Research Paper

High-Resolution Flow Cytometry: a Suitable Tool for Monitoring Aneuploid Prostate Cancer Cells after TMZ and TMZ-BioShuttle Treatment

Klaus Braun[1] [#] [✉], Volker Ehemann[2] [#], Manfred Wiessler[1], Ruediger Pipkorn[3], Bernd Didinger[4], Gabriele Mueller[5], Waldemar Waldeck[5]

1. German Cancer Research Center, Dept. of Medical Physics in Radiooncology, INF 280, D-69120 Heidelberg, Germany
2. University of Heidelberg, Institute of Pathology, INF 220, D-69120 Heidelberg, Germany
3. German Cancer Research Center, Central Peptide Synthesis Unit, INF 580, D-69120 Heidelberg, Germany
4. Radiation Oncology, University of Heidelberg, INF 400, D-69120 Heidelberg, Germany
5. German Cancer Research Center, Division of Biophysics of Macromolecules, INF 580, D-69120 Heidelberg, Germany

[#] The authors contributed by equal parts to this work

[✉] Correspondence to: Dr. Klaus Braun, Medical Physics in Radiology, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel: +49 6221 42 2495; Fax: +49 6221 42 3326. k.braun@dkfz.de

Abstract

If metastatic prostate cancer gets resistant to antiandrogen therapy, there are few treatment options, because prostate cancer is not very sensitive to cytostatic agents. Temozolomide (TMZ) as an orally applicable chemotherapeutic substance has been proven to be effective and well tolerated with occasional moderate toxicity especially for brain tumors and an application to prostate cancer cells seemed to be promising. Unfortunately, TMZ was inefficient in the treatment of symptomatic progressive hormone-refractory prostate cancer (HRPC). The reasons could be a low sensitivity against TMZ the short plasma half-life of TMZ, non-adapted application regimens and additionally, the aneuploid DNA content of prostate cancer cells suggesting different sensitivity against therapeutical interventions e.g. radiation therapy or chemotherapy. Considerations to improve this unsatisfying situation resulted in the realization of higher local TMZ concentrations, sufficient to kill cells regardless of intrinsic cellular sensitivity and cell DNA-index. Therefore, we reformulated the TMZ by ligation to a peptide-based carrier system called TMZ-BioShuttle for intervention. The modular-composed carrier consists of a transmembrane transporter (CPP), connected to a nuclear localization sequence (NLS) cleavably-bound, which in turn was coupled with TMZ. The NLS-sequence allows an active delivery of the TMZ into the cell nucleus after transmembrane passage of the TMZ-BioShuttle and intra-cytoplasm enzymatic cleavage and separation from the CPP. This TMZ-BioShuttle could contribute to improve therapeutic options exemplified by the hormone refractory prostate cancer. The next step was to syllogize a qualified method monitoring cell toxic effects in a high sensitivity under consideration of the ploidy status. The high-resolution flow cytometric analysis showed to be an appropriate system for a better detection and distinction of several cell populations dependent on their different DNA-indices as well as changes in proliferation of cell populations after chemotherapeutical treatment.

Key words: TMZ-BioShuttle, Prostate Cancer Cells, Flow Cytometry

Introduction

Prostate cancer (PCa) is the most common solid tumor in men. In 2007, there will be approximately 220.000 men diagnosed with prostate cancer in the U.S. [1]. The annual incidence of the male population in West-Europe and the U.S.A averages approximately 88 per 100.000 men [2]. As the wide range of the PCa's aggressiveness shows: while some patients are being able to live symptom-free and without any treatment for many years, there are aggressive forms with rapid growth and early metastatic spreading. The current therapeutic options in the treatment of PCa are: i) Radical excision of prostate and seminal vesicles [3, 4]. ii) Percutaneous radiation therapy with high energetic photons (6-23 MeV) [5, 6]. iii) Interstitial radiation therapy with temporarily or permanent radioactive implants (brachytherapy) [7]. iv) The standard initial systemic therapy for locally advanced or metastatic disease is hormonal or androgen deprivation therapy (ADT) that may be performed by bilateral orchiectomy or pharmaceutical means. The androgen-sensitive period in patients with metastatic disease lasts a median of 14–30 months [8]. v) Chemotherapy for hormone refractory prostate cancer (HRPC) is possible using mitoxantrone plus prednisone [9] or with taxane-containing agents [10]. Despite manifold combined therapeutic approaches for a successful HRPC control in the past, metastatic AIPC and HRPC is difficult to treat [11-18]. Unfortunately, all current therapeutic options for patients with HRPC turned out to be poorly effective and chemotherapy had low response rates with median survivals of up to only 12 months [14]. Therefore, chemotherapy has not traditionally been offered to patients with HRPC as a routine treatment due to its treatment-related toxicity and poor response [19]. Despite initially encouraging results of tumor growth control were reported in other tumor types than brain tumors [20], the results of a phase II study of TMZ in PCa have been discouraging [21]. One of the reasons for this could be the presence of aneuploid cell fractions, which offers a broad spectrum of cells from highly sensitive up to therapy resistant [22]. In addition, the therapy resistant cell fraction gains a selective advantage after therapeutic intervention. The DU 145 cell line, presenting a cellular heterogeneity, suggests behaviour's similarity to advanced HRPC tumors. As demonstrated by karyotypic analyses DU 145 show an aneuploid human karyotype with a modal chromosome number of 64. Marker chromosomes like translocated Y chromosome, metacentric minute chromosomes and three large acrocentric chromosomes have been identified [23, 24] which in their entirety can be responsible for the constricted sensitivity against alkylating agents.

It is clear that effort is necessary to search for new forms of treatment modalities like drug delivery and targeting systems realizing high local TMZ concentrations in the nuclei of target cells. To eradicate the target cells, without considering the HRPC cells resistance against therapeutic interventions it is necessary to find convenient methods which allow the monitoring of the therapy progress and success at the cellular level. Flow cytometric analysis should be able to detect several cell populations dependent on their different DNA-indices, which are corresponding to different amount in chromosome numbers. By this method, the genetic integrity and stability can be analyzed [25].

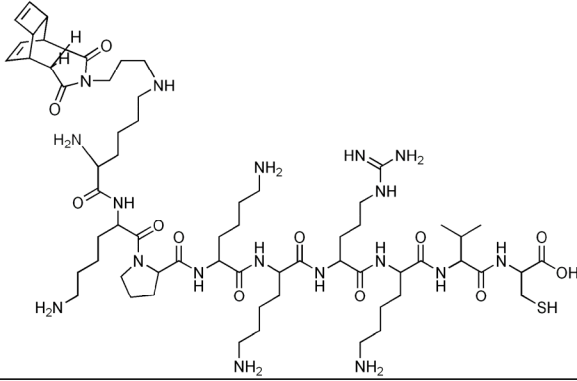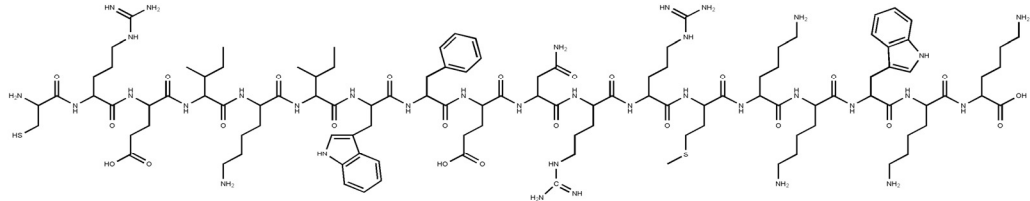## Materials & methods

Cell culture

The hormone refractory adherent prostate cancer cell line DU 145 [24] was cultivated and maintained in RPMI cell medium (Gibco, Germany) supplemented with 5% fetal calf serum and 4 mM glutamine (Biochrome, Germany) at 37°C in 5% CO_2 atmosphere.
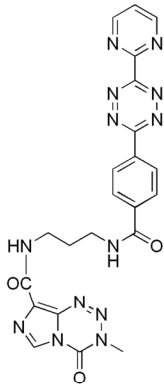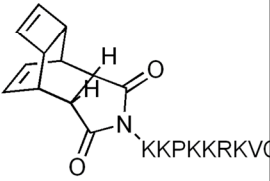
Chemical procedures

The syntheses of the investigated peptide-based functional modules like the cell penetrating peptide (CPP) and the nuclear localization sequence (NLS) as well as the syntheses of tetracyclo-[$5.4.2^{1,7}.O^{2,6}.O^{8,11}$]3,5-dioxo-4-aza-9,12-tridecadien acting dienophile and the tetrazoline-derivatization of the active compound temozolomide to (TMZ-tetrazine diene) (Table 2, left column), and in the end, the both ligation procedures, firstly the ligation of the dienophile-NLS module (Table 1, upper row) with cell penetrating peptide (CPP) (Table 1, lower row) by a reversible disulfide-bridge formation, and secondly, the compounds for the ligation in virtue to the Diels-Alder-Reaction with inverse electron-demand extensive described by Braun [26] and Pipkorn [27] are illustrated in Table 2.

All reactions and procedures were carried out under normal atmosphere conditions. The Figure 1 exhibits the constitutional formula of investigated active TMZ-NLS component.

Table 1   Schematic ligation pattern of the K(TcT)-NLS-Cys & CPP-Cys modules by disulfide bridge formation. Itemized modules of the investigated TMZ-BioShuttle. The upper line shows the chemical structure of the tetracy-clo-[5.4.2^{1,7}.O^{2,6}.O^{8,11}]3,5-dioxo-4-aza-9,12-tridecadien (TcT) acting as dienophile compound in the DAR$_{inv}$. It is connected via the ε-amino-coupling of the lysine spacer to the nuclear address sequence (NLS). At the right site of the table the CPP module in the single letter code mode is represented. In the upper line the corresponding module in the single code mode is represented.

Modules of the TMZ-BioShuttle			
K(TcT)$^{*)}$-NLS module		NLS-address-peptide with K(TcT)$^{*)}$ - dienophile	
			[K[TcT]KKPKRKV]$^{**)}$
			$^{*)}$ Tetracyclo-[5.4.2^{1,7}.O^{2,6}.O^{8,11}]3,5-dioxo-4-aza-9,12-tridecadien $^{**)}$ Single letter code
Cell penetrating peptide (CPP)		CRQIKIWFQNRRMKKWKK$^{**)}$	

Table 2   Middle and right part of the table represents the dienophile-educt for the DAR$_{inv}$: K(TcT)-NLS-S⌒S-CPP and the TMZ tetrazine spacer derivatized acts as a diene compound as a cargo (left column).

DIENE	DIENOPHILE		
TMZ-cargo with tetrazine-Spacer	NLS-peptide with K(TcT)$^{*)}$-dienophile	Disulfide bridge	cell penetrating peptide (CPP)
	KKPKKRKV(	-S⌒S-	CRQIKIWFQNRRMKKWKK
	$^{*)}$ Tetracyclo-[5.4.2^{1,7}.O^{2,6}.O^{8,11}]3,5-dioxo-4-aza-9,12-tridecadien		
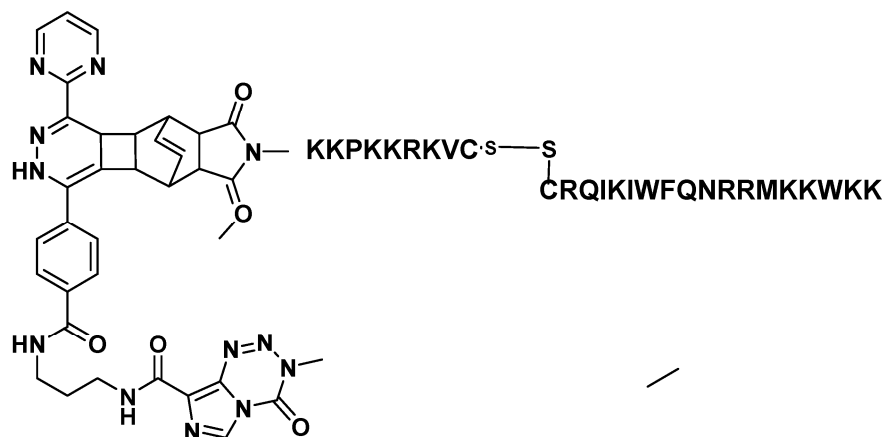
Figure 1   Constitutional formula of the investigated TMZ-BioShuttle

Cell Cycle and Cell Death Analysis

The effects on the cell viability and the cell cycle distribution were determined by DNA flow cytometry.

Flow cytometric analyses were performed using a PAS II flow cytometer (Partec, Munster/Germany) equipped with mercury lamp 100 W and filter combination for 2, 4-diamidino-2-phenylindole (DAPI) stained single cells. From native sampled probes the cells were isolated with 2.1% citric acid/ 0.5% Tween 20 according to the method for high resolution DNA and cell cycle analyses [28] at room temperature with slightly shaking. Phosphate buffer (7.2g $Na_2HPO_4 \times 2H_2O$ in 100ml H_2O dist.) pH 8.0 containing 2, 4-diamidino-2-phenylindole (DAPI) for staining the cell suspension was performed. Each histogram represents 30.000 cells for measuring DNA-index and cell cycle. For histogram analysis we used the Multicycle program (Phoenix Flow Systems, San Diego, CA).

Human lymphocyte nuclei from healthy donors were used as internal standard for determination the diploid cell population. The mean coefficient of variation (CV) of the diploid lymphocytes was 0.8 - 1.0.

Cell viability and apoptotic cells were assessed by flow cytometry with propidium iodide (PI)-method. For detection of apoptotic cells and viability a FACS Calibur flow cytometer (Becton Dickinson Cytometry Systems, San Jose, CA) was used with filter combinations for propidium iodide. For analyses and calculations the Cellquest program (Becton Dickinson Cytometry Systems, San Jose, CA) was used. Each histogram represents 10.000 cells. After preparation according to Nicoletti with modifications [29, 30] measurements were acquired in Fl-2 in logarithmic mode and calculated by setting gates over

the first three decades to detect apoptotic cells. The effect of the used solvent acetonitrile on the viability of lymphocytes was without pathological findings.

Preparation of short term lymphocytes

Human lymphocytes were isolated from 30ml native venous blood from a healthy donor by a lymphocyte preparation with Lymphoprep™ gradient (AXIS-Shield PoC AS, Norway) under sterile conditions. A short term culture was established with lymphocytes using RPMI 1640 medium, containing 10% foetal calf serum and Phytohemagglutinin (PHA-P) (5mg/ml) in phosphate buffer solution PBS (Sigma, Germany) at 37°C and 5% CO_2 for 144 hours.

Treatment of lymphocytes was followed by identical procedures according to the DU 145 cells. The suspension culture was harvested by centrifugation at 800rpm for 10min, rinsed in PBS and marked with propidium iodide (PI) before measurement in a FACS-calibur flow cytometer (Becton & Dickinson, Germany) equipped with a 488nm argon laser and emission filter combinations for red fluorescence (610nm) using the Cell Quest acquisition and analyses software (Becton & Dickinson, Germany). For analysis a minimum of 10.000 cells were counted and the results were presented as histograms in logarithmic-modus. The specific fluorescence intensity was calculated as the ratio of the geometric mean fluorescence values obtained with the specific PI-uptake.

Results

The aims of the study were:

1. to achieve a rapid and high local concentration and an accumulation of TMZ derivative within the cell nuclei using the TMZ-BioShuttle as delivery and targeting system.

2. the presentation, distinction and evaluation of the cell cycle behaviour of aneuploid DU 145 prostate cancer cells after treatment with TMZ alone and with its TMZ-BioShuttle derivative.

Control DU 145 cells form a continuous monolayer, while treated DU 145 cells show loss of adhesion primarily in the TMZ-BioShuttle treated cells combined with spread and attached to the well-plate. Whereas in the TMZ treated cells, the cell closeness was declined and accompanied with an increase of amount of dead cells in the supernatant.

Using the flow cytometry technique not only a differentiation in the cell cycle state of DU 145 cells but also a schedule line of the diploid (**red**) and an aneuploid (**blue**) DNA content of the DU 145 cells is demonstrated as shown in Figure 2 and table 3 A, graphically visualized in 3B.
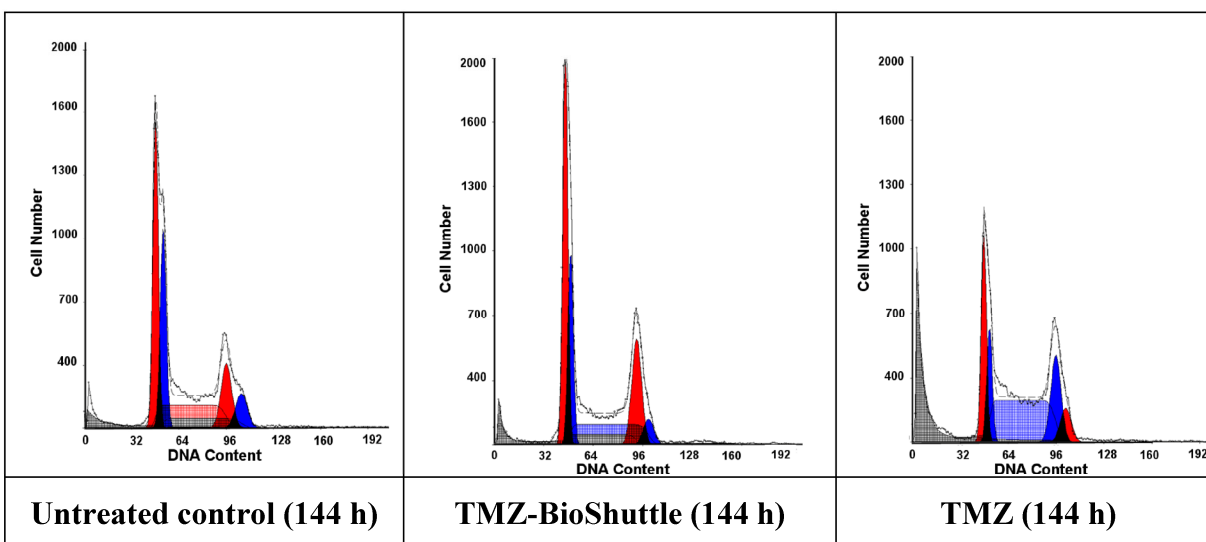

Figure 2 The figure shows the cell cycle distribution of DU 145 cells: In the left part of the figure the plot of the untreated control is demonstrated, the middle and the right plot show the cell cycle distribution 144 h after treatment with TMZ-BioShuttle and TMZ respectively. The prostate cancer cell line exhibits two cell fractions: a **diploid** (DNA-index of 1.0)[**red** coloured] and an **aneuploid** fraction (DNA-index 1.1)[**blue** coloured], close to the diploid. The G1 and the G2M peaks show a diploid and an aneuploid DNA content respectively. S-phase: After TMZ treatment (right plot) the cell number of the aneuploid cells is 10% higher (59 %) compared to aneuploid S-Phase cells in the TMZ-BioShuttle treated probe (middle plot). This in turn was 1.8 fold increased (49 %) compared to the corresponding cell fraction of the control (27 %). The relative amount of the diploid cell fraction differs from the aneuploid fraction: The control shows 35 % diploid cells. The aneuploid fractions reveal decreased amounts 15% (TMZ-BioShuttle) and 3% (TMZ). G2/M phase: The cell cycle behaviour of both cell fractions, the diploid and the aneuploid, show partly opposing effects. In comparison to the control which shows identical percentage of the diploid and the aneuploid cells, the TMZ and the TMZ-BioShuttle treated probes display an increased diploid cell fraction in which the TMZ-BioShuttle probe shows the highest cell contingent. The fraction of aneuploid cells is reduced to 11% whereas the diploid part is increased to 28% in the TMZ-BioShuttle treated probe. The amount of cells in the G2/M phase is increased in a similar ratio of 25 % diploid and 24 % aneuploid cells in the TMZ probe. G1-phase: The comparison of the amount of aneuploid and diploid cells in the G1 phase shows a ratio of 49 % and 54%. The cells in the G1 phase of the TMZ treated probe was increased to 73 % (diploid) and 57 % in the TMZ-BioShuttle probe. The aneuploid cell fractions exhibit an opposite result: the aneuploid cell fraction is decreased to 41% (TMZ-BioShuttle) and 17 % (TMZ).

Table 3   The relative appointment [%] of the particular cell fractions of the cell cycle in DU 145 cells is listed in the table **3A** and vertical bar chart **3B**. Diploid (**red**) and aneuploid (**blue**) DNA contents are demonstrated. The varying cell fraction's properties are clarified by connecting lines.

A

Cell fraction	Untreated control	TMZ-BioShuttle	TMZ
G1 [%]	49*/54**	57/41	73/17
G2/M [%]	19/19	28/11	25/24
S [%]	35/27	15/49	3/59
			* diploid /**aneuploid

B
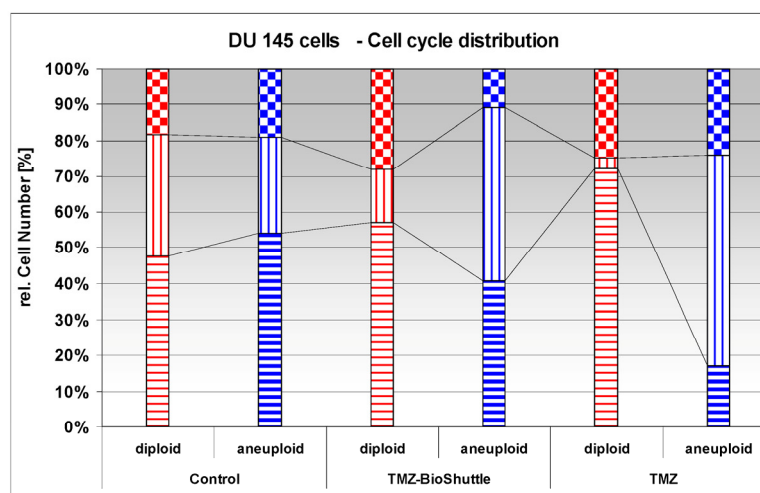
The cell cycle distribution of the untreated control cells shows two different cell fractions harbouring a diploid (49%) and an aneuploid part (54%) in the G1 phase. Both cell fractions have a rate of 19% in the G2/M phase respectively. The cell fraction in the S phase possesses a diploid / aneuploid ratio of 35% to 27%.

The cell cycle distribution of the diploid and aneuploid cell fractions after treatment with TMZ alone and with TMZ-BioShuttle shows different patterns. The amount of the diploid cell fraction and of the aneuploid fraction is opposed in untreated cells compared with the cells in the G1 phase of the TMZ-BioShuttle and TMZ cell fractions (table 3A).

In contrast to the untreated control cell fractions in the G1-phase, the diploid part of the TMZ-BioShuttle treated cells in the G1 phase is slightly increased to 57% whereas the aneuploid fraction is decreased to 41%. This different behaviour

could be caused by an arrest of diploid cells in the G1 phase, whereas the cells of the aneuploid fraction pass from the G1 into the S phase.

This finding is confirmed by the investigation of DU 145 cells in the S phase: TMZ-BioShuttle treated cells show in the S phase cell fraction a decreased amount of diploid (15%) but an increased amount of aneuploid cells (49%). The increase of diploid cells to 28% in the G2/M phase and a parallel decrease of the amount of aneuploid cells to 11% measured after TMZ-BioShuttle treatment exhibit a similar cell cycle behaviour of both cell fractions. This amount of diploid cells suggests an arrest in the G2/M phase, while the aneuploid cell fraction runs through this phase into the S phase.

The DU 145 cells treated with TMZ show different cell cycle behaviour and a different DNA-index-dependant sensitivity. The treatment results in increasing amounts of cells of the diploid

(25%) and of the aneuploid cell (24%) fraction in the G2/M phase.

The amount of diploid TMZ treated cells increased to 73 % (!) featuring an arrest of diploid cells in the G1 phases in contrast to the aneuploid fraction which is reduced to 17%. This could be a hint for a block by TMZ-sensitivity in the G1 phase. The aneuploid cell fraction however proves to be insensitive against TMZ and the G1 phase seems to be reduced because the cells reach the S phase as shown in the measurements. The diploid cells in the S phase are reduced to 3%, whereas the part of aneuploid cells exhibits an extreme increase to 59%!

Moreover, this strong increase of the aneuploid cell fraction in the S phase could be an evidence for a cell cycle block in the S phase.

Lymphocytes treatment

Undesired effects of the TMZ on peripheric lymphocytes of patients often show a leukopenia like hemogram. A reformulation of TMZ should circumvent these adverse reactions, limiting the therapeutic outcome.

Under the aspect of a potential future use of the TMZ-BioShuttle in patients, we investigated fresh human lymphocytes for survival of the treatment in the used concentrations and with our solvent. The life/dead cells data are depicted in Figure 3.

For studies cells were seeded at a density of 1.8×10^6 cells/ml. After incubation with TMZ and TMZ-BioShuttle in a final concentration of 50μM respectively, DU 145 cells were incubated and measured after 48 hours.
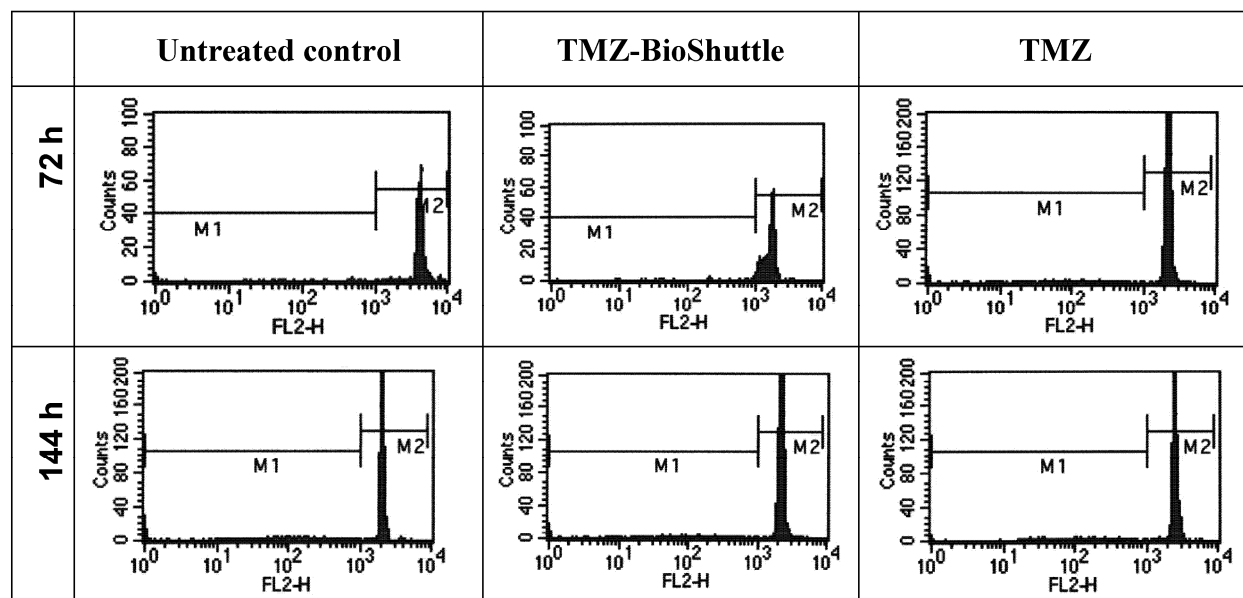
Figure 3 Histograms of lymphocytes of one healthy proband is represented exemplarily. The histogram shows in a log-mode the relative fluorescence intensities of human lymphocytes marked with PI. By setting the gate **M1** apoptotic cells are marked, morphologically intact cells with intact DNA content could be observed with higher relative fluorescence intensity in the gate **M2**.

The observation of effects of the TMZ molecule and its TMZ-BioShuttle respectively in blood of four healthy test persons permits an estimation of the toxicity. 72 hours after treatment the ratio of the fraction of dead cells was nearly constant at a median of 4.7 % and 4.5 % in the untreated control as well as in the TMZ-BioShuttle treated cells. The average of the TMZ treated lymphocytes was increased to 6.2 %. Six days after treatment the amount of dead cells was increased but constant on the untreated lymphocytes and in the TMZ-BioShuttle at 10.2 % whereas the

TMZ-probe showed 14.2 % dead cells.

It turned out that high-resolution flow cytometric measurements are suitable for monitoring the therapeutic success at the cellular level.

The achievement of relevant high local concentrations of therapeutic substances at the side of action, like in the nuclei of tumor cells was realized using delivery systems like the modularly composed peptide-based BioShuttle which brings chemical agents like TMZ into cells and in a second step into the cell nuclei. Regardless the cell's aneuploid state, respon-

sible for the restricted pharmacological effects of chemotherapeutic agents, the local concentration is sufficient to overcome the intractability for cell killing under protection non-affected cells and the surrounding healthy tissue.

Aneuploidy is considered as the primary cause of the high rates and wide ranges of drug resistance in cancer cells.

Discussion

The outcome after chemotherapy still shows poor results with respect to overall survival in the treatment of advanced HRPC [31-33]. The resistance against therapeutic interventions is not completely understood, but various factors may be considered:

The behaviour of this form of PCas could be explained by either the loss of the homoeostasis's controls between cell proliferation and programmed cell death (apoptosis) [34, 35]. Tumorigenesis and progression are independent processes initiated and boosted by aberrant activation of cell cycle activating pathways but also by the inactivation of cell death associated signals resulting in the loss of the proliferation control and in the augmented resistance against apoptosis respectively [36]. Additionally recent data indicate that the inhibition of apoptosis is not associated with the transformation process to malign cells [37]. But affected cells show a prolonged cellular survival time and rate [38] compared with normal tissue. Both events can be detected in highly aggressive prostate cancer resistant to chemo- or /and radiation therapy. Anti-angiogenesis strategies avoiding the disappointing results are discussed [39]. The increasing understanding of molecular mechanisms and of the complex regulatory cellular network gives reason for several molecular approaches with high sensitivity and specificity for successful therapeutic intervention with lower side effects. Several approaches, like siRNA [40], Human-Antigen R (HuR) [41], [A+U]-rich element (ARE) [42], opener/closer mediated [43, 44] gene regulation [45] could be promising strategic approaches [46] in the treatment of HRPC.

But we are at the beginning and up to the clinical practice large scores of hurdles must to be taken. Until then, it must be resorted to reliable currently available drugs like the alkylating agent temozolomide (TMZ). The TMZ new-formulation with the focus enhancing the TMZ transport into the almost untreatable glioblastoma multiforme (GBM) is well documented and revealed higher pharmacological effect as TMZ alone.[47] Recent data indicate, compared to different primary glioblastoma cells, a lower sensitivity of DU 145 prostate cancer cells against TMZ treatment whereas all probes TMZ-BioShuttle treated showed dramatic cell cycle responses and diminished cell viability. [48]

The increased amount of diploid cells in the G1 phase after TMZ treatment suggests a cell trapping of the diploid cells in the G2/M phase. It is well documented that both cell cycle phase points G1 and G2/M represent check points for control and repair maintaining the genomic DNA-integrity.[49-51] Therefore among other things, both phases are characterized by low sensitivity against DNA-damaging interventions like exposition to ionizing radiation and after chemotherapeutic alkylation [52], whereas the latter part of the S phase is highly sensitive against DNA-damaging effectors.[53] Presumably the block of the diploid cell fraction of the DU 145 cells in the G1 phase allows the DNA-repair and subsequently the re-entry in the cell cycle. Therefore these cells turn out to be refractory against TMZ as shown in the TMZ-based therapy of advanced CaP.

In contrast to the described DNA fragmentation of glioma cells after TMZ treatment [54] the TMZ-BioShuttle treated glioma cells exhibited a deviant pattern: no comet formation indicating DNA single-strand breaks but cells swollen were observed.[48] This results from a loss of plasma membrane integrity which suggests nuclear chromatin decondenzation considered as necrosis biomarker.[55, 56] The observation, that the TMZ and the TMZ-BioShuttle influence unequally the cell cycle behaviour of DU 145 prostate cancer cells could indicate a mode of action different from the documented methylation of the O^6 position of guanine in the genomic DNA [57].

It remains to speculate to which extent the aneuploidy state of the DU 145 cells could influence the pharmacological effect of the TMZ on the proliferation behaviour. Flow cytometric cell cycle studies exhibit an enhanced fraction of S phase cells of the numeric aberrant chromosomes harbouring cells. DU 145 karyotype analyses show the threefold existence of the chromosome 8. The impact of alterations of chromosome 8 and the high-grade state of advanced prostate carcinoma is well documented and appears to be associated with poor prognosis [58]. We would like to emphasize that alterations of the chromosome 8 indicate an early critical step in the prostate tumorigenesis [59]. C-*myc* is localized at chromosome 8 [60] and its overrepresentation is associated with prostate cancer progression [61]. Due to the fact that prostate cancer is one of the leading causes of death in the industrialized world, the need of new approaches for control the CaP is the major goal in the current research.

As the results show the DNA-cytometry proves to be a dedicated diagnostic tool in the cytopathology by measurements of the DNA-content in cells and tissues. Within the scope of the tumor diagnostics, objective and valid gradiations of the malignant potential of cells of different tumors and inside of a tumor (in process control) are possible. For the purpose of the malignant grading the extent of the DNA-aneuploidy must be quantified. Given the fact that different tumor identities present different secondary and tertiary aberrations of chromosomes during the tumor progression, the prognostic interpretation of the DNA-distribution must be realized tumor-specifically. In case of CaP (early state) the DNA malignant grading allows relevant early therapeutic decisions. High resolution flow cytometry is an appropriate tool not restricted to the monitoring of the therapeutic effect. DNA aneuploidy, as determined with high-resolution flow cytometry, has been shown to be an excellent and independent predictor of cell survival [62].

Conflict of Interest

The authors have declared that no conflict of interest exists.

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