Measurement of Total Cytokeratin 18 (CK18) and Apoptosis-Specific Caspase Cleaved CK18 (ccCK18), as Specific Cell Death Markers in Anticancer Drug Development

1 Introduction

Most of the pharmaceuticals that are currently used to treat cancer patients were originally discovered due to their toxicity to cells. Examples are cisplatin, discovered in experiments where electrical currents were sent though bacterial cultures [1], and cyclophosphamide, a chemical warfare agent found to induce leukopenia. These drugs are among the most effective of current clinical use.

In recent years, there has been a renewed interest from the pharmaceutical industry to use cell based screening as a tool to identify novel anticancer agents. Cell based screens can be set up in different settings to identify compounds specifically cytotoxic to tumor cells (over normal cells), to certain tumor cell types, to certain phenotypes etc. Different assay read-outs can be used in cell-based screening experiments. Viability assays such as MTT assays are commonly used. These assays have the advantage that they detect antiproliferative activity “by any cause” and also detect proliferation arrest (i.e. fewer cells in treated wells compared to untreated control). A disadvantage of viability assays is that they detect cell death by acutely toxic agents (e.g. membrane active agents) that are unlikely to have a therapeutic window.

Most of the clinically used anticancer agents induce apoptosis (programmed cell death). Apoptosis is also an attractive outcome of treatment with novel therapeutic drugs in oncology. Direct screening for compounds that induce apoptosis is therefore an attractive option.

In this paper, we compare current methods to assess cytotoxicity to tumor cells, in research, with novel assays that specifically measure total epithelial cell death and cell death by apoptosis. We propose measuring the accumulation of total keratin[1] 18 (K18) as an integrative measure for epithelial cells undergoing cell death by any cause, and caspase-cleaved K18 (ccK18) as an integrative measure for epithelial cells undergoing cell death by apoptosis. Keratin 18 is also referred to as cytokeratin 18 (CK18) oftentimes in the literature, and throughout the remainder of the article will be referred to as CK18.

CK18 is an intermediate filament required for maintenance of the cytoskeletal architecture, representing about 5% of total protein in epithelial tissues. The release of CK18 fragments into the extracellular space occurs as a consequence of proteolytic cleavage by members of the caspase family of proteases during the early and intermediate stage of apoptosis. M30 represents a proteolytic CK18 neoepitope specific for caspase cleavage and therefore serves as a highly specific marker for apoptosis [2]. M65 represents an epitope present on both intact and caspase-cleaved CK18, and therefore serves as a marker for total cell death independent of death mode (necrosis or apoptosis).

2 Current assays to identify novel anticancer agents

i. Viability assays

Viability assays are commonly used to determine the ability of cells or tissues to survive under certain conditions. These assays have the advantage that they reflect the combined outcome of cell proliferation and cell death (by any cause). A main application allows assessing the viability by cell counting and the proliferation of cells by cell culture assays. As these methods are low throughput they are less suitable for massive drug screening programs.

As an alternative, assays measuring mitochondrial activity are often used. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays are colorimetric viability assays that measure the activity of enzymes that reduce MTT or close dyes (XTT, MTS, WSTs) to formazan dyes, giving a purple color. Reduction is mediated by mitochondrial

[1] Keratin 18 (K18), also called Cytokeratin 18 (CK18); caspase-cleaved K18 = ccK18, ccCK18)

reductases, and therefore conversion is often used as a measure of viable (living) cells. Unfortunately, changes in metabolic activity can give large changes in MTT or MTS results while the number of viable cells by cell counting techniques is in fact constant. A further disadvantage of MTT and other viability assays is that they detect cell death by acutely toxic agents (e.g. membrane active agents) that are unlikely to be applicable in clinical situations. Most of the clinically used anticancer agents induce apoptosis and direct screening for compounds that induce apoptosis is therefore an attractive option.

ii. Apoptosis assays

Apoptosis is the major cell death pathway for removing unnecessary cells during embryonic development, tissue homeostasis and immune regulation. The process of apoptosis leads from initiation via internal changes to blebbing, apoptotic bodies and finally secondary necrosis of these apoptotic bodies. Most anticancer therapies rely on the activation of apoptotic pathways and various assays are available for assessing drug-induced apoptosis.

These assays monitor apoptosis related biochemical events, mainly A) caspase activation; B) membrane alterations; C) DNA fragmentation; D) release of cytochrome C from mitochondria (mitochondrial changes). Not all of these biochemical events are highly specific to apoptosis, and not all events occur in all stages of apoptosis (fast or late apoptosis). Therefore, it is critical to choose the biochemical events and corresponding assays wisely to be certain that tumor cell death occurs exclusively by this pathway. For example, if the measurement is based on the fact that a specific protein (e.g. Annexin V) cannot pass the membrane during apoptosis, this passage will occur later, when the apoptotic bodies die by secondary necrosis. Therefore, the Annexin V assay measurement is just a snapshot of the distribution between living, apoptotic and (secondary) necrotic cells/bodies. Chosen a late timepoint, all apoptotic cells will have passed on and are stained during secondary necrosis. A measurement at a single late time point will show no difference between cells that die by acute or secondary necrosis.

Like Annexin V, many methods cannot be regarded as integrative measures of apoptosis; they reflect the ongoing apoptotic process at a specific timepoint. Given fast apoptosis induction, the measurement at a single specific timepoint is a snapshot and will return a misleading value with these assays. Multiple measurements (thus establishing the kinetics) are therefore required. In contrast, when a test measures a stable apoptosis-specific product, measurements can be done hours to days after induction of apoptosis, and independent of the kinetics.

Finally, many of these research assays use low throughput and/or qualitative (QL) methods such as western blotting, microscopy or flow cytometry. Low throughput, QL methods, kinetic measurements and doubts about apoptosis specificity are all highly undesirable characteristics for anticancer drug screening.

We here present an alternative to above apoptosis assays that is based on the quantification of a product of caspase activation, a cornerstone of the apoptotic process. This method measures the caspase cleaved CK18 (ccCK18), which only is present if the cell has undergone the apoptotic process of internal protein cleavage (see also 3.2), independent of secondary necrotis. Moreover, the presence of ccCK18 is independent of the course and duration of the apoptosis process.

The Table below summarizes the characteristics for commonly used assays for apoptosis. Measuring ccCK18 in the drug development process meets all critical criteria: the results are apoptosis-specific, integrative, quantitative, and the ELISA method is high throughput and suitable for automation.

Assay	Method	Measures	Specificity	High/Low Throughput	QT/ QL[1]	Integrative Measure[2]
Caspase Activation						
ccCK18	ELISA	M30 neoepitope of caspase-cleaved CK18 (ccCK18)	Highly specific for epithelial apoptosis, only cleaved fragment is detected	H	QT	Yes
CK18	ELISA	Total and caspase-cleaved CK18	Total epithelial cell death (necrosis + apoptosis)	H	QT	Yes
Antibodies against caspase cleaved substrates	Western blot, Immuno-precipitation, immune-histology	PARP-1 intact (113 kDa) & caspase cleaved fragment (89 kDa)	Specific for apoptosis, but subjective as non cleaved PARP is also detected	L	QL	No
	Immuno-fluorescence,	Caspase-cleaved CK18 (using M30 CytoDEATH™	Highly specific for epithelial apoptosis,	L	sQT	Yes

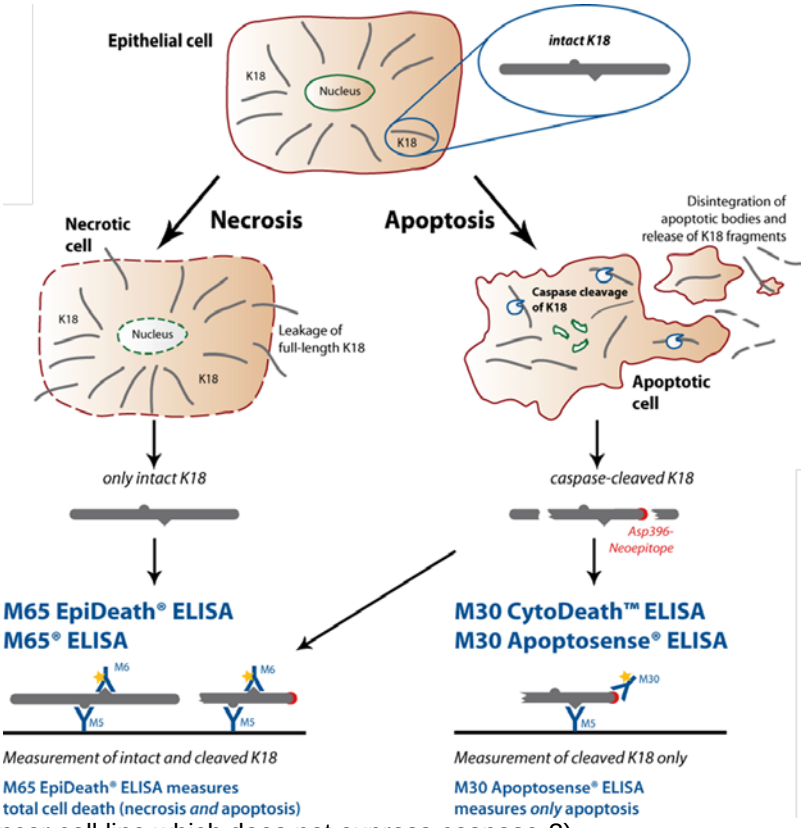
	flow cytometry, fluorescence/ light microscopy	monoclonal antibody)	only cleaved fragment is detected			
Activation of caspases	Immunoblot IHC Immuno-fluorescence	Cleaved (and thus activated) pro-caspases	Specific for apoptosis	L	QT	No
Caspase activity	Caspase enzyme assay	Quantitative detection of caspase activity in cellular lysates	Apoptosis specific	H	QT	No
Membrane alteration						
Annexin V	Flow cytometry, Fluorescence or light microscopy	Translocation of PS to the outer cell membrane. Double staining with membrane- impermeable DNA dyes such as PI. Healthy cells are doubly negative to annexin V and PI, whereas cells in the early pha- ses of apoptosis are annexin-V-positive but PI-negative, and necrotic + secondary necrotic cells are double positive.	Apoptosis specific, but interpretation of results is subjective	L	sQT	No
LDH release	ELISA	Quantitation of LDH activity released from damaged/dying cells	Not specific for apoptosis	H	QT	Yes
DNA fragmentation						
DNA ladder	Gel electro-phoresis	Apoptotic cells show a characteristic DNA ladder due to internucleosomal DNA degradation (multiples of 180 base pairs in size), while necrotic cells reveal a smear of randomly degraded DNA	180 bp fragments are specific for apoptosis	L	QL	Yes
Hypodiploid DNA	Flow cytometry	Hypodiploid nuclei from apoptotic cells	Not fully specific for apoptosis	L	QL	No
Cytoplasmic nucleosomes	ELISA (Cell Death Detection plus)	Determination of nucleosomes in the cytoplasmic fraction of cell lysates. Not specific for the 180 bp apoptotic DNA fragments.	Not specific for apoptosis, Nucleosome- like DNA fragments can also result from other types of cell death [3]	H	QT	No
TUNEL	Light/ fluorescence microscopy or Flow cytometry	Endonuclease-generated DNA breaks are enzymatically labelled by terminal transferase with UTP derivatives coupled to fluorochromes or biotin.	Not fully specific for apoptosis	L	sQT	No
Mitochondrial changes						
MPT	Flow cytometry or Fluorescence Microscopy	Loss of the inner mitochondrial transmembrane potential, causing a pore.	Not specific for apoptosis, MT damage may occur in late apoptosis, but also during necrosis	L	QL/ sQT	No
Cytochrome C	Immuno-cytochemistry High Content Analysis Immuno-fluorescence	Release of Cytochrome C from the intermembrane space into the cytosol	Not specific for apoptosis	L	QL/ sQT	No

PS: *phospholipid phosphatidylserine;* ***PI:*** *propidium iodide;* ***PARP-1:*** *poly ADP-ribose polymerase;*
TUNEL: *terminal dUTP nick end-labeling;* ***MPT:*** *mitochondrial permeability transition;* ***LDH:*** *lactate dehydrogenase*
1) ***QL:*** *qualitative;* ***QT:*** *quantitative;* ***sQT:*** *semi-quantitative*
2) Integrative measure is a test measuring a stable product, measurements can be done hours to days after induction of apoptosis

3 ccCK18 (M30) and CK18 (M65) as research markers for drug-induced tumor cell death

i. Background

The ccCK18 ELISAs are based on the monoclonal antibody M30. This antibody recognizes a neoepitope on CK18 which is exposed after caspase cleavage at residue Asp396. M30 detects only caspase-cleaved fragments of CK18 (ccCK18; CK18Asp396) but not the native protein. The M30 assays have been used in a number of scientific publications for drug screening and for characterization of the response to various drugs [4–8]. Antigen generation is strictly dependent on caspase activation (activity is inhibited by broad-spectrum caspase inhibitors). A number of different caspases are able to cleave CK18 at Asp396, primarily caspase-3, -7 and -9; activity is therefore not dependent on the action of a *specific* caspase. For example, ccCK18 is released by apoptotic MCF7

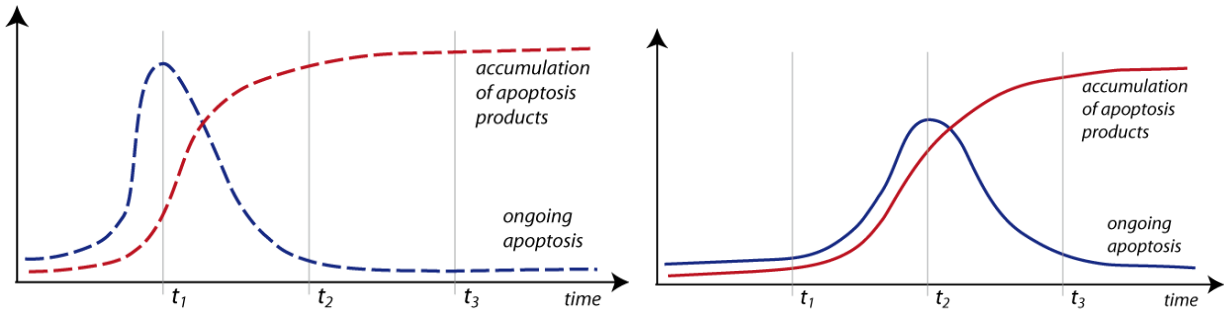


cells (a breast cancer cell line which does not express caspase-3).

Intracellular macromolecules are released from dying epithelial cells and may be detected in cell culture and patient blood. Cytokeratins are suitable biomarkers for epithelial cell death since these proteins are only expressed in this cell type. CK18 is cleaved by caspases during apoptosis; the presence of ccCK18 in plasma therefore shows epithelial cell apoptosis. In analogy, the presence of total (cleaved and native) CK18 reflects the amount of total epithelial cell death (regardless of death mode).

ii. Use of the M30 ELISA to measure ccCK18 provides an integrated measurement of apoptosis.

The M30 assay has major advantages for screening and for characterization of apoptotic responses. The method combines high-throughput with end-point measurements. The assay quantifies the accumulation of an apoptosis-generated product which is semi-stable in cells and culture media; the M30 assay provides integrated measurement of apoptosis. The signal from cells that have undergone apoptosis remains in the culture medium or in blood, also after all apoptotic processes are completed and all cells are dead. A late time point (t_3 in the chart below) is sufficient to quantify the intensity of the apoptotic stimulus regardless of its kinetic. In contrast, methods that measure the number of apoptotic cells at t_1 or t_2 will often return incorrect results (see figure below).

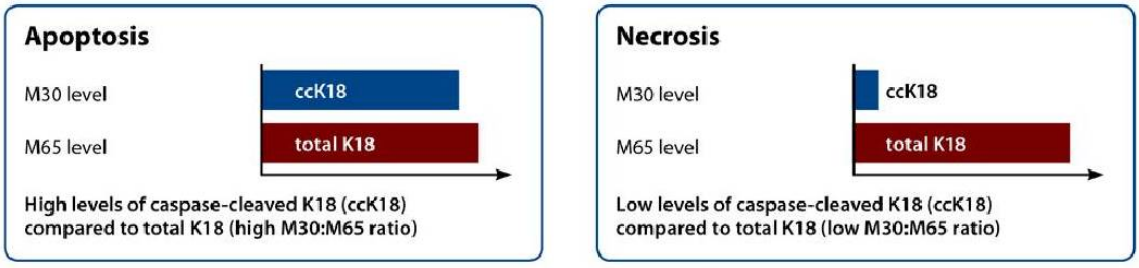


Fast induction of apoptosis **Slow induction of apoptosis**

Red lines: *signal in the M30 ELISA*
Blue lines: *signal of ongoing apoptosis (e. g. Annexin V)*
Dotted lines: *example of fast induction of apoptosis*
Solid lines: *example of slow induction of apoptosis*
t₁: *peak of the apoptotic rate for a quickly acting stimulus, but still very low signal upon treatment with slower induction.*
t₂: *peak of the apoptotic rate at slower apoptosis induction, but in the fast acting example, the apoptotic process in almost completed and leads to low signal.*
t₃: *late time point, where the apoptotic process is completed for both treatments; the signals of ongoing apoptosis are very low, but the concentrations of the accumulated apoptotic product measured by theM30 assays are at its plateau.*

i. Combined use of M30 and M65 ELISAs gives clear information on the contribution of apoptosis to the total degree of tumor cell death

The M30 and M65 ELISA assays measure two forms of the same antigen and are calibrated against an identical reference. Therefore, the combination of the two assays allows determining the relative contribution of apoptosis to the total degree of tumor cell death and thus the quantification of cell death mode. The analysis of cell death mode is based on the calculation of a M30:M65 ratio. High M30:M65 ratios indicate predominance of apoptosis, whereas low ccCK18 (M30) concentrations compared to the level of total CK18 (M65) indicates necrosis.



Note: M30:M65 ratios should only be calculated for samples with increased concentrations of CK18, and should be calibrated for the specific cell line or tumor type analyzed.

iii. Available assays for measuring ccCK18 and CK18

Three assays are available for measuring ccCK18 (M30) and total CK18 (M65). As the kits are based on the measurement of CK18, they are specific for epithelially derived cells. Most solid tumors, such as liver, lung, intestines, breast and prostate, are of epithelial origin. CK18 is not expressed by neurons, skin, muscles, connective tissue, bone marrow, immune system and blood cells.

The M30 Apoptosense® ELISA is for quantitative detection of apoptosis by measuring the concentration of ccCK18. The assay is intended for human sera or plasma and detection of tumor apoptosis in mouse xenograft models using plasma samples.

The M30 CytoDeath™ ELISA is for apoptosis measurement in cell culture applications. The assay has a dynamic range and sensitivity suitable for *in vitro* characterization of apoptosis-inducing drugs, including establishment of time course kinetics and dose-response relationships. The effects of large numbers of siRNAs on apoptosis can be tested in the 96-well format and the assay can quantify apoptosis of epithelially derived cells in multicellular spheroids and organ culture.

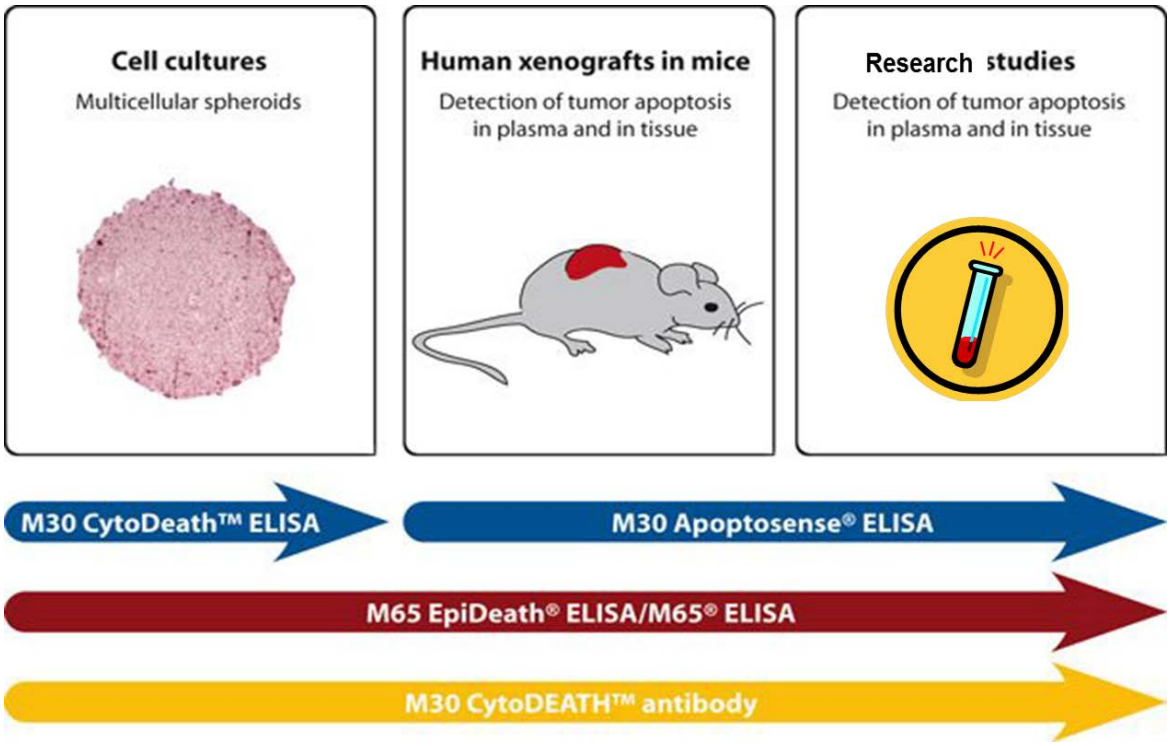
The M65 EpiDeath® ELISA (M65® ELISA) measures soluble CK18 released from dying cells in cell culture supernatants, serum and plasma. The CK18 levels reflect the amount of total epithelial cell death, regardless of the cause of death and can be used to assess overall cell death (due to apoptosis and necrosis). The M65® ELISA is primarily intended to be used together with the M30 Apoptosense or M30 CytoDeath ELISAs, which specifically measure apoptosis.

For details about application of the three assays, see the Table below.

Assay	M30 CytoDeath™ ELISA	M30 Apoptosense® ELISA	M65 EpiDeath® ELISA/ M65® ELISA
Antigen	ccCK18 neo-epitope M30 (CK18-Asp396-Ne, ccCK18)		Total CK18
Sample Type	In vitro samples Cell culture tissue Tissue slices spheroids	Serum/plasma In vitro samples Samples from mouse/rat models with human xenografts Cell culture tissue Tissue slices spheroids	
Cell death mode	Apoptosis of epithelial cells		Total cell death (apoptosis + necrosis) of epithelial cells
Quantification	End point measurement of accumulated cell death products		
sensitivity	250 U/L	25 U/L	25 U/L
range	0-3000 U/L	0-1000 U/L	0-5000 U/L / 0-2000 U/L
Species reactivity	Human, monkey*, bovine*	Human, monkey*	human, monkey*

*: based on identical CK18 protein sequence; to be determined

The cell death assays cover the whole range of anticancer drug development, from cell culture based tests to clinical research studies:

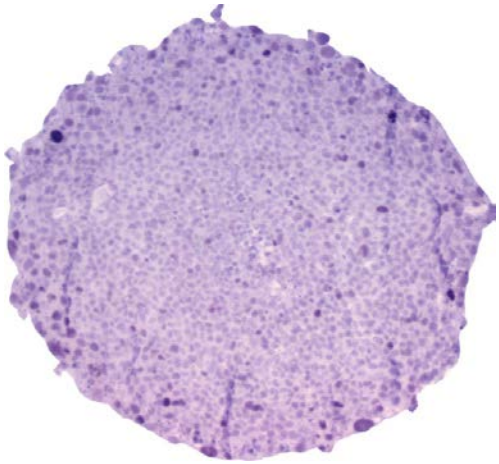


4 Applications

i. ccCK18 measurement in 3D cultures and mixed cultures

Two-dimensional (2D) monolayer cultures are universally used in experimental studies in biology. Monolayer cultures are convenient to use but do not provide accurate models for the microenvironment of tumors. Whereas nutrients and oxygen will be easily accessible to all tumor cells in 2D culture, this is not the situation in 3D tumor tissue. A large number of studies have reported differences in cell proliferation and phenotypic properties between cells grown in 2D or 3D culture.

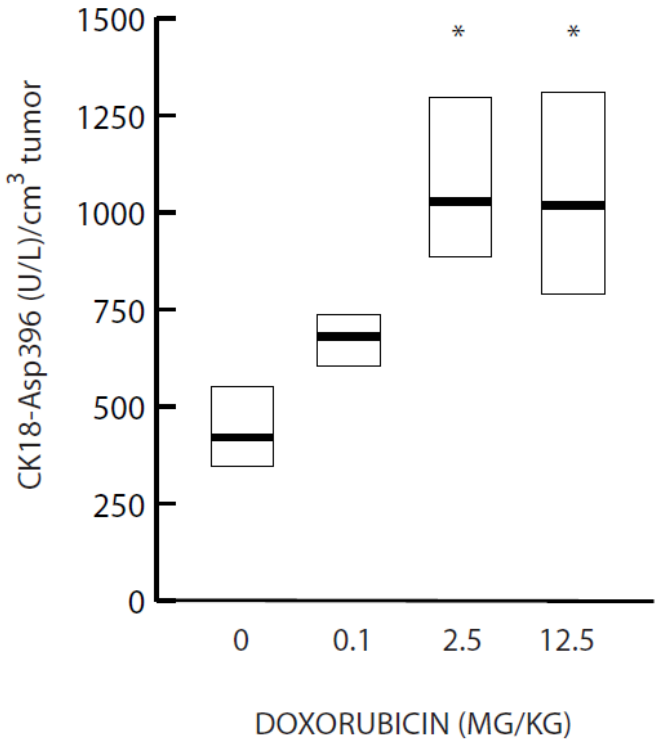
The interest of the scientific community to use *multicellular spheroids* as *in vitro* models of tumors has increased in recent years. Multicellular spheroids are known to better mimic the 3D conditions of tumors compared to monolayer cultures. The inner spheroid cell layers contain non-proliferating, quiescent cell populations that respond poorly to radiation and chemotherapy.



Spheroids can be used for drug screening and lead optimization. Herrmann *et al.* [9] have demonstrated excellent performance characteristics of a screening assay based on colon carcinoma spheroids and measurement of caspase-cleaved CK18 (Z' values of > 0.5). The M30 CytoDeath™ ELISA method provides the opportunity to restrict the number of primary hits in cell-based screening campaigns to compounds with good tumor penetration. Therefore, it is an ideal assay for primary screening of new anticancer compounds as well as for a secondary analysis of drug candidates identified in monolayer screenings.

A HCT-116 colon cancer spheroid after 7 days of incubation; diameter ca. 500 µm.

ii. Determination of tumor apoptosis in plasma from rodents with human xenografts



Caspase-cleaved CK18 generated in apoptotic cells is released into the extracellular compartment. Such extra-cellular fragments will reach the circulation. The M30 Apoptosense ELISA assay detects human but not mouse/rat CK18 [10]. The detection of ccCK18 in the blood of a mouse carrying a human tumor xenograft is therefore specifically due to apoptosis of the human tumor cells. The M30 Apoptosense ELISA is therefore a convenient pharmacodynamic assay for development of apoptosis-inducing compounds.

- Cell death in tumor xenografts will lead to increases of human CK18 in the blood of the mouse.
- The M30 Apoptosense ELISA and M65 EpiDeath ELISA will only measure tumor cell death, and not mouse liver toxicity thanks to the specificity to human CK18.

Release of CK18Asp396 into plasma of SCID mice carrying FaDu xenografts following doxorubicin treatment. SCID mice were injected with FaDu cells and tumors were allowed to grow to approximately 0.2 – 1 cm^3 and then treated with 5 mg/kg doxorubicin (intraveneous injection). Plasma samples were collected before (from the tail vein) and after treatment at the times indicated from all mice. ccCK18 concentrations measured with the M30 Apoptosense ELISA were adjusted to tumor size

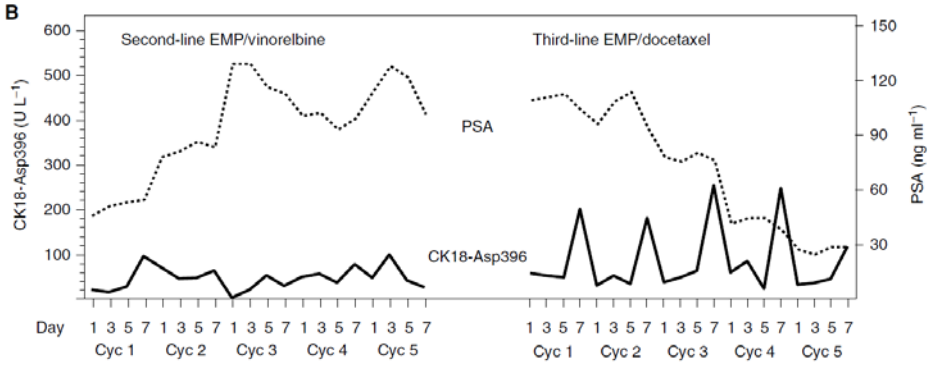
*(in cm^3). Data is expressed as medians (25^{th} – 75^{th} percentile; 3 – 4 mice per group; * $p < 0.05$).*

iii. Measurement in human serum and plasma

i. Measuring ccCK18 and CK18 to assess efficacy during clinical research studies.

The M30 Apoptosense ELISA and M65 EpiDeath ELISA can be used to determine whether a drug induces apoptosis or cell death of epithelial cells in research studies. These determinations are obviously complicated by the possibility that a drug may induce liver toxicity. Hepatocytes express CK18, and apoptosis/cell death of hepatocytes will lead to increases in circulating ccCK18/CK18. It is therefore useful to determine standard liver markers in the same samples (e.g. ALT) to evaluate liver damage. In the absence of liver toxicity, increases of ccCK18/CK18 levels are a very a strong indication of tumor death [Hou 2009, Olofsson 2007]. On the other hand, absence of a ccCK18 increase, suggests a lack of the efficacy of the drug. For the evalutaion of the efficacy of the drug, tumor regrowth needs to be taken into account.

Below, an example is shown, where ccCK18 is induced during each cycle of chemotherapy in research subjects. Commonly used cancer therapeutics such as estramustine phosphate (EMP) in combination with docetaxel did not show any effect of the treatment (shown by stable ccCK18 serum levels and increasing PSA concentrations). Switching to EMP-docetaxel therapy induced increases of ccCK18 levels in serum that correlate with decreases of PSA.



Generally, increases in ccCK18 levels in serum/plasma are observed 24–72 hours after initiation of treatment [11, 12]. Increases in serum/plasma ccCK18 are associated with response to treatment [13].

ii. Assessment of drug-induced epithelial toxicity in research subjects with tumors of non-epithelial origin by measuring CK18.

A recent example of this application was published by Greystoke et al. [14]. They investigated toxicity in subjects with lymphomas. The majority of lymphomas are treatable and many patients are potentially curable with modern treatment approaches. There is, however, a need to mitigate the risk of life-threatening treatment toxicity and to overcome the poor prognosis associated with treatment failure. Biomarkers that report the efficacy/toxicity of treatments and enable the development of individualized treatment strategies are needed. As CK18 is not expressed in cells of lymphoid origin, elevated circulating CK18 levels were expected to reflect epithelial damage as reported in several non-malignant conditions including sepsis, hepatitis and ischaemic heart disease.

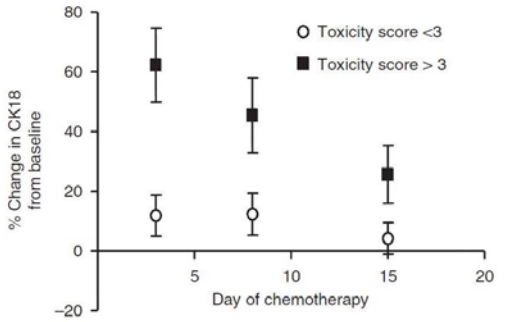


Figure 4 Changes in circulating CK18 following chemotherapy in patients with lymphoma according to CTCAE epithelial toxicity score.

The CK18 biomarker as measured by the M65 ELISA was able to measure host toxicity objectively; rises in CK18 levels give early warning of epithelial toxicity. In early clinical research studies CK18 measurement seems attractive to assess the potential for future problematic toxicity.

5 Investigated Cell Lines

Cell lines	Activity observed
Human breast cancer	
MDA-MB-231	+++
MCF-7	+++
HBL100	+++
Human colon cancer	
WiDr	++
HCT 116	+++
HT29	++
Human cervical cancer	
HeLa	++
Huamn melanoma	
AA	+
DFW	+
RPMI-8322	+
Human embryonic kidney	
HEK 293	++
Human head & neck cancer	
FaDu	+++
SCC9	++
SCC25	++
Mouse colon cancer	
CT51	-

6 References

1. Rosenberg et al. Inhibition of Cell Division in Escherichia coli by Electrolysis Products from a Platinum Electrode. Nature 1965, Vol 205, page 698.
2. Leers et al. Immunohistochemical detection and mapping of a Cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 1999;187:567–572.
3. Holdenrieder et al. Therapy Control in Oncology by Circulating Nucleosomes. Annals of the New York Academy of Science. Volume 1022 page 211-216, June 2004 .
4. Erdal et al. Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis. Proc Natl Acad Sci U S A. 2005;102(1):192–7.
5. Shukla et al. Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther. 2006 Apr; 5(4):843–52.
6. Duan, et al. SD-1029 inhibits signal transducer and activator of transcription 3 nuclear translocation. Clin Cancer Res. 2006; 12:6844–52.
7. Berndtsson et al. Induction of the lysosomal apoptosis pathway by inhibitors of the ubiquitin-proteasome system. Int J Cancer. 2009; 124:1463–9.
8. Brnjic et al. Chemical biology suggests a role for calcium signaling in mediating sustained JNK activation during apoptosis. Mol Biosyst. 2010; 6:767–74.
9. Herrmann et al. Screening for compounds that induce apoptosis of cancer cells grown as multicellular spheroids. J Biomol Screen. 2008;13:1–8.
10. Olofsson et al. Specific demonstration of drug-induced tumor cell apoptosis in human xenografts models using a plasma biomarker Cancer Biomarkers 5 (2009) 117–125 117
11. Kramer et al. Differentiation between cell death modes using measurements of different soluble forms of extracellular Cytokeratin 18. Cancer Res. 2004;64:1751–6.
12. Kramer et al. Docetaxel induces apoptosis in hormone refractory prostate carcinomas during multiple treatment cycles British Journal of Cancer (2006), 1 –7
13. Hägg Olofsson et al. Cytokeratin-18 is a useful serum biomarker for early determination of response of breast carcinomas to chemotherapy. Clin Cancer Res. 2007;13:3198–206.
14. Greystoke et al. Assessment of circulating biomarkers for potential pharmacodynamic utility in patients with lymphoma. British Journal of Cancer (2011) 104, 719 – 725.