Effects of the novel polyphenol conjugate DPP-23 on head and neck squamous cell carcinoma cells in vitro

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Abstract. Despite partial advances in therapy for patients suffering from head and neck squamous cell carcinomas (HNSCC), prognosis still remains poor with minimal improvement in survival for over the last several decades. Some agents found are known to cause cancer cell death in vitro by promoting cellular reactive oxygen species (ROS) accumulation. This is particularly of interest as some cancer cells are more sensitive to ROS than normal cells. It could be shown that the novel polyphenol conjugate (E)-3-(3',5'-Dimethoxyphenyl)-1-(2'-methoxyphenyl) prop-2-en-1-one (DPP-23) offers antitumor effects by the selective generation of ROS without an indication of toxicity in normal tissues in vitro and in vivo. In order to further evaluate the role of DPP-23 as a potential agent in head and neck oncology, the present study investigated its cytotoxic effects on well-established HNSCC cell lines such as HLaC 78 and FaDu, as well as primary human bone marrow stem cells (hBMSCs) and human peripheral blood lymphocytes in vitro. As DPP-23 is not commercially available, it was synthesized via a ‘cold’ procedure of the Claisen-Schmidt condensation. Following cell treatment with DPP-23 for 24 h, viability and apoptosis were measured via a MTT assay and the Annexin V-propidium iodide test. The results suggest a dose-dependent cytotoxicity in the tested HNSCC tumor cell lines, as well as in hBMSC and lymphocytes. In contrast to previous findings, these preliminary results indicate that the cytotoxic effects of DPP-23 in benign cells may be notably greater than previously suspected. This may indicate a limitation for in the feasibility, or at least of the systemic application, of DPP-23 for patients with HNSCC.

Introduction

With >600,000 new cases diagnosed yearly, head and neck cancer is the sixth most common cancer worldwide (1). It is associated with impairments like pain, severe disfigurement and difficulties in swallowing, breathing and speech. Most tumors of this group are head and neck squamous cell carcinomas (HNSCC). Despite partial advances in therapy of patients suffering from head and neck cancer, prognosis still remains poor with minimal improvement in survival over the past several decades (2). Thus, new treatment modalities are needed for a better outcome of patients with this aggressive disease.

In the past, the increase of reactive oxygen species (ROS) has been seen as a disadvantageous circumstance associated with carcinogenesis and cancer progression. In contrast, there are agents known to kill cancer cells in vitro by promoting cellular ROS accumulation (3-5). Additionally, since some cancer cells are more sensitive to ROS than normal cells, (6) selective generation of ROS may be a promising strategy for killing cancer cells without significantly harming benign tissue (7-9).

Shin et al (10) synthesized the novel polyphenol conjugate (E)-3-(3'',5''-dimethoxyphenyl)-1-(2'-methoxyphenyl) prop-2-en-1-one (DPP-23). They assessed its effects on cell lines of human colon cancer, glioblastoma, breast cancer and pancreatic cancer in vitro as well as in vivo on the human colon cancer cell line HCT116 in athymic nude mice. Their data suggests that DPP-23 may be a promising therapeutic agent with antitumor effects by the selective generation of ROS and targeting the unfolded protein response in the endoplasmatic reticulum, resulting in a growth inhibition of cancer cells via caspase-dependent apoptosis. Furthermore, DPP-23 induced autophagy in various cancer cell types. Notably, there was no indication of toxicity in normal tissues in vivo. (10).

In a recent study of Kim et al (11), the selective killing of HNSCC cells and increased cisplatin antitumor activity in resistant HNSCC cells by interfering with Nrf2 antioxidant...
systems, via activation of p53 expression and accumulation of cellular ROS could be shown in vitro and in vivo. For their investigations, they used oral keratinocytes, fibroblasts and HN3, HN4 and HN9 cells (11).

Due to their high phenotypic and cellular plasticity, human mesenchymal stem cells like human bone marrow stem cells (hBMSC) are a suitable model for in vitro toxicological assessment of various biological and chemical agents (12). Furthermore, since systematically applied substances come into contact with peripheral blood lymphocytes, the latter is relevant for toxicological evaluations as well.

Hence, the present study was performed to further elucidate the role of DPP-23 as a possible agent in head and neck oncology, and to investigate its cytotoxic effects on well-established HNSSC cell lines like HLaC 78 and FaDu, as well as primary hBMSC and human peripheral blood lymphocytes in vitro.

Materials and methods

Chemical synthesis and analysis of DPP-23

Reagents and devices. All reagents used were of commercial quality. Organic solvents were dried and distilled prior to use. 2′-Methoxyacetophenone and 3,5-dimethoxybenzaldehyde (Sigma-Aldrich, Steinheim, Germany). Reactions were monitored by thin-layer chromatography (TLC) on aluminum plates coated with silica gel 60 F254 (Merck, Darmstadt, Germany). Column chromatography was performed on Merck silica gel (0.063-0.200 mm).

General methods. Melting points (uncorrected) were determined using a Reichert-Jung Thermovar hot-stage apparatus. Infrared spectra (IR) were obtained with a Jasco FT/IR-410 spectrophotometer (Jasco, Inc., Easton, MD, USA), and are reported in wave numbers (cm⁻¹). Proton and carbon-13 spectra were recorded on a Bruker Avance spectrometer (Bruker, Billerica, MA, USA) at 400 MHz (for 1H NMR), and at 100 MHz (for 13C NMR) at ambient temperature. Chemical shifts (δ) are reported in parts per million (ppm) with the proton or carbon-13 signals of chloroform (1H, δ = 7.26 ppm; 13C, δ = 77.16 ppm) in the deuterated solvent used as internal reference. Coupling constants (J) are given in Hertz (Hz). The following abbreviations are used: s, singulet; d, doublet; dd, double doublet; t, triplet. Electron ionization mass spectra (70 eV) were obtained using a Finnigan MAT 8200 mass spectrometer in the positive mode (70 eV), the relative intensities are given in brackets. Matrix-assisted mass spectra (MALDI) were measured in the positive mode via a Bruker Autoflex II mass spectrometer using DCTB as the matrix. High-resolution electrospray ionization mass spectra (HRESIMS) were determined with a Bruker microOTOF spectrometer.

Synthesis, purification, and characterization of DPP-23.

DPP-23: The title compound was synthesized according to a published procedure (13), which was slightly modified: To a solution of 1.0 eq. of 2′-methoxyacetophenone (1.25 g, 8.22 mmol) and 1.0 eq. of 3,5-dimethoxybenzaldehyde (1.37 g, 8.22 mmol) in 30 ml of anhydrous ethanol, 2.0 eq. of KOH (923 mg, 16.5 mmol) was added. The reaction was allowed to proceed at room temperature (RT) for 45 min with stirring, and monitored by TLC (elu: n-Hexan/Et2O, 1:1). The reaction mixture was then treated with water (20 ml), and extracted with ethyl acetate (2x20 ml). The combined organic layers were dried over anhydrous Na2SO4, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (elu: n-Hexan/Et2O, 3:1). The remaining yellowish oil was then crystallized from methanol at 4°C by storage of the solution overnight in a refrigerator, providing pure DPP-23 (2.20 g, 7.36 mmol, 90% yield) as pale yellow crystals. The spectroscopic data (1H NMR, 13C NMR) were in agreement with those reported in the literature (14) and were found to be as follows:

1H NMR (400 MHz, CDCl3): δ = 3.82 (s, 6H), 3.89 (s,3H), 6.50 (d, J = 2.3, 2.3 Hz, 1H), 6.72 (d, J = 2.3 Hz, 1H), 6.73 (d, J = 2.3 Hz, 1H), 7.00 (dd, J = 8.4, 1.0 Hz, 1H), 7.04 (dd, J = 7.5, 1.0 Hz, 1H), 7.31 (d, J = 15.9 Hz, 1H), 7.47 (dd, J = 8.3, 7.4, 1.9 Hz, 1H), 7.52 (d, J = 15.9 Hz, 1H), 7.61 (dd, J = 7.6, 1.8 Hz, 1H). 13C NMR (101 MHz, CDCl3): δ = 55.58, 55.90, 102.57, 106.43, 111.75, 120.87, 127.72, 129.33, 130.45, 132.03, 137.16, 134.41, 151.21, 161.10, 193.19. M.p. 55-57°C (MeOH). IR (ATR) ν/cm⁻¹: 2939 (w), 2837 (w), 1652 (w), 1587 (s), 1483 (m), 1456 (m), 1425 (m), 1347 (w), 1288 (s), 1240 (m), 1202 (s), 1151 (s), 1113 (m), 1059 (m), 1018 (m), 977 (m), 925 (w), 834 (m), 752 (s), 671 (m), 637 (m). EI-MS (70 eV) m/z (%): 77 (36), 154 (54), 152 (28), 239 (49), 267 (49), 298 (100), 299 (20). MALDI calculated for C6H15NO2 [M + H]+: 299.128; found: 299.074. ESI-MS (positive) exact mass calculated for C18H16O2Na [M + Na]+: 321.10973; found: 321.10920.

Cell lines and culture. HNSSC cell lines FaDu, HLaC 78 and Cal 27 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 lg/ml streptomycin, 1% 100 mM sodium pyruvate and 1% of a 100-fold concentration of non-essential amino acids (Biochim, Berlin, Germany). The cells were maintained in a tissue culture incubator equilibrated with 95% air and 5% CO2 at 37°C in 150 cm² flasks.

Human bone marrow cells were harvested from six voluntary donors undergoing surgery in the Department of Orthopedics, and written informed consent was obtained from all of the individuals included. The study was approved by the Ethics Committee of the Medical Faculty, University of Wuerzburg (Wuerzburg, Germany; 12/06). As previously described, (15) hBMSC were isolated from fresh bone marrow aspirates by several washing and centrifugation steps. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Invitrogen, Karlsruhe, Germany) expansion medium (DMEM-EM) supplemented with 10% FCS and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C and 5% CO2. At a confluence of >70%, cells were trypsinized, resuspended and subcultured at a concentration of 2,000 cells/cm².

Human lymphocytes were obtained by venous puncture from healthy volunteers. Lymphocytes were separated by density-gradient centrifugation (10 min, 1,000 x g) at RT on equal amounts of Ficoll (Biochrom), using a membrane containing 50 ml cell tube (Greiner Bio-One, Frickenhausen, Germany). After washing twice in PBS, lymphocytes were resuspended in RPMI (Biochrom) containing the supplement of bovine serum albumin (Linaris, Wertheim-Bettingen, Germany), 1% sodium-pyruvate, 1% non-essential amino acids,
and 1% penicillin-streptomycin (all Biochrom). The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian-University Wuerzburg, (Wuerzburg, Germany) and all participants gave written informed consent.

**MTT assay.** For evaluation of the cytotoxic effects of DPP-23 on tumor cells and hBMSC the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT; Sigma-Aldrich) colorimetric staining method according to Mosmann (18) was used. Cells were seeded onto 24-well plates at a concentration of 1x10^5 cells/ml and treated with DPP-23 at various concentrations for 24 h. Respective concentrations of dimethyl sulfoxide were added to controls. After subsequent incubation with 100 µl of MTT solution (1 mg/ml), MTT was removed and 100 µl of isopropanol was added for 30 min. Then, the absorption values of the blue formazan dye were determined with a multi-plate reader at a wavelength of 570 nm (Titertek Multiskan PLUS MK II; Labsystems, Helsinki, Finland). All measurements were performed in triplicate.

**Annexin V-propidium iodide test.** Apoptosis and necrosis were evaluated by flow cytometry using an Annexin V-propidium iodide kit (BD Bioscience, Heidelberg, Germany) according to the manufacturer's manual. Cells were washed twice with cold PBS and resuspended in an Annexin V binding buffer containing 10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM CaCl_2 at a concentration of 1x10^6 cells/ml. 100 µl aliquots of this cell suspension (1x10^5 cells) were then incubated with 5 µl of Annexin V-APC and 5 µl of propidium iodide (PI for 15 min in the dark at RT). After resuspension with 400 µl 1:10 binding buffer, viable (Annexin V^-/PI^-), apoptotic cells (Annexin V^+/PI^-) and necrotic cells (Annexin V^+/PI^+) were measured using a flow cytometer.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 6.0c for Mac (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). For determination of the half maximal inhibitory concentration (IC_{50}), dose response curves were generated by nonlinear regression analysis.

For detection of significant differences between treated and untreated samples, the paired Student's t-test was applied. P<0.05 was considered to indicate a statistically significant difference. All results are expressed as the mean ± SD.

**Results**

**Chemical characterization of DPP-23.** The chalcone DPP-23 was synthesized via a 'cold' procedure of the Claisen-Schmidt condensation (13), by running the reaction of 2-methoxyacetophenone with 3,5-dimethoxybenzaldehyde in ethanol under basic conditions at RT (Fig. 1). The resulting chalcone was obtained as a pure compound in high chemical yields (ca. 90%) after purification of the crude reaction mixture by chromatography on silica gel, followed by subsequent crystallization of the resulting pale yellow oil from methanol. As expected, the substance was found to possess a molecular formula of C_{18}H_{18}O_4 due to the most abundant molecular mass peaks of m/z 321.1092 [M + Na]^+ and m/z 619.2299 [2M + Na]^++. This was evidenced from high-resolution electrospray ionization mass spectrometry (HRESIMS) (Fig. 2). The selective, and thus exclusive formation of DPP-23 with an (E)-configuration at the double bond, as outlined in Scheme 1, was unequivocally confirmed by the large coupling constant of 15.9 Hz between H-2 and H-3, as determined by NMR measurements (Fig. 3).

**Cytotoxicity.** The MTT test indicated a reduction in cell viability on FaDu, HLaC 78 and Cal 27 cells, as well as...
hBMSC after treatment with DPP-23 in a dose-dependent manner (Fig. 4). There was a cytotoxic effect at a concentration of 10 µM of DPP-23, respectively. The calculated IC₅₀ was 5.9 µM in FaDu, 10.4 µM in HLaC 78, 6.9 µM in Cal 27 and 12.1 µM in hBMSC.

The results of the MTT test were confirmed by the Annexin V-propidium iodide test. The latter showed a dose-dependent enhancement of cellular apoptosis by DPP-23 in FaDu and lymphocytes. In detail, FaDu cells were subdivided into 92.3% viable, 2.0% apoptotic and 5.1% necrotic...
cells in the control group, whereas in the treatment group (40 µM DPP-23) there were 79.8% viable, 4.6% apoptotic and 13.1% necrotic cells. Flow cytometry of lymphocytes revealed 83.8% viable, 8.0% apoptotic and 8.1% necrotic cells in the control group, whereas there were 52.3% viable, 44.2% apoptotic and 3.4% necrotic cells in the treatment group (Fig. 5).

Discussion

Novel therapeutic approaches with lower adverse effects than with currently performed treatments are needed for HNSCC (16). In the present study, the chalcone DPP-23 was synthesized according to the structural formula published by Shin et al (10), and using this substance to test a novel idea, we assessed its effects on well-established HNSCC tumor cell lines and primary non-malignant cells. In particular, the hBMSC test system used in this study is considered to be very suitable for the prediction of toxicological behavior since it provides a more accurate simulation of in vivo conditions than traditional in vitro systems with transformed or immortalized cell lines. Furthermore, hBMSC are highly proliferative and can be cultivated over several passages. DNA stability was demonstrated over up to 10 passages (17). In contrast to other primary cells, they are suitable for long-term toxicological evaluations, especially for the determination of DNA fragmentation and repair capacity. In addition, functional properties like cytokine secretion, migration or differentiation can be assessed (12). Therefore, hBMSC are an optimal cell entity for further investigations of the effects of DPP-23 on non-malignant cells.

The data from the MTT assay and the Annexin V-propidium iodide test suggest a dose-dependent cytotoxicity in the tested HNSCC tumor cell lines, as well as in hBMSC and lymphocytes. The applied concentrations of DPP-23 are similar to the doses reported by Shin et al (10), whereas Kim et al (11) partially administered lower doses in combination with longer exposure times (up to 72 h). As a limitation of the MTT assay, it should be mentioned that its results do not directly reflect the cell viability, but the activity of the mitochondria (18). Nevertheless, the Annexin V-propidium iodide test allows a precise estimation of apoptotic incidence (19). In contrast to the findings of Shin et al (10) and Kim et al (11), our preliminary results suggest surprisingly higher cytotoxic effects of DPP-23 in benign cells than suspected. This may indicate a limitation in the feasibility, or at least the systemic application of this substance. One reason for the discrepancy between the findings of Shin et al (10) and Kim et al (11) vs. the results of the present study could be on the one hand due to different cell types and in vitro test systems, and on the other hand due to different surrounding conditions of in vivo and in vitro settings. Compensatory mechanisms like maintaining the redox balance via the cells’ antioxidant systems should be discussed as a possible explanation for the differences. However, a substance with less cytotoxic effects in mesenchymal stem cells may be a more favorable candidate for a chemotherapeutic agent with presumably low adverse effects.

Future investigations should address the cytotoxicity of DPP-23 using additional in vitro test systems like spheroids. These three-dimensional cell culture models can be constructed for stem cells and tumor cells. Due to the possibility of intercellular communication, they are a very distinct test system for evaluating xenobiotics in human cells. Furthermore, the comparison between multilayer cellular test systems for human squamous cell carcinomas and multilayer systems for the human oropharyngeal mucosa will provide more detailed information about the underlying mechanisms of DPP-23-induced toxicity (20).
Taken together, our results show that prior to the application of DPP-23 in clinical studies, further molecular evaluations are warranted to understand the effects and mechanisms of DPP-23 in malignant as well as benign cell populations.

References