Anti-proliferative activity of epigallocatechin-3-gallate and silibinin on soft tissue sarcoma cells

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Abstract. Disseminated soft tissue sarcomas (STS) present a therapeutic dilemma. The first-line cytostatic doxorubicin demonstrates a response rate of 30% and is not suitable for elderly patients with underlying cardiac disease, due to its cardiotoxicity. Well-tolerated alternative treatment options, particularly in palliative situations, are rare. Therefore, the present study assessed the anti-proliferative effects of the natural compounds epigallocatechin-3-gallate (EGCG), silibinin and noscapine on STS cells. A total of eight different human STS cell lines were used in the study: Fibrosarcoma (HT1080), liposarcoma (SW872, T778 and MLS-402), synovial sarcoma (SW982, SYO1 and 1273) and pleomorphic sarcoma (U2197). Cell proliferation and viability were analysed by 5-bromo-2'-deoxyuridine and MTT assays and real-time cell analysis (RTCA). RTCA indicated that noscapine did not exhibit any inhibitory effects. By contrast, EGCG decreased proliferation and viability of all cell lines except for the 1273 synovial sarcoma cell line. Silibinin exhibited anti-proliferative effects on all synovial sarcoma, liposarcoma and fibrosarcoma cell lines. Liposarcoma cell lines responded particularly well to EGCG while synovial sarcoma cell lines were more sensitive to silibinin. In conclusion, the green tea polyphenol EGCG and the natural flavonoid silibinin from milk thistle suppressed the proliferation and viability of liposarcoma, synovial sarcoma and fibrosarcoma cells. These compounds are therefore potential candidates as mild therapeutic options for patients that are not suitable for doxorubicin-based chemotherapy and require palliative treatment. The findings from the present study provide evidence to support *in vivo* trials assessing the effect of these natural compounds on solid sarcomas.

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Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of solid tumours arising from transformed cells of mesenchymal origin. They may occur throughout the body and represent ~1% of all adult malignancies (1). In patients with primary diagnosed STS without distant metastasis, standard treatment involves surgical resection with negative margins, typically followed by adjuvant radiation to decrease the risk of recurrence (2,3). However, almost half of all patients with STS develop distant metastases, rendering them unsuitable for surgery (4,5). If metastasis has occurred, the median survival time regardless of chemotherapeutic treatment is <12 months (6,7). A limited number of chemotherapeutic agents, including doxorubicin and ifosfamide, are effective for the treatment of metastatic STS (2). However, the response rates of these agents are poor and often do not result in significant extension of survival (8). Doxorubicin is the predominant chemotherapeutic agent used for the treatment of metastatic STS, and has a response rate of ~30% (9,10). The combination of doxorubicin and ifosfamide exhibits greater response rates compared with doxorubicin alone; however, it is associated with severe short- and long-term adverse effects, including bone marrow suppression and cardiomyopathy (11-13).

A multicentre analysis by the European Organisation for Research and Treatment of Cancer (trial 62012) on 455 patients with advanced STS indicated that an intensified combination treatment with doxorubicin and ifosfamide is not suitable for treatment of locally advanced or metastatic STS as a result of the serious adverse effects, and should therefore only be used with a view to tumour shrinkage (13). Furthermore, the versatility of doxorubicin is limited by dose-associated and cumulative myocardial toxicity, particularly in older patients with a history of cardiac disease (14). However, the incidence of STS increases markedly >50 years of age, when the prevalence of cardiac diseases is also greater (15). Currently, there are no efficacious and safe agents for the palliative treatment of patients who may not undergo doxorubicin-based chemotherapy due to underlying cardiac disease. Therefore, the

development of novel therapeutic agents is required for the treatment of STS.

A review of the literature reveals various potential well-tolerated and natural phytochemicals that exhibit anti-neoplastic effects on malignant cells, including the compounds epigallocatechin-3-gallate (EGCG), silibinin and noscapine. EGCG is the most abundant catechin in green tea and demonstrates anti-inflammatory, antioxidant and antineoplastic activities (16-18). Various *in vitro* studies have revealed that EGCG exhibits anticancer activity in lung (19), prostate (20), colon (21), gastric (22), breast (23) and cervical carcinoma cells (24). To date, EGCG has undergone various phase II trials and has been demonstrated to be well-tolerated following oral administration (25-29). The most frequent adverse reactions observed were gastrointestinal reactions, including nausea and vomiting. In rare cases, patients presented with elevated serum alanine aminotransferase levels following the administration of high doses of oral EGCG; however, liver function tests returned to baseline following discontinuation of ECGC (30). Therefore, EGCG is considered to be a safe and well-tolerated agent for the treatment of cancer patients (31,32).

Silibinin is the primary active constituent of silymarin, a standardized extract from the seeds of the milk thistle plant (*Silybum marianum*). Silibinin is available as a therapeutic agent in various European countries and is used for the treatment of toxic liver damage, particularly due to *Amanita phalloides* intoxication (33). It is well tolerated in cancer patients (34,35) and has demonstrated anti-neoplastic effects in various malignant cell lines including HT1080 fibrosarcoma cells (36-40).

Noscapine is a naturally occurring opium alkaloid and a widely used antitussive drug that is non-addictive and has a low toxicity profile (41). As a tubulin-binding agent, various preclinical studies have established its tumour-inhibitory effects in a wide range of malignancies (42-45). Currently, noscapine is undergoing phase II clinical trials for cancer chemotherapy (46).

Based on these results, the present study aimed to investigate the anti-proliferative activity of EGCG, silibinin and noscapine on eight different STS cell lines, including fibrosarcoma, liposarcoma, synovial sarcoma and pleomorphic sarcoma cells.

Materials and methods

Cell lines. Eight different human STS cell lines were used in the present study: HT1080 (fibrosarcoma), SW872 (liposarcoma), T778 (liposarcoma), MLS-402 (liposarcoma), SW982 (synovial sarcoma), SYO1 (synovial sarcoma), 1273 (synovial sarcoma) and U2197 (pleomorphic sarcoma/malignant fibrous histiocytoma). HT1080, SW872 and SW982 were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and were cultured in Dulbecco's modified Eagle's medium (DMEM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin (100 U/ml) and 1% streptomycin (100 μg/ml; PAN-Biotech GmbH). The well-differentiated T778 liposarcoma cell line and the MLS-402 myxoid liposarcoma cell line were donated by Professor Pierre Åman (University of Gothenburg, Gothenburg, Sweden) and Professor Ola Myklebost (Oslo University Hospital, Oslo, Norway), respectively. T778 and MLS-402 cells were cultured in RPMI (PAN-Biotech GmbH) supplemented with 10% FBS and 1% penicillin/streptomycin as previously described (47,48). The SYO-1 and 1273 cell lines were donated by Dr Akira Kawai (National Cancer Center, Tokyo, Japan) and Professor Olle Larsson (Karolinska Institutet, Stockholm, Sweden) (49,50). The SYO-1 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 0.5% sodium pyruvate. The 1273 cells were cultivated in Ham's F12 (PAN-Biotech GmbH) supplemented with 10% FBS and 1% penicillin/streptomycin. The U2197 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and was cultured in minimum essential medium (PAN-Biotech GmbH) supplemented with 20% FBS, 0.165% sodium bicarbonate and 1% penicillin/streptomycin (51). All cultures were maintained at 37˚C in a humidified 5% CO_2 atmosphere.

Phytotherapeutic agents. EGCG, silibinin and noscapine were obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). The stock solution was dissolved in dimethyl sulfoxide (DMSO; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and further diluted in DMEM to concentrations of 50 μM (EGCG), 150 μM (silibinin) and 30 μM (noscapine) for all assays. These concentrations have been demonstrated to inhibit proliferation and induce apoptosis in various malignant cell lines (36,52,53).

Cell viability assay. Metabolic activity was measured using an MTT assay. Cells were seeded in 96-well plates (Corning Incorporated, Corning, NY, USA) at $1x10^4$ cells per well. The following day, the three agents were added in the aforementioned concentrations for 24 h. Subsequently, 50 μl 0.5 mg/ml MTT (Sigma-Aldrich; Merck Millipore) was added for 4 h. MTT is a yellow dye that is reduced to purple formazan in the mitochondria of vital cells. Cells were lysed following the addition of 200 μl DMSO and 25 μl glycine buffer (containing 0.1 M glycine and 0.1 M NaCl, adjusted to pH 10.5 with NaOH) per well. The quantity of integrated dye represented the level of metabolism and was measured at a wavelength of 562 nm using an Elx808 Ultra Microplate Reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany).

Proliferation assay. To quantify the effects of EGCG, silibinin and noscapine on cell proliferation, a colorimetric cell proliferation 5-bromo-2'-deoxyuridine (BrdU)-ELISA assay (Roche Diagnostics GmbH, Mannheim, Germany) was performed according to the manufacturer's protocol. Briefly, cells were seeded at $1x10^4$ cells/well in 96-well plates and cultured for 24 h. The phytotherapeutic agents were subsequently added in the appropriate concentrations for 24 h. The BrdU labelling solution was added and incubated for a further 24 h. BrdU, a pyrimidine analogue, integrates into the DNA of proliferating cells. The level of proliferation was quantified by the light emission detected via an Orion Microplate Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). Cell proliferation was determined in quadruplicate. The results are expressed as a percentage of the proliferation of DMSO-treated control cells.

Table I. Summary of the cytostatic effects of EGCG, silibinin and noscapine, as assessed by MTT and BrdU assays and RTCA.

		EGCG			Silibinin			Noscapine		
Subtype	Cell line	MTT	BrdU	RTCA	MTT	BrdU	RTCA	MTT	BrdU	RTCA
Fibrosarcoma	HT1080	+	+	+	+	+	+	+	+	-
Liposarcoma	SW872	+	+	+	+	-	+	+	+	-
	T778	+	+	+	+	+	+	+	+	-
	MLS-402	+	+	+	+	+	+	+	-	-
Synovial sarcoma	SW982	+	+	+	+	+	+	+	+	-
	SYO1	-	+	+	+	+	+	+	-	-
	1273	+	+	-	+	+	+	+	+	+
Pleomorphic sarcoma	U2197	+	+	+	+	+	-	-	+	-

+, cytostatic effect; -, no cytostatic effect. EGCG, epigallocatechin-3-gallate; BrdU, 5-bromo-2'-deoxyuridine; RTCA, real-time cell analysis.

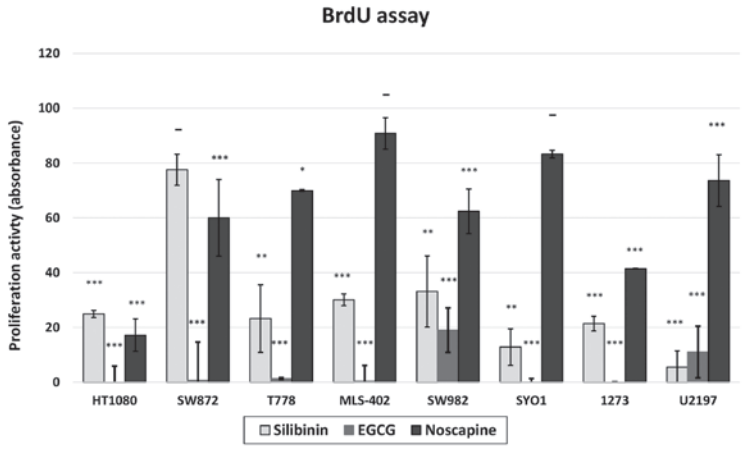

Figure 1. Effects of silibinin, ECGC and noscapine on cell proliferation. The proliferative activity of all cell lines was measured by BrdU assay. The assay was performed following 24 h of treatment with DMSO, silibinin, ECGC or noscapine. For clarity, the BrdU-labelling index of the DMSO-treated control cells was adjusted to 100 for each cell line and is not included. The indices for the different treatment groups were subsequently calculated as follows: Index treatment group i=(mean absorbance rate treatment group i x100)/mean absorbance rate DMSO control. Data are presented as the mean ± standard deviation. $^{-}$P>0.05, *P<0.05, **P<0.01 and ***P<0.001 vs. DMSO-treated control cells. EGCG, epigallocatechin-3-gallate; BrdU, 5-bromo-2'-deoxyuridine; DMSO, dimethyl sulfoxide.

Real-time cell analysis (RTCA). Cells were seeded in two 8-well plates with an integrated microelectronic sensor array in 600 µl culture medium (iCELLigence Real Time Cell Analyser; ACEA Biosciences, San Diego, CA, USA). After 24 h, the therapeutic agents were added for a total volume of 50 µl. The cell proliferation and survival were monitored in real-time by measuring the cell-to-electrode responses of the seeded cells. In each individual E-well, the cell impedance was measured and converted to cell index (CI) values by the RTCA software version 1.2 (Roche Diagnostics GmbH) (54). The graphs were generated in real-time by the iCELLigence system. Untreated and DMSO-treated cells served as controls.

Statistical analysis. Data analyses were performed using the statistical program SPSS 16 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Comparisons between the experimental groups in BrdU and MTT assays were performed using one-way analysis of variance followed by *post-hoc* Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

EGCG significantly inhibits the proliferation and viability of STS cell lines. As indicated by the BrdU assay, the proliferation of all eight human STS cell lines was inhibited by EGCG (Fig. 1). By MTT analysis, EGCG decreased the viability of seven cell lines (Fig. 2). To evaluate the proliferation and viability of cells continuously over a longer time period, RTCA was performed. The viability, adhesion and proliferation of the cells were monitored prior to and during EGCG treatment in real time for 160 h (Figs. 3-5). EGCG markedly decreased the CI of all STS cell lines except the 1273 synovial sarcoma cell line. The administration of EGCG reduced the CI of the HT1080 fibrosarcoma cell line and the U2197 pleomorphic sarcoma cell line (Fig. 3). All three liposarcoma cell lines (SW872, T778 and MLS-402) exhibited a continuously decreased CI during EGCG treatment compared with untreated or DMSO-treated cells (Fig. 4), as did the remaining two synovial sarcoma cells lines (SW982 and SYO1; Fig. 5).

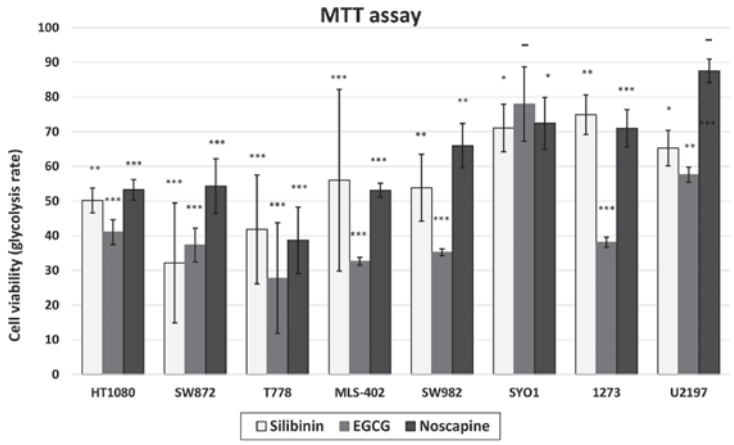

Figure 2. Effects of silibinin, ECGC and noscapine on cell viability. The cell viability of all cell lines was measured by MTT assay. The assay was performed following 24 h of treatment with DMSO, silibinin, ECGC or noscapine. For clarity, the MTT-index of the DMSO-treated control cells was adjusted to 100 for each cell line and is not included. The indices for the different treatment groups were subsequently calculated as follows: Index treatment group i=(mean absorbance rate treatment group i x100)/mean absorbance rate DMSO control. Data are presented as the mean ± standard deviation. $^{-}$P>0.05, *P<0.05, **P<0.01 and ***P<0.001 vs. DMSO-treated control cells. EGCG, epigallocatechin-3-gallate; DMSO, dimethyl sulfoxide.

Silibinin significantly decreases the proliferative activity and viability of STS cell lines. Treatment with silibinin significantly reduced the proliferation of seven STS cell lines (Fig. 1), and significantly decreased the cell viability of all eight assessed STS cell lines, as analysed by MTT assay (Fig. 2). By RTCA, silibinin was the only compound that exhibited a strong inhibitory effect on all three synovial sarcoma cells (Fig. 5). In addition, silibinin reduced the CI of all liposarcoma cell lines; however, not to the extent of EGCG. Only the U2197 pleomorphic sarcoma cell line did not respond to silibinin treatment.

By RTCA, STS cell lines are unaffected by noscapine treatment. Noscapine exhibited cytostatic effects on STS cells, as assessed using BrdU (Fig. 1) and MTT (Fig. 2) assays at 24 h. However, these effects could not be validated by RTCA over a longer time period. The proliferation inhibition resulting from noscapine treatment in six cell lines at 24 h did not result in a continual decrease of the CI. In all cell lines, the CI of noscapine-treated cells increased steadily and was comparable to the CI of DMSO-treated or untreated control cells during the 160 h of real-time analysis (Figs. 3-5).

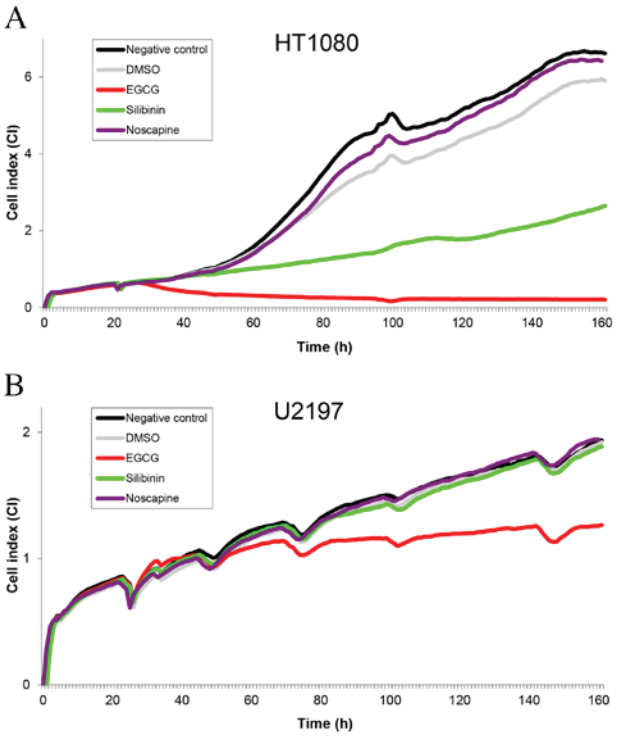

Figure 3. Real-time cell analysis of fibrosarcoma and malignant fibrous histiocytoma cells. (A) HT1080 fibrosarcoma cells and (B) U2197 pleomorphic sarcoma/malignant fibrous histiocytoma cells were seeded in 8-well plates with an integrated microelectronic sensor array. The CI reflecting the number of viable cells was monitored continuously in real-time. The compounds were applied to the wells after 24 h resulting in a bend of the CI curve as impedance measurements were transiently disrupted by the addition of the solutions. In EGCG-treated HT1080 cells, CI decreased and remained at a low level. Silibinin slightly decreased the CI of HT1080 cells. U2197 cells were unaffected by silibinin and responded only poorly to EGCG. EGCG, epigallocatechin-3-gallate; CI, cell index; DMSO, dimethyl sulfoxide.

Discussion

STS are a heterogeneous group of rare mesenchymal malignancies. To date, systemic treatment options are limited following metastasis. Patients with distant metastases have a median survival time of less than one year despite systemic chemotherapy (6,7). Due to the infrequent and heterogeneous nature of STS the development of novel systemic therapeutic agents is challenging and novel chemotherapy strategies are lacking. Therefore, the development of well-tolerated and effective chemotherapeutic agents for the treatment of STS is required.

The present study assessed the cytostatic effects of the naturally occurring compounds noscapine, silibinin and EGCG on eight STS cell lines. By RTCA, noscapine did not exhibit any relevant anti-proliferative effects (Table I). In contrast, silibinin and EGCG exerted cytostatic effects in almost all examined STS cell lines, as assessed by BrdU, MTT and RTCA. Administration of EGCG decreased proliferation and viability of all liposarcoma cell lines and two synovial sarcoma cell lines for more than five days. In addition, it

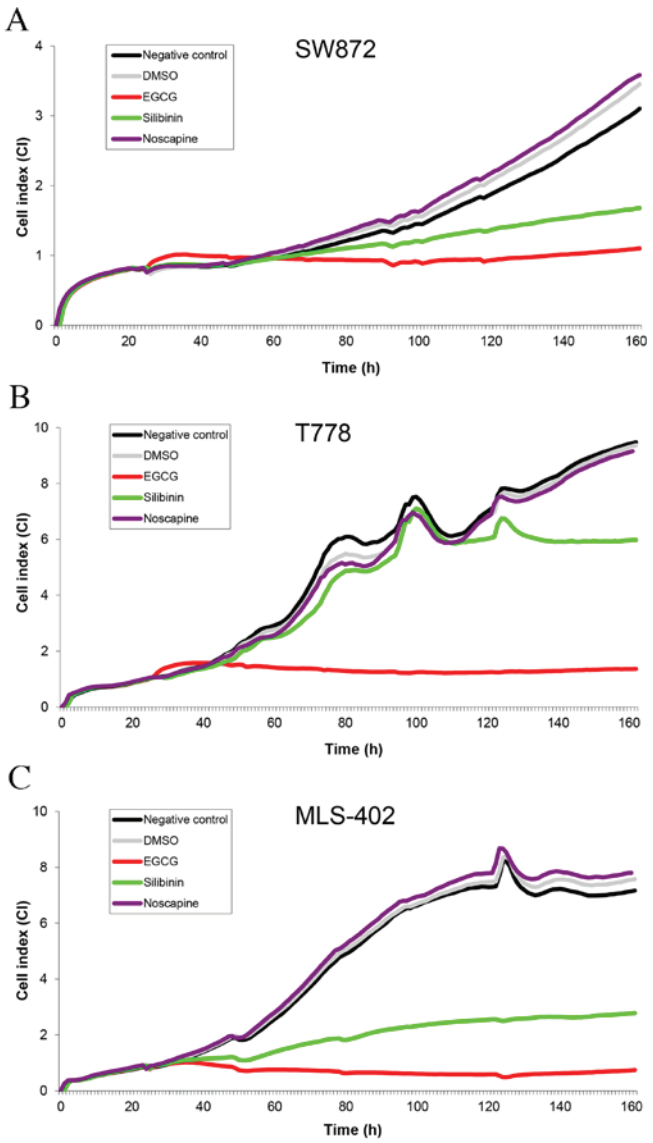

Figure 4. Real-time cell analysis of liposarcoma cell lines. (A) SW872, (B) T778 and (C) MLS-402 liposarcoma cells were seeded in 8-well plates with an integrated microelectronic sensor array. The CI reflecting the number of viable cells was monitored continuously in real-time. The compounds were applied to the wells after 24 h resulting in a bend of the CI curve as impedance measurements were transiently disrupted by the addition of the solutions. The CI curve of SW872 cells was slightly decreased by silibinin. T778 and MLS-402 cells exhibited a moderate response to silibinin, and a strong response to EGCG. EGCG, epigallocatechin-3-gallate; CI, cell index; DMSO, dimethyl sulfoxide.

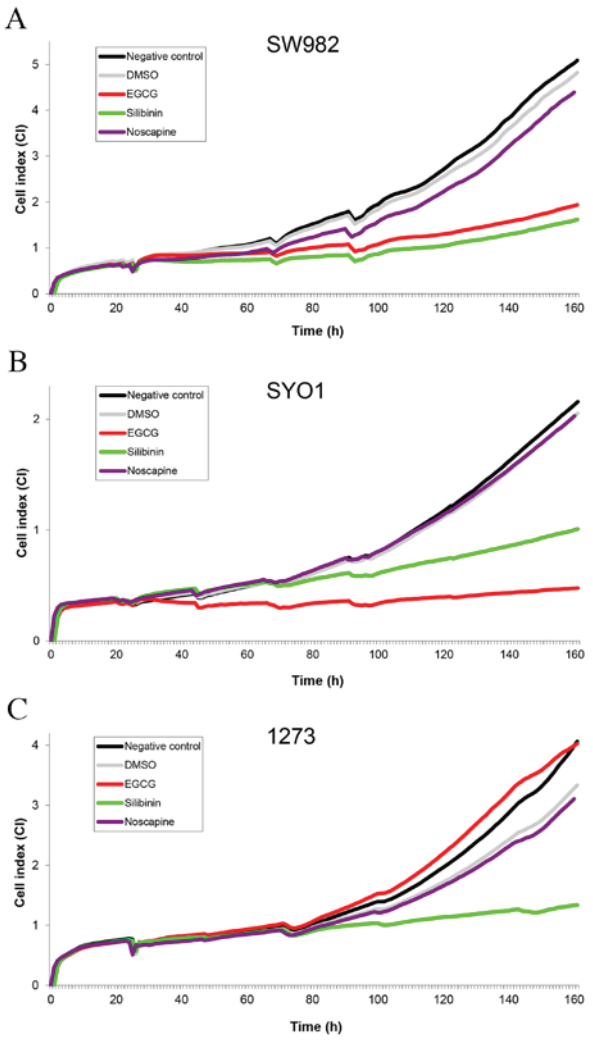

Figure 5. Real-time cell analysis of synovial sarcoma cell lines. (A) SW982, (B) SYO1 and (C) 1273 synovial sarcoma cells were seeded in 8-well plates with an integrated microelectronic sensor array. The CI reflecting the number of viable cells was monitored continuously in real-time. The compounds were applied to the wells after 24 h resulting in a bend of the CI curve as impedance measurements were transiently disrupted by the addition of the solutions. EGCG reduced the CI gradient of SW982 and SYO1 cells, but had no effect on 1273 cells. Silibinin markedly decreased the viability of all three synovial sarcoma cell lines. EGCG, epigallocatechin-3-gallate; CI, cell index; DMSO, dimethyl sulfoxide.

inhibited HT1080 fibrosarcoma and U2197 pleomorphic sarcoma cells. Of the three analysed compounds, EGCG exerted the greatest anti-proliferative activity in the three assessed liposarcoma cell lines, rendering it a potential agent of interest. Liposarcomas represent the most frequent somatic STS subtype and respond poorly to anthracycline-based chemotherapy, with well-differentiated and de-differentiated tumours exhibiting response rates of only 12 and 13%, respectively (55). Pleomorphic liposarcomas are the least responsive to chemotherapy, with a response rate of 5%, whereas myxoid liposarcomas have been revealed to be the most sensitive to chemotherapy, exhibiting response rates of 44-48% (56-58). In the present study, EGCG exhibited a distinct inhibitory effect on T778 cells from a well-differentiated liposarcoma, SW872 cells from a pleomorphic liposarcoma and MLS-402 cells from a myxoid liposarcoma. Although these findings were *in vitro*, they suggested a potential anti-proliferative activity of EGCG on liposarcoma cells that should be further investigated *in vivo*.

In comparison with EGCG, the inhibitory effect of silibinin was reduced in liposarcoma cells, but greater in synovial sarcoma cells. Silibinin significantly decreased proliferation and viability in all three synovial sarcoma cell lines. Although synovial sarcomas have typically been considered relatively chemosensitive, the European Organisation for Research and Treatment of Cancer recently reported a chemotherapy response rate of only 28% for patients with advanced synovial sarcoma (59). Therefore, there remains a requirement for

alternative cytostatic agents for the treatment for synovial sarcomas, and the *in vitro* effects of silibinin demonstrated in the present study should be further examined *in vivo*.

A literature review revealed that the green tea polyphenol EGCG has further notable properties. Various *in vivo* studies have confirmed that EGCG mitigates doxorubicin-induced cardiotoxicity by suppressing oxidative stress (60-63). The oxygen free radical scavenging ability of EGCG has been demonstrated to protect cardiomyocytes from doxorubicin-mediated cardiotoxicity according to histopathological analysis (64). Furthermore, EGCG has been revealed to synergistically enhance the anticancer activity of doxorubicin in various *in vivo* studies on prostate and liver cancer (65-67). Notably, similar chemosensitizing and chemopreventive activities have been described for silibinin; *in vivo* studies revealed that silibinin synergistically enhances the apoptosis-inducing activity of doxorubicin and ameliorates doxorubicin-induced cardiotoxicity (68-73). Therefore, EGCG and silibinin may additionally function as chemopreventives and chemosensitizers for doxorubicin, which remains the first-line cytostatic for the systemic treatment of disseminated STS.

In conclusion, the present *in vitro* study demonstrated that EGCG and silibinin inhibit the proliferation and viability of liposarcoma, synovial sarcoma, fibrosarcoma and pleomorphic sarcoma cells. Liposarcoma cell lines responded particularly well to EGCG while synovial sarcoma cell lines were more sensitive to silibinin. To the best of our knowledge, this is the first study to assess the effects of EGCG and silibinin on such a wide range of STS cell lines, including liposarcoma, synovial sarcoma, fibrosarcoma and pleomorphic sarcoma cells. EGCG and silibinin are not intended to supplant doxorubicin for the treatment of patients with disseminated STS; however, they may be a potential therapeutic option for patients who require palliative treatment but are considered unsuitable for chemotherapy. The present study provides evidence to support *in vivo* trials to examine the effects of these natural compounds on STS.

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