Original Article
Monitoring cytotoxicity of gemcitabine and cisplatin in T24 bladder cancer cells by the use of F-18-FDG and F-18-FMC

Jens Rabenstein[1*], Dagmar-C Fischer[2*], Oliver W Hakenberg[1], Daniela Jahn[1], Wolfgang Rutz[3], Alexander Hohn[3], Martin Heuschkel[3], Philipp Cuber[3], Bernd J Krause[3]

Departments of [1]Urology, [2]Paediatrics, [3]Nuclear Medicine, Rostock University Medicial Centre, Rostock, Germany. *Equal contributors.

Abstract: Purpose: We elucidated the utilization of [18F]-Fluor-deoxy-glucose (FDG) and [18F]-Fluor-methyl-choline (FMC) as imaging biomarkers for the T24 bladder cancer cell line for therapy monitoring. Methods: In T24 cells Michaelis-Menten kinetics for FDG and FMC were obtained. Inhibition of FDG uptake was assessed in the presence of α-D-glucose. In case of FMC uptake sodium and hemicholinium-3 (HC-3) as well as tetraethylammonium (TEA), i.e. an inhibitor of the organic cation transporter (OCT), were used as competitors. Changes in FDG and FMC uptake caused by gemcitabine (gc), cisplatin (cis) and the combination of both were evaluated. The glucose transporter (GLUT1) and choline transporter-like protein (CTL1) were detected via Western blotting. Results: Michaelis-Menten kinetics revealed a saturable transport of FDG and FMC with K_m-values of 111.8 µM glucose and 70.8 µM choline, respectively. Uptake of FDG was faster and the total amount internalized was about three times higher compared to FMC. FMC uptake was Na^+-independent and half-maximal inhibition was achieved with 166 M HC-3 and 53 M TEA, respectively. Western blot analysis confirmed the presence of CTL1 and GLUT1 in T24 cells. These transporters are the target structures for molecular imaging by FMC and FDG, respectively. Treatment of cells with IC_{50} concentrations of gc, cis and the combination of both caused a significant increase of FMC uptake compared to FDG. Conclusions: In T24 cells FMC uptake is realized through a Na^+-independent and HC-3 sensitive intermediate affinity choline transporter-like protein CTL1. A 24 h pre-incubation with gemcitabine (gc), cisplatin (cis) or gc+cis increased the uptake of imaging biomarkers most probably due to early and unspecific changes preceding apoptosis rather than clonal selection of chemoresistant cells.

Keywords: 18F-deoxy-glucose, 18F-methyl-choline, T24 bladder cancer cells, chemotherapeutics, gemcitabine, cisplatin, FDG-PET, FMC-PET

Introduction

With an incidence of approximately 110.000 per year, bladder cancer (BCa) is one of the most common malignancies of the urinary tract in Europe [1]. At time of diagnosis, about 75% and 25% of patients present with non-muscle-invasive-cancer (NMIBCa) and muscle-invasive-cancer (MIBCa), respectively [1-4].

10 to 30% of patients with MIBCa are at risk of regional lymph node metastasis and an associated poor prognosis [5, 6]. Therefore, reliable staging/restaging of patients with high risk BCa or MIBCa and an indication for radical cystectomy are essential for optimization of treatment strategy [7].

Positron emission tomography (PET) in combination with computerized tomography (CT) has been established as a powerful imaging tool for staging/restaging and FDG as well as ^{11}C-choline are currently the preferred tracers in patients with BCa [8-12]. In tumor cells, internalization of FDG is mediated mainly via the insulin independent transporter protein GLUT-1 [13, 14]. In case of FMC the situation is less clear as a variety of protein-mediated systems for uptake of choline are documented [15].

While FDG-PET/CT scanning is able to detect metastatic lesions of urinary bladder cancer with high accuracy, detection of primary bladder wall lesions proved to be difficult due to

renal clearance and concomitant urinary accumulation of FDG [12, 16, 17]. To overcome this problem, various interventions like bladder irrigation, increased hydration or forced diuresis with furosemide increased sensitivity and specificity reasonably [8-10, 18]. In contrast, the renal clearance of ^{11}C-choline is almost negligible and thus this compound is by far better suited to detect bladder tumors [19].

Because morphological changes in response to treatment are often seen only after several months, imaging biomarkers for detecting early treatment responses are of crucial interest. In urooncology, FDG-PET/CT is a sensitive and well established method to monitor glucose metabolism after androgen ablation in prostate cancer patients [20]. Furthermore, in a xenograft model with PC3 and LNCaP cells, PET/CT with ^{11}C-choline as tracer revealed a delay of tumor growth already one week after initiation of cytotoxic therapy [21].

By contrast, very little is known about tracer uptake and its modulation by cytotoxic therapy in BCa cells. Therefore, we used T24 bladder cancer cells to characterize the uptake of FDG and FMC with and without concomitant gemcitabine and/or cisplatin therapy.

Materials and methods

Materials

^{18}F and FDG were purchased from Eckert & Ziegler Radiopharma GmbH, Berlin, Germany and a gamma counter 2470 Wizard^2 (Perkin Elmer, Waltham Massachusetts, USA) was used.

Methods

Cell culture: The T24 human bladder cancer cell line was purchased from LGC Standards (ATCC, Wesel, Germany). Cells were maintained in DMEM high glucose supplemented with 10% fetal bovine serum, 100 U/l penicillin, and 100 mg/l streptomycin (Life Technologies, Carlsbad CA, USA) in an humidified atmosphere (37°C and 5% CO_2) and splitted twice a week. For this purpose, cells were treated 2 min. with ice-cold trypsin-EDTA (0.5% trypsin/0.2% EDTA; Sigma-Aldrich Munich, Germany). Cells were counted automatically with a CASY Modell TT cell counter (OLS, Bremen, Germany).

Determination of IC$_{50}$-concentrations of gemcitabine and cisplatin: To determine the IC$_{50}$-concentrations of gemcitabine (gc; Fresenius Kabi, Bad Homburg, Germany) and cisplatin (cis; TEVA, Ulm, Germany), T24 cells were used after 5 days of culturing. Cells were plated in 6-well plates (1.6*10^6 cells/well) and each well was supplemented with 4 ml complete DMEM (see above). After 24 hours the medium was replaced by 4 ml of complete DMEM containing either gc (0.5 to 4 ng/ml; 0.002 - 0.015 nmol/ml) or cis (50 ng/ml to 2 µg/ml; 0.17 - 6.66 nmol/ml) or the combination of both (total concentrations between 50.35 ng/ml and 2014 ng/ml). Cells were maintained under these conditions for 5 days and all concentrations were assayed in duplicate. Subsequently, cells were harvested, counted and IC$_{50}$-concentrations were calculated using Origin 6 software (OriginLab Cooperation, Northampton, USA).

Radiopharmaceutical uptake in the absence and presence of cytotoxic treatment: FMC was synthesized with a GRP-Modul (Scintomics, Fuerstenfeldbruck, Germany) and a FMC-Kit (ABX, Radeberg, Germany). For uptake studies, 2×10^5 subconfluent T24 cells were incubated with 1 MBq (15.78 fmol) of FDG or FMC in a total volume of 200 µl PBS without Ca^{2+} and Mg^{2+}. The mixture was kept in a shaking incubator (37°C, 140 rpm) and uptake was allowed to proceed for 30 to 240 min. Reactions were terminated by immediate transfer into ice-cold PBS (1 ml). The mixture was stored on ice for at least 30 min prior to centrifugation (5 min at 5000 rpm and 0°C). The cell pellet was washed three times (1 ml per wash) with ice-cold PBS, carefully dried and submitted to γ-counting. To determine Na$^+$-dependency of FMC uptake, PBS was replaced by uptake buffer (25 mM Tris/HCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, 5 mM glucose, pH 7.4), supplemented either with 140 mM NaCl or 140 mM N-methyl-D-glutamine (NMDG). To investigate the effect of cytotoxic treatment, 5*10^5 T24 cells were plated on large culture dishes (147 mm Ø). After 24 hours, medium was replaced by 20 ml of medium containing the IC$_{50}$-concentrations of gc, cis, or both. After 1 day of cytotoxic treatment, cells were used in uptake procedures as described above. Cellular protein content was determined according to the method of Bradford (Bio-Rad, Munich, Germany) and uptake is expressed as fmol/mg cell protein.
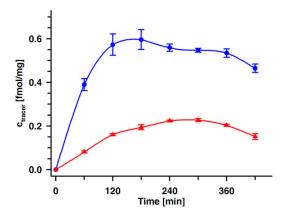
Figure 1. Uptake of FDG (blue) and FMC (red) in T24 cells. Cells were incubated with either compound under standard conditions for 7 hours.
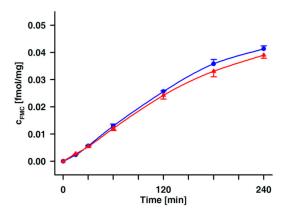

Figure 2. FMC uptake by T24 cells in the presence (blue) or absence (red) of NaCl. Cells were treated under standard uptake conditions and PBS was substituted by uptake buffer supplemented with either 140 mM NaCl (blue) or 140 mM NMDG (red).

Inhibition of FMC uptake: FMC inhibition assays were performed with varying concentrations of either HC-3 (4 nM to 40 mM) or TEA (100 nM to 2.4 mM). HC-3 is described as a specific competitive inhibitor of the high-affinity choline transporter (CHT1) while TEA is known to inhibit all enzymes from the organic cation transporter (OCT) family [22, 23]. After preincubation of cells (2×10^5) with inhibitors for 15 min (37°C and 140 rpm) FMC (1 MBq) was added and uptake was allowed to proceed for 90 minutes. Termination of FMC uptake and quantification was achieved as described above.

Calculation of Michaelis-Menten kinetics and IC$_{50}$-concentrations for α-D-glucose and choline: FDG- and FMC-uptake was assessed in the presence of increasing amounts of specific inhibitors. Per well 2×10^5 cells were incubated at 37°C with 1 MBq FDG or FMC in the presence of either α-D-glucose (0.01 µM to 20 mM) or unlabeled choline (1 µM to 10 mM), respectively. Uptake was allowed to proceed for 90 min. and after the amount of tracer incorporated per cell was determined as described above. K_m-values and IC$_{50}$-concentrations were calculated using Prism 4 (GraphPad Software, San Diego, USA) and Origin 6, respectively.

Preparation of protein extracts and western blot: T24 bladder cancer cells were grown to confluency (ca. 1.5×10^7 cells per 147 mm culture dish), harvested with a sterile cell scraper and washed with cold PBS (4°C). Subsequently, 400 µl of RIPA-buffer (10 mM sodium phosphate pH7.0, 150 mM NaCl, 2 mM EDTA, 1% Na-deoxycholate, 1% NP-40, 0.1% SDS; Sigma-Aldrich, Munich, Germany) supplemented with complete™ protease inhibitor (Roche, Mannheim, Germany) was added, the mixture was kept on ice for 30 min, and 10 µl of the lysate were submitted to SDS-PAGE (1.5 mm, 10% PAA separation-gel, 4.7% PAA loading-gel, 40 mA) and semi-dry blotting (0.8 mA/cm^2, 1 h) on a conditioned PVDF-membrane (immersing in ethanol, 10 s). For blocking of unspecific binding sites 5% non-fat milk powder in TBS (1 h, room temperature) was used. GLUT1 and CTL1 were visualized with a primary polyclonal anti-GLUT1 antibody (ab137656, abcam, Cambridge, England) and a primary polyclonal anti-CTL1 antibody (ABIN565086, antikoerper-online, Aachen, Germany). Antibodies were diluted appropriately in TBS containing 2% non fat-milk powder and incubation was done at 4°C over night. Unbound antibodies were washed off (TBS, 3×5 min) and bound antibodies were visualised using HRP-conjugated donkey anti-rabbit-antibodies and donkey-anti-mouse-antibodies (Dianova, Hamburg, Germany) in conjunction with the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce, Thermo Scientific, Waltham, MA, USA). Images were recorded with the Gel Logic 1500 Imaging System (Kodak, Rochester, NY, USA).

Statistics

Each value represent the mean ± standard deviation of at least three measurements in quadruplicate and results of the uptake experi-
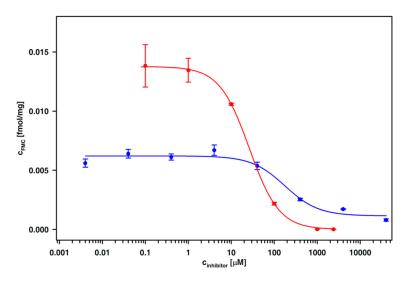
Figure 3. Inhibition of FMC uptake with hemicholinium-3 (HC-3; blue) and tetraethyl-ammonium (TAE; red). 2×10^5 cells were pretreated with various concentrations of HC-3 or TEA for 15 min and used for 30 min uptake experiments under standard conditions.
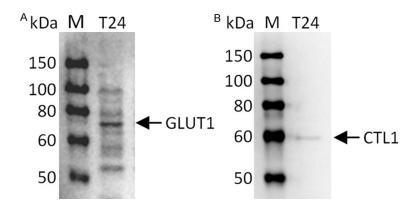

Figure 4. Detection of GLUT1 (A) and CTL1 (B) via Western Blot. For GLUT1 a 73 kDa band and for CTL1 a 60 kDa band could be detected.

ments are given as mean ± standard deviation of at least three measurements in quadruplicate. Statistical significance between groups was determined by Mann-Whitney-U-Test, since the Kolmogorov-Smirnov test revealed non-normal distribution of the data; $P < 0.05$ was considered statistical significant.

Results

FDG and FMC uptake kinetics and NaCl dependence of FMC uptake in T24 cells

Total uptake of FDG in T24 cells was significantly higher than uptake of FMC (**Figure 1**). FDG uptake increased dramatically within the first 2

hours and reached its maximum of 0.59 fmol/mg cell protein after 3 hours, whereas the uptake of FMC increased moderately and was at maximum (0.23 fmol/mg cell protein) after 5 hours. FMC uptake in T24 cells was monitored for 240 min in uptake buffer supplemented with either NaCl or NMDG. Under these conditions uptake velocity and maximum amount of FMC per mg cell protein was almost identical (4.13×10^{-2} and 3.9×10^{-2} fmol/mg). This indicates that FMC uptake in T24 cells is sodium-independent (**Figure 2**).

Competitive inhibition of FMC uptake

Competitive inhibition of FMC uptake using HC-3 (4 nM to 40 mM) or TEA (100 µM to 2.4 M) resulted in sigmoid inhibition curves (**Figure 3**). From this, IC_{50}-concentrations of 165.8 µM for HC-3 and 53.1 µM for TEA were calculated. FMC uptake could be inhibited almost completely (99.7% ± 0.02%) at TEA concentrations of 2.4 M while even with the highest HC-3 concentrations (40 mM) an inhibition of only 85.8% ± 1.9% was achieved. These findings together with the sodium independency point to CTL1 as the main mediator of FMC uptake and western blot analysis revealed the presence of the choline transporter-like protein 1 in T24 cells (**Figure 4**).

Determination of Michaelis-Menten kinetics

Uptake of FMC and FDG was measured in the presence of increasing amounts of unlabeled choline and α-D-glucose and data were used to calculate K_m-values (**Figure 5A**) and IC_{50}-concentrations (**Figure 5B**). Choline uptake increased with concentrations between 0.001 and 0.1 mM and reached a plateau between 0.250 mM and 1 mM. A K_m-value of 70.8 µM with a
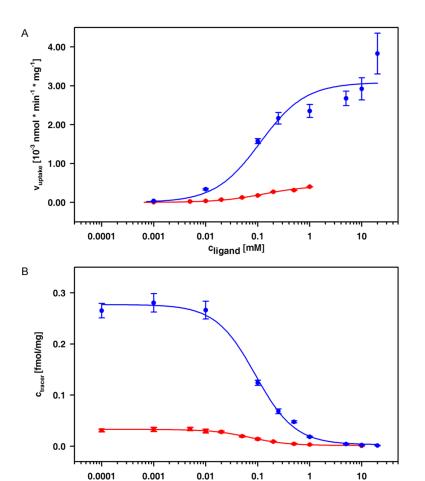
Figure 5. Calculating Michaelis-Menten kinetics (A) for FMC (red) and FDG (blue) in T24 cells and determination of IC$_{50}$-concentrations (B) for choline (red) and α-D-glucose (blue). Uptake was performed under standard conditions with increasing concentrations of unlabeled choline and α-D-glucose for 90 min.

in 5 days (**Figure 6**). Pre-incubation (24 h) of cells with these dosages prior to addition of radiopharmaceuticals increased the uptake of FDG and FMC by roughly 10% and 45% (**Table 1**).

Discussion

In patients suffering from bladder cancer tumor staging at time of diagnosis and assessment of response to chemotherapeutic drugs as early as possible is of outmost importance to spare treatment associated sequelae to patients with non-responding tumors. In bladder cancer, treatment related morphological changes are often seen only after several months. PET techniques are increasingly recognized as valuable tools for staging and restaging of cancer. In bladder cancer patients the utilization of ^{18}F-labeled tracers require additional maneuvers like bladder irrigation, increased hydration or forced diuresis to discriminate between urinary and bladder wall accumulation [8-10, 12, 16-18].

corresponding IC$_{50}$ of 70.2 µM choline were calculated. Uptake of α-D-glucose rose steeply at concentrations between 0.001 and 0.250 mM and remained almost constant with glucose concentrations of more than 4 mM. The corresponding values for half-maximum uptake velocity and inhibition were 111.8 µM and 94.9 µM, respectively.

Determination of IC$_{50}$-concentrations for gemcitabine and cisplatin and effect of cytotoxic treatment on FDG and FMC uptake

In bladder cancer, gc, cis, and the combination of both have narrow therapeutic windows. In our hands, 4.9 nM gc, 610.5 nM cis as well as 3.6 nM gc+442.5 nM cis were sufficient to reduce the number of viable cells by 50% with-

Recently, we were able to show that in a xenograft model of prostate cancer ^{11}C-choline in combination with PET/CT is a sensitive measure to evaluate early effects of cytotoxic treatment [21, 24]. Now we have extended these studies and evaluate the suitability of FDG and FMC as imaging biomarkers in T24 bladder cancer cells. Our results indicate, that uptake of FDG and FMC by T24 bladder cancer cells is saturable and decreases as the concentration of unlabeled competitors, i.e. glucose and HC-3, increases.

With either imaging biomarker a slightly decreasing uptake is noted when cells are exposed to radiopharmaceuticals and uptake medium for more than 6 hours. This might be due to the onset of cell damage characterized
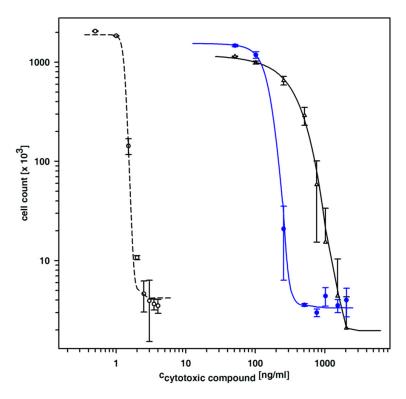
Figure 6. The number of viable T24 cells after 5 days of treatment with increasing concentrations of gemcitabine (0.5-4 ng/ml; dashed line), cisplatin (50.0 ng/ml-2 µg/ml; solid line) or a combination of both (absolute concentrations from 50.35 ng/ml up to 2.01 µg/ml; blue line). IC$_{50}$-concentrations of 1.29 ng/ml (4.9 nM) for gc, 183.2 ng/ml (610.5 nM) for cis and 0.9 ng/ml gc+132.8 ng/ml cis (3.6 nM gc+442.5 nM cis) were calculated.

Table 1. Uptake of FMC and FDG in T24 bladder cancer cells after 24 h pre-treatment relative to untreated cells

	FDG uptake	FMC uptake	P
gc	11.8% ± 3.0%	32.8% ± 3.2%	0.008
cis	14.2% ± 1.4%	45.7% ± 6.2%	0.008
gc+cis	11.3% ± 3.5%	52.4% ± 4.2%	0.003

IC$_{50}$-concentrations of gemcitabine (gc), cisplatin (cis), and the combination of both (gc+cis) were used.

by disturbances of membrane integrity and leakage of cytosolic compounds including previously internalized tracers. Consequently, these are washed off during separation of cell-bound from free radioactivity, while most of the proteins associated with (even incomplete) damaged cells sedimentation during centrifugation and contribute to the protein content of the pellet. These effects are hard to control and therefore we limited co-incubation of cells with radiopharmaceuticals to a maximum of 4 hours.

Although T24 cells had an apparently higher affinity to FMC compared to FDG, the uptake of FDG was much faster and on a molar scale about 2 to 3 times higher than for FMC. This points to an excess of glucose-specific over choline-specific cell membrane transporters and is in line with previous reports noting increased numbers of cell surface glucose transporter proteins and intracellular enzymes promoting glycolysis in tumor cells (for review see [13, 14]). Most commonly, the insulin independent GLUT-1 is overexpressed in tumor cell membranes and western blot analysis confirmed the presence of GLUT-1 in T24 bladder cancer cells. However, other members of the GLUT-family might have contributed to the internalization of FDG as well.

Several protein-mediated and saturable systems for uptake of choline are documented and data on Michaelis-Menten kinetics are available (for review see [15, 23, 25]. Apart from the high affinity choline transporter (CHT1) which is mostly confined to cholinergic nerve endings, members of the family of low affinity organic cation transporters and choline transporter like protein CTL1 (also known as SLC44A1 and CDw92) contribute to the internalization of choline and its labeled derivatives [15, 25]. CHT1 is Na$^+$-dependent, displays K$_m$ values between 0.5 and 3 µM, and can be inhibited already with minute amounts (1-3 µM) of HC-3 [26]. Our results are in sharp contrast to this and we can exclude almost completely that CHT1 contributes to FMC uptake in T24 bladder cancer cells. Furthermore, the requirement of about 166 µM HC-3 for half-maximal inhibition of FMC uptake makes even thecontribution of the low-affinity choline transporter CHT2, rather unlikely [21]. For CHT2 IC$_{50}$ of approximately 100 µM were described recently.

Thus, in this particular cell line members of the organic cation transporter family and/or CTL1

may have contributed to FMC uptake [23, 27-29]. Therefore, we investigated FMC uptake in the presence of TEA, a broad-band inhibitor for OCTs [22, 23]. In T24 bladder cancer cells, the concentration of TEA required for half maximal inhibition of FMC uptake was approximately three times higher than the specific inhibitor HC-3 and we can reasonably exclude an important contribution of OCTs. Instead, Michaelis-Menten kinetics together with Western blot analysis point to CTL1 as the main choline transporter in T24 bladder cancer cells. This contrasts sharply to findings in the prostate cancer derived PC3 and LNCaP cell lines [30, 31].

Cisplatin and gemcitabine are well established cytotoxic drugs inducing apoptosis via formation of DNA adducts and inhibition of DNA synthesis, respectively [32, 33]. Our data indicate, that in terms of IC_{50} gemcitabine is approximately 150 times more potent than cisplatin in T24 bladder cancer cells. The combination of both drugs is even more effective and under this circumstances the IC_{50} of either compound is about 26% less than what is required for monotherapy. Although not investigated in detail, our findings after 5 days of cytotoxic treatment argue against an even partial resistance and escape from chemotherapy induced cell death. However, 24 h of cytotoxic treatment most likely stimulate cell metabolism and are characterized by an increased uptake of FDG and FMC, respectively. Such an effect has been described previously in cell lines derived from breast (MCF7), prostate (LNCaP, PC3) and colon (SW 707) cancer, respectively [24, 34-37]. In this study, we used gc and cis in concentrations sufficient to induce cell death after an incubation period of 5 days. Thus, it is rather unlikely, that a reasonable number of cells underwent apoptosis within 24 hours. Instead, the increased uptake of FDG and FMC at this particular time point may indicate an overall enhanced cellular metabolism and energy demand secondary to cytotoxic cellular stress [24, 38]. However, unspecific changes of the cell membrane with concomitantly increased permeability for the imaging biomarkers have to be considered as well.

Conclusions

Our results indicate that FMC uptake in T24 cells is Na^+-independent, HC-3 sensitive and occurs mainly via the intermediate-affinity choline transporter like protein CTL1. A participation of the high-affinity choline transporter CHT1 or another member of the OCT-family can be excluded. Uptake of FDG and FMC increases early after application of cytotoxic drugs and most probably reflect early and unspecific changes preceding apoptosis.

Acknowledgements

The authors would like to thank Anja Rahn for technical support.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Dagmar-C Fischer, Department of Paediatrics, Ernst-Heydemann-Str. 8, 18057 Rostock, Germany. Tel: + 49-381-4947041; Fax: + 49-381-4947044; E-mail: dagmar-christiane. fischer@med.uni-rostock.de

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