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Activity of *Artemisia annua* and artemisinin derivatives, in prostate carcinoma

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ABSTRACT

Background: *Artemisia annua* L, artemisinin and artesunate reveal profound activity not only against malaria, but also against cancer *in vivo* and clinical trials. Longitudinal observations on the efficacy of *A. annua* in patients are, however missing as of yet.
Methods: Clinical diagnosis was performed by imaging techniques (MRT, scintigraphy, SPECT/CT) and blood examinations of standard parameters from clinical chemistry. Immunohistochemistry of formalin-fixed, paraffin-embedded tumor material was performed to determine the expression of several biomarkers (cyclooxygenase-2 (COX2), epidermal growth factor receptor (EGFR), glutathione S-transferase P1 (GSTP1), Ki-67, MYC, oxidized low density lipoprotein (lectin-like) receptor 1 (LOX1), p53, P-glycoprotein, transferrin receptor (TFR, CD71), vascular endothelial growth factor (VEGF), von Willebrand factor (CD31)). The immunohistochemical expression has been compared with the microarray-based mRNA expression of these markers in two prostate carcinoma cell lines (PC-3, DU-145).
Results: A patient with prostate carcinoma (pT3bN1M1, Gleason score 8 (4+4)) presented with a prostate specific antigen (PSA) level >800 μg/l. After short-term treatment with bacalitumide (50 mg/d for 14 days) and long-term oral treatment with *A. annua* capsules (continuously 5 × 50 mg/d), the PSA level dropped down to 0.98 μg/l. MRT, scintigraphy and SPECT/CT verified tumor remission. Seven months later, PSA and ostase levels increased, indicating tumor recurrence and skeletal metastases. Substituting *A. annua* capsules by artesunate injections (2 × 150 mg twice weekly *i.v.*) did not prohibit tumor recurrence. PSA and ostase levels rose to 1245 μg/l and 434 U/l, respectively, and MRT revealed progressive skeletal metastases, indicating that the tumor acquired resistance. The high expression of MYC, TFR, and VEGFC in the patient biopsy corresponded with high expression of these markers in the artemisinin-sensitive PC-3 cells compared to artemisinin-resistant DU-145 cells.
Conclusion: Long-term treatment with *A. annua* capsules combined with short-term bicalitumide treatment resulted in considerable regression of advanced metastasized prostate carcinoma. Controlled clinical trials are required to evaluate the clinical benefit of *A. annua* in prostate cancer.

Introduction

In addition to their antimalarial activity, artemisinin from *Artemisia annua* L. and its derivatives also exert remarkable anticancer effects towards cell lines from a broad variety of tumor types (Efferth et al., 2001; Efferth et al., 2002; Efferth et al., 1996; Efferth et al., 2003) including prostate carcinoma (He et al., 2010; Morrissey et al., 2010; Willoughby et al., 2009). Importantly, artemisinin-type drugs are also active against diverse syngeneic animal tumors (Disbrow et al., 2005; Lai and Singh, 2006; Moore et al., 1995) and human xenograft tumors in nude mice (Dell'Eva et al., 2004; Du et al., 2010; Li et al., 2007; Ma et al., 2011). Artesunate also protects from inflammatory and oxidative tissue injury *in vivo* caused by carcinogens (Ng et al., 2014). Compassionate uses of artemisinins and *Artemisia annua* preparations for cancer therapy of veterinary and human tumors encouraged the performance of several clinical phase I/II trials

Abbreviations: COX2, cyclooxygenase 2; CRP, C-reactive protein; EGFR, epidermal growth factor receptor; GSTP1, glutathione S-transferase P1; LOX1, oxidized low density lipoprotein (lectin-like) receptor 1, MRT, magnetic resonance tomography; PSA, prostate-specific antigen, SPECT, single-photon emission computed tomography; TFR, transferrin receptor (CD71); VEGF, vascular endothelial growth factor.

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(Berger et al., 2005; Breuer and Efferth, 2014; Jansen et al., 2011; Krishna et al., 2015; Krishna S, 2014; Rutteman et al., 2013; Singh, 2002; Zhang et al., 2008). A recent placebo-controlled, randomized and double-blind phase II trial in colorectal carcinoma patients demonstrated that patients taking artesunate tablets in addition to standard surgical therapy had a survival advantage (Krishna et al., 2015).

Despite clinical activity of artemisinins against cancer, longitudinal studies on the efficacy of artemisinin and *Artemisia annua* preparations upon longer application times are missing. In the present report, we describe a patient suffering from progressive prostate carcinoma. He was short-term treated with bicalitumide and long-term treated with *A. annua p.o.* and artesunate *i.v.* Initially, this patient responded remarkably well to *A. annua* capsules, but developed artesunate-resistant bone metastases later on.

Patient and methods

Clinical management

The patient was born November 9th 1934. Medical records have been provided by the patient himself with written consent to scientifically evaluate and publish them (letter dated from January 13th 2015).

Magnetic resonance tomography (MRT) was performed at the Kernspinzentrum Europa-Passage 20095 Hamburg, Germany. The histological appraisal of prostate fine needle biopsies were done at the Asklepios Clinics St. Georg (academic teaching hospital of the University of Hamburg), 20099, Hamburg, Germany. Whole body scintigraphy (after injection of 690 MBq Tc-99m-MDP) and SPECT/CT were done at the Radiological Clinic, Uelzen, Germany. Blood parameters were measured by Dr. von Froreich Bioscientia GmbH, Hamburg, Germany.

Treatment with vitamin C, glutathione (Ridolex, 600 mg), citric acid was done in the clinic of Dr. Jörg Schwarzkopf (Hitzacker, Germany). Treatment with *Artemisia annua* was performed by Dr. Walter Weber (Hamburg, Germany) (Artemisinin, Eura-Nutrador, Landgraaf, Netherlands; 50 mg *Artemisia annua* concentrate (with tricalcium phosphate, microcrystalline cellulose, silicium dioxide and magnesium stearate as adjuvants). Artesunate injections were obtained from Dr. Miller GmbH (Hamburg, Germany; 2 × 150 mg, 2 × weekly).

Immunohistochemistry

For determination of protein expression the UltraVision polymer detection method (kit from Thermo Fisher Scientific GmbH, Dreieich, Germany), method was used as previously described. Formalin-fixed, and paraffin-embedded sections were deparaffinated (2 × 2 min xylol) and rehydrated. For antigen retrieval, sections were submerged in Target Retrieval Solution (Thermo Fisher Scientific) for 20 min at 95–99 °C. Afterwards, slides were allowed to cool down at room temperature and washed in phosphate buffered saline (pH 4) for 10 min. Endogenous peroxidase activity was blocked by immersing the slides in 3% H_2O_2 for 10 min at room temperature. After rinsing for 5 min in PBS, non-specific binding was blocked by Ultra Vision Block (Thermo Scientific) for another 5 min. Slides were incubated in a humidified chamber for 1 h at room temperature with primary antibody: EGFR (RM-2111-S0, dilution 1:50, Thermo Scientific), p53 (M3629, dilution: 1:100, DAKO GmbH, Hamburg, Germany), (RM-2111-S0, dilution 1:50, Thermo Scientific), c-Myc (MS-139-PCL, dilution 1:50, Thermo Scientific), Ki-67 (ab16667, dilution 1:100, Abcam, Cambridge, UK), CD31 (MS-353-S0, dilution 1:50, Thermo Scientific), P-glycoprotein (CD243, AM05632PU-N, dilution 1:200, Acris, Herford, Germany), GSTP1 (AP02100SU-S, Acris), COX2 (AM11127PU-N, dilution 1:100, Acris), LOX1 (HPA050798, dilution 1:300, Sigma), TFR (CD71; MS-1096-S0, dilution 1:50, Thermo Scientific), and VEGF (Ab-7, clone VG-1, which recognized 121,165, and 189 isoformes of VEGF; dilution 1:150, Thermo Scientific). After rinsing for 5 min in PBS, Primary Antibody Amplifier Quanto (Thermo Scientific) was applied for 10 min at room temperature. Slides were washed in PBS for 5 min and then HRP Polymer Quanto (Thermo Scientific) was applied for 10 min, a wash step (5 min) followed. Afterwards, 30 μg/l diaminobenzidine (DAB) Quanto chromogen (Thermo Scientific) was mixed with 1 ml DAB Quanto substrate and applied to the slides for 5 min. After washing in PBS for 5 min, the tissues were counterstained in hemalaun solution (Merck KGaA, Darmstadt, Germany) and rinsed in PBS for 5 min, followed by running tap water (10 min). Tissue sections were dehydrated (2 × 1 min 70% ethanol, 2 × 1 min 96% ethanol, 2 × 1 min 100% ethanol, 2 × 5 min xylol, 1 × 2 min xylol) and embedded using Entellan (Merck).

The immunostained slides were scanned by Panoramic Desk (3D Histotech Pannoramic digital slide scanner, Budapest, Ungary) and interpreted (Quantification of immunostained slides) by panoramic viewer software (NuclearQuant and MembraneQuant, 3D HISTECH) in which positive stained nucleus or membrane were counted in each defined annotated area. Evaluation parameters included number of overall detected objects (nucleus or membrane) in each annotated area, average of positivity and intensity. Nuclear stainings (Ki-67, p53, c-Myc, TUNEL) were quantified using the NuclearQuant software and membrane-bound and cytosolic stainings were quantified by the MembraneQuant software (3D HistoQuant).

Cell lines

PC-3 and DU-145 prostate cancer cell lines are part of the drug screening panel of 60 cell lines of the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD, USA). The cytotoxicity of 10 artemisinin-type compounds (artemisinin, artemether, arteether, artesunate, artenimol, arteanuin B, one artesunate derivative, and three artemisinin dimers (chemical structures, see Fig. 4) towards PC-3 and DU-145 cells was measured by the sulforhodamine B assay (Monks et al., 1991). The 50% inhibition concentrations calculated from dose response curves and converted to logarithmic values ($\log_{10}IC_{50}$) have been deposited in the NCI database (http://dtp.nci.nih.gov).

Cluster analyses of microarray data

The mRNA microarray hybridization of the NCI cell lines has been reported and deposited at the NCI website (http://dtp.nci.nih.gov) (Amundson et al., 2008; Scherf et al., 2000). For hierarchical cluster analysis, objects were classified by calculation of distances according to the closeness of between-individual distances by means of hierarchical cluster analysis. All objects were assembled into cluster trees (dendrograms) by the algorithm included into the WINSTAT program (Kalmia Co, MA, U.S.A.). The cluster analyses were run using the WARD method. Previously, cluster models have been validated for gene expression profiling and for approaching molecular pharmacology of cancer (Efferth et al., 1997; Villeneuve and Parissenti, 2004; Zeeberg et al., 2011).

Results

Initial clinical presentation

On January 19th 2014, a 80-year old man presented with abdominal pain, weakness, and fever (39.8 °C) on January 21st 2014, which were due to a prostate carcinoma (3.5 cm diameter) and extended ubiquitary skeletal metastases, but without hepatic metastasis as determined by MRT on January 29th 2014. A suspicious lymph node