Metallomics

PAPER

KRAS NF-κB is involved in the development of zinc resistance and reduced curability in prostate cancer

Cite this: *Metallomics*, 2014, **6**, 1240

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Received 28th February 2014,
Accepted 6th May 2014

DOI: 10.1039/c4mt00065j

www.rsc.org/metallomics

Zinc(II) ions are important components of many proteins and are involved in numerous cellular processes such as apoptosis or drug resistance. Prostate cancer has a unique relationship with zinc(II) ions. However, the relationship was examined only in short-term zinc(II) treatments. Therefore, the aim of this study was to create zinc-resistant prostatic cell lines at various stages of the disease (22Rv1 and PC-3) and a normal prostate epithelium (PNT1A) using a long-term zinc exposure. Consequently, the expression profile of the following genes was analyzed: *BAX*, *Bcl-2*, *Beclin-1*, *CFLAR*, *HIF1α*, *KRAS*, *mTOR*, *MT1A*, *MT2A*, *NF-κB1*, *p53*, *survivin*, *ZIP1*, *ZnT-1*. The resistance was verified using the MTT test; on average a 1.35-fold lower zinc(II) toxicity (higher IC_{50}) was determined in zinc(II)-resistant cells. The associated resistance to cisplatin was also determined; IC_{50} for cisplatin was 1.52-fold higher. With regard to the gene expression profiles, our results indicate that differential mechanisms participate in the short-term zinc toxicity regulation and long-term resistance; the short-term treatment was associated with *MT2A* ($p < 0.001$), *ZnT-1* ($p < 0.001$), and *MT1A* ($p < 0.03$) and the long-term resistance was associated particularly with *NF-κB1* ($p < 0.001$), *CFLAR* ($p < 0.001$), *KRAS* ($p < 0.001$), p53 ($p < 0.002$), *survivin* ($p = 0.02$), *ZIP1* ($p = 0.002$), *BAX* ($p = 0.005$), and *HIF1α* ($p = 0.05$). Therefore, the *KRAS-PI3K-NF-κB* pathway is expected to play a crucial role in the regulation of zinc resistance. In summary, compared to previous studies, identical mechanisms of resistance were demonstrated on multiple cell lines, both non-tumor and tumorous, derived both from primary and advanced secondary sites.

1 Introduction

Zinc(II) ions represent an essential nutrient and an important constituent of many metalloenzymes, transcription factors, and other key proteins. It is estimated that up to 10% of all human genes encode proteins containing a domain capable of interacting with these ions.[1] Zinc plays a crucial role in cellular processes such as oxidative stress, programmed cell death, cell differentiation and proliferation. It also participates in the regulation of DNA synthesis and mitosis by transmitting cellular signals.[2] Approximately 50% of intracellular zinc is localized in the cytosol and cytosolic organelles, 30–40% in the nucleus, and the rest is associated with the cell membranes.[3] The cellular concentrations of zinc are heavily regulated since disruption of the zinc homeostasis may have serious pathological consequences.

The prostate gland accumulates an unusually high amount of zinc (150 μg of zinc per 1 g of wet tissue compared with 20–50 μg g^{-1} in the other organs).[4] Such a substantial zinc accumulation requires integration of multiple zinc importing and exporting systems. A significant role in maintaining zinc homeostasis is carried out by zinc transporters, as zinc ions are hydrophilic and do not penetrate through the cell membranes by passive diffusion.[5] Three ZIP (Zrt–Irt like protein or zinc iron permease) proteins seem to be important in the prostate as zinc influx transporters involved in the special capability of prostate cells to possess high cellular zinc concentrations.[6] The expression of all three transporters is down-regulated in adenocarcinomatous prostate glands, which have lost the capability to accumulate zinc.[6b] Zinc concentrations found in the malignant prostate tissue tend to be 75% lower than those in the normal prostate.[6a,7]

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The ZnT transporter proteins (solute-linked carrier 30 (SLC30A)) reduce intracellular zinc levels by ensuring zinc efflux from the cells or zinc uptake into intracellular vesicles or organelles.[8] Most ZnT proteins occur in intracellular compartments (associated with the endoplasmic reticulum, Golgi apparatus, or endosomes). ZnT-1 seems to be the only ZnT transporter occurring within the plasma membrane, which is in concordance with its role of the primary regulator of cellular zinc efflux.[9]

A high zinc concentration in the normal prostate inhibits citrate oxidation through the Krebs cycle and results in the accumulation of citrate in prostatic fluids.[10] Conversely, in the transformed prostate cells, the zinc concentration is very low, citrate oxidation can take place, ATP is generated and provides an unrestricted energy flow for the growth of malignant cells.[11] At the same time, as the intracellular zinc levels diminish, the apoptogenic impact of zinc wanes, hence the proliferation of malignant cells is supported.[11a]

High zinc concentrations have been shown to be cytotoxic for prostate cancer cells,[7,12] which implies that the sustenance of cellular zinc homeostasis is not only the requirement of healthy cells, but it is important for malignant cells as well, especially if the ability to cope with the high zinc concentration present in healthy cells can be impaired in cancer cells. However, the effects of zinc on the prostatic cell lines were examined only by relatively brief exposures in up to date studies (the longest observation period being 7 weeks in the study published by Wong *et al.*[13]).

In order to examine the general role of zinc in cell death regulation and homeostasis maintained in the prostate cell lines, we established PC-3, 22Rv1, and PNT1A cell lines resistant to the long-term (at least 3 months of cultivation), high zinc concentrations in the medium. PC-3 is an androgen-independent and p53 null metastatic prostate cancer cell line. The 22Rv1 cell line represents a model of partially androgen-sensitive p53 expressing prostate cancer, and the PNT1A cell line is a normal prostate cell line model. The use of markedly dissimilar prostate cancer cell lines allowed us to inspect the effect of zinc resistance irrespective of underlying pathways and the grade of transformation. The selection of studied genes was adapted to cover all important pathways that affect cancer cell behavior. We focused on zinc homeostasis (*ZnT-1*, *ZIP1*, *MT1A*, *MT2A*), cell proliferation (*NF-κB1*, *KRAS*), autophagy (*Beclin-1*, *mTOR*), apoptosis (*BAX*, *Bcl-2*, *CFLAR*, *survivin*, *p53*), and redox regulation (*HIF1α*). The aim of our study was to describe the expression profiles of the newly created cell lines and to elucidate changes in their cancerous and malignant potential and curability.

2 Results

2.1 Cell viability analysis

First of all, we prepared prostatic cell lines continuously viable at high zinc(II) concentrations. Half-maximal inhibition concentration (IC_{50}) for zinc(II) of these long-term-treated zinc-resistant cells was determined and compared with the IC_{50} of wild-type (short-term zinc-treated) cells. Prostatic cell lines used for this experiment included non-tumor PNT1A and tumorous 22Rv1 and PC-3. In the previous experiments, the IC_{50} for zinc of these wild-type cell lines was determined as follows: 150.8 μM, 369.1 μM and 55.5 for PNT1A, 22Rv1, and PC-3, respectively.[12c,14] These concentrations for these particular cell lines were further designated as 1-fold baseline IC_{50}. Through a positive selection of zinc resistant cells, we created cell lines continuously viable at concentrations exceeding IC_{50} for zinc(II). The following concentrations of long-term treatments were used: 150 μM, 300 μM and 450 μM for PNT1A, 400 μM, 800 μM and 1200 μM for 22Rv1 and 50 μM, 100 μM and 150 μM for PC-3 (1-, 2- and 3-fold wild-type IC_{50}). The resistant cells were able to divide and grow at zinc concentrations that were even higher than a triple of the standard IC_{50}. A minimum duration of the zinc treatment required for the achievement of a 3-fold IC_{50} resistance was 3 months. The process of selecting the resistant cell lines is illustrated in Fig. 1.

The effect of zinc resistance was verified using MTT analysis. The viability of the ''zinc resistant'' cell lines after 24 h exposure of up to 2 mM zinc(II) ions was determined (Table 1). The IC_{50} values of zinc resistant cells were on average 1.2- ($p = 0.0002$), 1.4- ($p = 0.18$) and 1.4- ($p < 0.0001$) fold higher in PNT1A, 22Rv1 and PC-3, respectively as compared to the wild type cell lines.

In order to demonstrate that the long-term zinc(II) treatment induces resistance not only to zinc(II) ions, but also to cytostatics and thus that zinc resistance is associated with worse curability, IC_{50} was determined for cisplatin in the zinc-resistant cells and compared with WT cells. For a detailed MTT analysis of WT cells, see Gumulec *et al.*[15] Resistant cells markedly increased their IC_{50} for cisplatin: by 1.4-, 1.6-, and 1.6-fold for PNT1A, 22Rv1, and PC-3, respectively.

2.2 Expression analysis

The expression of all studied genes was related to the expression of the specific gene expression of the PNT1A cell line not exposed to zinc. Expression levels of the other cell lines and zinc(II) concentrations are expressed as a fold change of the PNT1A gene expression. For summary see Fig. 2.

To characterize the expression profile of zinc-resistant cell lines, the effect of zinc concentration, cell line, and zinc resistance was analyzed separately after the adjustment of all other variables using multivariate ANOVA (*i.e.* when analyzing the effect of zinc concentration, the effects of cell line and resistance were adjusted). The following genes were selected: for zinc transporters *ZnT-1* and *ZIP1*, zinc-binding metallothioneins *MT1A* and *MT2A*, regulators *p53*, *mTOR*, *HIF1α*, *KRAS*, and *NF-κB1*, and effectors of programmed cell death and autophagy (*Beclin-1*, *CFLAR*, *survivin*, *Bcl-2*, *BAX*).

There was a significant effect from the zinc concentration, $F(42, 12.63) = 4.15$, $p = 0.004$ and cell line, $F(28, 8) = 37.00$, $p < 0.001$ and no significant effect of zinc resistance on the expression of all genes assessed together. Despite this fact, zinc resistance affected the expression of a particular set of genes. More detailed results will be discussed in the following sections.

2.2.1 Effect of zinc(II) concentration. The effect of zinc concentration as a categorical factor was analyzed after the adjustment of all other variables using multivariate ANOVA.

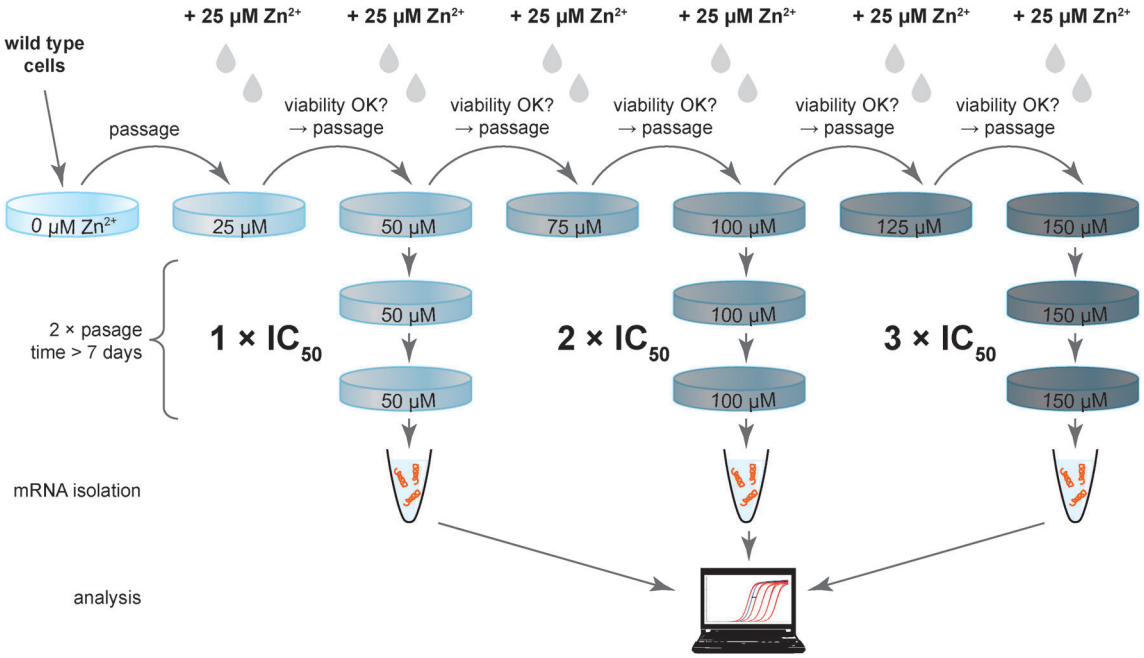

Fig. 1 Preparation of "resistant" cell lines from the wild type common cells. Concentrations mentioned in this figure reflect protocol for the resistant PC-3 cell line. For details regarding the concentrations used for the PNT1A and 22Rv1 preparation see the Experimental section. Viability was controlled before each passage and the time of the cultivation varied depending thereon. The least time required for gaining the resistance was three months.

Table 1 Analysis of half-maximal inhibition concentration (IC_{50}) of zinc

Factor	Cell line	Zinc(II) IC_{50} (μMμ)	Cisplatin IC_{50} (μMμ)
Wild type	PNT1A	150.8	44.0
	22Rv1	369.1	30.8
	PC-3	55.5	74.9
Zinc resistant	PNT1A	177.2 ± 10.9[a]	60.7 ± 19.4
	22Rv1	514.2 ± 204.0[c]	50.0 ± 31.0
	PC-3	83.0 ± 4.7[b,c]	116.7 ± 70.0

Analysis of wild-type cells is based on the previous studies.[12c,14,15] Data is displayed as mean ± standard deviation. [a] Significantly different compared to WT at $p = 0.0002$. [b] Significantly different compared to WT at $p < 0.0001$. [c] Significantly different compared to PNT1A at $p < 0.05$.

There was a significant increase in the expression of *MT1A* and *MT2A* (up to 3.1-fold at $p = 0.03$ and up to 2.5-fold at $p = 0.0001$ for *MT1A* and *MT2A*, respectively) and zinc transporter *ZnT-1* (2.6-fold at $p = 0.0007$) genes. There was also a barely significant decrease of *KRAS* gene expression (0.7-fold at $p = 0.045$). No other significant trend was observed in the expression of the remaining genes (Table 2).

2.2.2 Effect of cell line. The effect of cell line on the gene expression was analyzed after the adjustment of zinc concentration and zinc resistance. Compared to the effect of zinc concentration and zinc resistance, the effect of cell line affected the expression profile most intensively $F(28, 8) = 37.00$, $p < 0.001$. Significantly affected by the cell line were *p53*, *Bcl-2*, *CFLAR*, *ZIP1*, *KRAS*, *survivin*, *mTOR*, *MT1A*, and *HIF1α* gene expressions. As predicted, the expression of non-tumor PNT1A and tumorous 22Rv1 and PC-3 differed mutually. Firstly, the expression was analyzed in the tumorous cell line 22Rv1. Compared to PNT1A, this cell line exhibited rather an up-regulation of the gene expression; there was a significantly up-regulated expression of *Bcl-2* (7.6-fold, $p < 0.001$), *KRAS* (2.6-fold, $p < 0.001$), *mTOR* (2.0-fold, $p < 0.001$), *Beclin-1* (1.5-fold, $p = 0.04$), *MT2A* (1.8-fold, $p < 0.05$), *ZIP1* (1.9-fold, $p < 0.001$), *ZnT-1* (1.9-fold, $p = 0.04$), and *CFLAR* (1.5-fold, $p < 0.001$). Secondly, the expression of cell line derived from metastasis, PC-3, was analyzed. In contrast to 22Rv1, the expression profile of this cell line was rather down-regulated. Compared to PNT1A, there was a significant down-regulation of *p53* (0.2-fold, $p < 0.001$), *MT1A* (0.1-fold, $p < 0.001$), *Bcl-2* (0.4-fold, $p < 0.001$), *ZnT-1* (0.5-fold, $p = 0.04$), and *KRAS* (0.5-fold, $p < 0.001$). The following genes were significantly up-regulated in PC-3: *HIF1a* (2.2-fold, $p < 0.001$), *CFLAR* (2-fold, $p < 0.001$), and *survivin* (1.8-fold, $p < 0.001$) (Table 2).

2.2.3 Effect of long-term treatment (zinc resistance). In this step, the effect of "zinc resistance" (*i.e.* long-term *vs.* short term treatment comparison) was evaluated. Long-term treated cells are further denoted as "zinc-resistant", short-term-treated cells are designated as "wild-type". Despite the fact that there was no significant effect of this factor in the gene expression using a multivariate test, a set of significantly up-regulated genes in zinc-resistant cells was determined using univariate analysis. These include (in the order of significance) *NF-κB1* (1.6-fold increase of expression, $p < 0.001$), *CFLAR* (1.7-fold, $p < 0.001$), *KRAS* (1.7-fold, $p = 0.0005$), *p53* (1.5-fold, $p = 0.001$), *survivin* (1.4-fold, $p = 0.02$), *ZIP1* (1.3-fold, $p = 0.002$), *BAX* (1.4-fold, $p = 0.005$), and *HIF1a* (1.4-fold, $p = 0.05$). The remaining genes did not exhibit a significant trend (Table 2).

2.2.4 *BAX*/*Bcl-2* ratio. In the above sections, the expression of genes was analyzed separately depending on either zinc

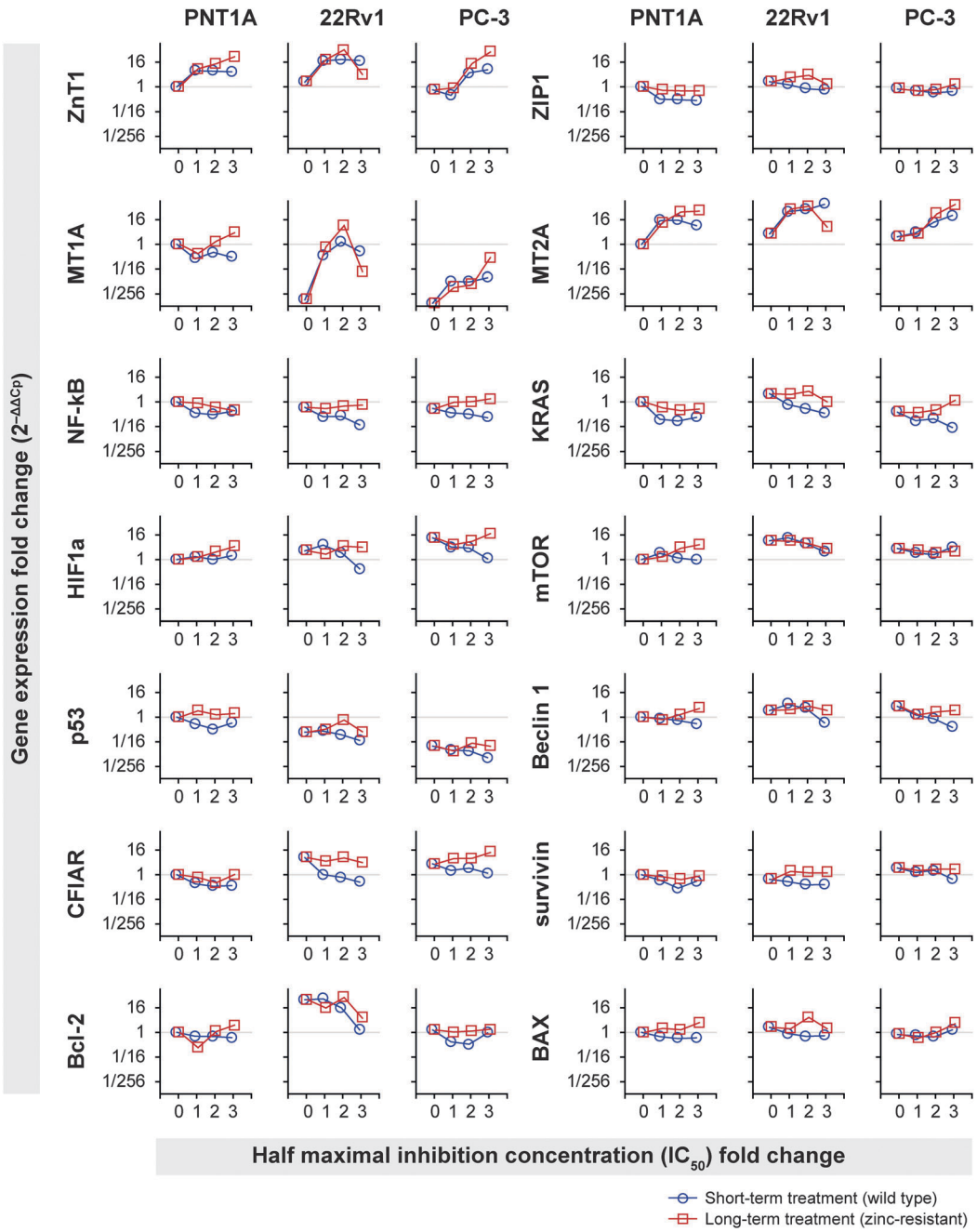

Fig. 2 Expression profile of genes. mRNA expression levels of all analyzed genes in the PNT1A, 22RV1, and PC-3 cell lines in reaction to the long-term ("resistant", red line) and short-term ("wild type", blue line) treatment with zinc(II) ions. The *y*-axis in log scale reflects the relative gene expression fold change of these genes, the *x*-axis reflects the fold change of IC_{50} concentrations.

resistance, zinc(II) treatment, or type of the cell line. Nevertheless, the *BAX*/*Bcl-2* ratio is crucial for the fate of cells. Therefore, a *BAX*/*Bcl-2* ratio was determined (Fig. 3). The most apparent trend was evident with regard to the cell line; while in the non-tumor PNT1A cell line, the *BAX*/*Bcl-2* ratio was above 1.0 (apoptosis triggering), the ratio in the tumorous cell lines 22Rv1 and PC-3 was below 1.0 (anti-apoptotic effect); this trend was significant using Tukey's post-hoc test. With regard to zinc resistance, the ratio was lower in WT cells, but below the level of statistical significance. As to the zinc(II) concentration, an increasing *BAX*/*Bcl-2* ratio was associated with increasing zinc(II) concentration but only up to a value of 0.92 of the *BAX*/*Bcl-2* ratio (no apoptosis triggering), which was achieved by a 2-fold IC_{50} zinc(II) concentration; see Fig. 3B.

2.2.5 Gene co-expression analysis. Previous analyses did not highlight whether there are some common trends in the expression profiles of individual genes. Therefore, correlations between the genes were determined, and the resistant and wild type cells were analyzed separately.

In both resistant and WT cell line forms, a correlation of *ZnT-1* with *MT1A* and *MT2A* was found ($r = 0.58$ at $p = 0.045$

Table 2 Multivariate analysis of the effect of zinc resistance, cell line and IC_{50} fold change

	Level of effect (gene expression fold change)													
Effect	*mTOR*	*Beclin 1*	*HIF1α*	*Bcl-2*	*MT2A*	*BAX*	*p53*	*MT1A*	*ZIP1*	*ZnT1*	*NF-κB*	*Survivin*	*CFLAR*	*KRAS*
Zinc resistance $F(14, 4) = 0.77$, $p = 0.67$														
Resist.	1.10	1.28	1.38[a]	1.24	1.11	1.40[a]	1.46[a]	1.31	1.34[a]	1.30	1.58[a]	1.35[a]	1.72[a]	1.70[a]
Cell line $F(28, 8) = 37.00$, $p < 0.001$														
22Rv1	2.02[a]	1.48[a]	0.85	7.56[a]	1.81[a]	1.29	1.19	1.43	1.87[a]	1.93[a]	0.77	0.77[a]	1.50[a]	2.55[a]
PC-3	0.89	1.00	2.15[a]	0.38[a]	0.77	0.84	0.17[a]	0.11[a]	0.88	0.53[a]	1.14	1.80[a]	2.05[a]	0.52[a]
IC_{50} fold change $F(42, 12.63) = 4.15$, $p = 0.004$														
1-fold	1.12	1.03	0.97	0.80	1.06	0.81	1.04	1.09	0.97	0.80	0.97	1.07	0.89	0.86
2-fold	0.97	1.06	1.06	1.08	2.50[a]	0.97	1.11	3.11[a]	0.91	2.60[a]	0.91	0.85	0.82	0.85[a]
3-fold	0.90	0.69	0.86	0.68	2.32[a]	1.24	0.76	2.12[a]	0.83	2.39[a]	0.78	0.88	0.82	0.71[a]

Displayed as a gene expression fold change after adjustment of all other variables, related to wild type in case of "zinc resistance" effect, PNT1A in case of "cell line" effect, and 0-fold IC_{50} fold change in case of "IC_{50} fold change" effect. [a] Indicates significant at $p < 0.05$. For exact *p*-values see Fig. 4A.

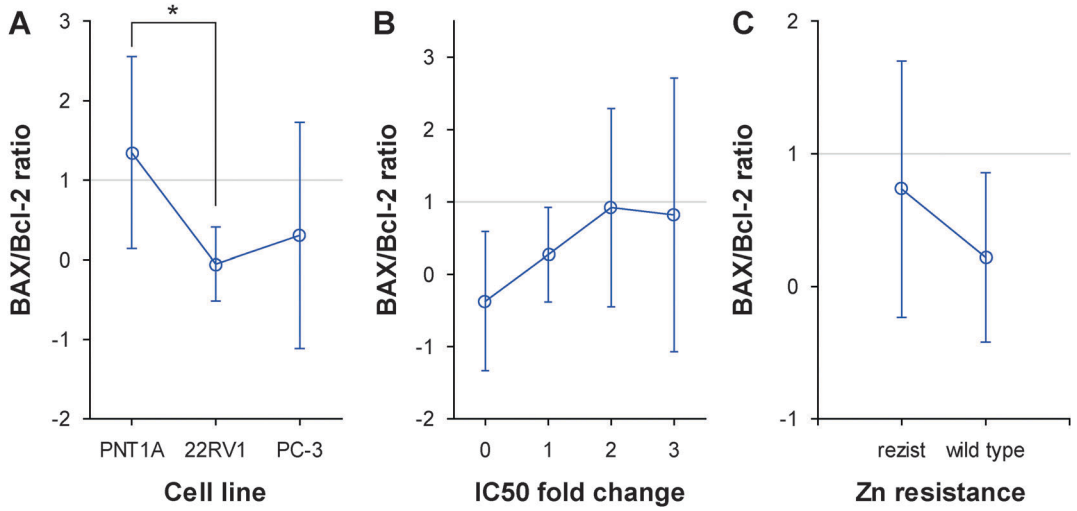

Fig. 3 *BAX/Bcl-2* ratio related to the cell line, zinc treatment, and zinc resistance. *Significantly different using Tukey's post-hoc testing at $p < 0.05$. Displayed as mean ± standard deviation. The *y*-axis reflects the ratio of relative gene expression fold changes of these genes; values > 1 indicate excess of *BAX* gene expression.

and $r = 0.91$ at $p < 0.001$ for WT; and $r = 0.68$ at $p = 0.014$ and $r = 0.97$ at $p < 0.001$ for the resistant cells, respectively). Additionally, there was a positive correlation between *KRAS* and *ZIP1* ($r = 0.75$ at $p = 0.005$ and $r = 0.95$ at $p < 0.001$ for WT and resistant cells). No other similar correlations between WT and resistant cell forms was observed; Wild type cells showed a negative relationship between *MT1A* and *CFLAR* ($r = -0.81$ $p = 0.001$) and a negative correlation between *NF-κB1* and *MT2A* ($r = -0.93$ $p < 0.001$). With regard to the resistant cells, strong positive correlations were determined between *BAX* and *ZnT-1* ($r = 0.80$ at $p = 0.002$), *ZIP1* and *KRAS* ($r = 0.95$ at $p < 0.001$) and between *Bcl-2* and *ZIP1* ($r = 0.85$ at $p < 0.001$), *Bcl-2* and *KRAS* ($r = 0.82$ at $p = 0.001$) and *Bcl-2* and *mTOR* ($r = 0.82$ at $p = 0.001$).

Regardless of zinc resistance, some specific co-expressions typical of particular cell lines were revealed. The results indicated a correlation between the *p53* and *Beclin 1* gene expression in the PC-3 cell line ($r = 0.88$ at $p = 0.004$). Furthermore, in the PC-3 cells, the *p53* expression correlated with *survivin* ($r = 0.89$ at $p = 0.003$), *KRAS* ($r = 0.79$ at $p = 0.02$), and *HIF1α* ($r = 0.88$ at $p = 0.004$) as well. Nevertheless, in the PC-3 cell line, no correlation was found between *p53* and *BAX* in contrast to the other two cell lines (PNT1A and 22Rv1). The 22RV1 cell line expressed *p53* in correlation with *BAX* ($r = 0.88$ at $p = 0.004$) and *ZIP1* ($r = 0.89$ at $p = 0.003$) genes. *CFLAR* and *NF-κB* ($r = 0.98$ at $p < 0.001$ for PC-3; $r = 0.95$ at $p < 0.001$ for 22Rv1) correlated with each other, but only in the cancerous cell lines; in the PNT1A cell line this connection was not found. In the 22Rv1 cell line, a significant correlation between *KRAS* and *BAX* was found ($r = 0.86$ at $p = 0.007$). Furthermore, in PC-3 *KRAS* correlated with *HIF-1α* ($r = 0.92$ at $p = 0.001$). *Survivin* was related to *HIF-1α* in the wild type cell lines ($r = 0.64$ at $p = 0.025$) and in both types of PC-3 cells ($r = 0.90$ at $p = 0.002$). These particular correlations were not found in the other cell lines.

However, a simple correlation does not make it possible for us to reveal the complex co-expression pattern and potential differences between long- and short-term treatments. Therefore, principal component analysis was performed. In addition to the correlation analysis, the component analysis made it possible for us to detect the structure of relationships between

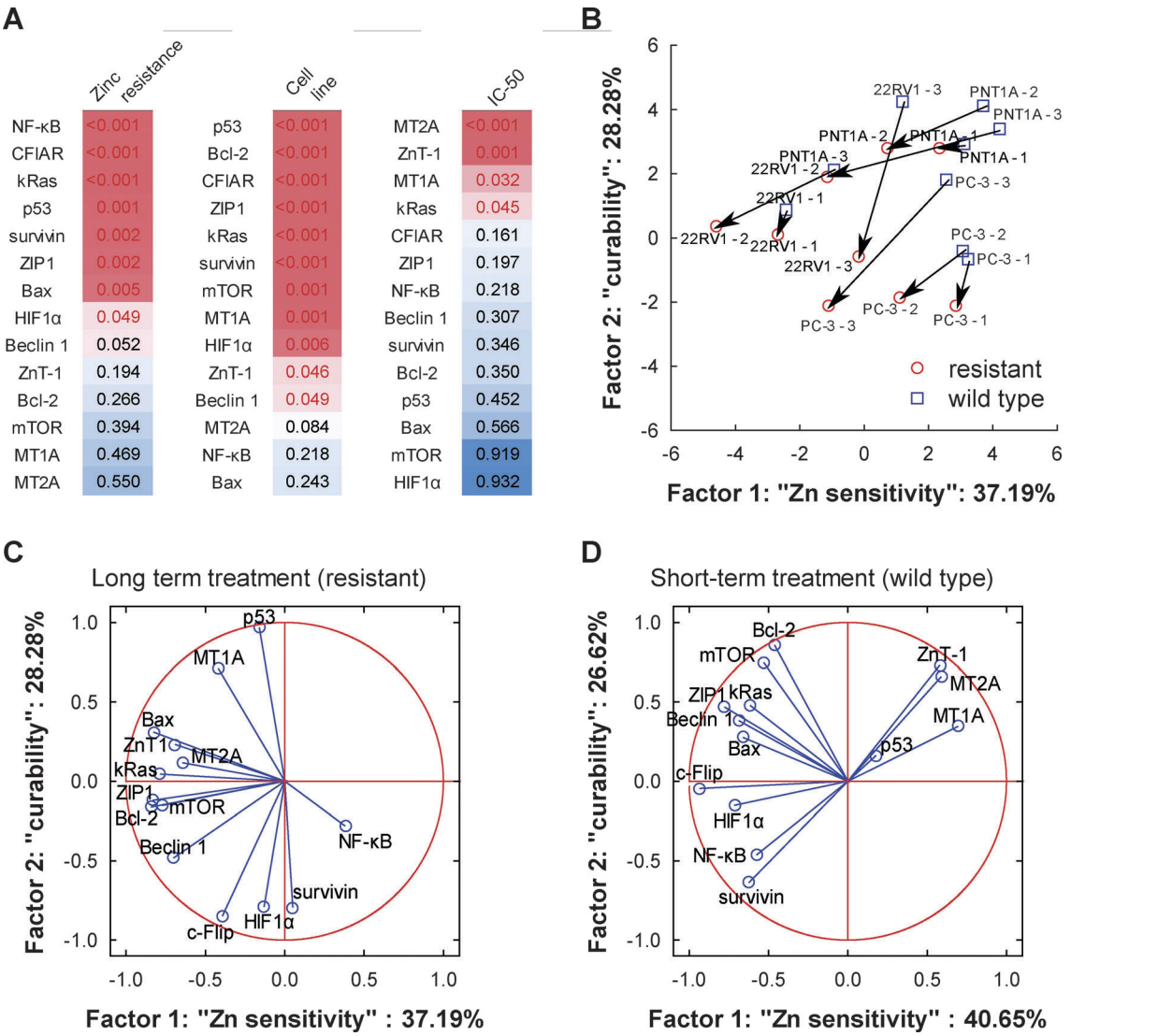

Fig. 4 Analysis of the effect of zinc resistance, cell line and zinc concentration. (A) *p*-values from multivariate ANOVA. Zinc resistance cell line, and IC_{50} fold change used as categorical predictors. Sorted by *p*-value level (genes with the most significant changes in a descending order). Color coding reflects statistical significance. Red-coded genes are significant at $p < 0.05$. (B) Principal component analysis: projection of cases (cell lines) on the two-factor-plane. Arrows indicate "shift" in the phenotype of the cells towards lower zinc(II) sensitivity (factor 1) and worse curability (factor 2). Numbers after the names of the cell lines represent IC_{50}-fold change. (C) and (D) Principal component analysis: projection of variables (genes associated with zinc sensitivity and "curability") on the two-factor plane. Factors in B, C, and D are identical. Distinction between long term (C) and short term (D) corresponds with the shift in Fig. 3B. For details see the Results section.

variables, thus helping us to assess variables in the WT and zinc-resistant groups. Moreover, this analysis allowed us to classify variables (cell lines of particular treatment and of particular zinc resistance condition) based on the expression profile. To illustrate the model of resistance, two-factor analysis was chosen with a total cumulative variance of 65.46% and eigenvalues 5.20 and 3.96 for factor 1 and factor 2, respectively. Firstly, the variables were projected based on resistance (resistant and WT), (Fig. 4C and D). Taking together, the results of multivariate ANOVA and the "direction" of genes in the principal component analysis, we designated the factor 1 of principal component analysis as "sensitivity" and factor 2 as "curability". These conclusions are based on the following findings: (a) *MT*s and *ZnT-1* are genes, which strongly correlate with the zinc concentration (Fig 4A), (b) factors in the "zinc resistance" analysis and "IC_{50} fold-change" refer to the "long-term treatment" and "short-term treatment", respectively; (c) *MT*s and *ZnT-1* are directed to the positive values of factor 1 in the short term treatment and (d) these genes are directed to the negative values in the zinc-resistant cells. Furthermore, the negative factor 1 in the resistant cells is associated with cell survival rather than with cell death. When these factors are used to project cases (*i.e.* cell lines), another apparent trend is evident: (e) cells derived from an advanced tumor, "aggressive" PC-3, are associated with the lower values of factor 2 and cells derived from the primary tumor, 22Rv1 with a lower aggressive potential are associated

with rather higher values of factor 2. However, (f) when the cells become resistant, they move toward the lower values of the two factors 1 and 2, *i.e.* they become less sensitive to zinc (as demonstrated by MTT), and less curable (as determined by the cisplatin treatment, for detail see the section "Cell viability analysis") (Fig. 4B).

2.3 Fluorescent staining

2.3.1 Determination of zinc. Fluorescent staining was performed to confirm that the resistant cell lines are affected by the high amounts of zinc(II) ions. Free zinc(II) ions were visualized by using a fluorescent probe *N*-(6-methoxy-8-quinolyl)-*p*-toluene sulphonamide, *i.e.* a probe specific to these ions. Differences found in the zinc(II) ions localized in all wild type cells and in the resistant (Fig. 5) cells were as follows: in both PNT1A and 22Rv1 cells, levels of intracellular free zinc(II) ions were closely connected with the zinc(II) ion treatment in a concentration-dependent manner. In the PC-3 cells, the difference between the non-treated and zinc(II)-treated cells was less obvious, although it was clear that all cell lines were able to accumulate zinc(II) ions. In the case of the PNT1A cell line, the localization of zinc(II) ions around the nuclei and irregularly within the nuclei was evident. Peripheral parts of the cytoplasm demonstrated only a weak emission, representing only low free zinc(II) levels in these localizations. In the 22Rv1 cells, the intensity of the fluorescence product emission significantly increased with increasing resistance to zinc(II) ions (Fig. 5). In PNT1A and 22RV1 resistant cells, free zinc(II) ions were localized around the nuclei and in the form of high emission spots. These spots are probably zincosomes, compartments of endoplasmic reticulum origin.[16]

2.3.2 Determination of free thiols. Compounds rich in –SH groups (low molecular mass peptides and proteins such as reduced glutathione and metallothionein, *etc.*) are responsible for binding metal ions within the intracellular space. The expression level of metallothionein was verified with 5-(bromomethyl)fluorescein, a probe that provides the formation of a fluorescent product after reaction with the –SH groups of thiols. The detection and cellular compartmentation of free thiols was made. In all cell lines (PNT1A, 22Rv1, and PC-3 see Fig. 5), the amount of free thiols increased within the zinc(II) ion resistant cells and some cells with an extremely high content of free thiols were detected. This fact was more evident in the PC-3 cells. In all cell lines, free thiols were localized around the nuclei and in the cytoplasm.

3 Discussion

In our previous studies, we demonstrated that zinc ions can change the expression levels of metallothionein and other genes connected with oxidative stress and apoptosis.[12c,14] However, the effect of zinc on the prostate cell lines was studied only at relatively short time exposures.[7,17] Through a positive selection of zinc resistant cells, we created cell lines continuously viable at concentrations exceeding the IC_{50} for zinc. Results of the fluorescent staining indicated that all cell lines were able to

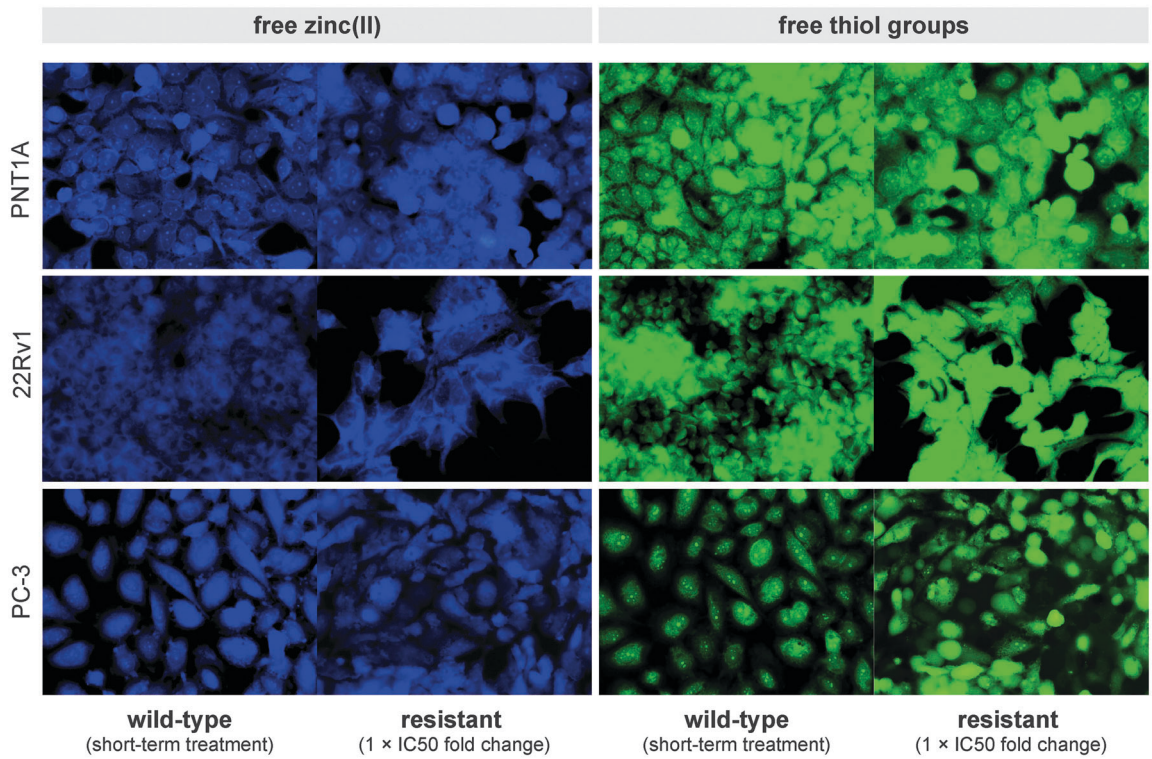

Fig. 5 Distribution of free zinc(II) ions and free thiol groups in the wild type and resistant cell lines. Displayed as pairs of wild-type and one-fold IC_{50} resistant cells. One-fold IC_{50} represents the following concentrations: 150, 400, and 50 μM for PNT1A, 22Rv1, and PC-3, respectively. For details see the Results section.

accumulate zinc(II) ions, which is in compliance with Costelo *et al.*,[11a] and in all cell lines, free thiols were localized around the nuclei and in the cytoplasm. This is considered the evidence for a possible role of free thiols in the transcriptional activity.[14] These results were in accordance with the expression analysis, where the production of *MT* mRNA increased with zinc(II) ions in a concentration-dependent manner. The resistant cells were capable of division at zinc concentrations that were three times higher than the standard IC_{50}. The PC-3 cell line was found to be the least tolerant to zinc (in both wild type and resistant form). In contrast, the most resistant 22Rv1 cells were able to grow at 1200 μM zinc concentrations.

3.1 Buffering of short-term zinc excess

When the intracellular concentration of zinc increases, the cells have a tendency to maintain homeostasis by reducing the impact of potential zinc toxicity. Such homeostasis can be achieved either through lower importing of zinc ions to the cells, by exporting the zinc ions from the cell or through the capability to reduce the amount of free zinc ions by binding it to thiol groups (for instance into metallothioneins). We expected *ZIP1* decreasing expression trends to participate in gaining the resistance, but our results did not turn out to be as good as the results of Wong *et al.*, who studied the LNCaP cell line.[13] Although the expression of *ZIP1* was conversely 1.34 fold higher in the resistant cell lines, this effect was caused mainly by the 22RV1 cell line. In this cell line, the expression of *ZIP1* increased (1.87-fold change). We also expected a negative correlation between *ZIP1* and *KRAS* expression according to previous studies, where a negative regulation was found.[18] Interestingly, however, we found strong positive correlations in all cases. Because significant changes in *ZIP1* expression levels due to increasing zinc concentrations were not found, the importance of the other two mechanisms can be assumed. In correlation with this hypothesis, a statistically significant link was found between *MT2A*, *ZnT-1*, and *MT1A* expression and IC_{50} fold change, which implies the key function of these genes in general zinc resistance regardless of the length of zinc treatment. Accordingly, the lowest *ZnT-1* levels and the slowest enhancement of *ZnT-1*, *MT1A*, and *MT2A* gene expression due to the zinc concentration increase were found in relatively zinc-sensitive PC-3 cells. *MT1A* expression levels in the PC-3 resistant form did not even reach wild-type PNT1A or 22Rv1 levels (see Fig. 2). *ZnT-1* was also identified as a gene liable for causing zinc resistance in baby hamster kidney cells,[9] but was not recognized as a resistance factor in the study published by Wong *et al.*[13] Nevertheless, Wong *et al.* studied *ZnT-1* expression levels at zinc concentrations lower than ours. Moreover, the expression of *ZnT-1* in our experiment highly correlated with the expression of *MT1A* and *MT2A* and with the zinc ion concentrations, which is in accordance with the fact that all these genes are regulated by the MTF-1 transcriptional factor.[19]

3.2 Long-term zinc resistance mechanisms include *PI3K* and *NF-κB* pathways

While the above-mentioned effects are identically exhibited in both WT and resistant cell line types, in the following section we outline mechanisms specific for resistance obtained due to the long-term zinc treatment. In our study, we found that the genes which are important for long-term zinc resistance are *NF-κB1* (p50/p105), *CFLAR*, *KRAS*, *p53*, *survivin*, *ZIP1*, *BAX* and *HIF1α*. On the other hand, these genes had only a small effect in short-term resistance. All these genes are involved in *PI3K* and *NF-κB* signaling pathways[20] (see Fig. 2). Inasmuch as *NF-κB* is a down-stream gene of *KRAS* (through the activity of *PI3K*/*AKT*) and *CFLAR*,[21] *survivin*, and *HIF1α* are the down-stream genes of *NF-κB*, we took the liberty to assume the involvement of the mentioned pathway. All these genes are able to support anti-apoptotic processes and cell survival.[22] Furthermore, a strong negative correlation between *NF-κB* and *MT2A* was found specifically in the wild-type cell lines, which indicates the existence of negative regulation mechanisms between these genes. Accordingly, *MT2A* was previously reported to play a tumor-suppressive role through inhibition of *NF-κB* signaling.[23] This negative correlation between *NF-κB* and *MT2A* expression was not observed in the resistant types of cells. One of the most important ways in which the *NF-κB* activity could influence cell survival at long-term high zinc concentrations is *via* the increased expression of antioxidant proteins such as manganese superoxide dismutase (MnSOD), copper–zinc superoxide dismutase, catalase, thioredoxin-1, and thioredoxin-2, glutathione S-transferase pi, glutathione peroxidase-1, or ferritin heavy chain.[24] Moreover, the activity of *NF-κB* and *PI3K* pathways seems to be enhanced due to the long-term treatment, which can be followed by a higher cell survival rate and by the activation of metastatic mechanisms. It seems presumable that these particular pathways can easily push the healthy cell phenotype toward higher zinc resistance and simultaneously towards lower curability (see Fig. 4B). It was previously demonstrated that extracellular zinc activates *MAPK*s and *PI3K* in different tissues and also in the prostate cancer cells.[25] Collaboration of activated *RAS*/*AKT*/*NF-κB* signaling with other oncogenic signaling pathways could easily change the phosphorylation profile of prostate cancer cells to be more aggressive.[26] Elevated *PI3K*/*AKT* signaling was found in nearly all prostate cancer types, and irregularities in the *RAS* signaling pathways existed in more than 40% of primary prostate tumors and 90% of prostate metastases.[27] Results of some studies indicate that zinc ions inhibit *RAS* and also *NF-κB* mediated signaling.[18,28] These inhibiting effects of zinc ions were observed in our study too, but only in the wild-type (short-term treated) cells, where decreasing trends in the expression of *KRAS*, *CFLAR*, *p53* and *NF-κB1* after zinc administration were found. Some of the other inhibiting effects of zinc related to *RAS*-mediated signaling were specific only for some cell lines (*HIF-1α* inhibition in 22Rv1 and PC-3 cells, *survivin* in 22RV1 cells, *Beclin-1* in PNT1A and PC-3 cells); however, almost all of these mentioned inhibition effects were not present in the resistant forms of cells. Therefore, nearly opposite effects of zinc could be seen due to the different length of the treatment. According to our results, a prolonged zinc treatment could actually induce a more aggressive behavior of the prostate cancer cells. The shift to increased resistance and worse curability status was observed in all the studied long-treated cell lines. These observations were corroborated by results of the MTT test

for cisplatin, which is a potent member of the anticancer drug family. The resistant cell lines were significantly less sensitive to the cisplatin treatment. This is in line with studies declaring that excessive zinc amounts help cells withstand the impact of other toxins.[29]

3.3 Modulation of apoptosis

One of the described effects of zinc on prostate cancer cells is the influence on intrinsic apoptotic pathways through increasing the *BAX* expression,[11b] affecting the *BAX/Bcl-2* proportion,[17a] and helping *BAX* to shift onto the mitochondrial membrane.[17d] In this experiment, both types (wild type and resistant) of the PNT1A cell lines gave a *BAX/Bcl-2* ratio indicating apoptosis triggering. The cell lines derived from cancerous tissues did not show any increase of this ratio. When the long-term and short-term treated cells were analyzed separately, the *BAX/Bcl-2* proportion continuously increased in the resistant cell lines along with the amount of zinc(II) ions. This could be due to the fact, that the cells do not lose the ability to induce mitochondrial apoptotic pathways, but they are also capable of neutralizing these processes with other factors like *survivin* or *CFLAR.* The C-Flip product of the *CFLAR* gene might be important in managing the extrinsic apoptotic pathways after long-term treatments, and obviously in gaining resistance. *CFLAR* is a target gene of *NF-κB* transcription factor[30] and indeed they correlated with each other, but only in the cancerous cell lines; in the PNT1A cell line, this connection was not found. *Survivin* is a potent caspase inhibitor and its expression protected the cells against high zinc concentrations.[22b] It was also shown that the production of *survivin* increases in aggressive prostate carcinoma,[31] which is in agreement with our study.

On the other hand, the wild type (short-treated) cells had an increasing *BAX/Bcl-2* ratio only at lower concentrations of zinc(II) ions. Consequently, zinc amounts exceeding double the IC_{50} concentration apparently do not trigger apoptosis in the WT cells, but rather necrosis. Unlike apoptosis, necrosis does not need functional ATP production.[32] Therefore, the decision between death by apoptosis or by necrosis depends upon the intensity of the cell injury and also upon the availability of intracellular ATP. Accordingly, the resistant cell lines seem to be able to preserve ATP production even at high zinc concentrations.

3.4 Cell lines as a major factor affecting the gene expression profile

Furthermore, we recorded differences in expression trends between the cell lines. The use of markedly dissimilar prostate cancer cell lines allowed us to inspect the effect of zinc resistance irrespective of underlying pathways and grade of transformation. Significantly affected by the cell line influence were almost all examined gene expressions, as we expected, but for instance not *NF-κB* and *MT2A*, which were found to be important in both long-term (*NF-κB*) and short-term (*MT2A*) zinc resistance in spite of the cell line effect. See Fig. 4A (results of multivariate ANOVA, comparing the effect of cell line *vs.* effect of zinc resistance). The greatest effect of the cell line was recorded on the *p53* transcription level. This gene was transcribed less in the metastatic cell line PC-3 as compared to PNT1A. The PC-3 cell line does not produce functional protein p53.[33] Moreover, the results indicated a correlation between *p53* and the pro-autophagic *Beclin1* gene expression in the PC-3 cell line. This suggests that the non-functional protein accumulates and is degraded during the process of autophagy.[34] Furthermore, in the PC-3 cells, the *p53* expression correlated with *survivin*, *KRAS*, and *HIF1α* as well. This implies that the anti-apoptotic mechanisms were triggered. The relationship between *p53* and *HIF1a* could be explained by the hypothesis that both *p53* and *HIF-1α* are degraded by *Mdm2*. This ubiquitin ligase normally prefers to cleave the *HIF-1α* protein, but in this case, the accumulated non-functional *p53* had priority.[35] The expression of *HIF1a* was the highest in the PC-3 cell line, which was in accordance with the previous findings. The malignant prostatic tissue shows a more positive redox status than the healthy tissue and this may explain the connection observed between *HIF-1α* expression and the staging of the tumor.[36] The resistant cell lines showed a higher expression of *HIF-1α* mRNA, which indicates the involvement of *HIF-1α* in the resistance mechanism. According to a study by Feng *et al.*, *HIF-1α* expression inhibits the apoptotic effects of zinc.[17d]

In contrast, in the PC-3 cell line, no correlation was found between *p53* and any pro-apoptotic factor unlike in the other two cell lines (PNT1A and 22Rv1), where *p53* is partially functional. The 22RV1 cell line expressed the wild-type *p53* in correlation with the pro-apoptotic *BAX*. It indicates that the cells were able to induce *p53* dependent apoptosis, but other effectors could oppose the process. The PNT1A cell line is transfected by the Simian Virus 40 vector that affects the *p53* activity;[37] some studies demonstrated, however, that the use of oxidants can negate this inhibition.[15,38] Zinc can also act like an oxidant and our results are in agreement with these findings, because the *p53* expression correlated with *BAX* expression levels in PNT1A. Higher expression of *p53* was found in the resistant forms of cell lines. Nevertheless, it was reported that zinc can cause increases in *p53* expression but interrupts *p53* binding to DNA.[39]

3.5 Overview of complex gene expression changes

In general, our findings indicate the great importance of the zinc treatment duration. Whereas the short-term (24–72 h) zinc treatment induces the apoptotic process in the prostate cancer cell lines,[14,17b,22b] the long term treated (several months) prostate cancer cells change their phenotype into a more aggressive and resistant form (Fig. 6). This shift in phenotype is clearly illustrated in Fig. 4B. The ''moving'' of zinc treated cell lines along the axis representing zinc sensitivity and tumor curability depicts a thin line between ''healthy'' phenotype and aggressive cancer characteristics. These findings are in line with those of Wong *et al.*, who revealed that the initial inhibitory effects of Zn(II) on LNCaP cell proliferation were reversed due to the seven week long presence of high intracellular zinc ions.[13]

Some studies claim that dietary zinc supplementation reduces cancer risk,[40] is protective against tumorigenesis[12a] or can sensitize the cells to cytotoxic agents.[28b] These effects of zinc in prostate

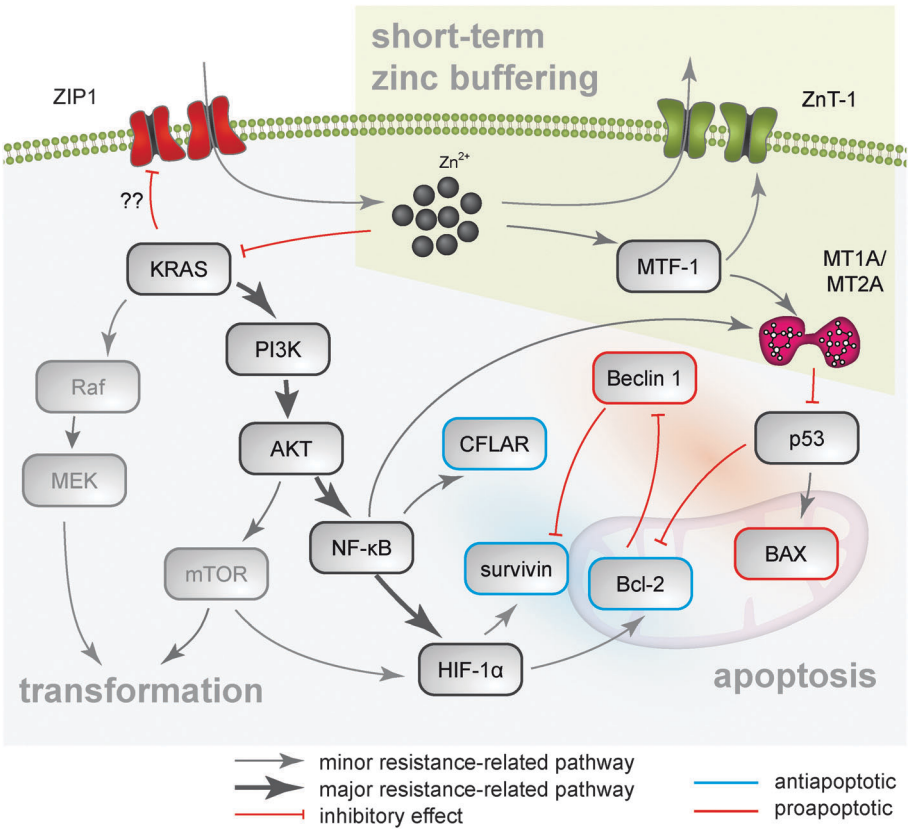

Fig. 6 Mechanisms of zinc resistance (long-term zinc buffering) and short-term zinc buffering. Synthesis of the effect of the zinc(II) ions and major pathways affecting tumor transformation, apoptosis, and short-term zinc buffering based on the results of this study and in accordance with literature (for details see Discussion section). Short-term zinc(II) buffering is indicated by a green background color, long-term zinc buffering displayed in the remaining parts of the scheme. The major *KRAS-PI3K-NF-κB* pathway and its effect on cell survival are accentuated by thick lines. Antiapoptic/proapoptic genes are outlined with blue/red, respectively.

cancer have become popular and many dietary supplements for humans contain zinc. However, according to our results, it can be deduced that zinc protecting abilities on the prostate tissue are not without exception. This suspicion has been also corroborated by several other studies demonstrating that zinc can cause the development of BPH and cancer,[41] or dietary zinc uptake correlates with the increased prostate cancer risk.[42]

4 Experimental

4.1 Chemical and biochemical reagents

RPMI-1640 medium, Ham's F12 medium, fetal bovine serum (FBS) (mycoplasma-free), penicillin/streptomycin and trypsin were purchased from PAA Laboratories GmbH (Pashing, Austria). Phosphate buffered saline PBS was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Ethylenediaminetetraacetic acid (EDTA), zinc(II) sulphate (BioReagent grade, suitable for cell cultures), cisplatin 0.5 mg ml^{-1} solution (Medac, Germany) and all other chemicals of ACS purity were purchased from Sigma Aldrich Co. (St. Louis, MO, USA), unless noted otherwise.

4.2 Cell cultures

Three human prostatic cell lines were used in this study. The PNT1A human cell line derived from normal adult prostatic epithelial cells immortalized by transfection with a plasmid containing SV40 genome with defective replication origin. The primary culture was obtained post mortem from the normal prostatic tissue of a 35 year old male. The 22Rv1 human epithelial cell line derived from a xenograft serially propagated in mice after castration, and the androgen sensitive. PC-3 human epithelial cell line established from a 4 grade prostatic adenocarcinoma, an androgen independent and unresponsive metastatic site in bone. All three cell lines used in this study were purchased from HPA Culture Collections (Salisbury, UK).

4.3 Cell cultivation

The PNT1A and 22RV1 cells were cultivated in RPMI-1640 medium with 10% FBS. The PC-3 cells were cultivated in Ham's F12 medium with 10% FBS. Both mediums were supplemented with antibiotics (penicillin 100 U ml^{-1} and streptomycin 0.1 mg ml^{-1}). The cells were maintained at 37 °C in the humidified (60%) incubator with 5% CO_2 (Sanyo, Japan). Passages of the PNT1A and 22RV1 cell lines ranged from 15 to 35; passages of the PC-3 cell line ranged from 30 to 40.

4.4 Zinc(II) treatment of cell cultures

We used two different treatments in this study. The first one was a short-term treatment. Cells confluent up to 50–60% were

washed with a FBS-free medium and treated in fresh medium with FBS and the required zinc concentration (one-fold to three-fold the value of IC_{50} for the cell line). The cells were cultivated under these conditions for 24 h. The resulting samples are called ''wild type'' in this study. The second type of treatment was a long-term one. Cells were cultivated at a constant presence of zinc(II) ions. Concentrations of zinc(II) sulfate in the medium were increased gradually by small changes of 25 or 50 μM. The cells were cultivated at each concentration no less than one week before harvesting and their viability was checked before adding more zinc. Zinc resistant cells were selected naturally by this process. These cells with the long-term exposure are denoted as ''resistant'' in this study. The resulting concentrations in the two types of treatment were 50; 100 and 150 μM for the PC-3 cell line, 150; 300 and 450 μM for the PNT1A cell line, and 400; 800 and 1200 μM for the 22Rv1 cell line. Concentrations of zinc(II) in media and FBS were taken into account.

4.5 RNA isolation, cDNA preparation

We used the high pure total RNA isolation kit (Roche, Basel, Switzerland) to isolate RNA from the treated cells. The cultivation medium was removed; the cells were washed with PBS and trypsinizated. After transporting the cells into the centrifugation tube, the cells were centrifuged at 2700 × *g* for 7 min at 4 °C. The pellet was resuspended in PBS and lysis and binding buffers were added. The whole process was carried out according to the manufacturer's instructions. The obtained RNA was used for cDNA transcription. Total RNA (600 ng) was transcribed using random hexamer primers in a Transcriptor First Strand cDNA synthesis kit (Roche). The prepared cDNA was diluted in water and analyzed using real-time PCR.

4.6 Real-time reverse-transcription polymerase chain reaction (RT-PCR)

We used the TaqMan gene expression assay with 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction was performed in triplicate. The amplified DNA was analyzed using the comparative Ct method using β-actin as an endogenous control. Real-time PCR was carried out under the following amplification conditions: total volume of 20 μl, initial incubation 50 °C/2 min, denaturation 95 °C/10 min, 40 cycles 95 °C/15 s, 60 °C/1 min. The primer and probe sets for β-actin (assay ID: Hs99999903_m1), *BAX* (Hs00180269_m1), *Bcl-2* (Hs00608023_m1), *Beclin-1* (Hs00186838_m1), *CFLAR* (Hs00153439_m1), *HIF1α* (Hs00153153_m1), *KRAS* (Hs00364284_g1), *MT1A* (Hs00831826_s1), *MT2A* (Hs02379661_g1), *mTOR* (Hs00234508_m1), *Nf-kB1* (Hs0076573_m1), *p53* (Hs01034249_m1), *survivin* (Hs04194392_s1), *ZIP1* (Hs00205358_m1) and *ZnT-1* (Hs00253602_m1) were selected from TaqMan gene expression assays (Life Technologies, USA).

4.7 Cell content quantification

We analyzed the total cell content on the Casy model TT system (Roche Applied Science, USA). The following protocol was used: calibration was made from the samples of viable and necrotic cells. 100 μl of necrotic cell suspension was mixed with 800 μl Casy Blue solution and incubated for 5 min at room temperature. The mixture was added 9 ml of Casy Tone. Viable cell standard was prepared from 100 μl of viable cell suspension and 10 ml Casy Tone. Measurements were made on a 100× diluted cell suspension (100 μl). Before each measurement, the background was subtracted and the capillary was cleaned. All samples were measured in duplicate.

4.8 Measurements of cell viability – MTT test

We used the MTT test to determine the cell viability in different conditions. We made 6 different MTT tests; all of them were made 8 times. Each cell line was treated with zinc and with cisplatin. The suspension of cells was diluted in the medium as required. The final density was 5000 cells per 1 well on the plate. The volume of the medium was 200 μl. Two wells for each experiment did not have cells as a control. The plates were incubated at 37 °C for two days. The medium was removed from the wells containing cells, then a new medium with the treatment was added to the cells (concentration ranged between 0 and 2000 μM of the tested substance). Subsequently, the plates were incubated at 37 °C for 24 h. From the wells containing cells and from one control cell, the medium was removed and 200 μl of fresh medium was added to 50 μl of MTT solution (5 mg ml^{-1} in PBS). The plates were subsequently incubated at 37 °C in darkness for 4 h. After this, MTT-formazan crystals were dissolved in 99.9% DMSO (dimethyl sulphoxide). 25 μl of glycine buffer was added to all wells and absorbance at 570 nm was determined (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA, USA).

4.9 Fluorescence microscopy and cell staining

The cells were cultivated directly on microscope glass slides (75 × 25 mm, thickness 1 mm, Fisher Scientific, Pardubice, Czech Republic) laid on the Petri dishes with the conventional medium as described above. After the treatment, the glass slides with a layer of cells were removed from the Petri dishes and washed with a zinc free medium and PBS buffer and directly used for staining and fluorescence microscopy. For staining the free –SH groups, we used 5-(bromomethyl)fluorescein (5-BMF, Sigma-Aldrich). This probe reacts with the thiols of peptides, proteins and thiolated nucleic acids. Its reaction is slow, compared to other probes, but its thioether bonds are stronger, remaining stable under the conditions of fluorescence microscopy. Both stock (4 mM, anhydrous DMSO) and working (diluting stock solution with PBS buffer, pH 7.6) solutions of 5-BMF are unstable. They were prepared immediately before use. The cells were incubated in the dark at 37 °C for 1 h. Then the glass was washed in PBS and observed using a fluorescence microscope (Axioskop 40, Carl Zeiss AG, Oberkochen, Germany) equipped with wideband excitation and a set of filters (FITC, DAPI, Carl Zeiss). Photographs were taken using a digital camera (Olympus Camedia 750, Olympus, Tokyo, Japan). The ''NIS-elements'' program was used to evaluate the intensity of emission and all values were recalculated to the control (100%). Ten random fields from each variant and replicate were evaluated.

A fluorescent probe *N*-(6-methoxy-8-quinolyl)-*p*-toluene sulphonamide (TSQ, Invitrogen) was used for free zinc(II) ion staining. The stock solution (10 mM, acetone) was diluted to give a working solution (10 μM, phosphate buffer pH 7.6). The cells were carefully washed with PBS buffer and stained in the working solution for 30 min at 37 °C in the dark. After staining, the cells were rinsed three times with PBS and observed under the fluorescence microscope (Axioskop 40, Carl Zeiss) equipped with FITC and DAPI filters (Carl Zeiss). Images were taken using the digital camera (Olympus Camedia 750, Olympus). The intensity of emission was evaluated using the NIS-element. All values were recalculated to the control (100%). Ten random fields from each variant and replicate were evaluated.

4.10 Statistical analysis

The data was checked for normality and log-normal data was transformed. Multivariate ANOVA with Tukey's post-hoc testing were used to reveal differences depending on categorical factors. Pearlson correlation was used to reveal dependencies between the variables. This analysis was completed with the principal component analysis to analyze patterns in the data. Software Statistica 12 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis. Unless noted otherwise, the level of statistical significance was set at $p < 0.05$.

5 Conclusions

A number of studies have demonstrated that zinc(II) levels change during the progression of numerous neoplasms, particularly in prostate cancer. However, the precise mechanisms are still unclear. Therefore, a model where zinc(II) buffering is affected across various stages of tumor progression may shed light on this issue. Through a positive selection of zinc resistant cells, we created cell lines continuously viable at concentrations exceeding their initial IC_{50} for zinc(II). The resistant cells were able to divide at zinc concentrations that were even higher than a triple of the standard IC_{50}. This study clearly illustrates that a mere gene expression analysis makes it possible for us to prove the mechanisms of acquired resistance to zinc ions. This fact is also confirmed and supplemented by fluorescence microscopy. Additionally, principal component analysis as a statistical tool commonly used to reduce data can even visualize changes in the expression of genes, thus enabling us to sort genes according to their involvement in the particular resistance mechanism. Based on these results, it can be confirmed that different ''groups'' of genes are involved in the regulation of short- and long-term zinc(II) increases. While metallothioneins and zinc transporters play an important role in the regulation of short-term zinc(II) buffering, *KRAS*, *NF-κB*, and *PI3K* are important factors in the modulation of long-term resistance. Furthermore, the results indicate, that the acquisition of zinc resistance is associated with a worse, more aggressive phenotype, less sensitive to cytostatics such as cisplatin. These results may therefore serve as a baseline for further research in to the downstream mechanisms of these genes.

Conflicts of interest

The authors declare they have no competing interests as defined by *Metallomics*, or other interests that might be perceived to influence the results and discussion reported in this paper.

Acknowledgements

Financial support from MUNI/C/0965/2012, MUNI/A/1003/2013, CEITEC CZ.1.05/1.1.00/02.0068 and doc CEITEC 02/2012 (JG) is greatly acknowledged.

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