Pro-apoptotic effects of pycnogenol on HT1080 human fibrosarcoma cells

KAMRAN HARATI[1], PAWEL SLODNIK[1], ANSGAR MICHAEL CHROMIK[2], BJÖRN BEHR[1],
OLE GOERTZ[1], TOBIAS HIRSCH[1], NICOLAI KAPALSCHINSKI[1], LUDGER KLEIN-HITPASS[3],
JONAS KOLBENSCHLAG[1], WALDEMAR UHL[2], MARCUS LEHNHARDT[1] and ADRIEN DAIGELER[1]

[1]Department of Plastic Surgery, Burn Center, Hand Center, Sarcoma Reference Center, BG-University
Hospital Bergmannsheil, Ruhr-University Bochum, D-44789 Bochum; [2]Department of General and
Visceral Surgery, St. Josef Hospital, Ruhr-University Bochum, D-44791 Bochum; [3]Institute of Cell
Biology (Cancer Research), University of Duisburg-Essen, D-45122 Essen, Germany

Abstract. Complete surgical resection with clear margins remains the mainstay of therapy for localised fibrosarcomas. Nevertheless, metastatic fibrosarcomas still represent a therapeutic dilemma. Commonly used chemotherapeutic agents like doxorubicin have proven to be effective in <30% of all cases of disseminated fibrosarcoma. Especially elderly patients with cardiac subdisease are not suitable for systemic chemotherapy with doxorubicin. Therefore we tested the apoptotic effects of the well-tolerated pine bark extract pycnogenol and its constituents on human fibrosarcoma cells (HT1080). Ten healthy subjects (six females, four males, mean age 24.8±6 years) received a single dose of 300 mg pycnogenol orally. Blood plasma samples were obtained before and 6 h after intake of pycnogenol. HT1080 cells were treated with these plasma samples. Additionally, HT1080 were incubated separately with catechin, epicatechin and taxifolin that are known as the main constituents of pycnogenol. Vital, apoptotic and necrotic cells were quantified using flow cytometric analysis. Gene expression was analyzed by RNA microarray. The results showed that single application of taxifolin, catechin and epicatechin reduced cell viability of HT1080 cells only moderately. A single dose of 300 mg pycnogenol given to 10 healthy adults produced plasma samples that led to significant apoptotic cell death *ex vivo* whereas pycnogenol-negative serum displayed no apoptotic activity. Microarray analysis revealed remarkable expression changes induced by pycnogenol in a variety of genes, which are involved in different apoptotic pathways of cancer cells [Janus kinase 1 (*JAK1*), *DUSP1*, *RHOA*, laminin γ1 (*LAMC1*), fibronectin 1 (*FN1*), catenin α1 (*CTNNA1*), *ITGB1*]. In conclusion, metabolised pycnogenol induces apoptosis in human fibrosarcoma cells. Pycnogenol exhibits its pro-apoptotic activity as a mixture and is more effective than its main constituents catechin, epicatechin and taxifolin indicating that the metabolised components interact synergistically. These results provide experimental support for *in vivo* trials assessing the effect of the pine bark extract pycnogenol.

Introduction

Soft tissue sarcomas are a heterogeneous group of solid malignant tumours which represent ~1% of all new cancer cases in Europe and the United States (1). Fibrosarcomas are rare soft tissue sarcomas originating from the intra- and intermuscular fibrous tissues, fascia and tendons and account for ~3% of all soft tissue sarcomas. Therapy for fibrosarcomas should be individualised and multimodal. The therapy of choice involves surgical resection with a wide margin of healthy tissue, usually followed by radiation treatment in order to decrease local recurrence (2,3). Unfortunately, ~50% of all patients develop distant metastases and are ineligible for surgical treatment (4,5). In cases of advanced metastatic disease the median survival time with and without chemotherapy treatment is <12 months (6,7). Few agents such as doxorubicin, ifosfamide and dacarbazine have proven to be effective in the therapy of soft tissue sarcomas (2). However, the results of these treatments are poor and often exhibit no significant improvements in overall survival (8). Doxorubicin, which has been the most frequently used chemotherapeutic agent in the treatment of soft tissue sarcomas, demonstrates response rates of 20-30% in disseminated disease (9,10). The combination of doxorubicin with ifosfamide is more effective, exhibiting higher response rates than doxorubicin alone, but is associated with severe short- and long-term toxicities, including cardiomyopathy and bone marrow suppression (11-13). The recently published EORTC 62012 trial which

Correspondence to: Dr Kamran Harati, Department of Plastic Surgery, Burn Center, Hand Center, Sarcoma Reference Center, BG-University Hospital Bergmannsheil, Ruhr-University Bochum, Buerkle-de-la-Camp-Platz 1, D-44789 Bochum, Germany
E-mail: kamran.harati@t-online.de

involved 455 patients with locally advanced, unresectable or metastatic high-grade soft tissue sarcomas concluded that an intensified therapy with doxorubicin and ifosfamide is not suitable for palliation of advanced soft tissue sarcomas because of the severe side-effects and should only be used when the specific goal is tumour shrinkage (13). Further, the utility of the first-line cytostatic doxorubicin is limited by dose-related and cumulative myocardial toxicity, especially in elderly patients with pre-existing cardiac disease (14). However, age is an important determinant of sarcoma occurrence and the incidence of soft tissue sarcomas increases dramatically at ages >50 years and above which are naturally associated with higher prevalence of cardiac diseases (15). To date, there are no effective and well-tolerated cytostatics for the palliative treatment of patients who are not suitable for aggressive anthracycline-based chemotherapy. Hence, there is still a need for alternative and well-tolerated compounds that exhibit antineoplastic effects in sarcoma cells.

Within the scope of this trial, we investigated the effects of the natural pine bark extract pycnogenol on human fibrosarcoma cells. Pycnogenol is a brand name for an extract obtained from the bark of the *Pinus pinaster* pine tree by a standardised process. It is manufactured by Horphag Research, Ltd. (Geneva, Switzerland) and is available as a nutritional supplement in the United States and in Europe. Pycnogenol is primarily composed of a mixture of flavonoids, mainly procyanidins and phenolic acids. It is standardised to contain ~65-75% procyanidins that consist of taxifolin, catechin and epicatechin subunits of varying chain length (16). Other constituents are polyphenolic monomers, cinnamic acids and their glycosides (17).

Since pycnogenol is a naturally occurring compound that is very well-tolerated with a high oral bioavailability, it has been highly studied for the treatment of many diseases including cancer (17,18). Several *in vitro* studies demonstrated the anticancer activity of pycnogenol in a wide range of malignant cell lines including leukemia, ovarian and breast cancer cells (19-21). Moreover, pycnogenol has been reported to alleviate adverse effects of oncologic treatment in a clinical trial with 64 chemotherapy patients (22). Patients receiving pycnogenol during chemotherapy treatment had a significant decreased incidence of side-effects such as nausea, vomiting, diarrhoea and weight loss when compared with patients from the control group.

Inspired by these findings, we examined in the following study the apoptosis-inducing activity of pycnogenol and its constituents on human fibrosarcoma cells.

Materials and methods

Volunteers. Ten healthy female (six) and male (four) subjects aged 18-31 years (mean age 24.8±6 years) participated in this study. All participants gave written informed consent. The study was reviewed and approved by the Ethics Committee of the BG-University Hospital Bergmannsheil, Ruhr-University Bochum, Germany with the permit no. 3162-08.

Protocol of pycnogenol intake. After a 24-h diet free of flavonoids (no vegetables, fruits, marmalades, tea, coffee, cocoa, wine and beer) blood samples were taken from the 10 volunteers. Subsequently, the volunteers received a single dose of 300 mg pycnogenol (Pycnogenol®; Horphag Research, Ltd., London, UK) *per os* with 200 ml water. Flavonoid-free diet was continued by the volunteers for another 6 h. Blood samples were taken again 6 h after pycnogenol intake. All blood samples were centrifuged and plasma was aliquoted, frozen and stored at -80˚C until further analysis.

Cell line. Human fibrosarcoma cells, HT1080, were purchased from American Type Culture Collection (ATCC) (cell line CCI 121; Wesel, Germany) and maintained in modified Eagle's medium (MEM) and non-essential amino acids (NEAA) + 10% fetal bovine serum (FBS) supplemented with 1% penicillin (100 U/ml) and streptomycin (100 μg/ml), 1% sodium pyruvate and 1% L-glutamine. The cells were cultured in a humidified atmosphere at 37˚C with 5% CO_2 in 25 cm^2 flasks.

Reagents. Pycnogenol was obtained from Horphag Research, Ltd. (Geneva, Switzerland). Catechin, epicatechin and taxifolin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in distilled water obtaining a concentration of 11.25 ng/ml (catechin), 6.25 ng/ml (epicatechin) and 6.25 ng/ml (taxifolin). The concentrations of catechin and taxifolin are achievable mean concentrations in human plasma several hours after single oral intake of catechin and taxifolin, respectively (16,23). There were no data available regarding pharmacokinetics and achievable concentrations of epicatechin in human plasma after oral intake.

Cell treatment. For every drug experiment, 80 μl of 3x10^6 cells/ml were placed in 6-well plates containing the medium. After 24 h, the medium was replaced and the drugs (catechin, epicatechin, taxifolin) or diluted plasma samples were added to each well at the above-mentioned concentrations. Different time points were chosen to identify the possible time dependency of the effects. All experiments were repeated for each of three consecutive passages.

Flow cytometric analysis. At the indicated incubation time, the floating cells were collected together with the supernatant and adherent cells, which were harvested by trypsinisation. The cells were pelleted by centrifugation, resuspended in 195 μl binding buffer (Bender MedSystems, Vienna, Austria) and incubated with 5 μl Annexin V (BD Biosciences, Heidelberg, Germany) and 10 μl propidium iodide (PI) (Bender MedSystems) following the manufacturer's instructions. The cells were analysed immediately using a FACSCalibur flow cytometer (BD Biosciences). For each measurement, 20,000 cells were counted. Dot plots and histograms were analysed using CellQuest Pro Software (BD Biosciences). Annexin V binds phosphatidylserine on the outer membranes of cells, which then becomes exposed on the surface of apoptotic cells. Thus, the Annexin V-positive cells are considered apoptotic. PI is an intercalating agent that cannot permeate through the cell membranes of viable or early apoptotic cells. Therefore, PI stains only the DNA of necrotic or very late apoptotic cells. In this study, Annexin V- and PI-positive cells were termed necrotic. Annexin V- and PI-negative cells were counted as viable.

Cell morphology. The morphology of the adherent and suspended cells was examined and documented using a phase contrast Zeiss Axiovert 25 microscope (Carl Zeiss, Jena, Germany).

Statistical analysis. The results of FACS analysis were used to determine the percentages of viable, apoptotic and necrotic cells, which are expressed as the means ± SD from at least three independent experiments and consecutive passages. In this study, comparisons between the experimental groups were performed using one-way measures of variance (one-way ANOVAs) over all time points (Tukey's test). Results were considered statistically significant for $p \leq 0.05$.

Oligonucleotide microarray analysis. To identify the changes in gene expression levels caused by the treatment with the tested substances or plasma samples, total RNA was purified from the cells after incubation with the appropriate agent for 6 h using a RNeasy kit from Qiagen (Hilden, Germany) as specified by the manufacturer. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). For microarray analyses, we applied the methods previously described by Daigeler *et al* (24). We used the Affymetrix GeneChip platform, employing a standard protocol for sample preparation and microarray hybridisation. A one-way ANOVA model followed by Tukey's honestly significant difference (HSD) test was used to verify the hypothesis that there were no differences in expression between the drug-treated and the control group. The multiplicity correction was performed using Benjamini-Hochberg procedure to control the false discovery rate (FDR) at 0.05%. In a pair-wise comparison of the differentially expressed genes between the control and the treated cells identified by the ANOVA analyses, a subset of genes was identified that displayed a conjoint regulation in the treated cells. Genes were placed in this latter group if they exhibited a mean ≥ 2-fold increase or decrease compared to the control cells. This subset of genes was subjected to the GeneTrail (25) software to identify any over-representation of genes associated with the regulatory pathways that are represented in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and TRANSPATH databases. Microarray data are deposited in the GEO public database (accession no. GSE59704). These methods fulfilled the MIAME criteria (http://www.mged.org/miame).

Results

Single applications of catechin, epicatechin and taxifolin are not effective in reducing cell viability of HT1080 fibrosarcoma cells. The viability of the HT1080 cells was moderately but significantly reduced by single treatment with taxifolin (Fig. 1). A total of 82.5±1.7% (mean ± SD) of the cells were detected as viable after 24 h treatment with taxifolin (vs. 94.9±0.6% in the control group, p=0.001). Single treatment with catechin led also to significant reduction of viable cells after 24 h of incubation, but only a slight decrease in cell viability was observed; the percentage of viable cells was reduced to 87.2±1.0% (p<0.001). Exposure to epicatechin alone decreased cell viability likewise to 87.1±0.9% (p<0.001).
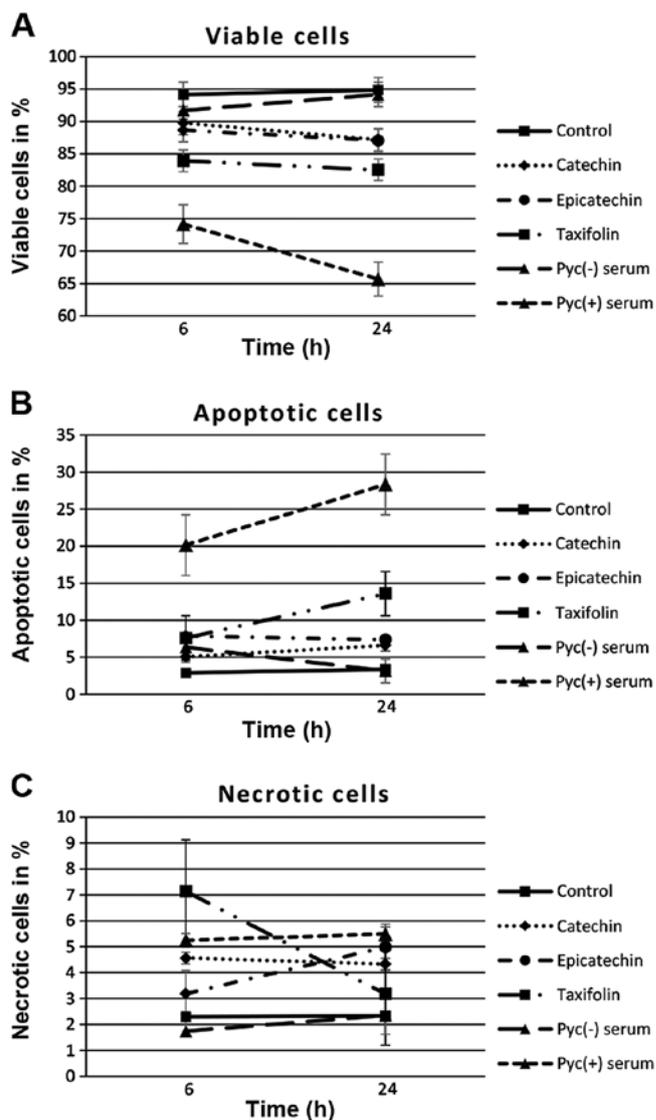

Figure 1. Effects of pycnogenol and its metabolites on viability, apoptosis and necrosis. HT1080 cells were incubated for 24 h with catechin, epicatechin, taxifolin, plasma samples after and before pycnogenol intake. The percentages of (A) viable, (B) apoptotic and (C) necrotic cells were determined using fluorescence activated cell sorting (FACS) analysis for Annexin V-FITC and propidium iodide (PI). The values indicate the means ± SD of three independent experiments and consecutive passages. Pyc(+), plasma sample after pycnogenol intake; Pyc(-), plasma sample before pycnogenol intake.

Plasma samples obtained after pycnogenol intake induced significantly apoptotic cell death. Application of plasma samples before pycnogenol intake had no significant effect on cell viability over all time points (Fig. 1). After 24-h treatment with pycnogenol-negative human plasma 94.2±1.0% of the cells were detected as viable. In contrast, the viability of untreated control cells was 94.9±1.4%. Strikingly, treatment of HT1080 cells with plasma samples after pycnogenol intake resulted in significant apoptotic cell death. The first significant apoptotic response was observed after 6 h of incubation with 20.1±2.9% of the cells left apoptotic and 74.2±1.4% remaining viable (p<0.001). Apoptosis reached a maximum after 24 h of treatment. Here, 28.3±6.0% were observed to be apoptotic (vs. 3.4±1.4% in control group, p<0.001) and 65.7±4.0% were left viable whereas the percentage of necrotic cells was only 5.5±3.6%.
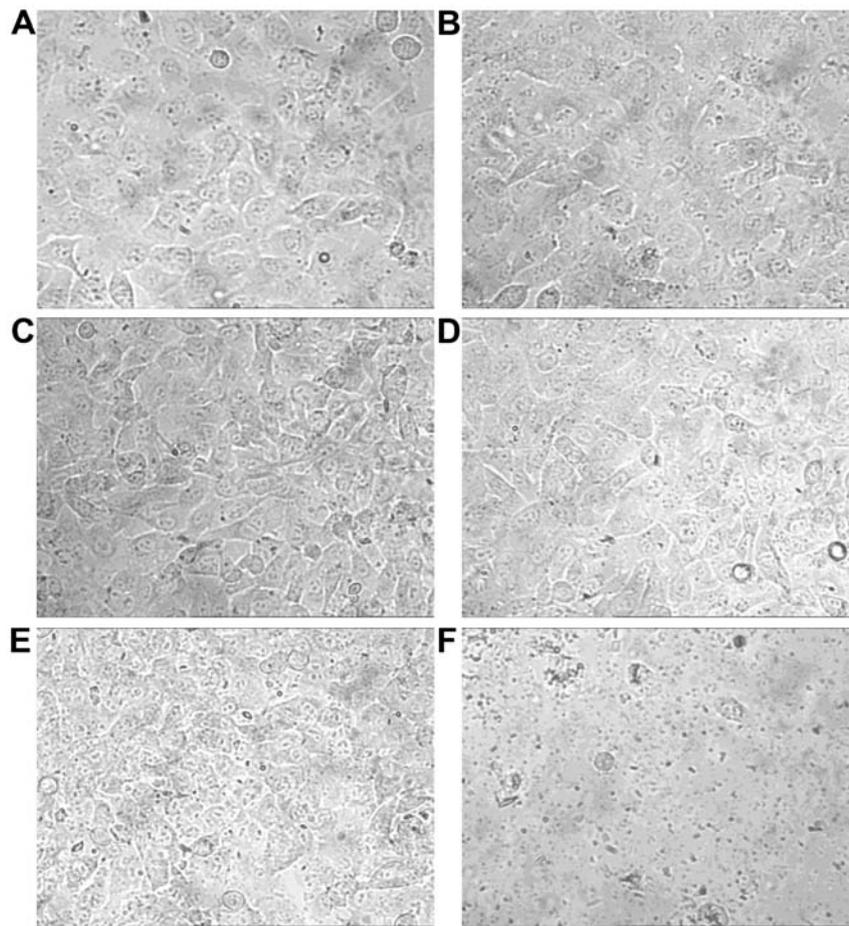
Figure 2. Morphological changes after treatment. Representative photographs (20-fold enlargement) demonstrating morphological changes in HT1080 cells induced by (B) catechin, (C) epicatechin, (D) taxifolin, (E) plasma samples before and (F) after pycnogenol intake following 24 h of treatment compared with (A) untreated control cells.

Table I. Microarray analysis using GeneTrail software identified significant changes in the following computed KEGG pathway categories with p<0.05 and FDR adjustments (Benjamini-Hochberg).

Selection of significant subcategories (α=0.05, FDR adjusted)	p-values	Expected no. of regulated genes	Observed no. of regulated genes
Apoptosis	0.002	55	84
MAPK signalling pathway	0.028	12	22
Pathways in cancer	0.002	16	33
P53 signalling	0.002	13	27
Cell adhesion	0.002	29	51
Metabolic pathways	0.004	54	32

The analysed genes in the subcategories were significantly up- or downregulated with a ≥2-fold mean change in the treatment group compared to the control. KEGG, Kyoto Encyclopaedia of Genes and Genomes; FDR, false discovery rate.

Only addition of plasma samples after pycnogenol intake induced morphological changes and cell detachment. Catechin, epicatechin, taxifolin and plasma samples before pycnogenol intake did not alter cell morphology and density as observed using bright-field microscopy (Fig. 2). However, plasma samples after pycnogenol intake reduced cell density of HT1080 fibrosarcoma cells indicating decreased rates of cell division and proliferation respectively. Further, it led to

shrinkage of cells and dissolution of confluent cell groups followed by complete cell detachment. Longer incubation resulted in obvious morphological aberrations.

Microarray analysis revealed differential gene expression patterns of HT1080 cells treated with plasma samples after pycnogenol intake. Based on comparison analysis algorithm, pycnogenol remarkably altered the expression levels of different combinations of probe sets. In cells treated with plasma samples

Table II. Summary of the expression changes of apoptosis-related genes for cells treated with human plasma samples after pycnogenol intake compared to untreated cells.

Gene ID	Gene title	Oncological relevance	Signal log ratio (compared to untreated cells)	Refs.
FN1	Fibronectin 1	Promotes pulmonary metastasis of human fibrosarcoma HT1080 cells in nude mice. Upregulation is associated with increased proliferation, adhesion and invasion of fibrosarcoma cells *in vitro*	-1.1	37,38
CTNNA1	Catenin α1	Associated with increased cell survival of synovial sarcoma cells	-1.1	39
LAMC1	Laminin γ1	Contributes to cancer cell migration and invasion in prostate cancer	-1.0	40
RHOA	Rho GDP dissociation inhibitor α	Enhances metastatic potential of different sarcoma cell lines *in vivo*	-0.9	47,48
ITGB1	Integrin, β1 (fibronectin receptor, β polypeptide)	Promotes human lung cancer cell invasion and metastasis *in vitro* and *in vivo*. Promotes proliferation and cell survival of colorectal carcinoma cells	-0.9	49,50
JAK1	Janus kinase 1	Inactivation of JAK1 in fibrosarcoma cells leads to loss of invasion *in vitro* and metastasis *in vivo*	-0.8	46
PIK3CB	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit β	Required for growth of phosphatase and tensin homolog (PTEN)-deficient coloncarcinoma cells	-0.3	51
PIK3RI	Phosphoinositide-3-kinase, regulatory subunit 1 (α)	Downregulation results in decreased proliferation, migration, and invasion in different malignant cell lines	-0.3	52,53
AKT3	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, γ)	Contributes to invasive migration and tumour metastasis in various malignancies	-0.3	54
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Overexpression promotes progression of metastatic fibrosarcoma *in vivo*	-0.3	55
DUSP1	Dual specificity phosphatase 1	Inhibits proliferation and induces apoptosis in human hepatocellular and pancreatic carcinoma	1.0	56,57
BCLAF	BCL2-associated transcription factor 1	Upregulation is associated with increased apoptosis and growth inhibition in bladder cancer cell lines	1.0	58
COX3	Cytochrome *c* oxidase III	Decreased expression is associated with apoptosis resistance in colon cancer cells	0.7	59
MAPK8	Mitogen-activated protein kinase 8	Contributes to apoptosis induced by cytostatics in different sarcoma cell lines	0.5	60

after pycnogenol intake, microarray analyses identified noticeable expression changes in 1,128 genes. Of these, 57.5% (649) were downregulated and 42.5% (479) upregulated.

To obtain an overview of the biological processes affected by pycnogenol, we analysed the regulated targets of the pathways that were over-represented in our data set using the GeneTrail application (25). Significant over-representation was detected in several pathway categories that included apoptosis, MAPK signalling pathway, pathways in cancer, p53 signalling, cell adhesion and metabolic pathways (Table I). To understand

the molecular details underlying the diverse modes of cell death in fibrosarcoma cells, we focused on the differentially expressed apoptosis-associated genes that were altered by plasma samples after pycnogenol intake (Fig. 3, Table II).

Discussion

Fibrosarcomas are rare tumours within the heterogeneous group of soft tissue sarcomas and respond poorly to conventional treatments, such as chemotherapy and radiation. Despite excellent rates of local disease control, treatment options in distant metastatic disease, especially in pulmonary locations, are very limited and have an associated median survival of <12 months (6,7). Due to the rarity of fibrosarcomas, the development of new therapeutics has been difficult, and the lack of novel chemotherapy protocols remains a major problem. Additionally, elderly patients with cardiac subdisease are ineligible for doxorubicin-based chemotherapy which is still considered as first-line treatment at metastatic disease stage. For these reasons, there was increasing interest in assessing whether the cardiotoxicity of doxorubicin could be mitigated by antioxidant compounds. In past studies, the maritime pine bark pycnogenol as well as its main constituent catechin were found to protect cardiomyocytes against doxorubicin-induced free radicals attenuating its cardiotoxicity in mice (26-28). Interestingly, pycnogenol and its metabolites are also known to exhibit anticancer activity in a wide range of human cancer cell lines (19-21). Because pycnogenol is extremely well-tolerated and no severe side-effects were ever reported, it is categorized as a nutritional supplement in most of the European countries and readily available. The numerous advantages of pycnogenol inspired us to analyse its anticancer activity in human fibrosarcoma cells.

In our study, plasma samples after pycnogenol intake significantly induced apoptosis in HT1080 cells *ex vivo* whereas plasma samples before pycnogenol intake did not exhibit any effect. Moreover, it led to decreased cell division and distinct morphological changes. Interestingly, pycnogenol was more effective in apoptosis induction than its main constituents catechin, epicatechin and taxifolin indicating that the metabolised components of pycnogenol interact synergistically.

To further elucidate the actions of metabolised pycnogenol on a molecular basis, we analysed changes in expression of apoptosis-related genes using microarray technology.

Notable gene alterations induced by pycnogenol were found in members of the PI3K/Akt signalling pathway (Table II). Interestingly, the PI3K/Akt pathway is widely dysregulated in many solid malignancies including several soft tissue sarcoma subtypes and many studies have shown this pathway to be vital to the growth and survival of cancer cells (29-32). Here, multiple mechanisms have been found to induce PI3K/Akt signalling, such as activating mutations of key genes such as phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit β (*PIK3CB*), phosphoinositide-3-kinase, regulatory subunit 1 (*PIK3RI*) and V-akt murine thymoma viral oncogene homolog 3 (*AKT3*) (33). In the current study, plasma samples after pycnogenol intake led to a downregulation of PIK3CB, PIK3RI and AKT3 when compared to untreated cells or cells treated with plasma samples before pycnogenol intake suggesting that the PI3K/Akt signalling pathway may play a role
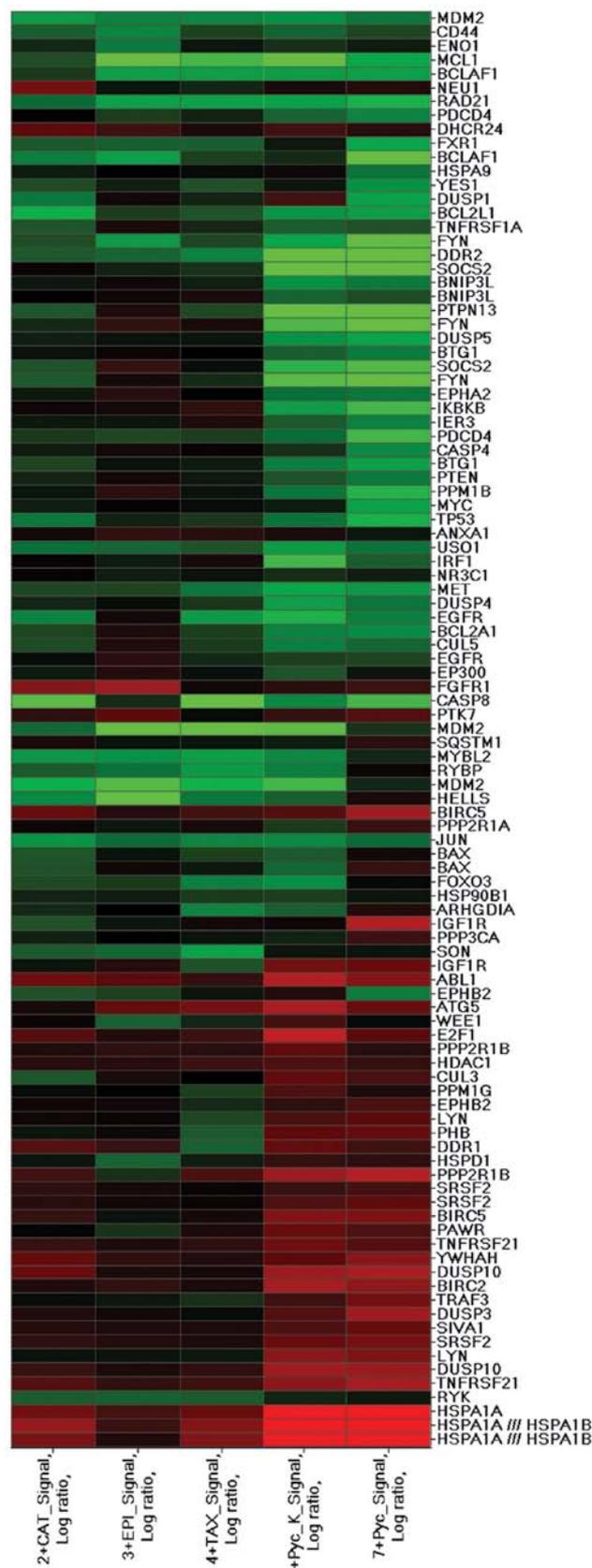

Figure 3. Hierarchical clustering: overall gene expression pattern of reliably measured probe sets. Horizontal rows represent individual probe sets/genes, vertical columns represent individual samples. 2+CAT, catechin; 2+EPI, epicatechin; 4+TAX, taxifolin; 6+Pyc_K, plasma sample before pycnogenol intake; 7+Pyc, plasma sample after pycnogenol intake. Color scale: black, mean (indicates unchanged expression); brightest green, 0.25x mean (indicates expression level below mean); brightest red, 4x mean (indicates higher expression level than mean).

in apoptosis induction in human fibrosarcoma cells (Table II). The only experimental study assessing the impact of PI3K/Akt pathway in fibrosarcoma cells demonstrated that inhibition of PI3K via small molecular inhibitors decreased remarkably the invasive potential of HT1080 cells *in vitro* (34). However, the role of PI3K/Akt pathway in human fibrosarcoma is still unknown and warrants further research because the novel and well-tolerated group of PI3K inhibitors could be potentially useful therapeutic options.

Interestingly, we found a correlation between apoptotic efficacy of metabolised pycnogenol and downregulation of several genes encoding for cell adhesion proteins such as fibronectin 1 (FN1), catenin α1 (CTNNA1) and laminin γ1 (LAMC1) (Table II). Cell adhesion and its underlying pathways play a crucial role in growth, metastasis and development of fibrosarcomas (35,36). In past experimental studies, upregulation of FN1 was shown to increase the invasive potential of HT1080 cells *in vitro* and to promote their pulmonary metastasis *in vivo* whereas overexpression of CTNNA1 and LAMC1 were associated with increased cell survival of different malignant cell lines (37-40). However, retrospectively we cannot conclude whether downregulation of these cell adhesion proteins itself led to apoptosis or vice versa. Thus, the appealing hypothesis that disruption of cell adhesion leads to apoptosis in human fibrosarcoma cells requires further experimental support.

Plasma samples with metabolised pycnogenol led also to a downregulation of Janus kinase 1 (JAK1). The JAK/STAT signalling pathway is a key signal transduction pathway implicated in the pathogenesis of many human cancers including several soft tissue sarcoma subtypes (41,42). Constitutive JAK/STAT activity has been demonstrated to cause tumourigenic inflammation and increased proliferation in a wide range of malignant diseases, including malignant fibrous histiocytoma (43-45). In fibrosarcomas, inactivation of JAK1 led to loss of invasion *in vitro* and metastasis *in vivo* (46). Recently, pharmacological inhibition of JAK1 was shown to induce apoptosis in rhabdomyosarcoma cells *in vivo* (42). However, understanding the complex role of JAK1 in sarcoma cell death may provide new opportunities for rational pathway-based therapies and drug development. Several novel JAK/STAT-inhibitors have been tested in clinical trials which could be promising agents in the therapy of metastatic soft tissue sarcomas.

In conclusion, this *in vitro* study demonstrates that the natural pine bark extract pycnogenol has the potential to induce apoptosis and alter gene expression in fibrosarcoma cells. Although a wide variety of genes and pathways were involved, the PIK3/Akt signalling pathway appears to play a key role in mediating apoptosis of HT1080 cells via pycnogenol metabolites. Pycnogenol is not meant to replace doxorubicin-based chemotherapy in patients with metastatic fibrosarcoma, but it could be a potential mild therapeutic option for patients that are not suitable for chemotherapy and have to undergo palliative treatment. The encouraging results of this study provide experimental support for *in vivo* trials assessing the effect of pycnogneol in soft tissue sarcomas.

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