Use of the 1-mm micro-probe for metabolic analysis on small volume biological samples

Natalie J. Serkova a, Amy S. Freund b, Jaimi L. Brown a, Douglas J. Kominsky a, *

aBiomedical MRI/MRS Cancer Center Core, University of Colorado Health Sciences Center, Denver, CO, USA 
bBruker BioSpin Corporation, Billerica, MA, USA

Received: February 20, 2008; Accepted: August 1, 2008

Abstract

Endogenous metabolites are promising diagnostic end-points in cancer research. Clinical application of high-resolution NMR spectroscopy is often limited by extremely low volumes of human specimens. In the present study, the use of the Bruker 1-mm high-resolution TXI micro-probe was evaluated in the elucidation of metabolic profiles for three different clinical applications with limited sample sizes (body fluids, isolated cells and tissue biopsies). Sample preparation and 1H-NMR metabolite quantification protocols were optimized for following oncology-oriented applications: (i) to validate the absolute concentrations of citrate and spermine in human expressed prostatic specimens (EPS volumes 5 to 10 µl: prostate cancer application); (ii) to establish the metabolic profile of isolated human lymphocytes (total cell count 4 × 10^6: chronic myelogenous leukaemia application); (iii) to assess the metabolic composition of human head-and-neck cancers from mouse xenografts (biopsy weights 20 to 70 mg: anti-cancer treatment application). In this study, the use of the Bruker 1-mm micro-probe provides a convenient way to measure and quantify endogenous metabolic profiles of samples with a very low volume/weight/cell count.

Keywords: quantitative 1H-NMR ● micro-probe ● body fluids ● biopsy extract ● cell extract ● oncology

Introduction

Today, systems biology is rapidly developing in the various areas of biomedical research, including human oncology. So far, functional genomic strategies have largely centred on gene-expression studies (genomics and transcriptomics) or protein level (proteomics). In addition, the biochemistry of a tumour, especially glucose uptake and metabolism, is very different from that of a normal cell: mitochondrial metabolism is impaired and cytosolic glycolysis is elevated with a subsequent increase in glucose uptake (Warburg’s effect). In the past years, various specific metabolites have been reported to be associated with cancer development and progression, including citrate, myo-inositol, poly-unsaturated fatty acids (PUFA), nucleotides, phosphocholine and other cell membrane constituents [1]. This makes nuclear magnetic resonance (NMR) (1H, 31P and 13C NMR spectroscopy) one of the most valuable techniques to evaluate cancer metabolism and efficacy of the treatment since multiple metabolic pathways can be assessed simultaneously [2–5]. Understanding tumour-related processes through metabolic profiling has been widely used in the last decades to differentiate between different cancer cell lines and to monitor metabolic processes that occur in cancer cells during events such as apoptosis, down-stream-pathway regulation and enzyme abnormalities [6–8]. Despite the successful use of high-resolution NMR-based metabolic analysis in cell culture models as well as in human blood or urine, clinical application of NMR is often limited by the fact that this technology has rather low sensitivity, compared with other approaches. In the clinical setting, the volume of collected samples is rather low. For examples, fine needle aspirates, solid tumour biopsies or specific body fluids often do not exceed 10–20 µl volumes. For conventional high-resolution (liquid) methods, which are based on the use of 3–5 mm probes, this amount of study sample is below the limit of detection, not to mention their limits of quantification. Various novel technological advances are developed to overcome the ‘small volume sample’ problem [9], such as...
high-resolution magic angle spinning (HR-MAS) probes for solid state NMR, cryo-probes or micro-probes.

In the present article, we report of the use of Bruker 1-mm TXI (triple-resonance $^1$H/$^{13}$C/$^{31}$P inverse) micro-probe (Bruker Biospin, Billerica, MA, USA) for small volume biological samples. We present our sample preparation protocols and quantitative metabolic results for three different study designs, including small volume human body fluids, small volume human cell samples and small volume biopsy samples. All NMR spectra were obtained using high-resolution Bruker 500 MHz Avance and DRX spectrometers equipped with Bruker high-resolution inverse 1-mm TXI probes with or without automated tuning and matching (ATM) option.

**Experimental**

All human and animal studies were approved by the Institutional Review Board (IRB) of Human Research and the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado, Health Sciences Center, respectively.

**Sample preparation protocol on human prostatic fluids**

Five to 10 ml of prostatic fluids were collected after prostate massage and immediately put onto dry ice (−78.5°C). Thirty ml of deuterium oxide (D$_2$O) with ca. 0.03 wt.% 3-(trimethylsilyl)propionic-2,2,3,3,-d$_4$ acid sodium salt (TMSP, Aldrich, Milwaukee, WI, USA) was added to each sample, resulting in the final volume of 35–40 µl. Samples were centrifuged at 4000 × g for 10 min. (4°C) to remove proteins. Thirty µl of supernatant were transferred into a Bruker 1-mm glass capillary using 1-ml syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

**Cell isolation and acid extraction protocol for human lymphocytes**

Twenty millilitre whole blood was collected from healthy subjects into plastic heparin preserved serological tubes. Lymphocytes were isolated from the fresh whole blood by Ficoll gradient centrifugation using a Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA). Briefly, the blood was mixed with an equal amount (1:1 vol/vol) of balanced salt solution. In another centrifuge tube, an appropriate amount (3:4 vol/vol of blood/salt mixture) of Ficoll-Paque Plus solution was added to the bottom of a centrifuge tube. The blood/salt mixture was carefully layered on top of the Ficoll-Paque Plus and centrifuged at 4000 × g for 40 min. at 20°C. The top layer of plasma was removed. The layer of lymphocytes was transferred to a new centrifuge tube and re-suspended in three-times volume of balanced salt solution. The cells were centrifuged at 60 × g for 10 min. at 20°C to wash the leucocytes and remove platelets. Isolated lymphocytes in the supernatant (4 × 10$^6$ cells) were subsequently re-suspended in 10 ml of RPMI 1640 medium containing 5 mMol [1-15C] labelled glucose and 10% foetal bovine serum (FBS) and incubated at 37°C for 4 hrs. The lymphocytes were washed twice with 1 ml ice-cold isotonic NaCl, centrifuged (5 min. at 400 × g and 4°C) and frozen in liquid nitrogen. The cells were then extracted with 2 ml of ice-cold PCA (12%) [10]. The samples were centrifuged (15 min. at 1300 × g and 4°C) and the aqueous phase was removed and neutralized (pH 5.7) using KOH. The samples were centrifuged again and lyophilized overnight. The lyophilized leucocytes extracts were dissolved in 30 µl D$_2$O with TMSP and centrifuged at 8000 × g for 5 min. at 4°C. The supernatants were transferred into Bruker 1-mm glass capillaries using 1-ml syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

**Methanol/chloroform and acid extraction protocols for tissue biopsies**

Two human head-and-neck squamous cell carcinoma (HNSSC) cell lines – UMSSC2 and HN31 – were used in a nude mouse xenograft model. After 28 days, tumour xenografts were harvested, immediately frozen in liquid nitrogen and subsequently underwent dual methanol/chloroform or acid extraction for protein precipitation and separation of water-soluble from lipid metabolites. The tumour mass was between 20 and 80 mg per sample. Two extraction protocols were tested: a dual methanol/chloroform extraction for HN31 tumours and a 12% perchloric acid extraction for UMSSC2 tumours. Methanol/chloroform extraction: Frozen tissues were weighed, powdered in the presence of liquid nitrogen and homogenized in 0.5 ml of ice-cold methanol. Ice-cold chloroform (0.5 ml) was added and samples were vortexed. Additional 0.5 ml of ice-cold water was added to the samples and vortexed. The samples were kept at 4°C overnight. The samples were centrifuged for 30 min. at 1400 × g at 4°C. Upper methanol phase (water soluble extract) was transferred into a lyophilizing glass and freeze-dried overnight. Lipids in the lower organic phase were evaporated to dryness under a stream of nitrogen or, alternatively, using a vacuum speed centrifuge. Perchloric acid extract: Again, frozen tissues were weighed, powdered in the presence of liquid nitrogen and homogenized in 2 ml ice-cold 12% PCA. The extracts were then vortexed and centrifuged at 1300 × g for 10 min. at 4 degree. The supernatant was transferred to a new centrifuge tube and 1 ml ice-cold 12% PCA was added to the pellet, vortexed and centrifuged again. The supernatants were then combined and neutralized with 1.0M KOH to a pH of 7.2. The extracts were centrifuged again at 1300 × g for 10 min. at 4 degree. The supernatant (water-soluble phase) was transferred into a lyophilizing glass and freeze-dried overnight. The pellets (lipid fraction) were re-dissolved in 2 ml water, the pH was adjusted to 7.2 and freeze-dried overnight. All dried water-soluble extracts were dissolved in 40 µl D$_2$O with TMSP and centrifuged at 8000 × g for 5 min. at 4°C. The lipid extracts were dissolved in 4 µl of deuterated chloroform + 0.03 wt.% TMSP/deuterated methanol mixture (2:1 vol/vol). The re-dissolved water-soluble and lipid extracts were transferred into Bruker 1-mm glass capillaries using 1-ml syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

**NMR experiments**

All NMR experiments were performed on Bruker 500 MHz spectrometers (operating frequency 500.15 MHz) using Bruker 1-mm high-resolution inverse TXI ($^1$H/$^{13}$C/$^{31}$P) Z-gradient micro-probes (Bruker BioSpin, Billerica, MA, USA). The samples were maintained at 287 K, as measured...
with a thermocouple internal system. Field homogeneity was achieved by coil-shimming using 1D water pre-saturation experiment in interactive mode as control. To assist 1H-NMR peak assignment and metabolite identification in expressed prostatic specimen (EPS), leucocyte extracts and biopsy extracts, two-dimensional gradient (2D)-H/H-COSY (correlation spectroscopy) and (2D)-H/C-HSQC (heteronuclear single quantum correlation) NMR techniques were used for metabolite identification. The COSY acquisition parameters were: standard Bruker pulse sequence ‘cosygpqf’; ns = 64 scans across 256 increments with ds = 16 dummy scans, spectral width SW (F1) = SW (F2) = 6666 Hz and TD = 2048 data points; using 90 degree pulse and recovery delay of d1 = 1.5 sec. HSQC spectra were also acquired, with a standard Bruker pulse sequence ‘hsqcefgp’, with 512 increments (echo/anti-echo) and ns = 320 and ds = 16 scans per increment; SW (F1, 13C) = 17,608 Hz and SW (F2, 1H) = 5000 Hz; TD = 2048 data points; using 90 degree pulse and a recovery delay of 1 sec. All spectra were Fourier transformed and lactate (Lac3, CH3) was used as an internal chemical shift reference for both carbon (21 ppm) and proton (1.32 ppm) axes. Methanol was used as an internal chemical shift

---

**Fig. 1** $^1$H-NMR spectrum of 20 mM metabolite solution in D$_2$O supplemented with TMSP (ca. 0.05 wt%) for precise TMSP concentration calculations.

**Table 1** TMSP concentration calculations based on the 1H-NMR spectrum of 20 mM metabolite solution

|   | Start(ppm) | Start(Hz) | End(ppm) | End(Hz) | Integral | TMSP Mean ± S.D. |
|---|-----------|-----------|----------|---------|----------|-----------------|
| Inositol 1 | 4.1091 | 2055.53 | 4.035 | 2018.49 | 959.3 | 2.08 ± 0.07 |
| Creatine 2 | 3.9808 | 1991.35 | 3.9155 | 1958.7 | 2010.26 | 1.99 |
| Alanine 1 | 3.818 | 1909.92 | 3.7425 | 1872.14 | 897.35 | 2.23 |
| Inositol 2 | 3.6736 | 1837.66 | 3.5922 | 1796.94 | 1919.22 | 2.08 |
| Inositol 2 | 3.5782 | 1789.97 | 3.513 | 1757.33 | 1885.62 | 2.12 |
| Inositol 1 | 3.3282 | 1664.9 | 3.2512 | 1626.38 | 925.94 | 2.16 |
| Creatine 3 | 3.0899 | 1545.69 | 3.0034 | 1502.4 | 2984.37 | 2.01 |
| Citrate 2 | 2.7093 | 1355.32 | 2.6199 | 1310.57 | 1864.53 | 2.15 |
| Citrate 2 | 2.5949 | 1298.1 | 2.5128 | 1257.02 | 1858.63 | 2.15 |
| Alanine 3 | 1.5251 | 762.94 | 1.443 | 721.86 | 2813.2 | 2.13 |
| TMSP 9 | 0.0301 | 15.04 | 0 | 10.64 | 900 |

Alanine, citrate, creatine and myo-inositol (each 20 mM) were used to calculate the final concentrations of TMSP in D$_2$O in this experiment. $^1$H-NMR spectrum is presented in Fig. 1.
reference for lipid spectra (3.35 ppm). For metabolite quantification, one-dimensional ¹H-NMR spectra were obtained from each sample, with a standard water pre-saturation pulse program ‘zgpr’. The total number of acquisitions varied from 40 to 128, depending on the sample volume, with 2 scans were collected into TD = 32K data points, resulting in total acquisition time of 10–32 min. Conventional ¹H acquisition parameters were: power level pl1 = 20 dB; power angle p1 = 6.3 msec (90 degree pulse); power level for water pre-saturation pl9 = 77 dB; water suppression at O1P = 4.76 ppm; spectral width SW = 5000 MHz; and the pulse delay of 12.75 sec. (calculated as 5*T1) was applied between acquisitions for fully relaxed ¹H-NMR spectra. The external standard TMSP was used as a chemical shift reference (0 ppm).

In addition, for precise calculation of TMSP concentrations in D₂O, we recorded ¹H-NMR spectra of the 20 mM metabolite mixture in 40 µl D₂O with TMSP. The ¹H-NMR acquisition parameters were identical to the experimental set-up with the study samples, the number of transients for metabolite/ D₂O/ TMSP standard solutions was ns = 40.

Spectral analysis and metabolite quantification

All 2D data were processed using XWINNMR 3.5 or TopSpin software. Tissue metabolites were identified based on the results from our chemical
shift database and/or referred to the Human Metabolome Database from the University of Alberta (http://www.hmdb.ca/). After performing Fourier transformation (with line broadening LB/H110050.2 Hz) on one-dimensional 1H-NMR spectra and making phase and baseline corrections, each identified 1H peak was integrated using Bruker 1D WINNMR program. The final TMSP concentration in the capillary was calculated prior to metabolite calculations in the study extracts using a 20 mM metabolite standard solution. TMSP concentrations were calculated based on NMR intensities (from the fully relaxed 1H-NMR spectra) of each metabolite according to equation (1) (Fig. 1) and the final concentration of TMSP (given as mmol/ml) represented a mean value (Table 1).

$$C = \frac{20\text{mM} \times I_{\text{tmsp}} \times N_{\text{met}}}{I_{\text{tmsp}} \times N_{\text{tmsp}}}$$  \hspace{1cm} (1)$$

where
- $C$ = TMSP concentration
- $I_{\text{tmsp}}$ = integral of $^1\text{H}$ TMSP peak at 0 ppm
- $N_{\text{tmsp}}$ = number of protons in $^1\text{H}$ TMSP peak ($N = 9$)
- $I_{\text{met}}$ = integral of $^1\text{H}$ peak of a metabolite
- $N_{\text{met}}$ = number of protons in $^1\text{H}$ peak of a metabolite
- 20 mM = metabolite concentration in the standard solution

The absolute concentrations of single endogenous metabolites in the study sample were then referred to the TMSP integral and calculated according to the equation (2):

$$C_x = \frac{I_x \cdot N_x \cdot C}{1:9} \times \frac{I}{V \cdot M_{\text{sample}}}$$  \hspace{1cm} (2)$$

where
- $C_x$ = metabolite concentration
- $I_x$ = integral of endogenous metabolite $^1\text{H}$ peak
- $N_x$ = number of protons in metabolite $^1\text{H}$ peak (from CH, CH2, CH3, etc.)
- $C$ = TMSP concentration (see above for TMSP concentration calculation)

$V$ = total volume of the sample with D2O (0.02 ml)

Accordingly, all metabolite concentrations are given as mean ± S.D. from multiple experiment sets as [µmol/ml] for all EPS samples and as [µmol/g] for cell and tissue extracts.

### Results and discussion

**Metabolic analysis on human prostatic fluids: potential application for prostate cancer research**

Total of nine randomized EPS samples from healthy male volunteers were analysed by $^1$H-NMR using a Bruker 1-mm TXI micro-probe. The sample volume was between 5 and 10 µl. We compared the signal to noise ratios of the CH2-signal of citrate at 2.65 ppm in an EPS sample using a 1-mm TXI micro-probe versus a conventional 5-mm TXI probe. The signal to noise ratio for 10 µl EPS in 500 µl D2O (1 scan, line-broadening 0.2 Hz applied) using 1-mm TXI micro-probe was 35:1. The signal to noise ratio for 10 µl EPS in 30 µl D2O (1 scan, line-broadening 0.2 Hz applied) was 7:1. Even though the same amount of the metabolite was analysed, the signal to noise ratio was significantly decreased because of a dilution factor. In addition, the use of the 1-mm TXI micro-probe also facilitated a better solvent suppression.

All men were presumed cancer-free at the time of EPS collection. Using two-dimensional HSQC and COSY spectra, the

| Table 2 Absolute concentrations [µmol/ml] of endogenous metabolites in human EPS fluids from healthy male volunteers calculated from $^1$H-NMR spectra |
|-----------------------------------------------|
| EPS #1 | Alanine | Citrate | Inositol | Lactate | PCholine | Spermine | Valine |
| 1.65   | 270.51  | 19.18   | 2.01     | <LLQ    | 47.83    | 11.59    |
| 1.86   | 376.63  | 16.92   | 2.27     | 0.55    | 79.92    | 19.54    |
| 1.50   | 339.20  | 17.16   | 1.10     | <LLQ    | 50.09    | 15.34    |
| 0.10   | 764.47  | 41.85   | 0.57     | 0.39    | 168.24   | 37.70    |
| 0.82   | 326.86  | 21.24   | 1.26     | 0.19    | 36.62    | 23.36    |
| 3.29   | 309.93  | 18.72   | 3.61     | 0.14    | 56.88    | 18.66    |
| 2.27   | 125.88  | 11.87   | 2.07     | 0.16    | 22.84    | 17.97    |
| 0.47   | 292.34  | 17.36   | 0.28     | 0.24    | 53.26    | 7.72     |
| <LLQ   | 161.59  | 7.70    | <LLQ     | <LLQ    | 18.87    | 10.97    |

Volume of EPS samples was between 5 and 10 µl, and diluted with D2O to the final volumes of 30–40 µl (based on the ‘sensitive volume’ and the lowest limit of quantification of the TXI micro-probe).

Abbreviations: LLQ, low limit of quantification (0.10 µmol/ml)

The intra-sample variation (multiple sample preparations from the aliquots of the same sample) yielded error of ±5% [17, 18].
peaks for alanine, citrate, myo-inositol, lactate, phosphocholine, spermine, valine were identified and quantified on one-dimensional $^1$H-NMR spectra (Fig. 2A and B). Specifically, the ‘normal’ EPS profile had pronounced NMR peaks for the amino acid citrate (concentrations range 161.6 to 764.5 µmol/ml), as well as the polyamine spermine (18.9 to 168 µmol/ml) and the osmolyte myo-inositol (7.7–42 µmol/ml), and diminished peaks for alanine, lactate, phosphocholine and valine. There was some age-dependency for higher concentration ranges of citrate and spermine corresponding to younger subjects – a tendency which will be confirmed and validated in a large ongoing clinical study.

In prostate cancer research, previous ex vivo as well as in vivo NMR / MRSI studies have demonstrated a linear correlation between the pathological Gleason and the magnitude of the decrease of citrate and the elevation of choline in prostatic gland [11]. The advantage of using EPS versus prostate biopsies for cancer detection and characterization would be that the sampling procedure for EPS is relatively non-invasive, and can be performed multiple times without the risk of bleeding or infection. Moreover, EPS analysis may lead to a more ‘global’ sample of the prostate gland relative to the ‘hit-or-miss’ approach of random biopsy sampling. One of the shortcomings for clinical use of
1H-NMR-based metabolic assay on human EPS was a low-volume sample size. Use of a high-resolution Bruker 1-mm TXI micro-probe allows for precise 1H-NMR metabolite quantification in body fluid samples as small as 5 µl with minimum non-destructive sample preparation and total acquisition time of 32 min. We aim to apply this method for metabolite validation in healthy, benign hyperplasia and prostate cancer patients with various Gleason scores.

**Fig. 4** Representative NMR spectra from human HNSCC xenograft extracts: (A) one-dimensional 1H-NMR on the lipid fraction of HN31 xenograft biopsy (25 mg); (B) one-dimensional 1H-NMR on the water-soluble fraction of HN31 xenograft biopsy (33 mg); and (C) two-dimensional H,H-COSY on UMSCC2 xenograft extract (biopsy size 55 mg).

Peak assignment: (1) cholesterol; (2) CH₃-total fatty acids; (3) (CH₂)ₙ-total fatty acids; (4) CH₂-total fatty acids; (5) poly-unsaturated fatty acids (PUFA); (6) phospholipids; (7) triacylglycerol (TAG); (8) mono-unsaturated fatty acids (with PUFA and TAG); (9) valine, leucine, isoleucine; (10) lactate; (11) alanin; (12) CH₃-acetyl groups; (13) glutamate; (14) succinate; (15) glutamine; (16) glutathione; (17) aspartate; (18) creatine, phosphocreatine; (19) taurine; (20) glycero phosphocholine; (21) phosphocholine; (22) glycine; (23) myo-inositol; (24) glucose.

**Metabolic analysis of isolated human lymphocytes: chronic myelogenous leukaemia application**

Chronic myelogenous leukaemia (CML) has served as a prototype neoplasm for basic research as well as for clinical studies designed to develop curative cancer treatment. Beside their genetic instability (Philadelphia chromosome) and molecular abnormality (activity of BCR-Abl oncoprotein), human CML cells show an abnormal
Table 3 Absolute metabolite concentrations (µmol/g) calculated from 1H-NMR spectra (Fig. 4A and B) from HNSCC biopsy extracts (both water-soluble and lipid fractions) using Bruker 1-mm TXI micro-probe

| Metabolite                        | NH31 (n = 4) | UMSCC2 (n = 3) |
|-----------------------------------|-------------|---------------|
| CH3-Acetyl groups (peak 12)       | 9.25 ± 0.34 | 4.99 ± 0.61   |
| Alanine (11)                      | 3.12 ± 1.78 | 1.62 ± 0.42   |
| Aspartate (17)                    | 0.33 ± 0.15 | 0.32 ± 0.15   |
| Cholesterol (1)                   | 4.43 ± 0.56 | 3.12 ± 1.08   |
| Creatine-PCreatine (18)           | 1.65 ± 0.91 | 1.14 ± 0.09   |
| Total fatty acids (2)             | 91.00 ± 3.25| 77.24 ± 4.99  |
| Glucose (24)                      | 1.46 ± 0.49 | 0.47 ± 0.18   |
| Glutamate (13)                    | 6.24 ± 2.43 | 3.56 ± 0.61   |
| Glutamine (15)                    | 0.53 ± 0.22 | 1.37 ± 0.05   |
| Glutathione (16)                  | 0.68 ± 0.24 | 1.30 ± 0.25   |
| Glycine (22)                      | 0.43 ± 0.19 | 1.30 ± 0.08   |
| Glycerophosphocholine (20)        | 0.44 ± 0.05 | 0.20 ± 0.03   |
| myo-Inositol (23)                 | 0.31 ± 0.12 | 0.76 ± 0.65   |
| Lactate (10)                      | 5.66 ± 1.02 | 7.51 ± 1.77   |
| Mono-unsaturated fatty acids (8)  | 3.23 ± 0.78 | 1.22 ± 0.33   |
| Phosphocholine (21)               | 0.94 ± 0.21 | 2.15 ± 1.30   |
| Phospholipids (6)                 | 13.21 ± 1.02| 7.11 ± 1.77   |
| Poly-unsaturated fatty acids (5)  | 5.25 ± 0.66 | 3.23 ± 0.89   |
| Succinate (14)                    | 1.94 ± 0.46 | 1.26 ± 0.10   |
| Taurine (19)                      | 2.61 ± 0.17 | 2.58 ± 0.17   |
| Triacylglycerol (7)               | 9.23 ± 1.07 | 6.23 ± 1.01   |
| Valine, Leucine, Ile (9)          | 3.90 ± 0.23 | 4.06 ± 0.06   |

The data are given mean ± S.D. with n = 4 for NH31 and n = 3 for UMSCC2 cells. All metabolite assignments were made based on two-dimensional NMR experiments.

Metabolic analysis on human head-and-neck cancer biopsies: potential application for anti-cancer therapies

Metabolic cancer markers can be assessed not only in tumour cells in vitro, but can be quantified in human biopsies ex vivo. Unfortunately, the tumour mass that can be obtained during clinical biopsy sampling or from the orthotopic or xenograft animal models is usually very limited. In this study, we analysed 20–70 mg human HNSCC biopsies from mouse xenograft models. Metabolite quantification was not possible using a 5-mm conventional probe due to low signal-to-noise ratios (below 3:1) and low spectral resolution. Using a 1-mm TXI micro-probe for methanol/chloroform or acid extracts, high-quality one-dimensional 1H-NMR, Fig. 4A and B) and two-dimensional (COSY, Fig. 4C) spectra were obtained. Absolute concentrations of endogenous metabolites from two different HNSCC tumour types, calculated from 1H-NMR spectra (64 scans for water-soluble and 40 scans for lipid extracts), are presented in Table 3. Important markers, such as phosphocholine (NH31: 0.94 ± 0.21 µmol/g; UMSCC2: 2.15 ± 1.30 µmol/g), glycerophosphocholine (0.24 ± 0.05 and...
0.20 ± 0.03 µmol/g), glucose (1.46 ± 0.49 and 0.47 ± 0.18 µmol/g), lactate (5.66 ± 1.02 and 7.51 ± 1.77 µmol/g) and glutathione (0.68 ± 0.25 and 1.30 ± 0.25 µmol/g) were easily detected and quantified using the 1-mm TXI micro-probe. Both tumour types are EGFR over-expressed head-and-neck tumours. Since a lot of attention to targeting of down-stream pathways in cancer cells has been shown in the last 5 years [14], currently we are investigating metabolic consequences of targeting EGFR in HN31 and UMSCC2 tumours using the same xenograft model and NMR approaches.

Conclusions

The use of a Bruker 1-mm TXI micro-probe provides a more convenient way to measure and quantify samples with a very small volume/weight/cell count in biological samples. The micro-probe allows for a remarkable increase of the signal to noise ratio and therefore a significant decrease in the experiment time while improving spectral resolution and solvent suppression. The signal overlap – one of the major limiting factors in NMR-based metabolomics – was in the same range as for conventional 5-mm NMR probes and can be further addressed by applying modern NMR sequences [15]. Alternatively, spectral segmentation for multivariate analysis (PCA, PLS-DA) can be performed in a similar way as for conventional NMR probes, to overcome individual metabolite separation and identification [16]. In the present study, this first capillary NMR probe for discrete samples was applied for three different kinds of biological samples and may provide useful future applications:

1. 1H-NMR on small volume biofluids (e.g. prostatic fluids), after an appropriate validation of prostate-specific metabolites, e.g. citrate, inositol and spermine, may serve as an alternative non-invasive assay for early detection of prostate cancer;
2. 1H-NMR spectroscopy on low cell number extracts from small blood volumes can be used as a clinical assay for early metabolic detection of CML and treatment failure in CML patients;
3. 1H-NMR-based quantitative assessment on small volume tumour biopsies may be used to metabolically monitor the efficacy of anti-tumour and anti-angiogenic treatment and therefore improve the treatment regiments with expensive targeted agents.

Acknowledgements

The studies were supported through the grants from the National Cancer Institute (NCI R21 CA108624) and the Department of Defense (DOD PC041000). We appreciate the help of all our clinical and basic science collaborators for helping us in obtaining biological samples.

References

1. Griffin JL, Shocker JP. Metabolic profiles of cancer cells. Nat Rev Cancer. 2004; 4: 551–61.
2. Leibfritz D. An introduction to the potential of 1H-, 31P- and 13C-NMR-spectroscopy. Anticancer Res. 1996; 16: 1317–24.
3. Mountford CE, Doran S, Lean CL, et al. Cancer pathology in the year 2000. Biophys Chem. 1997; 68: 127–35.
4. Arias-Mendoza F, Zakian K, Schwartz A, et al. Methodological standardization for a multi-institutional in vivo trial of localized 31P MR spectroscopy in human cancer research. In vitro and normal volunteer studies. NMR Biomed. 2004; 17: 382–91.
5. Evelhoch JL, Gillies RJ, Karczmar GS, et al. Applications of magnetic resonance in model systems: Cancer therapeutics. Neoplasia. 2000; 2: 152–65.
6. Hakumaki JM, Kauppinen RA. 1H NMR visible lipids in the life and death of cells. Trends Biochem Sci. 2000; 25: 357–62.
7. Griffiths JR, Stubbs M. Opportunities for studying cancer by metabolomics: preliminary observations on tumors deficient in hypoxia-inducible factor 1. Adv Enzyme Regul. 2003; 43: 67–76.
8. Giunde K, Roman V, Mori N, et al. RNA interference-mediated choline kinase suppression in breast cancer cells induces differentiation and reduces proliferation. Cancer Res. 2005; 65: 11034–43.
9. Martinez-Granados B, Monleon D, Martinez-Bisbal MC, et al. Metabolite identification in human liver biopsies by high-resolution magic angle spinning 1H NMR spectroscopy. NMR Biomed. 2006; 19: 90–100.
10. Gottschalk S, Anderson N, Hainz C, et al. Imatinib (STI571)-mediated changes in glucose metabolism in human leukemia BCR-ABL positive cells. Clin Cancer Res. 2004; 10: 6661–8.
11. Kurbanevicz J, Swanson MG, Nelson SJ, et al. Combined magnetic resonance imaging and spectroscopic imaging approach to molecular imaging of prostate cancer. J Magn Reson Imaging. 2002; 16: 451–63.
12. Franks SE, Smith MR, Arias-Mendoza F, et al. Phosphonoester concentrations differ between chronic lymphatic leukemia cell and normal human lymphocytes. Leukemia Res. 2002; 26: 919–26.
13. Serkova N, Boros LG. Detection of resistance to imatinib by metabolic profiling. Am J Pharmacogenomics. 2005; 5: 293–302.
14. Chung YL, Troy H, Banerji U, et al. Magnetic resonance spectroscopic pharmacodynamic markers of the heat shock protein 90 inhibitor 17-allylamino, 17-demethoxygeldanamycin (17AAG) in human colon cancer models. J Natl Cancer Inst. 2003; 95: 1624–33.
15. Stoyanova R, Nicholls AW, Nicholson JK, et al. Automatic alignment of individual peaks in large high-resolution spectra data sets. J Magn Reson. 2004; 170: 329–35.
16. Serkova NJ, Rose JC, Epperson LE, et al. Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. Physiol Genomics 2007; 31: 15–24.
17. Serkova NJ, Zhang Y, Coatey JL, et al. Early detection of graft failure using the blood metabolic profile of a liver recipient. Transplantation. 2007; 83: 517–21.
18. Serkova NJ, Gamito EJ, Jones RH, et al. The metabolites citrate, myo-inositol, and spermine are potential age-dependent markers of prostate cancer in human expressed prostatic secretions. Prostate. 2008; 68: 620–8.