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Metabolic Signatures in Apoptotic Human Cancer Cell Lines

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Abstract

Cancer cells have several specific metabolic features, which have been explored for targeted therapies. Agents that promote apoptosis in tumors are currently considered as a powerful tool for cancer therapeutics. The present study aimed to design a fast, reliable and robust system for metabolite measurements in cells lines to observe impact of apoptosis on the metabolome. For that purpose the NBS (newborn screen) mass spectrometry-based metabolomics assay was adapted for cell culture approach. In HEK 293 and in cancer cell lines HepG2, PC3, and MCF7 we searched for metabolic biomarkers of apoptosis differing from that of necrosis. Already nontreated cell lines revealed distinct concentrations of metabolites. Several metabolites indicative for apoptotic processes in cell culture including aspartate, glutamate, methionine, alanine, glycine, propionyl carnitine (C3-carnitine), and malonyl carnitine (C3DC-carnitine) were observed. In some cell lines metabolite changes were visible as early as 4 h after apoptosis induction and preceeding the detection by caspase 3/7 assay. We demonstrated for the first time that the metabolomic signatures might be used in the tests of efficacy of agents causing apoptosis in cell culture. These signatures could be obtained in fast high-throughput screening.

Introduction

Cell fate

Generally, the cell fate could be proliferation, differentiation or death. Cell death could be regulated by different mechanisms: necrosis or apoptosis (Danial and Korsmeyer, 2004). Necrosis is the end result of homeostasis decay initiated mainly by cellular accidents such as toxic or physical challenge (Proskuryakov et al., 2003). This accidental cell death is characterized by vacuolization of the cytoplasm, breakdown of the plasma membrane, and unorganized chromatin condensation and fragmentation of DNA into a continuous spectrum of base pair sizes (Wyllie, 1997). Cells undergoing necrosis induce inflammatory response during release of cellular contents and pro-inflammatory molecules (Iyer et al., 2009). In turn, apoptosis is characterized by nuclear condensation and fragmentation, cleavage of chromosomal DNA into fragments with 200 bp DNA length, plasma membrane blebbing, and cell shrinkage (Schwartzman and Cidlowski, 1993). Necrosis and apoptosis reveal therefore different features with different tolerability to the host organism.

Apoptosis in cancer

Several reports demonstrated that the disruption of apoptosis-induced tumorogenesis stimulated tumor progression and resistance to the therapy (Edinger and Thompson, 2004). Agents that promote apoptosis in tumors are currently considered as powerful tool for cancer therapeutics (Call et al., 2008). Most patient-tolerable cancer therapies require induction of apoptosis in tumor cells. Selective triggering and follow-up of apoptosis is an important goal in cancer therapies (Call et al., 2008). Therefore, monitoring of early apoptosis biomarkers in clinical trials of anticancer therapies is urgently required. The ideal biomarkers should be specific for the biological process and noninvasive. Additionally, the biomarkers detection should be rapid, reliable, and robust (Ward et al., 2008). However, detection and monitoring of apoptosis in therapies is still a challenge.

Metabolomics contributions

A new perspective in this field is provided by metabolomics, which is the quantitative measurement of the dynamic metabolic response of living systems to pathophysiological stimuli, environmental modulation, or genetic modifications (Griffiths and Wang, 2009). Monitoring of small molecule concentrations (such as amino acids, lipids, steroids, sugars, etc.) in cells, tissues, or body fluids (plasma, urine) could give information about disease progression or response to the treatment. To increase resolution specific biochemical pathways or organisms have been analyzed. Examples for that are metabolomics of lipids (lipidomics; Griffiths and Wang, 2009; Oresic, 2009), steroids (sterome; Ceglarek et al., 2010), yeast or plant metabolomics (Bais et al., 2010; Snyder and Gallagher,

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2009), microbe–host interactions (Goodacre, 2007), nutritional challenges (Altmaier et al., 2009), or drug development (Gallagher et al., 2009; Xu et al., 2009). Metabolomics request a substantial support from bioinformatics to handle large data sets (Kastenmüller et al., 2010). Not only metabolomic signatures characteristic for a given biological process could be depicted, but as well kinetics of biochemical pathways or metabolite "flux" (Rios-Estepa et al., 2008). Metabolomic and genetic approaches could be even combined like in case of GWAS (genome-wide association studies) (Illig et al., 2010) where new "genetically determined metabotypes" were discovered in humans.

Present different approaches include profiling-, targeted-, or untargeted metabolomics. *Profiling* metabolomics refers mostly to discovery analyses with a very high mass resolution, low throughput, and rather identification than quantification using gas chromatography-mass spectrometry (GC-MS), NMR, LC-FT-ICR, or UPLC-MS (Griffiths et al., 2007; Zhao et al., 2010). *Targeted* metabolomics has a dedicated approach with high throughput (e.g., 1,000 samples per week), metabolite quantification of previously chosen metabolites (several hundreds at the same time), is used in diagnostics and is based on liquid chromatography mass spectrometry (LC-MS), GC-MS or flow injection analysis (FIA)-MS (Ellis et al., 2007; Illig et al., 2010; Weljie et al., 2006). The *untargeted* metabolomics approach provides a high throughput with a large number of analytes quantified, but requests high degree of parallelism (i.e., simultaneous analyses on LC- and GC-MS) and special identification algorithms with specific databanks (Lawton et al., 2008; Ohta et al., 2009).

Metabolomics in cancer

Cancer cells have several specific metabolic features (for an overview see http://www.metabolic-database.com/html/tumor_metabolome_overview.html), which have been explored for targeted therapies (Griffin & Shockcor, 2004). One of the first described was the Warburg effect, a shift from oxidative phosphorylation in mitochondria towards glycolysis (Weljie & Jirik, 2010). Metabolomics has been instrumental in finding further susceptibility points or biomarkers for cancer (Kim et al., 2009; Koulman et al., 2009; Sreekumar et al., 2009).

A metabolite profile of HL60 leukemia cells measured with ^{1}H-NMR was already reported (Rainaldi et al., 2008; Tiziani et al., 2009), but it was not based on LC-MS experiments. Such method may be very attractive for study in challenged cells in regard of higher sensitivity when compared to ^{1}H-NMR method (Cuperlovic-Culf et al., 2010).

Aim of the study

The present study aimed to design a fast, robust, reliable, and affordable system for metabolite measurements in cells. For that purpose the NBS (newborn screen) assay, originally used for diagnostic metabolite measurements from dried blood spots (Baumgartner et al., 2004; Chace et al., 2003; Rashed et al., 1997), was adapted by us for cell culture approach. Further goal of our study was to find early apoptosis biomarkers in cancer cell lines. To achieve that, apoptosis or necrosis were induced (by staurosporine or heating, respectively) in four different cell lines HEK 293, HepG2, PC3, and MCF7. Cell fate was determined by viability (MTT) and caspase 3/7 assay. To find metabolic biomarkers characteristic for apoptosis, metabolite concentrations of vehicle treated, apoptotic, and necrotic cells have been compared. Metabolomic signatures present only in apoptotic cells were analyzed for specific biomarkers. We report for the first time several metabolites indicative for early apoptotic processes in cell culture.

Materials and Methods

Cell culture

All cells were cultured at 37°C and 5% CO_2 in humidified atmosphere. HepG2 (hepatocellular carcinoma) and HEK 293 (human embryonic kidney) cells were purchased from the German collection of microorganisms and cell cultures (DSMZ) and grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Human prostate adenocarcinoma, PC3 (also from DSMZ) and human breast adenocarcinoma MCF 7 cells (kindly provided by Dr. T. Penning, Department of Pharmacology, University of Pennsylvania, Philadelphia, USA) were cultivated in RPMI 1640 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells grown over 12 passages were used in the experiments.

Stimulation of apoptosis or necrosis

Twenty-four hours prior to treatment the cells were seeded at a density of 2×10^5 cells in 12-well and 0.2×10^5 cells in 96-well plates, respectively. Medium volume was 1 mL in 12-well and 100 μL in 96-well cavities. After 24 h cells had reached confluency of 80% and medium was completely removed. To induce apoptosis fresh medium containing staurosporine (2 and 4 μM, respectively, in DMSO) was added. Cells for control of apoptotic cells were supplied with fresh medium containing vehicle (DMSO) without staurosporine. For induction of necrosis fresh medium was given to the cells subsequently heated for 20 min at 57°C on a hot plate. Cells considered as control for necrotic cells were only supplied with fresh medium. In all cases cells were further incubated for 4, 12, or 24 h and harvested or treated differently as described below, depending on the subsequent analyses. Experiments were done in triplicates.

MTT-assay to determine cell viability

To monitor the cell viability during stimulation of apoptosis and necrosis we performed the MTT assay (Mosmann, 1983). Cells were grown in 96-well plates. Four, 12, and 24 h after treatment 10 μL of 5 mg/mL solution of MTT (Roche, Indianapolis, IN, USA) in PBS buffer were added to each well and cells were incubated at 37°C for 2 h. Medium was then completely removed and the cells were frozen at −80°C for at least 1 h. To each well 100 μL of DMSO was given and plates were incubated for 40 min at room temperature (RT) under constant shaking to lyse the cells. Optical absorbance of reduced MTT, reflecting the viability of the cells, was measured at 590 nm using a GENiosPro plate reader (Tecan, Switzerland).

Caspase 3/7 assay to detect apoptosis

For detection of apoptosis cells were grown in 96-well plates. The enzymatic activity of caspase 3 and 7 was deter-

mined using the Caspase-Glo 3/7-assay (Promega, Madison, WI, USA). Four, 12, and 24 h after treatment 100 μL of Caspase-Glo reagent was added to the 100 μL cell culture supernatants according to manufacturer's instructions and luminescence, being directly proportional to the caspase activity, was measured using a GENiosPro plate reader (Tecan).

Adapted newborn screening (NBS) assay for metabolite measurements in cultured cells

The NBS assay kit from Chromsystems (Germany), originally developed for quantification of 42 amino acids and acylcarnitines in blood samples, was adapted for use with cultured cells. For this, assay cells grown in 12-well plates were used. Adherent and floating cells were recovered in 1.5-mL reaction tubes at three time points (4, 12, and 24 h after treatment). Attached cells were washed twice with phosphate-buffered saline (PBS) and afterward not trypsinized but scraped off the plate wells. For metabolite extraction pelleted cells were mixed with 300 μL of 20% MetOH in H_2O, placed into to 4°C precooled vials containing glass beads (Precellys-Glas-Kit, 0.1 mm, Peqlab, Germany) and homogenized three times for 20 s at 5500 rpm with the homogenizator Precellys24 (Peqlab) at 4°C. Procedures for metabolite extraction were validated as described (Römisch-Margl, Adamski et al., submitted). Homogenized samples were centrifuged for 10 min at 18,000×g at RT and 3.5 μL of the supernatants were placed on filter paper (Whatman filter paper 10538018) of 3-mm diameter in 96-well plates. The loaded filters were left overnight at RT for drying.

Metabolite quantification by mass spectrometry

Metabolite detection and quantification was performed according to the NBS assay kit instructions (Chromsystems, Germany). Briefly, the dried filter papers were extracted for 20 min with 200 μL MeOH containing isotopically labeled amino acids and acylcarnitines as internal standards. After extraction, the solvent was evaporated at 60°C and derivatized for 18 min at 72°C with 60 μL of derivatization reagent. After another evaporation step 100 μL of reconstitution solution were added, mixed for 10 min at RT, and samples were measured using FIA-MS/MS (4000 QTrap, AB Sciex, Darmstadt, Germany). For MS/MS analysis, 10 μL of reconstituted sample were directly injected (FIA) into the mass spectrometer and 40 amino acids and acylcarnitines were assayed in a total analysis time of 1.7 min per sample. For derivatized amino acids, a neutral loss scan ($m/z = 102$) was conducted in positive ion mode. Other amino acids like glycine, ornithine, arginine, and citrulline were quantified in positive ion mode by multiple reaction monitoring (MRM). Carnitine and acylcarnitines were assayed in positive ion mode by precursor ion ($m/z = 85+$) scan. Supplementary Table 1 provides an overview on metabolites and detection methods. Data analysis was performed with ChemoView software (AB Sciex).

For normalization purposes, cell number was determined in a parallel assay. Cells were cultivated and treated as for the metabolite analysis experiment but harvested by trypsinization and counted with a counting chamber under the microscope. For direct comparison, all metabolite concentrations were normalized to the number of cells (4×10^5) found in the control experiment (nontreated cells) at the 4-h time point.

Statistical evaluation of metabolite data

Statistical data analysis was performed with *metaP* server at Helmholtz Center Munich (http://metabolomics.helmholtz-muenchen.de/metap2/) providing automated and standardized data analysis for quantitative metabolomics data (Kastenmüller et al., 2010). For testing the association of metabolite concentrations with multiclass categorical phenotypes, the nonparametric Kruskal-Wallis test was used. For visualizing potential associations, the server creates box plots. Only those metabolite phenotype associations are marked as significant that showed a p-value below indicated levels after Bonferroni correction ("*" denotes a significance level of 5% and "**" a significance level of 1%).

Results

The main goal of this work was to find candidates for early biomarkers of apoptosis differing to that of necrosis. To accomplish this, the changes in concentrations of 42 amino acid and acylcarnitines in staurosporine-treated and control cells were monitored in comparison to the values in heat-treated cells. Cell lines chosen for the experiments were human cancer cell lines HepG2, PC3 and MCF 7 beside immortalized human embryonic kidney HEK 293.

Cell viability assays

Prior to metabolomic analyses we tested the sensitivity of the four cell lines to treatments by staurosporine (inducer of apoptosis) (Alberici et al., 1999) or heating (induction of necrosis) (Proskuryakov et al., 2003) by MTT assay. Figure 1 shows the influence of vehicle (DMSO), staurosporine (2 and 4 μM), and the heating on cell viability. Values were normalized to the viability of untreated cells. Staurosporine caused a time- and dose-dependent decrease in cell viability in all examined cell lines with the strongest effect after 24 h of treatment with 4 μM agent. In MCF7 cells a marked decrease in the number of viable cells was apparent already after 4 h of treatment with 4 μM staurosporine. A high sensitivity to this proapoptotic agent was also found in HEK 293 and PC3 cells, where viability decreased dramatically to 20 and 40%, respectively, but not until 12 h of treatment. In case of HepG2 cells only a moderate decrease in metabolic activity (60% in comparison to control).

The effect of heat treatment on the cell lines was also different between the four cell lines. Already 4 h after of incubation HEK 293 and HepG2 cells pushed to necrosis showed a dramatic decrease in cell viability (below 20% in comparison to control). In contrast, the decrease in viability of heated PC3 and MCF7 cells was not as drastic as in HEK 293 and HepG2. The strongest effect has been observed only as late as after 24 h of treatment. Cells treated with vehicle (DMSO) did not show any changes in viability in comparison to nontreated cells.

Apoptosis validation

To estimate the extent of apoptosis induced in the four cell lines caused by the different treatments caspase 3 and 7 activities were analyzed (Fig. 2). Values were normalized to the caspase 3/7 activity of nontreated cells. The four analyzed cell lines show different responses to staurosporine. In HEK 293 and HepG2 a caspase assay-detectable apoptosis could only be induced by the higher concentration of staurosporine

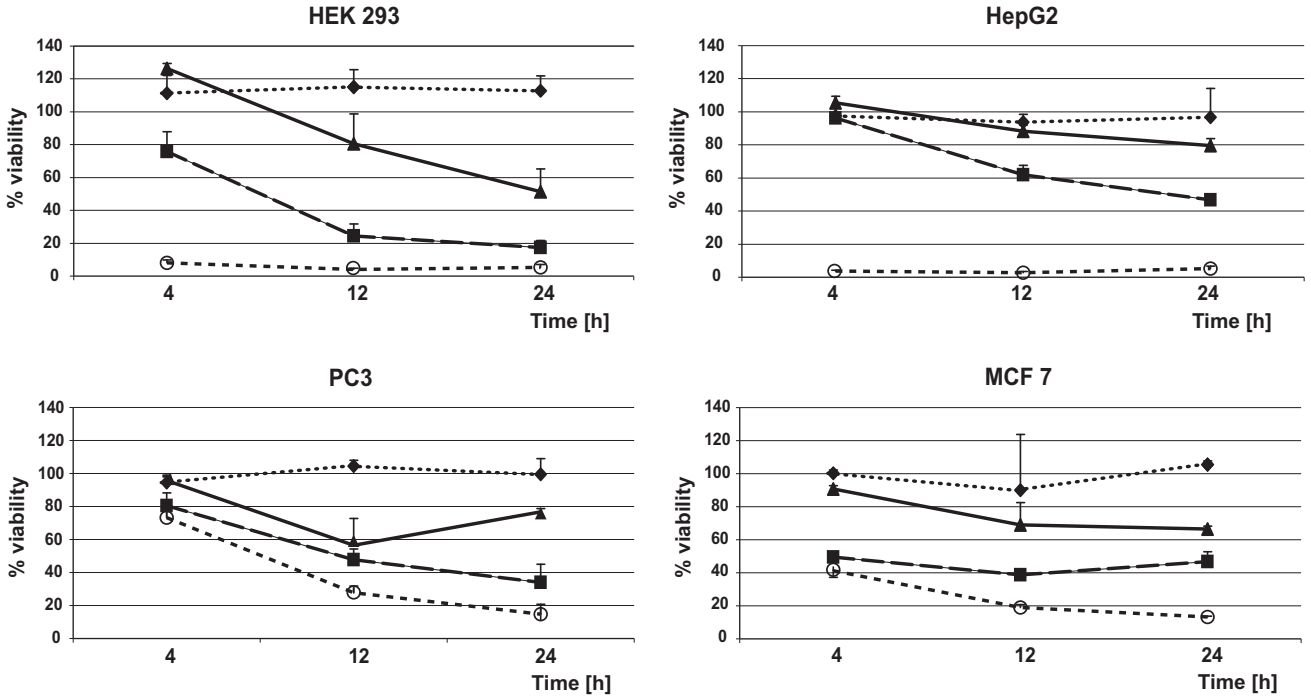

FIG. 1. Effect of staurosporine and heating on the cell viability of cell lines HEK 293, HepG2, PC3, and MCF7. Cell viability was measured with MTT assay after treatment with vehicle (DMSO) [◆], staurosporine 2 μM [▲], or 4 μM [■], or by heating [○] at indicated time points. Values represent the mean ± SD of triplicates from three independent experiments and were normalized to the viability of nontreated cells, taken as 100%.

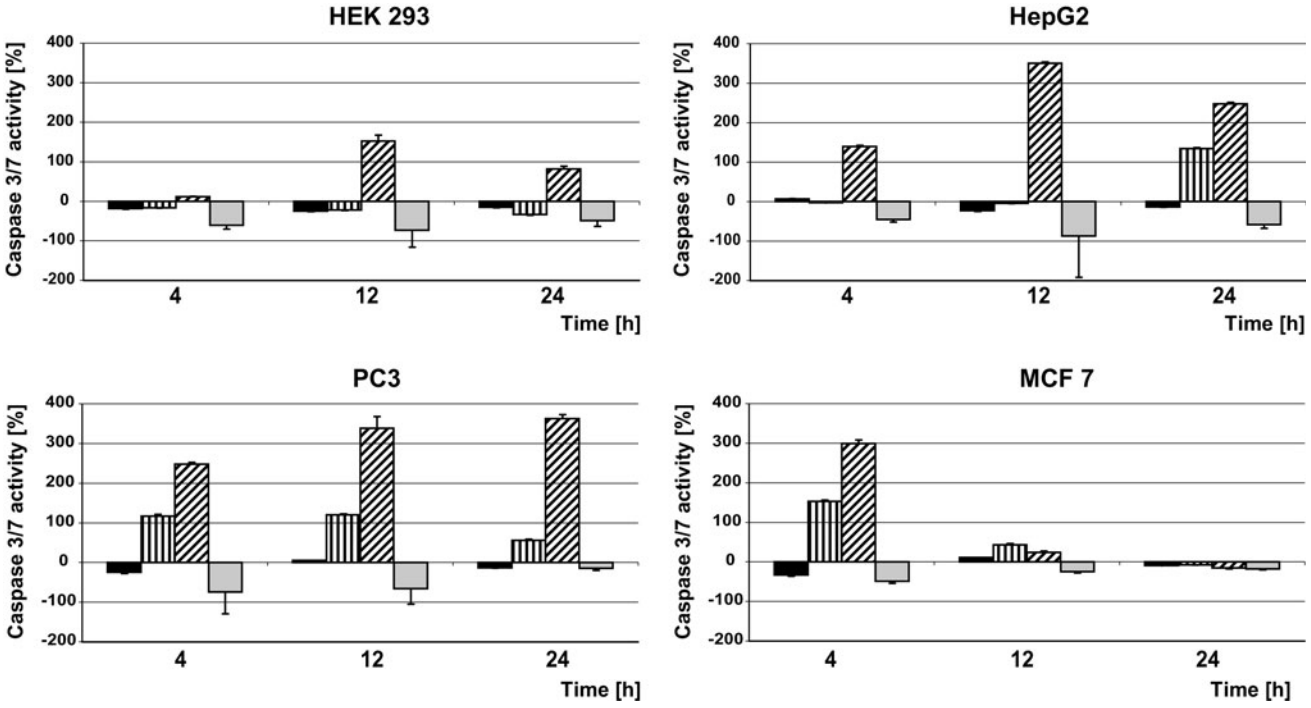

FIG. 2. Effect of staurosporine and heating on the Caspase 3/7 activity in cell lines HEK 293, HepG2, PC3, and MCF7. Caspase 3/7 activity was measured using the luminescence Caspase-Glo 3/7 kit after treatment with vehicle DMSO [solid bars], 2 μM [striped bars] or 4 μM [crossbred bars] staurosporine or by heating [gray bars] at indicated time points. The data shown are the mean ± SD from three independent experiments carried out in triplicates and were normalized to the caspase 3/7 activity of nontreated cells.

(4 μM). In the same cell lines the enzymatic activity of the caspase 3 and 7 activity increased with time. A maximum was observed at the 12-h time point, after which activity was lower again. In PC3 cells the caspase activity was already high 4 h after treatment with staurosporine and further increased with time. Distinct results were observed for the MCF7 cells. In this cell line activity of caspases 3 and 7 reached its maximum already after 4 h of treatment with staurosporine, but was dramatically decreased afterward and was abolished at 24 h (these effects were most pronounced at a concentration of 4 μM staurosporine). In none of the cells treated with heat or vehicle (DMSO) any activation of caspase 3 and 7 has been observed.

Distinct metabolite signatures of nontreated cell lines

In the next step we checked the change of metabolites upon staurosporine (4 μM) and heat treatment in the four cell lines to find biomarker candidates of apoptosis. We have chosen the NBS assay, which was originally developed for the fast and simultaneous detection and quantification of 42 amino acids and acylcarnitines in blood samples (Rashed et al., 1997), and adapted the assay for cell culture. All metabolite concentrations were normalized to the number of cells (4×10^5) in the control experiment (nontreated cells).

When comparing the concentration levels of the metabolites of the control groups (nontreated cells) differences could be observed between the concentration levels in the four analyzed cell lines. Metabolites that were found to be significantly different between HEK 293, HepG2, PC3, and MCF7 cells in their concentration levels at zero time point included alanine (Ala), glutamate (Glu), phenylalanine (Phe), methionine (Met), glycine (Gly), free carnitine (C0-carnitine), acetyl carnitine (C2-carnitine), and propionyl carnitine (C3-carnitine) and are summarized in Table 1. To illustrate these effects the differences in concentration of Glu and C3-carnitine are presented in the form of box-plots (Fig. 3). From the four analyzed cell lines in HepG2 the highest concentration of Glu, Phe, Met, Gly, and C2-carnitine has been found. Furthermore, Ala and C3-carnitine reached the highest level in HEK 293 cells. The PC3 cells distinguished from other cells by lowest concentration of Ala, Glu, Phe, Gly, and C2-carnitine. The lowest concentration of Met was specific for MCF7 cells.

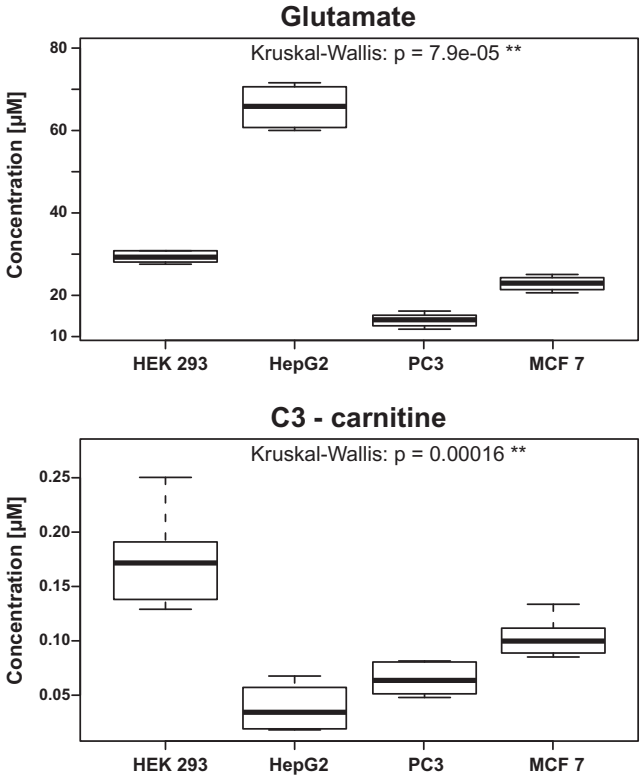

FIG. 3. Differences in glutamate and C3-carnitine (propionylcarnitine) concentrations of untreated cell lines HEK 293, HepG2, PC3, and MCF7. The metabolite concentrations are visualized in form of box plots obtained after statistical analysis with metaP server. The *p*-value calculated by using Kruskal-Wallis are shown in each diagram, "**"denotes a significance level of 1% after Bonferroni correction. The medians were calculated from two experiments carried out in triplicate. Values were normalized to the number of 4×10^5 cells.

Biomarker candidates for apoptosis

In our experiment setup we compared the metabolite levels of apoptotic cells with these of control and necrotic cells. We assumed that any changes that occur in apoptotic, but not in

Table 1. Differences in Metabolite Concentrations of Nontreated HEK293, HepG2, PC3, and MCF7 Cells

		Cells				
	Metabolite	*HEK 293*	*HepG2*	*PC3*	*MCF7*	p-*value*
Metabolite concentrations [μM]	Ala	27.74	24.57	4.11	8.49	(*) $3.6*10^{-4}$
	Glu	29.27	65.86	14.07	22.96	(**) $7.9*10^{-5}$
	Phe	3.24	5.59	1.58	1.90	(*) $8.0*10^{-4}$
	Met	1.87	2.53	1.13	1.07	(*) $7.5*10^{-4}$
	Gly	42.88	76.60	17.36	20.63	(*) $2.9*10^{-4}$
	C3-carnitine	0.17	0.03	0.06	0.09	(**) $1.6*10^{-4}$
	C2-carnitine	0.34	1.14	0.26	0.56	(*) $2.8*10^{-4}$
	C5-carnitine	0.01	0.04	0.03	0.02	(*) $6.9*10^{-4}$

Metabolite concentrations were normalized to the number of 4×10^5 cells. The medians were calculated from two experiments carried out in triplicate.

The statistical evaluation was performed with *metaP* server (see Materials and Methods) including data of the four control groups (non treated cells). The *p*-values were calculated according to Kruskal-Wallis: (*) denotes a significance level of 5% and (**) a significance level of 1% after Bonferroni correction. Abbreviations: Ala, alanine; Glu, glutamate; Phe, phenylalanine; Met, methionine; Gly, glycine; C3-carnitine, propionyl carnitine; C2-carnitine, acetyl carnitine; C5-carnitine, isovaleryl carnitine.

necrotic and control cells, will be specifically related to apoptosis and could be considered as its biomarkers.

For the identification of early biomarkers of apoptosis, a statistical evaluation was performed using Kruskal-Wallis analyses. Metabolite concentration data of four groups—staurosporine-treated (apoptosis induced), heat-treated (necrosis induced), vehicle treated, and nontreated cells—were included.

Seven (five amino acids and two acylcarnitines) of the 42 analyzed metabolites were significantly changed in treated cell lines as can be seen in Table 2. These were Asp, Glu, Met, Ala, and Gly, as well as C3-carnitine and malonyl carnitine (C3DC-carnitine). However, we observed distinct patterns of metabolic changes in different cell lines.

As depicted in Table 2, the cell lines HEK 293 and HepG2 are the most similar in the reaction to apoptosis and necrosis induction. In most time points the metabolites Asp, Glu, Met, Gly, Ala, and C3DC-carnitine increased at apoptosis conditions and decreased in case of necrosis. For most of these six metabolites, this effect appeared already at the 4-h time point and seemed to remain throughout the 24 h of the experiment. In case of Ala this regulation was found first after 24 h of treatment. The C3-carnitine was the only metabolite that was downregulated in the two cell lines independently of the kind of treatment.

The cell lines PC3 and MCF7 both revealed different signatures to that of HEK 293 and HepG2 cells as well as among each other. In both cell lines many of the seven metabolites were either up- or downregulated regardless of the different treatment.

In the PC3 cells all seven metabolites were up-regulated at the 4-h time point, but only with the metabolites Asp and C3-carnitine apoptosis and necrosis could be distinguished. At later time points this effect with C3-carnitine was no longer observed. In case of Asp, the difference between apoptotic and necrotic PC3 cells was still visible 12 h after treatment, but not detected later on. Met and Ala appeared to be different between apoptotic and necrotic PC3 cells only at the later time points (12 and 24 h).

In MCF7 cells a totally different metabolic signature was observed during apoptosis and necrosis. The majority of regulated metabolites were either down- or upregulated in comparison to controls but independent of treatment. Only Met and C3DC-carnitine showed a difference in reaction to the nature of treatment, but at different time points of 12 h and 24 h, respectively.

We closely analyzed the early signatures measured after 4 h of treatment to evaluate candidates for early biomarkers. Indeed, distinct values become already apparent at this time and are depicted for the most significant metabolites (Fig. 4). In the cell lines HEK 293, HepG2, and PC3 the levels of Asp, Glu, Met, Gly, Ala, C3-carnitine, and C3DC-carnitine (Fig. 4A–G) were upregulated in apoptotic versus necrotic cells. In the analyzed time window the cell line MCF7 has not revealed significant changes of metabolite concentrations differentiating apoptosis and necrosis. On the overall seven metabolites were characteristic for apoptosis as early as 4 h after the challenge.

Discussion

Disturbed balance between cell proliferation and incidence of apoptosis in cancer cells cause abnormal proliferation, tumor invasion, and metastatic potential (Olopade et al., 1997). Apoptosis is an important phenomenon in cancer therapies and represents a common mechanism of drug effect (Salomons et al., 1999). Therefore, introduction of early apoptosis biomarkers in theranostics (Picard and Bergeron, 2002) of cancer therapies is required. We used metabolites as markers of genomic changes or readouts of apoptotic process (Zhivotovsky and Kroemer, 2004) because of their robust quantification possibilities. In the present study we applied metabolomics as method for the survey of candidates for early

Table 2. Overview of Metabolite Level Changes as Detected by FIA/MS-MS in Cell Lines HEK 293, HepG2, PC3, and MCF7 Undergoing Apoptosis or Necrosis

	Aspartate			Glutamate			Methionine			Glycine			Alanine			C3 - carnitine			C3DC - carnitine		
Time [h]	4	12	24	4	12	24	4	12	24	4	12	24	4	12	24	4	12	24	4	12	24
A_HEK 293																					
N_HEK 293																					
A_HepG2																					
N_HepG2																					
A_PC3																					
N_PC3																					
A_MCF7																					
N_MCF7																					

Statistical evaluation was performed with *metaP* server (see Materials and Methods) including data of the four groups staurosporine-treated (induction of apoptosis), heat-treated (induction of necrosis), vehicle-treated, and nontreated cells. Changes in metabolite concentrations and indicate increase (shown in gray), decrease (black), or no change (white).

C3-carnitine, propionyl carnitine; C3DC-carnitine, malonyl carnitine; "A_," apoptosis, "N_," necrosis. Underlying data for 4-h time point are shown in Figure 4 in more detail.

biomarkers of apoptosis detection and classification of cell death. We demonstrated that the discrimination of apoptosis from necrosis is possible by metabolic profiling.

Analyses of metabolite concentration of challenged cells were already performed by ^{1}H-NMR technique (Garcia-Alvarez et al., 2009; Griffin and Shockcor, 2004; Tiziani et al., 2009). Nevertheless, the MS-based approaches (like LC-MS) are more sensitive than ^{1}H-NMR spectroscopy, and can potentially detect metabolites at a concentration two orders of magnitude below that of NMR (Griffin and Shockcor, 2004). Therefore, in our study FIA-MS/MS as detection method of changes in metabolite concentrations was chosen.

To determine cell death, metabolic activity (MTT) as well as caspase 3 and 7 assays were performed. We observed that staurosporine at 4 μM induced cells apoptosis in all four cell lines (HEK 293, HepG2, PC3 and MCF7). It was unexpected to us to observe apoptosis in PC3, a cell line known as staurosporine-insensitive (Marcelli et al., 2000). This sensitivity of PC3 to staurosporine can be due to an acquired mutation, which was not further investigated. However, the metabolite profile of PC3 was further examined. In the case of heating the cells, no increase of caspase 3/7 activity has been detected, clearly showing that heating does not induce apoptosis in the cell lines (Fig. 2). In parallel, the heated cells were proven to be necrotic as they lost viability by this treatment (Fig. 1).

We applied staurosporine for several reasons. This agent triggering apoptosis has been reported to be effective in HEK 293, HepG2, and MCF 7 cells (Miyamoto et al., 2004; Nagata et al., 2005; Xue et al., 2003). One unique property of staurosporine is its ability to rapidly and completely drive virtually all mammalian cells into apoptosis (Bertrand et al., 1994; Stepczynska et al., 2001). Furthermore, the staurosporine-induced apoptosis involves mitochondrial caspase activation (Caballero-Benitez and Moran, 2003), which can be easy detected by caspase 3/7 assay. Moreover, staurosporine and

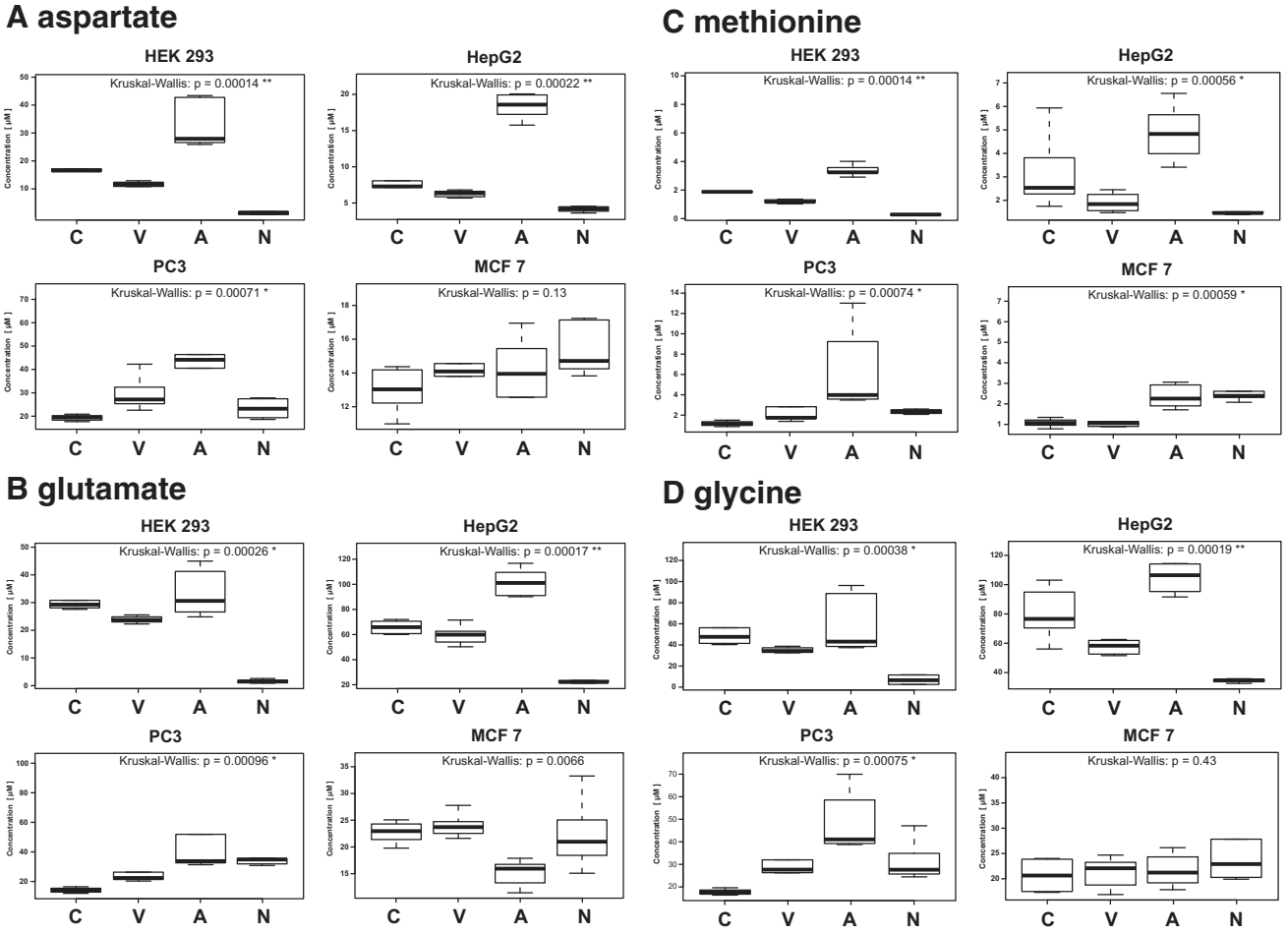

FIG. 4. Effect of staurosporine and heating on metabolite concentrations in HEK 293, HepG2, PC3, and MCF7 cells. Metabolites were determined after treatment with DMSO (vehicle) at 4-h time point, 4 μM staurosporine (apoptosis) by heating (necrosis) and for untreated cells (control). Panel lettering: (**A**) aspartate, (**B**) glutamate, (**C**) methionine, (**D**) glycine, (**E**) alanine, (**F**) C3-carnitine—propionyl carnitine, (**G**) C3DC—malonyl carnitine. Concentrations are given in μM (Y-axis), group definitions are: C—control, V—vehicle, A—apoptosis, N—necrosis (X-axis). Statistical evaluation was performed with the metaP server (see Materials and Methods). The *p*-value calculated by using Kruskal-Wallis are given in each diagram, "*" denotes a significance level of 5% and "**" a significance level of 1% after Bonferroni correction. The medians were calculated from two independent experiments carried out in triplicate. Values were normalized to the number of cells in the control experiment (nontreated cells), which were determined to be 4×10^{5} cells.

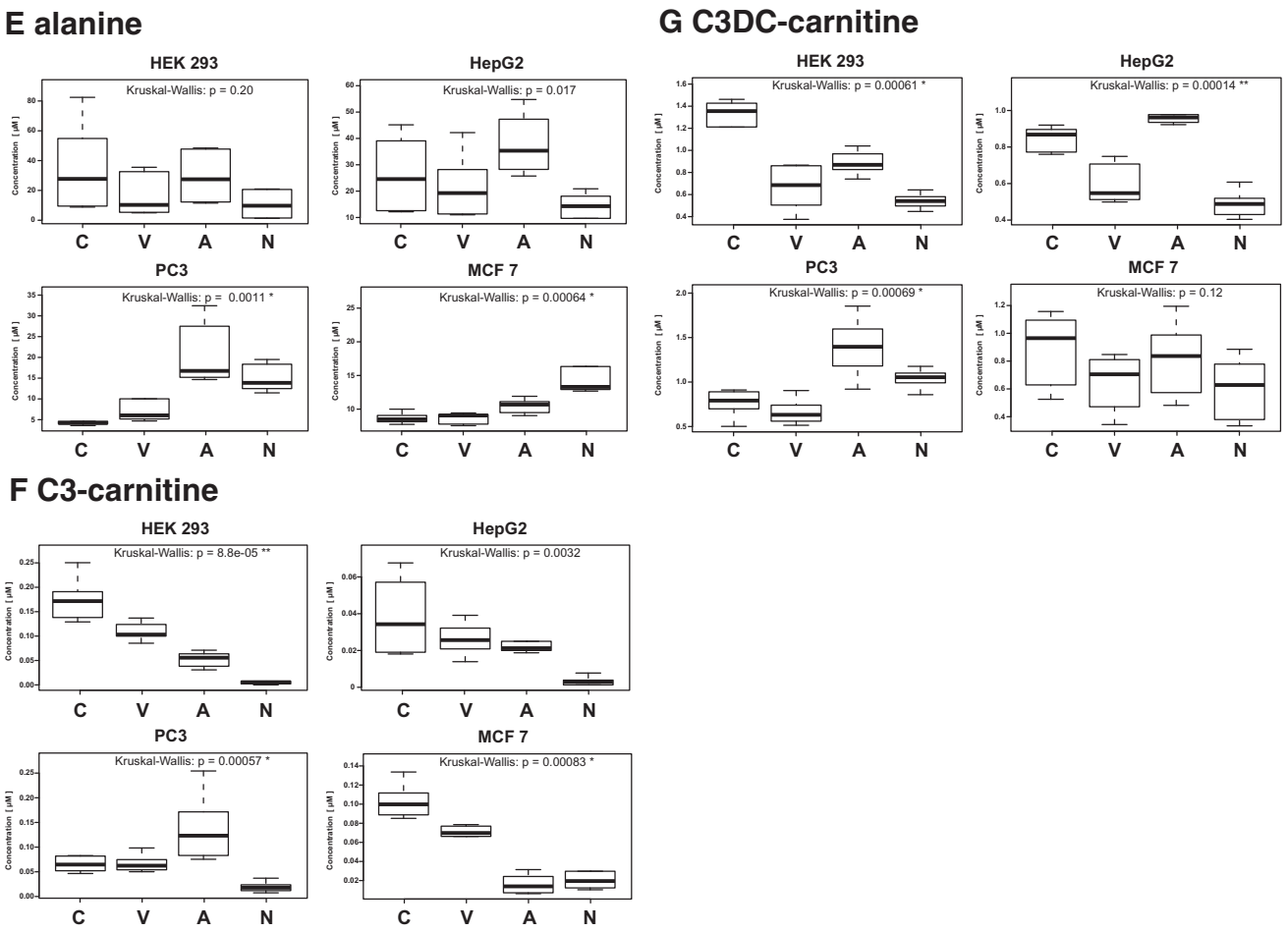

FIG. 4. *(Continued).*

derivatives has been used in clinical trials for cancer therapies (Edelman et al., 2007; Perez et al., 2006).

Necrosis can be induced by heat (Rainaldi et al., 2008) or starvation with PBS (Mirbahai et al., 2010). Here we used heating of the cells at 57°C as we aimed for a fast necrotic process.

The NBS assay has been performed for untreated, DMSO, staurosporine, or temperature challenged HEK 293, HepG2, PC3, and MCF7 cell lines. This assay, commonly used for screening infants to manage treatable, genetic, and endocrine diseases, performed well in our hands with cell cancer lines. Beside its robustness and efficiency, the metabolite panel of NBS covers amino acids and acylcarnitines, which were shown to be altered in cancer. Already early reports revealed that valine, isoleucine, and glutamine are preferentially metabolized by tumor cells (Kovacevic and Morris, 1972; Wagle et al., 1963). Carnitines have been shown to reveal a protective effect on cisplatin mediated toxicity (Altun et al., 2009) and intracellular transport of carnitines is linked to apoptosis (Mazzarelli et al., 2007). Besides, carnitines play a vital role in energy production and fatty acid metabolism and a nutritional substitution is being explored for beneficial effects (Flanagan et al., 2010). With the NBS assay we were able to measure a panel of 42 metabolites (amino acids and acylcarnitines) in less than 3 min from a small sample volume. To our knowledge, the present study is the first that describes the effective usage of the NBS for cell culture purposes.

Comparing only the control groups (nontreated cells) we already observed significant differences in the basal metabolite levels between the four cell lines. For example, the highest concentration of glutamate was found in HepG2 cells. In case of neoplastic cells, glutamine is an important energy source and products of its metabolism include glutamate and glutathione, molecules that play a crucial role in tumor proliferation, invasiveness, and resistance to therapy (Szeliga and Obara-Michlewska, 2009). High concentrations of glutamate in HepG2 cells could therefore be due to increased proliferation of this cell line. To our knowledge, the highest concentration of glutamate in comparison with other control groups (HEK 293, PC3, and MCF7) was not reported before.

The current study shows that apoptosis compared to necrosis caused distinct and significant changes in concentration levels of seven metabolites (Asp, Glu, Met, Ala, and Gly, as well as C3-carnitine and C3DC-carnitine) in the human cell lines HEK 293, HepG2, PC3, and MCF7. Changes in concentration of glutamate and aspartate can be linked to disordered energy metabolism in apoptotic or necrotic cells (Rainaldi et al., 2008).

In case of necrosis metabolite levels mostly decreased drastically. This could be explained by the loss of structural integrity of necrotic cells and the associated dissipation of metabolites. The decrease in concentration of glutamate in necrotic cells is also corroborated by a report on cells examined with ^{1}H-NMR (Mirbahai et al., 2010). In reaction to staurosporine the change in metabolite concentrations in the analyzed cell lines was very individual. Only HEK293 and HepG2 responded mostly in the same manner while MCF7 and PC3 showed very unique behavior. This is in concordance with the different caspase 3 and 7 activities observed after apoptosis induction in the cells. The cell lines used in this work were derived from various different epithelia and the cells have therefore distinct metabolic activity. A different response to apoptosis is thus not unexpected and has to be considered in biomarker development.

Conclusions

To our knowledge, the present study is the first to describe the effective usage of NBS assay for metabolite measurements in cells. During this study we successfully adapted the NBS assay for monitoring of apoptosis in cell culture. Moreover, we demonstrated that measured metabolites could represent highly sensitive markers for genomic differences between cell lines (like the observed highest concentration of glutamate in HepG2 or C3-carnitine in HEK 293). Our observation clearly demonstrated differences between cell line-specific responses to stimuli like staurosporine. Additionally, we found that first changes in metabolite concentrations (e.g., upregulation in aspartate or glutamate) as soon as 4 h after treatment are characteristic for challenge type. Especially the aspartate and glutamate should be further examined in the context of general usage as new apoptosis biomarkers. This report gives a new light on NBS (and targeted metabolomics) assay as a robust method for metabolite measurements and theranostics. The potential of metabolomics in animal and human studies is yet to be demonstrated.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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