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Inverse-electron-demand Diels-Alder reaction as a highly efficient chemoselective ligation procedure: Synthesis and function of a BioShuttle for temozolomide transport into prostate cancer cells‡§

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Hormone-refractory prostate cancer (HRPC), insensitive to most cytostatic interventions, features low response rates and bad prognosis. Studies with HRPC treated with temozolomide (TMZ) showed a poor response and the results were discouraging. Therefore, TMZ has been considered to be ineffective for the treatment of patients with symptomatic and progressive HRPC. A solution to this problem is demonstrated in this study by combining proper solid-phase peptide synthesis and a chemoselective new 'click' chemistry based on the Diels–Alder reaction with 'inverse-electron-demand' (DAR_{inv}) for the construction of a highly efficient TMZ-BioShuttle in which TMZ is ligated to transporter and subcellular address molecules. The transport to the targeted nuclei resulted in much higher efficiency and better pharmacological effects. The reformulation of TMZ to TMZ-BioShuttle achieved higher *in vitro* killing of prostate cancer cells. Accordingly, the potential of TMZ for the treatment of prostate tumors was dramatically enhanced even in a tenfold lower concentration than applied normally. This TMZ-BioShuttle may be well suited for combining chemotherapy with other cytostatic agents or radiation therapy.

Supporting information may be found in the online version of this article

Keywords: BioShuttle; chemoselective ligation; inverse Diels-Alder reaction; prostate cancer; temozolomide

Introduction

In recent years, the redesign of established and old-fashioned drugs to highly active pharmaceuticals of improved therapeutic index has attracted great attention [1–4]. In the field of brain tumors, especially in the therapy of glioblastoma multiforme (GBM), temozolomide [(TMZ), 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3*H*)-one] is a widely used anticancer drug. In a current phase III study it has been shown that TMZ improves survival in GBM when accompanied by radiotherapy [5]. Encouraging data [6] support its application in other tumor therapies such as prostate cancer. The action of TMZ has been extensively studied [7,8]. It is rapidly absorbed into the bloodstream and spontaneously decomposed at physiological pH to the cytotoxic methylating agent 5-(3-methyltriazeno)-imidazole-4-carboxamide (MTIC). The average half-life and apparent oral systemic clearance values are 1.8 h and 97 ml/min/m^2, respectively. Pharmacokinetic analysis performed for TMZ and its active metabolite MTIC revealed neutropenia and thrombocytopenia with dose limits of 1.0 mg/m^2 [9]. The maximum tolerated dose (MTL) of TMZ depends upon the indication with 750 mg/m^2 in the therapy of advanced cancer and lymphoma [10], and 300 mg/m^2 in patients with recurrent brain tumors [11]. The half-life of TMZ in plasma [12] and the non-target-gene-specific alkylating mode of action can lead to undesired adverse reactions which hamper the therapeutic efficiency and in the worst cases require interruption of the therapy. Therefore, TMZ represents a promising candidate for a drug redesign as new

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§ *This article is dedicated to the winner of the Nobel Price Award 2008 in Medical Sciences Harald zur Hausen, the retired Head of the German Cancer Research Center.*

TMZ derivatives could well-bypass these drawbacks, but retain as second-generation alkylating agents the high therapeutic efficacy.

It is well established that the cell membrane permeability of molecules coupled to a nuclear localization signal (NLS) is poor and that permeabilized cells are required [13]. To improve the membrane transport without perturbing cells, different laboratories have proposed viral vectors for the transport [14–18] and non-virally-based transport vehicles such as cell penetrating peptides (CPPs) [19–24]. Our approach to this problem is schematically outlined in Figure 1 and consists of coupling of TMZ to the nuclear localization sequence NLS(SV40-T), which is disulfide-bridged to the CPP pAntp$_{43-58}$ resulting in an active nuclear targeting. Such a nonviral construct, named BioShuttle, was expected to facilitate the cellular entry and nuclear targeting of TMZ as previously observed in our laboratory for the delivery of peptide nucleic acids (PNA) [25]. Indeed, after the passage of the TMZ-BioShuttle across the cellular membrane into the cytoplasm, the peptide-module responsible for the transmembrane passage discharges its TMZ-NLS(SV40-T)-Cys-OH module containing the cargo TMZ by cytoplasmic glutathione or disulfide reductases. Thereby the NLS sequence serves as a substrate for importin/Ran-mediated active transport into the cell nucleus [26,27] to enrich the pharmacologically active molecule at the target site inside the nucleus.

Among the various chemoselective ligation chemistries for peptides and proteins, the normal-electron-demand Diels–Alder cycloaddition has been proposed [28], while the inverse-electron-demand Diels–Alder (DAR$_{inv}$) reactions have been only most recently applied for bioconjugation. During preparation of the manuscript, a related publication has appeared [29].

Tetrazines as dienes react exceptionally fast and irreversibly with dienophiles and N_2 is produced as the only by-product upon subsequent retro-[4-2]cycloaddition as shown in Figure 2 [30,31]. Since 3,6-diaryl-tetrazines react in water with dienophiles, we have selected such derivatives for the assembly of the TMZ-BioShuttle.

In the present study, we report the high efficiency of this ligation chemistry in aqueous solution without interference of the amino acid functionalities of peptides and thus proteins. Accordingly this cycloaddition reaction via constrained unsaturated ring systems, i.e. the Diels–Alder reaction with 'inverse-electron-demand' (DAR$_{inv}$) may well represent a promising biorthogonal ligation method [32].

With the newly designed TMZ-BioShuttle (**8**) a significantly increased efficiency of the drug in the human prostate cancer cell line DU-145 was observed.

Materials and Methods

All chemical reactions and procedures were carried out under normal atmospheric conditions. Pure TMZ, the educts as well as all solvents for chemical syntheses were purchased from Sigma–Aldrich, Germany. The chemicals used for peptide synthesis and purification were purchased from Roth, Germany. The solvents were of reagent grade and used without further purification. Amino acids, derivatives and coupling agents were purchased from Merck Bioscience, Germany. Cleavage reagents were from Fluka–Sigma-Aldrich, Buchs, Switzerland). RPMI cell culture medium, fetal calf serum (FCS) and glutamine used for biological studies were obtained from Becton and Dickinson, (B&D), Germany. Mass spectra were recorded with laser desorption mass spectrometer TSQ 7000 (ThermoFinnigan) (ESI).

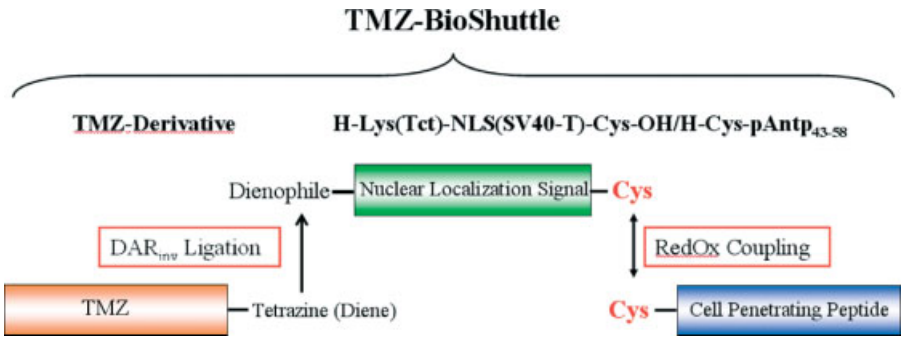

Figure 1. Schematic representation of the modular organization of the TMZ-BioShuttle and its assembly. It consists of a CPP module that facilitates the transport across cell membranes (blue) connected via a redox cleavage site (disulfide bridge) to the NLS address module (green) carrying a cyclooctatetraene as the dienophile. Coupling of this dienophile with the cargo TMZ (ocher) as tetrazine derivative (diene component) occurs via DAR$_{inv}$ with formation of pyridazine as spacer.

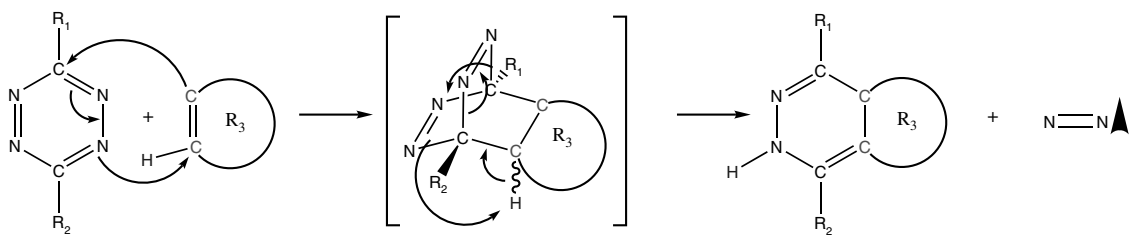

Figure 2. Mechanism of the inverse-electron-demand Diels–Alder reaction. In a first step, the reaction sites of the diene and the dienophile react in a [**4** + **2**] cycloaddition as indicated by the arrows. Formation of gaseous nitrogen makes this reaction irreversible and produces the dihydropyrimidine derivatives as exclusive reaction products. R_1, R_2 and R_3 indicate appropriate substituents at the reaction sites of diene and dienophile.

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Synthesis of the TMZ-Bioshuttle

N-(2-Aminopropyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (4)

4-(6-(Pyrimidin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)benzoic acid (**3**) was prepared from compounds **1** and **2** by reaction with hydrazine and then oxidized with sodium nitrite to the tetrazine derivative following known procedure [33]. The tetrazine derivative was converted with thionyl chloride under standard conditions to the chloride. To a suspension of this acid chloride (2 mmol) in 20 ml CH_2Cl_2, a solution of *N*-Boc-1,3-diaminopropane (2 mmol) and TEA (2 mmol) in 10 ml CH_2Cl_2 was slowly added at 0–5 °C. The resulting deeply colored solution was maintained for 4 h at room temperature. Then the organic phase was washed with water, followed by 1 N HCl and again water. The organic layer was dried over Na_2SO_4 and evaporated. The resulting residue was chromatographed on silica gel by elution with chloroform/ethanol (9 : 1) and further purified by recrystallization from acetone. Yield: 50–70% depending on the quality of the carboxylic acid. ESI MS: *m/z* 437.2 $[M]^+$ (Supporting Information, Figure S1). The Boc-protected derivative was treated with TFA (5 ml) for 30 min at room temperature and isolated by evaporation to a solid residue (**4**) (ESI: *m/z* 337.2 $[M]^+$).

3-Methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxylic acid chloride (5)

3-Methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxylic acid was converted to the corresponding chloride (**5**) as described by Arrowsmith [34]. The acid (2 mmol) was refluxed with thionyl chloride (10 ml) until the acid was completely dissolved. The excess of thionyl chloride was evaporated under vacuum and the resulting solid was stored over NaOH in a desiccator.

3-Methyl-4-oxo-*N*-(3-(4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamido)propyl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (TMZ-tetrazine diene, 6)

Compound **4** (0.5 mmol) and the chloride **5** (0.5 mmol) were dissolved in 5-ml chloroform and 5 ml-TEA at 0–5 °C. After 4 h at room temperature, the solution was washed with water, 1 N HCl and with water again. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by chromatography (silica gel); chloroform/ethanol (9.5/0.5). Yield: 68%; ESI: *m/z* 536.3 $[M + Na]^+$ (Supporting Information, Figure S1).

Boc-Lys(Tct)-OH (12)

The Reppe anhydride (**11**) was prepared from 42 mg of (1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene (**9**) and 44 mg maleic anhydride (**10**) in chloroform as described by Reppe [35]. The anhydride (10 mmol) was reacted with Boc-Lys-OH (10 mmol) in 50-ml methanol under reflux over 3 h. After evaporation of the solvent, the residue was crystallized from methanol; yield 79%; ESI: *m/z* 536.3 $[2M - H]^-$ (Supporting Information Figure S2).

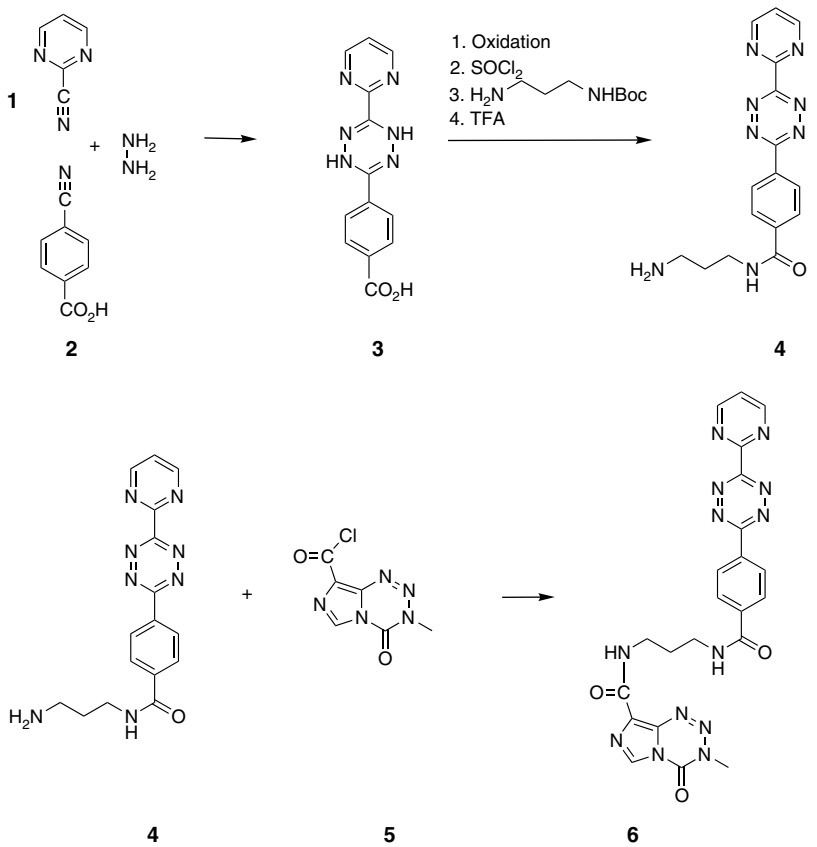

Figure 3. Synthetic route for the preparation of the TMZ-tetrazine diene (**6**).

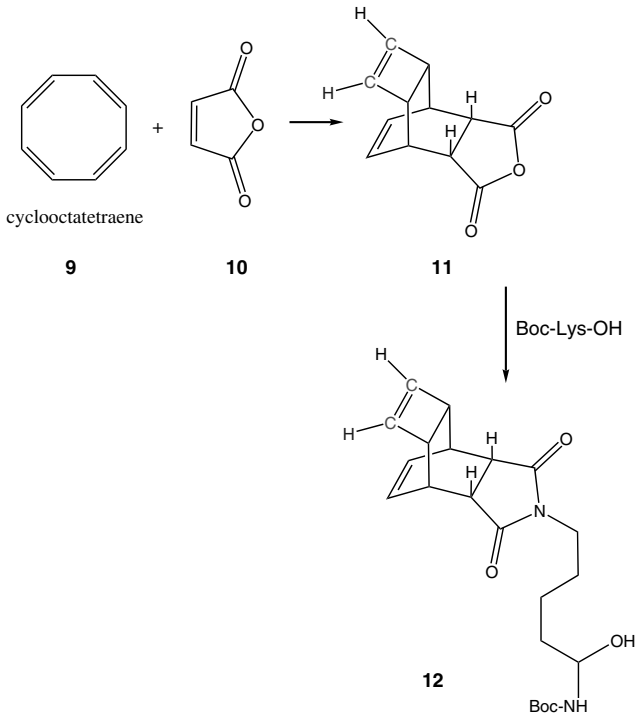

Figure 4. Scheme of synthesis of the Reppe anhydride which was used for the preparation of the lysine derivative **12**.

Syntheses of the Lys(Tct)-NLS(SV40-T)-Cys Module and of Cys-pAntp$_{43-58}$ as CPP Module

For solid-phase synthesis of the Lys(Tct)-Lys-Lys-Pro-Lys-Lys-Arg-Lys-Val-Cys-OH [Lys(Tct)-NLS(SV40-T)-Cys] and of the Cys-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-OH (Cys-pAntp$_{43-58}$), the Fmoc-strategy was applied in a fully automated multiple synthesizer (Syro II,MultiSynTech, Witten) [36]. The synthesis was carried out in an 0.05 mmol scale on a Fmoc-Lys(Boc)-polystyrene resin (1% cross-linked) with 0.053 mmol/g loading and Fmoc-Cys(Trt)-polystyrene resin (1% cross-linked) with 0.005 mmol/g loading. As coupling reagents, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/HOBt/DIPEA (1 : 1 : 1) was used. Cleavage/deprotection of the peptide-resin was performed with TFA/ethanedithiol/thioanisole/phenol (90 : 5 : 2.5 : 2.5) for 2.5 h at room temperature. The products were precipitated with ether. The crude products were purified by preparative HPLC on a Kromasil 300-5C18 reverse phase column (20 × 150 mm) using as eluents 0.1% TFA in water (A) and 60% acetonitrile in water (B). The peptides were eluted with a linear gradient from 25% B to 60% B in 40 min at a flow rate of 20 ml/min. The fractions containing homogeneous peptides were lyophilized; yields: 9.1 mg of H-Lys(Tct)-[SV40-T]-Cys-OH (14%); ESI: *m/z* 1333.2 $[M]^+$; 22.5 mg of HO-Cys-pAntp$_{43-58}$ (19%); ESI: *m/z* 2350.1 $[M]^+$.

Disulfide-Crosslinked H-Lys(Tct)-NLS(SV40-T)-Cys-S-S-Cys-pAntp$_{43-58}$- (7)

Oxidation of H-Lys(Tct)-NLS(SV40-T)-Cys-OH (9.1 mg; 6.8 µmol) and HO-Cys-pAntp$_{43-58}$ (16 mg; 6.8 µmol) in aqueous solution of 12.5 ml (2 mg/ml) concentration was performed with 20% DMSO for about 5 h. The oxidation progress was monitored by analytical C18 reversed-phase HPLC, and the disulfide cross-linked heterodimer was purified by preparative HPLC as described for the single peptides. Yield: 8 mg; 31, 7%. For HPLC and mass spectrum of the purified product see Figure 5 (ESI).

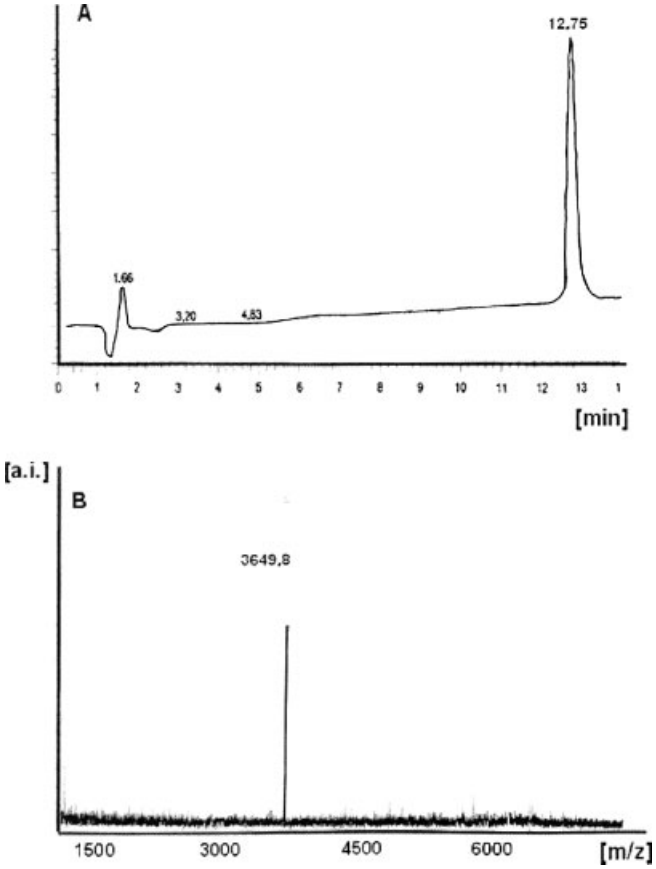

Figure 5. HPLC (**A**) and mass spectrum (ESI) (**B**) of the disulfide cross-linked H-Lys(Tct)-NLS(SV40-T)-Cys-S-S-Cys-pAntp$_{43-58}$ (**7**). For HPLC conditions see Experimental section.

Ligation of the Dienophile H-Lys(Tct)-Nls(SV40-T)-Cys-S-S-Cys-pAntp$_{43-58}$ with the TMZ-Tetrazine Diene (6)

Equimolar amounts of the TMZ-tetrazine conjugate (**6**) (1.03 mg; 2 µmol) and H-Lys(Tct)-NLS(SV40-T)-Cys-S-S-Cys-pAntp$_{43-58}$ (**7**) (7.3 mg, 2 µmol) were dissolved in aqueous solution and stored at room temperature for 24 h. The DAR$_{inv}$ reaction occurs at room temperature and was completed after the color changed from magenta to yellow. The product was isolated by lyophilization; yield: 98%; MS ESI: *m/z* 4165.5 $[M]^+$; calculated $C_{189}H_{289}N_{68}O_{35}S_3$ 4165.1; homogeneous on HPLC (Figure 7).

Cell Culture

The hormone nonsensitive adherent prostate cancer cell line DU-145 [37] was maintained in RPMI cell medium supplemented with 5% FCS and 4 mM glutamine at 37 °C in 5% CO_2 atmosphere. For studies and microscopic monitoring, cells were seeded at a

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density of 1.8×10^6 cells/ml. After incubation with TMZ and TMZ-BioShuttle at a final concentration of 50 μM, DU-145 cells were incubated for 144 h.

Chemotherapy Treatment of Prostate Cancer Cells

Pure TMZ was subdivided into two parts for subsequent processing. One part was followed up and the second part, after chemical transformation in the corresponding acid chloride, was used for coupling to our transporter molecules.

TMZ and TMZ-BioShuttle were dissolved in 10% aqueous solution of acetonitrile (Sigma–Aldrich, Germany). Control studies with acetonitrile were performed to exclude potential toxic effects of this solvent.

DU-145 cells were suspended (1.8×10^5 cells/ml) in RPMI (control), in RPMI containing appropriate amounts of TMZ and the TMZ-BioShuttle (**8**) (50 μM) and their behavior was analyzed up to 6 days.

Results

Synthesis of TMZ-BioShuttle

For ligation of TMZ to the peptidic portion of the BioShuttle construct via the DAR_{inv}, the cargo TMZ was linked to a suitable tetrazine derivative as shown in Figure 3. For this purpose a carboxy-functionalized 3,6-diaryl-tetrazine was coupled to a diamine spacer for subsequent covalent linkage of the TMZ via its carboxylic acid chloride to produce the highly reactive diene **6**. This key intermediate was obtained as well-characterized homogenous compound.

For the synthesis of the dienophilic peptidic NLS module, Boc-Lys-OH was reacted with the Reppe anhydride [33] to produce the maleinimido derivative Boc-Lys(Tct)-OH (**12**) as outlined in Figure 4. On the basis of our previous experience [38], we selected as NLS module the sequence of (SV40-T) [26] which was N-terminally elongated with the dienophile Lys(Tct) and C-terminally with a cysteine residue. As CPP portion of the BioShuttle, the hexadecapeptide sequence of $pAntp_{43-58}$ was N-terminally extended with a cysteine residue for subsequent disulfide cross-linking with the H-Lys(Tct)-NLS(SV40-T)-Cys-OH peptide derivative. Both peptides were obtained by standard solid-phase synthesis using the Fmoc-strategy. Their subsequent disulfide cross-linking was carried out by oxidation of an equimolar mixture of the two Cys-peptides with DMSO. Although a selective procedure for the disulfide formation was not applied, the heterodimer **7** could be isolated as homogeneous material with the correct mass (Figure 5).

As shown in Figure 6, in the last synthetic step, the diene **6** and the dienophile **7** were reacted in aqueous solution leading to practically quantitative ligation of the two components to produce the target TMZ-BioShuttle (**8**) as well-characterized compound (Figure 7).

The biological activity of the TMZ-BioShuttle (**8**) was tested in cell culture. In the following section, we show the degradation of the chromatin in treated prostate cancer cells.

Comet Formation in DU-145 Prostate Cancer Cells Induced by TMZ and TMZ-BioShuttle

The human prostate epithelium adenocarcinoma, herein referred as DU-145 (Figure 8) was used to investigate the pharmacological effect of TMZ and TMZ-BioShuttle (**8**). The DNA damage and cell death in the DU-145 cell line were evaluated after treatment with TMZ and TMZ-BioShuttle at a final concentration of 50 μM. Comet assays were performed in parallel probes (middle column and right column of the Figure 8). The untreated cultures of DU-145 prostate cancer cells (left column) did not exhibit fragmented DNA.

After 40-h treatment with TMZ (final concentration 50 μM), the DU-145 cells seem to be undisturbed and no visual change in the phenotype could be observed under the light microscope. Similarly, the comet assay does not reveal a higher sensitivity and exhibits no fragmented DNA in the probes treated with 50 μM TMZ after 40 and 70 h. This is in contrast to the probes treated with TMZ-BioShuttle, exhibiting comets and almost swollen nuclei 40 h after treatment. After 70 h, the probes display a deviant behavior.

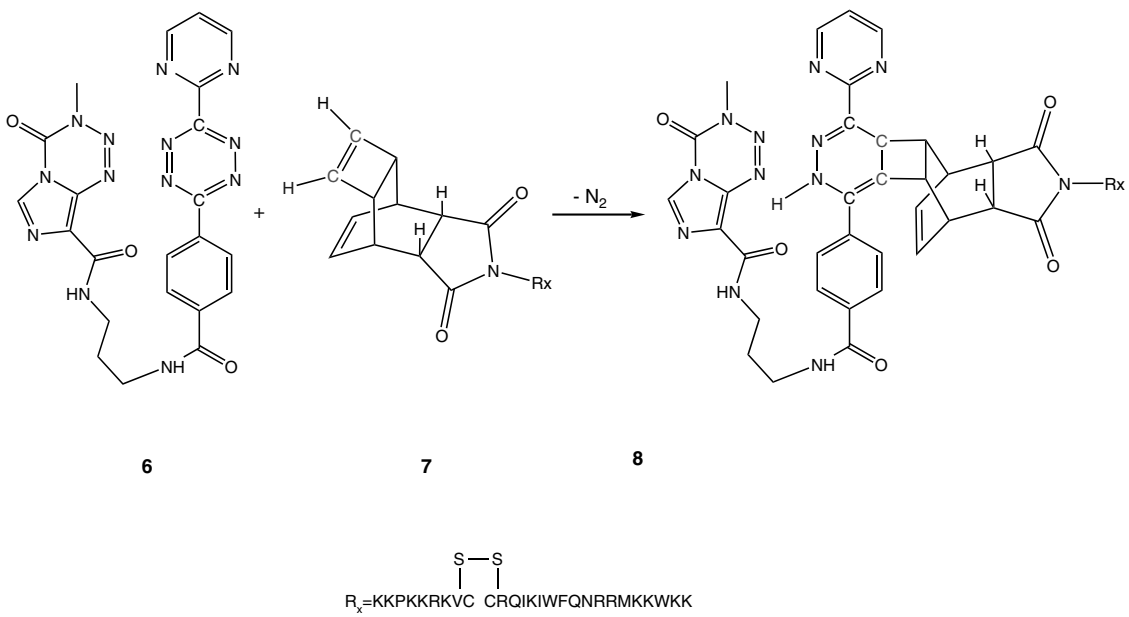

Figure 6. Chemoselective DAR_{inv} ligation of the diene component with the dienophile **7** to produce the target TMZ-BioShuttle (**8**).

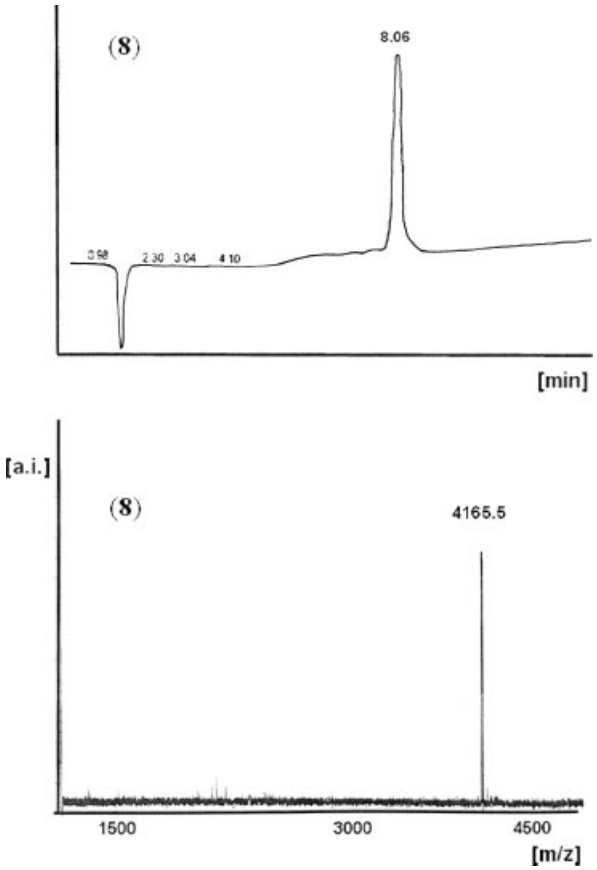

Figure 7. HPLC (for conditions see Experimental section) and ESI mass spectrum of the TMZ-BioShuttle (**8**).

The cells offer less DNA fragmentation but their nuclei are partly shrunken and disintegrated by damaged chromatin.

Discussion

As definitive treatments of prostate cancer, the leading cause of morbidity and mortality in man, remain surgery, radiation therapy, and hormonal therapy in case of metastatic progress. But the timing of treatment in all stages of the disease remains controversial and new therapies are needed [39,40]. Encouraging results of the chemotherapeutic TMZ treatment in brain tumors [41] remain unendorsed in the treatment of HRPC [42] and prostate cancer is difficult to treat.

The results of the present study demonstrate a close relationship of the reformulated drug with open clinical questions (as described in the Introduction) as a suitable solution for the patients. The TMZ ligation to peptidic components, which facilitate the cell membrane translocation as well as the targeting to subcellular compartments using appropriate address molecules holds tremendous potential to optimize therapeutic interventions of diseases. Enhanced cellular delivery and transport of active substances like TMZ into the cell nuclei, as site of pharmacological action, allow to expect lower application doses with concomitantly decreased side effects. The criteria to determine the applicability for targeting including accessibility, specificity, safety and subcellular precision are documented [43,44]. In order to fulfill all these biomedical aspects, the binding of the TMZ drug as a cargo to transporter molecules (TMZ-BioShuttle) needed proper chemical methods. These coupling reactions, however, can lead to novel properties of the TMZ and required a reformulation of the active drug TMZ. Chemoselective reaction conditions in aqueous solution and at room were obtained by chemical ligation of functional peptides via the DAR_{inv}. The 'Click' chemistry based on the DAR_{inv} with 'inverse electrons demand' resulted in an impressive efficiency. This reaction enhances the economics of the chemical reaction by several parameters: (i) the increase of the reaction rate, (ii) gentle reaction conditions at room temperature and (iii) the resulting reaction kinetics does not demand excess of the educts. Furthermore, by careful performance of the chemical reaction, pharmacologically active but sensitive and highly unstable pharmaceutical ingredients can be ligated without loss of function. The BioShuttle-mediated delivery and targeting platform that facilitates the transport of DNA derivatives into living cells [38,45] and of diagnostics into cytoplasm and cell nuclei, was documented [46,47]. Similarly, the TMZ-BioShuttle confirmed a high potential for patients treatment and represents an attractive drug for upcoming clinical combined chemotherapeutic approaches.

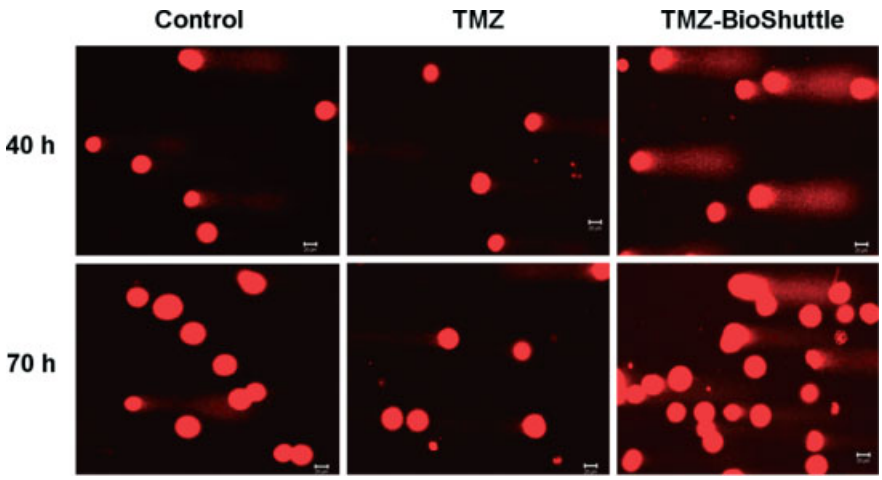

Figure 8. The comet formation of DU-145 prostate cancer cells by treatment with TMZ and TMZ-BioShuttle (final concentration 50 μM) after 40 h (upper line) and 70 h (lower line), respectively. The left column exhibits the untreated control cells. The scale bars (white) represent 20 μm.

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