflect a highly artificial cellular environment and thus have limited predictive value for clinical efficacy. Indeed, it is well known that many cells of normal and malignant origin lose some of their phenotypic and functional characteristics when grown in monolayer or suspension culture in vitro. The shortcomings of such assays thus lend strong support to the development and evaluation of complex, 3-dimensional (3-D) culture systems that are in principle known to better retain cellular and organotypic histomorphological features and to reflect the human tissue environment with increasing accuracy.[1-3] The application of such 3-D culture systems is increasingly discussed as to its potential to economically optimize preclinical and preanimal selection of the most active effectors from a large pool of drug candidates and to replace some animal test modules. However, 3-D assays have not yet been incorporated into mainstream drug development processes due to the more complex methodological requirements and lack of fully automated Read-Out systems.[2,4] Thus, scientists are encouraged to optimize tools for such advanced tissue-type, cell-based in vitro screening strategies.

In a recent article, we addressed the relevance and potential of 3-D in vitro systems before turning to whole-animal studies both for therapeutics development as well as in basic research, particularly in tumor biology. We focused on the screening for novel antitumor drugs and presented examples of 3-D culture models frequently applied in cancer research with emphasis on the spheroid model.[2] Indeed, multicellular tumor spheroids (MCTSs) are the classic 3-D culture system that has not only contributed essentially to the discovery of the contact effect in radiation resistance[5-7] but has progressively entered diverse areas of experimental therapeutics and considerably contributed to our knowledge of cellular response to a variety of treatment modalities.[8] The rationale for MCTS in antitumor drug testing is that they well reflect the cellular tumor micromilieu, volume growth kinetics,

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and cytoarchitecture, with cell-to-cell and cell-to-matrix interactions very similar to those of avascular tumor nodules, micrometastases, or intervascular regions of large solid tumors. Due to these characteristics, MCTSs have become a powerful tool to study therapeutic problems associated with metabolic and proliferative gradients. Accordingly, they provide a promising supplement to the use of monolayer systems, serving as a well-controlled, 3-D experimental model with intermediate complexity that supposedly better estimate in vivo antitumor effective dose ranges and treatment modalities.[2,9,10]

The particular aim of the present study was to provide an easy-handling, standardized protocol for rapid routine analysis with a technique that complies with the following requirements: 1) limited number of 3-D cultures per analysis, 2) appropriate for 3-D cultures with cell numbers ranging between 5×10^3 and 5×10^5, and 3) high sensitivity and specificity to allow for the discrimination of "normal" background in MCTSs with necrotic core. We explored the feasibility of various commercially available cytotoxicity/viability assays, some of which are routinely applied in high-throughput screening (HTS) with monolayer cultures, for application in 3-D MCTSs and found one of the assay systems to be a reliable platform for determining cytotoxic effects in single-spheroid cultures. The experimental protocol and 1st applications of this assay are particularly highlighted in the present article as it presents a small but substantial piece in the mosaic that contributes to the important establishment of advanced tissue-type in vitro systems in the antitumor drug screening scenario. The failure of other assay systems is briefly discussed.

MATERIALS AND METHODS

Cell line and spheroid culturing

Experiments were carried out with the human colon carcinoma cell lines HT29 and HCT-116 (ATCC, Manassas, VA). Cells were routinely thawed from frozen stocks and were subcultured for < 25 passages (cumulative population doublings [CPD] < 100). Dulbecco's Modified Eagle's Medium (DMEM) containing 1 g/L glucose, 1% sodium pyruvate, 1% L-glutamine, and 3.7% $NaHCO_3$, supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal calf serum (FCS), was used as "standard medium" for routine culturing (medium and all ingredients from PAN Biotech GmbH, Aidenbach, Germany). All cultures were kept in a humidified atmosphere with 5% CO_2 in air at 37 °C. Cell transfer and preparation of single-cell suspensions were performed by mild enzymatic dissociation using a 0.05% trypsin and 0.02% EDTA solution in phosphate-buffered saline (PBS; PAN Biotech GmbH). Stock cultures were passaged every 3 to 4 days by seeding 0.5 to 1×10^6 cells into T75 culture flasks. Spheroids were initiated in liquid overlay[11] by seeding 1.5×10^3 HT29 cells/well and 0.75×10^3 HCT-116 cells/well in a 200-µL medium using agarose-coated 96-well plates (50 µL 1.5% agarose/well).[11,12] After an initiation interval of 4 days, 50% of the supernatant was replaced by fresh, standard medium and every 48 h thereafter except for the 72-h drug treatment setup described in the Drug Treatment section (see below).

Cell counting and flow cytometry

Viable cell counts and cell volumes were routinely recorded with a Casy1 cell analyzer system (Schaerfe, Reutlingen, Germany) for culture quality assessment and to analyze cell growth kinetics as described earlier.[13] For the determination of viable cells in spheroids, 16 to 32 spheroids were collected and dissociated using a 0.1% trypsin and 0.04% EDTA solution in PBS, and the cell number was analyzed with the Casy1 cell analyzer system. In addition, appropriate cell aliquots were stained with the DNA intercalating dye propidium iodide (PI: 2 µg/mL per 10^6 cells), and the proportion of viable to membrane-damaged cells was analyzed on a FACSCalibur™ flow cytometer using the CellQuest/CellQuestPro software packages (BD Biosciences, San Jose, CA).

Microscopy and histology

Phase contrast imaging and morphological analyses of spheroids in 96-well microplates were carried out manually on a Zeiss Axiovert 200 microscope equipped with an AxioCam MRc camera or automated using a Zeiss Axiovert 200M equipped with an AxioCam MRm camera. Spheroid diameters and volumes were determined from images taken with a 10× objective using specific modules defined for the KS 300 and the AxioVison software (Zeiss, Jena, Germany), respectively. Spheroid histology was documented in spheroids of different size ranges following routine paraffin-embedding procedures, sectioning (5-6 µm), and H&E (hematoxylin/eosin) staining.

Acid phosphatase assay

A modified acid phosphatase (APH) assay, which is based on quantification of cytosolic acid phosphatase activity, was validated for determining cell viability in spheroids. Intracellular acid phosphatases in viable cells hydrolyze *p*-nitrophenyl phosphate to *p*-nitrophenol. Its absorption at 405 nm is directly proportional to the cell number in the range of 10^3 to 10^5 monolayer cells.[14] MCTS cultures grown in liquid overlay with 200 µL medium per well were transferred with supernatant into standard flat-bottom 96-well microplates and centrifuged for 10 min at 1500 UpM on a Heraeus Sepatech Varifuge 3.2RS to spin down spheroids, clusters, and single cells. The spheroid/cell pellet was washed by carefully replacing 160 µL of the supernatant with PBS. Centrifugation was repeated, and the supernatant was discarded to a final volume of 100 µL. Then, 100 µL of the assay buffer (0.1 M sodium acetate, 0.1% Triton-X-100, supplemented with ImmunoPure *p*-nitrophenyl phosphate; Pierce Biotech Inc., Rockford, IL) was added per well and incubated for 90 min at

37 °C. Following incubation, 10 µL of 1 N NaOH was supplemented to each well, and absorption at 405 nm was measured within 10 min on a Fusion Universal Microplate Analyzer (Packard, Meriden, CT).

Drug treatment

Treatment was performed with the following drugs: 5-fluorouracil (5-FU; Sigma-Aldrich, St. Louis, MO), Irinotecan (Toronto Research Chemicals, Ontario, Canada), and C-1311 (Symadex). The 5-(ω-aminoalkyl)-amino-8-hydroxyimidazoacridinone C-1311[15] was synthesized as described earlier[16,17] and generously provided by Marek Cholody (Avalon Pharmaceuticals Inc., Germantown, MD). Drugs (100-mM stock solutions in DMSO) were applied in the following final concentrations: 200 µM, 100 µM, 50 µM, 25 µM, 10 µM, 1 µM, and 0.1 µM in standard medium. Treatment with 10% Triton-X-100 (Sigma-Aldrich) in standard medium for 2 h at 37 °C leads to a 100% loss of cell membrane integrity in structurally intact spheroids and served as ultimate (positive) control.

For drug treatment, HT29 and HCT-116 monolayer culturing was carried out in 96-well plates (BD Biosciences/Falcon, Franklin Lakes, NJ) by plating 6×10^2 exponentially growing cells per well. Spheroids were grown in liquid overlay as detailed earlier. Both monolayer and spheroid cultures were treated 96 h after inoculation by replacing 50% (= 100 µL) of the culture supernatant with drug-supplemented fresh standard medium. The spheroid diameter at the onset of treatment was 380 to 400 µm. The routine treatment interval was 72 h. Untreated control cells and spheroids were always cultured in parallel as reference, using standard medium for refreshment that contained DMSO at the same concentration as present in the 200-µM drug dilution. Appropriate compound vehicle (negative) control experiments with 0.01% to 2% DMSO in standard medium revealed that the concentration range required for drug dilution (0.01%-0.2%) does not significantly alter cell viability/growth and assay signal.

Analysis of drug efficacy

Drug effects were recorded after 72 h of treatment via spheroid volume analysis and APH assay. All experiments were carried out at least in triplicate. APH data were corrected for background absorption at 405 nm as determined from wells containing medium alone. Drug effects were documented relative to untreated controls, taking into account the mean APH signal of $n = 8$ nontreated spheroids measured for each individual experiment. The absorption signals of medium background and nontreated spheroid controls within 1 experimental series varied by only 5% to 10%. Dose-response curves and IC_{50} values were calculated for each individual experiment via sigmoidal dose-response analysis using the Hill fitting equation in the Prism 4 software (GraphPad Software Inc., San Diego, CA). The individual dose-response curves and IC_{50} values for each drug were averaged and are documented as mean ± standard deviation (SD).

RESULTS

Characteristics of HT29 and HCT-116 colon carcinoma spheroids

Robust, single MCTSs of about 400 µm in diameter were obtained after an initiation interval of 96 h when 1.5×10^3 HT29 and 0.75×10^3 HCT-116 cells, respectively, were seeded per well. The interwell variation in spheroid diameter for each experiment was under 5%. The variation in the mean spheroid diameter at day 4 in culture for independent experiments was below 10%.

With the standard medium (classic DMEM containing 10% FCS) being refreshed every 48 h, spheroid volume increased as a function of time in culture according to the Gompertz equation, which mathematically describes tumor and also spheroid growth kinetics.[18-20] Maximum spheroid sizes of about 1 mm were reached after some 20 days for HCT-116 and 30 days for HT29 spheroids. The spheroid volume doubling was about 46 h for HT29 and 14 h for HCT-116 spheroids (**Fig. 1A**). Representative histological images of HT29 and HCT-116 spheroids of 2 size ranges are shown in **Figure 1B**. The 400-µm spheroids did not yet exhibit central secondary necroses. Cell death in the spheroid core developed at diameters of 520 to 580 µm in both spheroid types (data not shown). Central secondary necrosis is clearly visible in the representative 700- to 750-µm HT29 and HCT-116 spheroids documented in **Figure 1B**.

Viable cell count/spheroid was analyzed as a prerequisite for the evaluation of commercial cell viability/cytotoxicity assays. According to the spheroid volume, the number of viable cells per spheroid was determined following spheroid dissociation via automated counting amplified throughout spheroid culturing. For HT29 and HCT-116 spheroids, a positive linear correlation between spheroid size and viable cells/spheroid was documented (**Fig. 1C**). In parallel, the proportion of membrane-defective cells (PI positive) monitored via flow cytometry increased throughout spheroid growth, reflecting the onset and proceeding of cell death in the spheroid center at > 520 µm. However, spheroids with smaller sizes (e.g., 400 µm) contained only 6% to 9% membrane-defective cells, which were hypothesized to not critically affect selectivity or sensitivity of any viability assay. Sizes of 400 to 600 µm were chosen for a preliminary evaluation of various commercial cytotoxicity/viability assays. Among a series of assay systems that are discussed later (see Discussion section and **Fig. 6**), the quantification of cytosolic acid phosphatase activity showed the most convincing results and was thus further validated for reliable application in a spheroid drug screening assay.

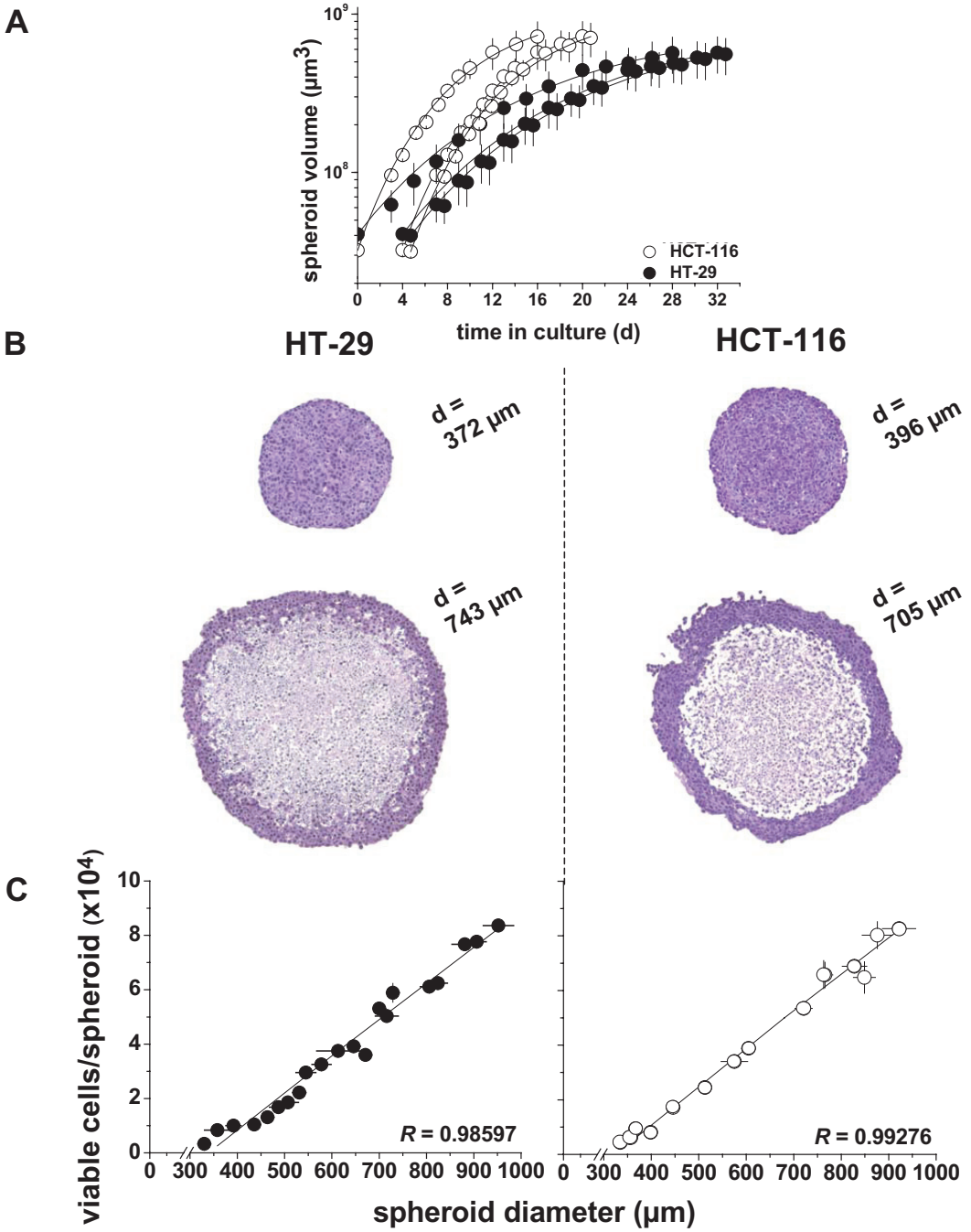

FIG. 1. Basic spheroid characteristics. (**A**) Volume growth of HT29 and HCT-116 spheroids as a function of time in liquid overlay culture with an initiation interval of 4 days and a seeding density of 1.5×10^3 HT29 and 0.75×10^3 HCT-116 colon cancer cells per well in Dulbecco's Modified Eagle's medium (DMEM) standard medium. Data points are mean spheroid volumes ± standard deviation (SD) for $8 \le n \le 16$ spheroids. Gompertz equation: $V(t) = V(0) \cdot \exp^{A/B(1-\exp^{Bt})}$ (for details, see Kunz-Schughart et al.[19] and Chignola et al.[20]). HT29: $V(0) = 4.07 \times 10^7$ µm^3; $A = 0.214$; $B = 0.069$. HCT-116: $V(0) = 3.23 \times 10^7$ µm^3; $A = 0.473$; $B = 0.133$. (**B**) Representative microscopic images of hematoxylin/eosin (H&E)–stained 5- to 6-µm paraffin median sections of HT29 and HCT-116 spheroids with and without central secondary necroses. (**C**) Number of viable cells in HT29 and HCT-116 spheroids as a function of the average spheroid diameter (± SD). Data points are mean cell numbers determined from 3 aliquots of 16 to 32 spheroids (± SD).

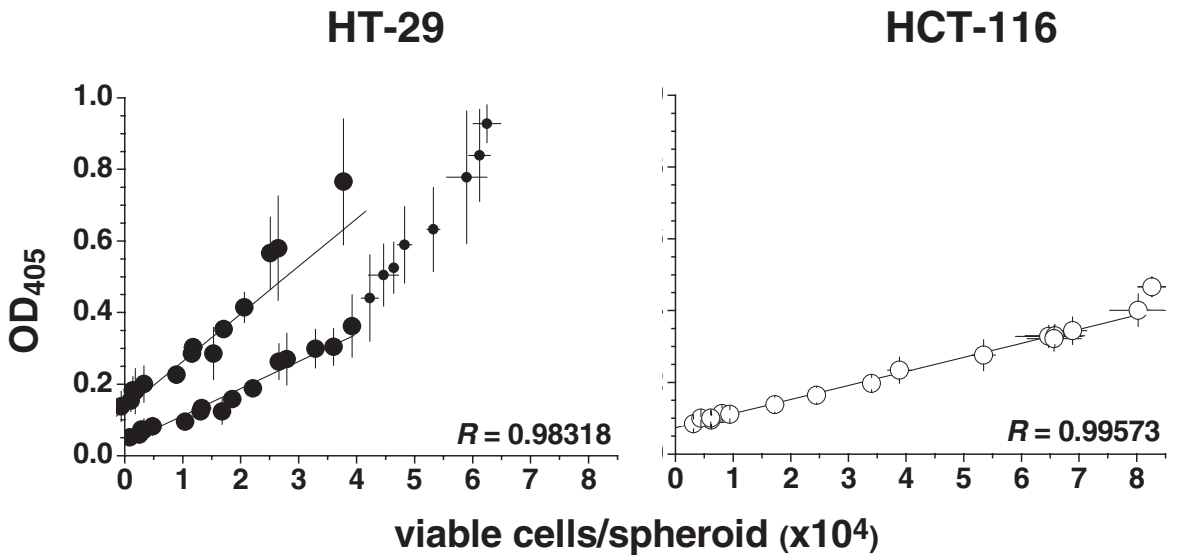

FIG. 2. Linearity of the acid phosphatase (APH) assay in HT29 and HCT-116 spheroids. Acid phosphatase colorimetric measurement in HT29 and HCT-116 spheroids (mean ± SD, $n \geq 8$) as a function of the average number of viable cells/spheroid, as determined by automated cell counting following spheroid dissociation (up to spheroid sizes of 650 µm and 900 µm, respectively); 3 aliquots of 20 to 25 spheroids were measured for each data point (± SD).

Applicability and linearity of the APH assay in spheroid cultures

Supernatants of adherent monolayer cells are in general removed prior to the addition of the APH assay buffer, and cells may even be washed once. However, with nonadherent spheroid cultures, this step is complicated and becomes even more problematic after drug treatment if the spheroid structure is partially disrupted. Here, detached cells or cell clusters may not necessarily and entirely be destroyed and should not be sucked off. Generally, the way to deal with suspension-cultured cells to avoid supernatant removal when using the APH assay is to add the reagents at higher concentrations. In a pilot study, we tried a nonremoval procedure but found the FCS to interfere with the determination of the acid phosphatase activity in our spheroid approach. To circumvent this dilemma, we developed a modified assay protocol as detailed in the Materials and Methods section.

Experiments with and without mild enzymatic dissociation of spheroids prior to performing the assay gave similar results with respect to absorption at 405 nm, indicating that preparation of single cells from spheroids is not required for the assay. The linearity of the APH assay signal in HT29 and HCT-116 spheroids as a function of the cell count/spheroid determined by automated viable cell counting is recorded in **Figure 2**. The absorption signal was linear in HT29 spheroids, with cell numbers from 8×10^2 to 4×10^4 cells/spheroid, thus covering spheroid diameters of up to 650 µm. HT29 spheroids with a size of > 650 µm containing more than 4×10^4 cells showed a steeper slope in the absorption signal at 405 nm and were thus excluded from the linear fitting in **Figure 2**. In HCT-116 spheroids, the APH signal was linear up to maximum cell numbers of 8×10^4 and diameters of about 900 µm. Supposedly, the development of secondary necroses at 520 to 580 µm in both spheroid types neither affects the specificity and sensitivity nor the linearity of the APH signal in MCTS. However, the phenomenon of enhanced APH signal in large HT29 spheroids is not yet understood.

Cytotoxicity in MCTS using the APH assay

Cell viability after drug treatment is one of the indexes of cytotoxicity. To further evaluate the applicability of the APH protocol for determining drug-induced cytotoxic/cytostatic effects in spheroids, HT29 MCTSs at day 4 in culture with a size of 380 to 400 µm, which consisted of about 0.9 to 1×10^4 viable cells without necrotic/apoptotic core and < 10% dead cells, were incubated with the well-established DNA antimetabolite 5-FU, which is current standard of care in clinical colon cancer therapy. Spheroids were analyzed after a 72-h treatment interval with 0.1 to 200 µM 5-FU for the following parameters: spheroid size and volume, APH assay signal, and cell count per spheroid following dissociation. The effect of 5-FU relative to untreated controls was calculated (in percentages), and the results of the APH assay and cell count analysis were compared (**Fig. 3**). Most important, both analyses showed a quite similar curve progression in the 5-FU concentration range applied, indicating that the APH assay indeed is a valuable and reliable tool to study drug efficacy in spheroids. However,

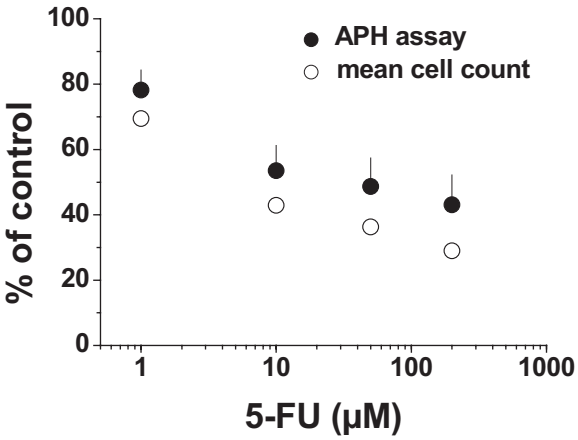

FIG. 3. Acid phosphatase (APH) activity reflects cell viability in HT29 spheroids upon treatment. Comparison of acid phosphatase activity (APH assay) and live cell counts following dissociation in HT29 spheroids after treatment with different concentrations of 5-fluorouracil (5-FU) for 72 h. Drug efficacy is documented relative to the respective untreated controls, which were averaged to define the 100% value. The APH absorption signal and cell count/spheroid in untreated controls varied by < 10% and < 3%, respectively. Data points are means ± SDs ($8 \leq n \leq 16$ for the APH assay; $n = 3$ for spheroid dissociation with 8 spheroids per sample).

the drug effect seems reproducibly higher by about 10% to 20% (in 3 independent experiments) in the automated cell count analysis of dissociated spheroids. This discrepancy ought to be discussed, taking into account that the assay-specific controls were independently set to 100%.

Drug efficacy in MCTS versus monolayer culture using the APH assay

Drug efficacy in a 3-D tissue-like cellular context supposedly differs from that in classic cellular in vitro assays. The APH assay applied in monolayer and spheroid culture is a new, robust tool to quantify and compare drug sensitivity in these cell systems and to establish or validate the potential of 3-D cultures with respect to in vivo and clinical predictability of drug efficacy. In a number of experimental series, 3 different antitumor drugs were thus compared herein as a 1st step toward routine application of the APH assay in spheroids in the process of drug development and validation. We chose the well-known compounds 5-FU and Irinotecan, in parallel to the new antitumor drug candidate C-1311 (Symadex), which has recently entered clinical trials. The drug solutions applied did not alter the background absorption. Dose-response curves were recorded in exponential HT29 as well as HCT-116 monolayer cultures and in spheroids with a size of 380 to 400 µm at the onset of treatment. Experiments were performed in a 96-well format using 0.1 to 200 µM 5-FU, Irinotecan, and C-1311.

Drug effects in HT29 cells were determined after 24-h and 72-h treatment intervals in 3 to 5 independent experiments per drug with $n = 8$ individual values per condition in each experiment. After 24 h of treatment with 200-µM drug concentrations, the acid phosphatase activity in monolayer cultures was reduced by > 60% by Irinotecan or C-1311 but only by about 30% to 40% by 5-FU (data not shown). The same concentration led to a reduction in acid phosphatase activity in spheroid cultures by only 20% to 40% for all drugs. Accordingly, IC_{50} values could not be determined in either monolayer cultures or spheroids. Therefore, data presentation herein focuses on the 72-h treatment modality for both spheroid types (**Fig. 4**).

After a 72-h treatment interval, the APH assay signal reproducibly decreased in a dose-dependent manner relative to untreated controls. The enzyme activity following treatment with 200 µM Irinotecan and C-1311, respectively, was consistently below 10% in both HT29 and HCT-116 monolayer cultures relative to control cells. Treatment with 200 µM 5-FU of HCT-116 monolayer cells resulted in comparable > 90% loss of cell viability, whereas HT29 monolayer cells in general showed a 30% survival with such high 5-FU concentrations. The IC_{50} values in HT29 monolayer cultures were 10.2 ± 2.3 µM for Irinotecan, 5.6 ± 3.1 µM for 5-FU, and 1.7 ± 1.2 µM for C-1311. HCT-116 monolayer cells showed IC_{50} values of 20.5 ± 3.9 µM for Irinotecan, 7.4 ± 2.9 µM for 5-FU, and 2.7 ± 1.3 µM for C-1311 (**Fig. 4**).

Spheroid cultures demonstrated clearly reduced sensitivities to the various chemotherapeutic agents independent of their different mode of action. For the highest drug concentrations of 200 µM, a > 50% reduction of cell viability (APH signal) was observed in HT29 spheroids only for C-1311 but not for 5-FU or Irinotecan (**Fig. 4**). The IC_{50} in spheroid cultures could thus only be estimated for C-1311 (56.7 ± 30.2 µM), which is higher by a factor of 30 to 50 than in monolayer cultures. Therefore, the efficacy of the drugs tested herein was in general lower in HT29 spheroids as opposed to monolayer cultures. In HCT-116 spheroids, about 30% of the cells survived the 72-h incubation interval with 200 µM 5-FU or Irinotecan, and about 45% were still viable after 200 µM C-1311, indicating that HCT-116 spheroid cells also show a reduced drug sensitivity as compared with the respective monolayers. The IC_{50} values were 28.7 ± 19.4 µM for 5-FU, 27.0 ± 3.7 µM for Irinotecan, and 30.6 ± 17.7 µM for C-1311. Thus, the difference in IC_{50} for HCT-116 monolayer and spheroid cells was not as pronounced as for the HT29 colorectal cancer cells but still detectable for the 5-FU and C-1311 treatment modalities.

Microscopic analyses were performed routinely prior to the APH assay to verify our hypothesis that spheroid volume following treatment is not necessarily indicative of APH signal intensity and cell viability, respectively. Representative phase contrast images of HT29 spheroids treated with either C-1311 or Irinotecan for 72 h are shown in **Figure 5**. It becomes evident that spheroid volume reduction and disintegration to some extent reflect a cell loss. However, spheroid disruption and shedding may occur

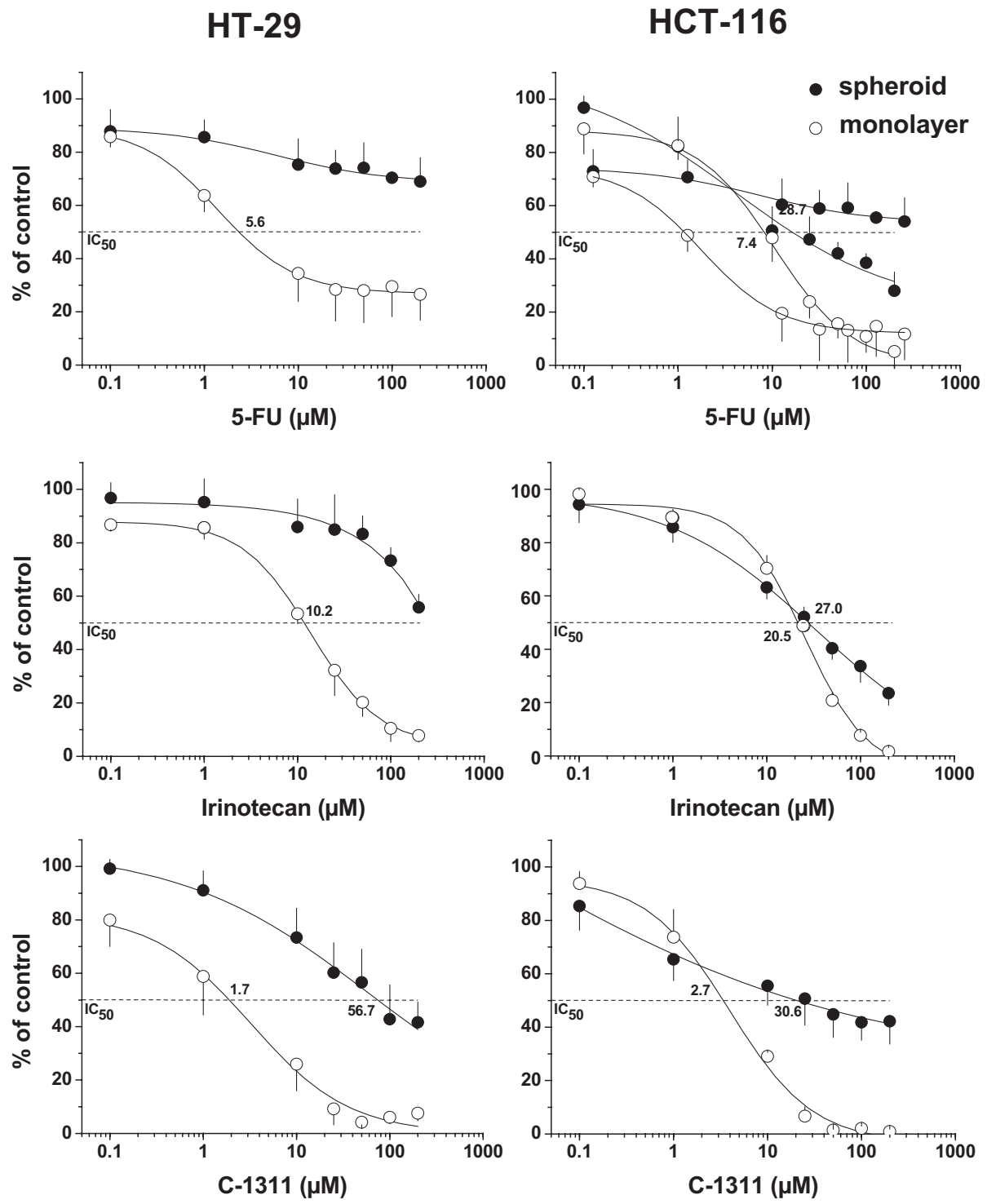

FIG. 4. Application of the acid phosphatase (APH) assay to determine drug effects in spheroid cultures. APH activity/cell viability in HT29 and HCT-116 monolayer and spheroid cultures after 72 h of treatment with 5-fluorouracil (5-FU), Irinotecan, or C-1311 (Symadex). Data points are means ± SDs for $n \geq 3$ individual experiments each with 8 spheroids treated and measured per condition. IC_{50} values were calculated to emphasize the difference of drug efficacy in 2-D versus 3-D culture and in the 2 different colorectal carcinoma cell lines.

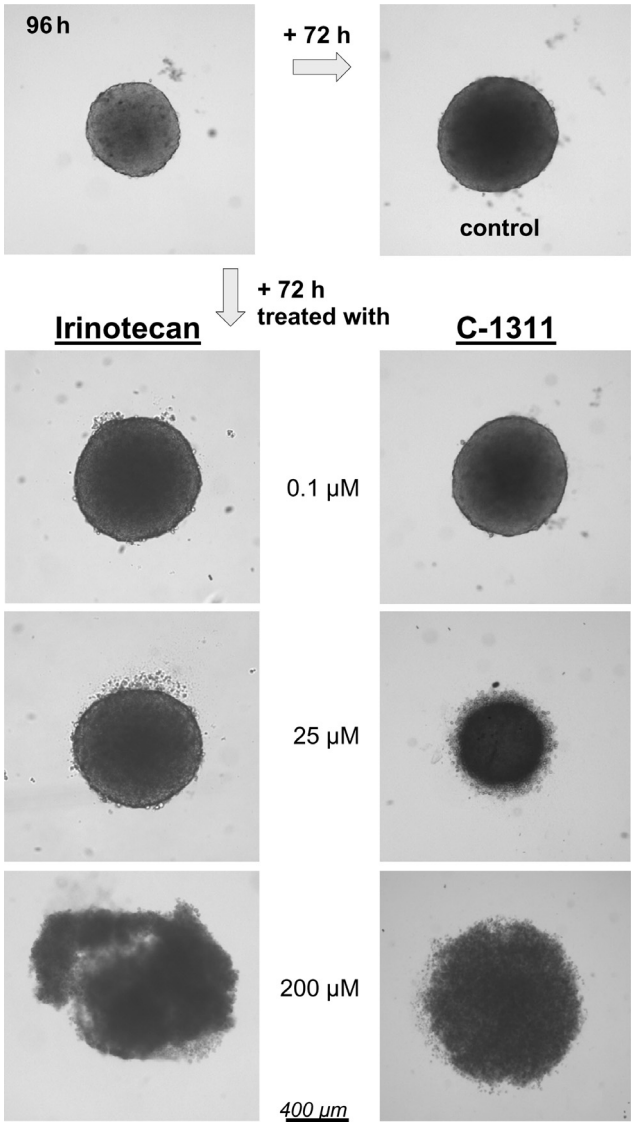

FIG. 5. Spheroid integrity following treatment with Irinotecan or C-1311 (Symadex). Representative phase contrast images of HT29 multicellular tumor spheroids (MCTSs) at the onset of treatment and after a 72-h treatment interval with 0 µM, 0.1 µM, 25 µM, and 200 µM Irinotecan or C-1311; magnification 10× objective.

(e.g., in spheroids treated with ≥ 25 µM C-1311 or 200 µM Irinotecan, but viable cells were still detected by the APH assay) (**Fig. 4**). Vice versa, structurally intact spheroids may contain large numbers of dead cells as, for example, found in the 10% Triton-X-100-treated control spheroids (data not shown).

DISCUSSION

Three-dimensional culture systems such as MCTS are known to better mimic important aspects of the in vivo tumor micromilieu and environment but can, in contrast to in vivo xenografts, easily be manipulated and exposed to specific treatment modalities. Here, we describe the essential evaluation of a cytotoxicity/viability assay for MCTS and show the potential of this application as a routine cell-based assay for HTS in 3-D cultures.

Several routinely used cytotoxicity/viability assays for monolayer cultures based on absorption, luminescence, or fluorescence were considered and tested in MCTS. Parameters determined included cellular uptake of membrane-impermeable dyes such as EtHD-1, release of substrates such as lactate dehydrogenase (LDH) or glucose-6-phosphate dehydrogenase (G6PD), activity of mitochondrial dehydrogenases or intracellular esterases, and cellular adenosine triphosphate (ATP) level and the ratio of ATP/adenosine diphosphate (ADP). All of these commercially available systems have been successfully applied in monolayer cultures in various tumor cell lines, including HT29 or HCT-116 colon cancer cells.[21-24] Unexpectedly, most of the approaches tested could not be easily adapted for application in 3-D cultures.

One set of assays, such as the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Carlsbad, CA) and the ApoGlow™ Adenylate Nucleotide Ratio Assay (Cambrex, East Rutherford, NJ), was checked for feasibility but was rapidly excluded from further validation due to handling difficulties as both assays required the initial dissociation of the spheroids. This experimental setup was too labor intensive, time consuming, and susceptible to artifacts to be considered for routine drug testing in particular because dissociation procedures and/or protocols would have to be optimized for each spheroid type.[25] Some of the observations that led to the exclusion of various other assays are documented in **Figure 6** and shall be discussed herein.

The determination of cell death via measurement of molecules released from membrane-defective cells into the supernatant has the advantage of potentially continued cell culturing. Thus, a same well or MCTS could be analyzed over a long period of time. Such assays would be preferable, but none of those under investigation were indicative for cell death processes in spheroids. One problem with this approach is the development of secondary necroses in spheroids as a function of culture time and spheroid size, respectively. This may or may not lead to an increase in background signal and may also critically depend on the spheroid feeding interval. As a prerequisite for reliable quantitation of cytotoxic effects with these technologies, the background signal should either reflect the real proportion of dead cells, including the secondary necrotic cell population in the spheroid, or should be negligible. We therefore compared the dead cell fraction calculated from the respective cytotoxicity assays with the proportion of membrane-defective, PI-stained cells in spheroids as determined by flow cytometric analysis following dissociation (**Fig. 6A**). The proportion of dead cells in HT29 spheroids with a size of 400 µm and 600 µm was about 6% to 10% and 18% to 22%, respectively. As an example, the dead cell fraction in spheroids calculated from the signals of a fluorescence-based assay to

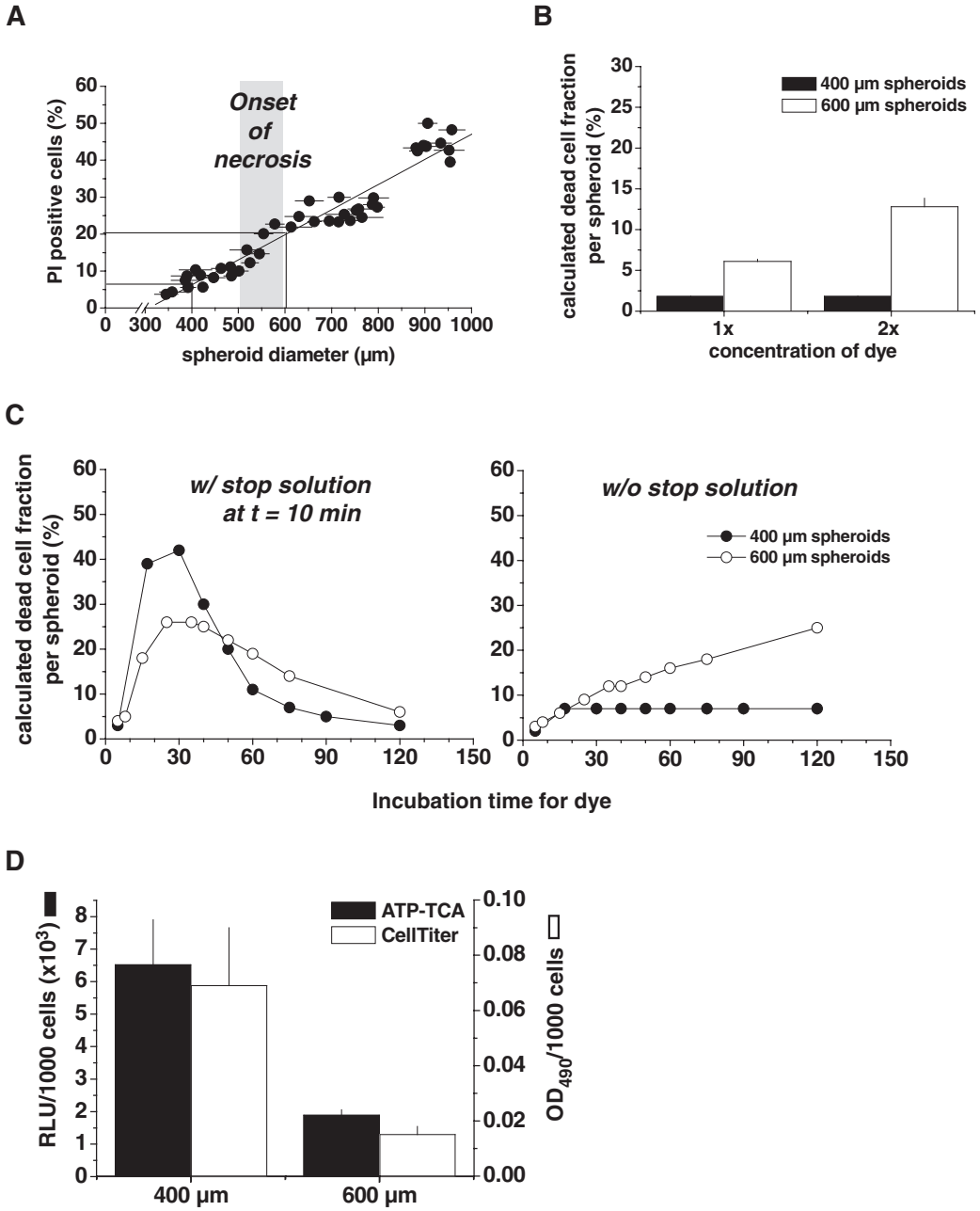

FIG. 6. Various routinely used cytotoxicity/viability assays for monolayer cultures fail in multicellular tunor spheroids (MCTSs). (**A**) Proportion of membrane-defective propidium iodide (PI)–positive cells in HT29 spheroids as a function of the average spheroid diameter (± SD) as determined by flow cytometry following dissociation of aliquots of 16 to 32 spheroids. (**B**) Estimated dead cell fraction in 400-µm and 600-µm HT29 spheroids as determined from the Vybrant® Cytotoxicity Assay (Molecular Probes) signal. Assay was performed with dye concentration given in the standard protocol (1 × x) and with a 2× dye concentration. Data points are means ± SDs for n ≥ 5 spheroids. The data neither correspond with the real proportion of membrane-defective cells in 600-µm spheroids, nor can they be subtracted as a constant (negligible) background. (**C**) Dead cell fraction in 400-µm and 600-µm HT29 spheroids estimated from the CytoTox-One™ Homogeneous Membrane Integrity Assay signals (Promega, Madison, WI). Assay was performed with (left panel) and without (right panel) addition of stop solution according to the manufacturer's instructions. Data points are means ± SDs for $n = 7$ spheroids. The signal does not stabilize as a function of time after addition of the dye. (**D**) Application of the ATP-TCA (DCS Innovative Diagnostic Systems, Hamburg, Germany) and the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (Promega) assays to determine cell viability in 400-µm and 600-µm HT29 spheroid cultures. Black bars show adenosine triphosphate (ATP) concentrations illustrated as relative light units (RLUs) per 1000 cells estimated via ATP-TCA assay; white bars show conversion of MTS to formazan, illustrated as absorption at 490 nm per 1000 cells estimated via CellTiter 96® AQ_{ueous}. Data are means ± SDs for $n = 7$ spheroids. The signal intensity per cell and per 1000 cells, respectively, critically decreases with spheroid size.

detect G6PD significantly increased with size but was consistently and critically too low as compared with the PI control values, even if the dye concentration (resazurin) was increased up to a factor of 2, as documented in **Figure 6B** (Vybrant® Cytotoxicity Assay, Molecular Probes).

Another membrane integrity assay was applied to determine LDH release but did not result in reproducible and constant signals. Here, various protocols ranging from measurement in supernatants of intact or dissociated spheroids were applied, and different lyses solutions for recording maximum signal intensities per well with all cells being membrane permeabilized were tested. The assay signals and the calculated dead cell fraction, respectively, changed as a function of incubation time with the resazurin dye, even if a solution that supposedly stops the continued generation of fluorescent product was added according to the manufacturer's instructions (CytoTox-One™ Homogeneous Membrane Integrity Assay, Promega). We therefore concluded that the red fluorescent end product resorufin does not reliably and reproducibly reflect release of LDH from membrane-defective spheroid cells, and chemical, yet undefined, side reactions during the required incubation intervals dramatically affect the assay signal. Interestingly, the fluorescence signal for 400-µm spheroids stabilized after an incubation interval with the resazurin dye of about 15 to 30 min if the stop solution was avoided. Here, the proportion of dead cells calculated from the signal indeed correlated with the PI-positive cell fraction. However, the signal continuously increased for spheroids with a diameter of about 600 µm, even after incubation with the dye of 120 min (**Fig. 6C**). The release of adenylate kinase was measured as a 3rd parameter in supernatants, supposedly correlating with membrane disintegration, but signal variations by a factor of 20 in spheroids with only 400 µm in diameter (ToxiLight® BioAssay Kit, Cambrex) indicated that this parameter is also not useful to measure cytotoxic effects in spheroid cultures.

Other assays under investigation are exclusively used to determine the number or proportion of viable cells relative to a control (e.g., untreated). Such cell viability assays include the APH assay detailed herein but also the determination of metabolically active cells via tetrazolium salt (MTS)–based systems (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) or the quantification of intracellular ATP (ATP-TCA assay, DCS Innovative Diagnostic Systems). If metabolic activity just reflects cell viability, assay signals should be independent of cell density and cell cycle distribution. However, all of the assays tested so far showed lower cellular signal intensities in confluent and spheroid cells as opposed to exponentially growing monolayer cells. This loss of sensitivity in spheroid cells is acceptable only if the signal/cell is constant throughout spheroid volume growth, a prerequisite that was exclusively found for the APH assay. In contrast, the fluorescence signals per cell resulting both from the NADH/NADPH-dependent production of formazan and from the intracellular ATP concentration were critically reduced in 600-µm as compared with 400-µm spheroids (**Fig. 6D**).

After this initial phase of assay testing, we focused on the most promising approach amenable to use in high-throughput settings for nondissociated spheroid cultures. Indeed, the APH assay frequently used for monolayer cultures[14,26-29] showed convincing sensitivity using a standardized protocol for rapid routine analysis of single spheroids. We verified that the APH assay is 1) applicable for single HT29 spheroids in 96-well plates, 2) does not require spheroid dissociation, 3) is linear and highly sensitive for HT29 spheroids up to 40,000 cells/spheroid (up to a size of 650 µm), and 4) can also be applied to other spheroid types such as HCT-116, which show APH signal linearity up to 80,000 cells/spheroid (~900 µm).

The biologically interesting phenomenon of enhanced APH signal in large HT29 spheroids is not yet understood. It remains speculation whether pathophysiological processes in this particular spheroid type, such as perpetual hypoxia and/or intensified cell-cell interactions, are causally related with this alteration in enzymatic activity. The observation that the cell viability in HT29 spheroids, determined by the acid phosphatase activity after treatment with different concentrations of 5-FU, slightly differs from the cell count analysis is an additional point of discussion. It was hypothesized that the APH signal may be overestimated in comparison to cell count analysis, an interpretation that seems to be supported by the observation of an offset of the APH signal as a function of the cell count/spheroid if the linear regression is extended to the axis of abscissa ($n = 0$) (**Fig. 2**). Such offset at $n = 0$ has also been observed in monolayer cultures but remains obscure.[14,26,29] The recording of drug efficacy relative to the respective controls both for the cell count and APH signal analyses should, however, have guaranteed the elimination of this APH assay offset from the dose-response curves. As an alternative, this discrepancy may also be attributed to some cell loss during spheroid dissociation (i.e., 5-FU-treated spheroid cells might show a higher sensitivity to trypsin/EDTA and/or other dissociation enzymes, resulting in artificial cell damage and reduced numbers of membrane-intact cells following spheroid dissociation). The APH assay may thus even more accurately reflect cellular integrity in MCTS. Despite these peculiarities, we found the APH assay in spheroids to be simple, rapid, and high-throughput compatible.

To establish a colon cancer–specific, spheroid-based screen, we evaluated the APH assay by treating HT29 and HCT-116 spheroids with drugs that are commonly used in clinical adjuvant chemotherapy regimens after surgery. The efficacy of 5-FU and Irinotecan in monolayer and spheroid culture was compared. As previously demonstrated via growth delay analyses and/or with other time-consuming or complex analytical tools (e.g., for MCF-7 breast cancer,[4,30] DLD-1 colon cancer,[30,31] and HT29 colon cancer spheroids and multilayers, respectively[32,33]), we expected a reduced sensitivity of spheroid cells to 5-FU or Irinotecan as opposed to monolayer cultures, which should be reflected not only in the spheroid integrity and size[34] but also in the APH signals and the APH-dependent IC_{50} values. This was indeed found to be the case. In our experimental series, monolayer controls

exhibited comparable dose-response curves and IC_{50} values as recorded in the literature, both for 5-FU and Irinotecan.[35-40] Drug effects in HT29 MCTS were clearly visible via the acid phosphatase activity assay, but the incubation interval necessary to reach the IC_{50} revealed that spheroids of the colorectal cancer cell line HT29 are less sensitive to 5-FU and Irinotecan than monolayers. Reduced drug efficacy is also seen in HCT-116 cells in the spheroid as opposed to monolayer culture. However, differences in the IC_{50} values are rather small (for 5-FU, factor of 4) or negligible (for Irinotecan). It also becomes evident that the near-diploid HCT-116 cells with the microsatellite-instable (MSI) phenotype are more sensitive to either 5-FU or Irinotecan in the spheroid culture than the aneuploid, chromosome-instable (CIN) HT29 spheroid cells. This cell line–dependent difference in drug sensitivity is not detected in monolayer culture.

The APH assay was then applied to also study the efficacy in spheroid versus monolayer cells of a new drug candidate that recently entered a phase II clinical trial in patients with metastatic breast and colorectal tumors (http://www.xanthus.com/products_symadex.htm). C-1311 (Symadex) belongs to the C-13xx imidazoacridinone series of compounds that showed convincing antitumor activity in vitro and in vivo in various tumor models, including HT29 colon cancer cells.[41-43] The in vitro studies, primarily performed with monolayer cultures, indicated multiple modes of action. Cell cycle perturbations and arrest in G_2M have been attributed to DNA intercalation and DNA topoismerase II inhibitor activity.[15,44-48] However, its potency as a selective FLT3 receptor tyrosine kinase inhibitor has been identified only recently.[49] The IC_{50} value for HT29 and HCT-116 monolayer cells after 72 h of treatment in our experiments was 1.7 μM and 2.7 μM, respectively, and corresponded to the literature data. However, in contrast to a previous spheroid approach showing similar dose responses in spheroid and monolayer cultures, we found significantly reduced drug efficacies in HT29 (IC_{50}: 56.7 μM) and HCT-116 (IC_{50}: 30.6 μM) spheroids using the APH assay. This discrepancy can be explained by the different cell types under investigation; that is, Skladanowski and coworkers[45] examined Chinese hamster lung fibrosarcoma (DC-3F) but not human carcinoma cells. We conclude that our colon cancer spheroid model more closely reflects the efficacy of new antitumor drugs in human epithelial tumors. With the APH assay, classic drug combinations such as 5-FU and Irinotecan, which already showed increased response rate, time to progression, and survival in patients,[50] can now be more easily monitored in spheroids and compared with monolayer data.[38,51-53] A particular prospective is the potential application of the spheroid-APH assay platform for the testing of single and combined therapeutic strategies with new, target, and/or pathway-specific treatment modalities. Further experiments to evaluate the feasibility of this approach are in progress.

In conclusion, validation of the APH assay for rapid and reproducible determination of cell viability in spheroid cultures is a prerequisite for the automated application of the MCTS model system for drug screening purposes and will essentially help to fill the gap between monolayer cultures and animal models in the process of antitumor drug development and testing.

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