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Efficacy screening of *Gloriosa superba* extracts in a murine pancreatic cancer model using $^{18}$F-FDG PET/CT for monitoring treatment response

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ABSTRACT

Purpose: *In vivo* efficacy of two herbal extracts of *Gloriosa superba* L. (Colchicaceae) was investigated in a murine pancreatic tumor model by tumor volume measurements and Positron Emission Tomography (PET) imaging using 2-deoxy-2-[18F]fluoro-D-glucose (18F-FDG).

Methods: A crude extract of *G. superba* seeds (GS) rich in colchicine and a colchicine-poor extract (GS2B) containing mostly colchicoside as a putative prodrug were prepared. PANC02-bearing C57BL/6 mice were treated with either placebo, gemcitabine or one of the extracts (three different doses) for 10 days. Tumor volume measurements were performed daily during treatment and additionally 18F-FDG PET/CT was acquired at baseline and after 7 days of treatment. Ki-67 and cleaved caspase-3 immunostaining was performed on the resected tumors.

Results: After 7 days of treatment, a dose-dependent tumor growth inhibition of both extracts was observed with the highest *in vivo* response at the highest dose of GS and GS2B and gemcitabine. A positive, significant correlation was found between Ki-67 scores and relative tumor volumes (RTV), and a negative, significant correlation between caspase-3 staining scores and RTV. A decrease in 18F-FDG uptake was clearly observed in all treatment groups.

Conclusions: The therapeutic efficacy of the two different herbal extracts was demonstrated in an *in vivo* pancreatic tumor model. 18F-FDG PET was able to detect an early response as overall lower 18F-FDG uptake was measured in the treated groups.
Introduction

Pancreatic cancer has a poor prognosis and is the fourth leading cause of cancer deaths. Most patients diagnosed with this condition die within one year while the 5-year survival is very low, 2 - 5%. Gemcitabine, a nucleoside analog, is the first-line treatment in patients with advanced pancreatic cancer. Other treatment options include a combination of gemcitabine with other chemotherapies (cisplatin, irinotecan, 5-fluorouracil) or radiotherapy. However, low objective response rates and low survival benefits obtained with the current treatment regimens make it necessary to investigate other potential approaches.

Gloriosa superba L. (Colchicaceae), commonly called glory lily, is native to tropical Africa, south-eastern Asia and India and is now widely cultivated throughout the world as an ornamental plant. G. superba has traditionally been used for various diseases. The tubers contain colchicine, a well-known medicine against gout. Colchicine is an inhibitor of microtubule polymerization by binding to tubulin and thus inhibiting cell mitosis and inducing cell death. It has also been used in the treatment of cancer, but nowadays it is considered too toxic for viable healthy tissue. In addition, related alkaloids such as 3-O-demethylcolchicine and the glycoside colchicoside are present (Fig.1). Because of the relatively high concentration of colchicoside, the putative prodrug of a biologically active colchicine-like aglycone, G. superba was selected in the present study for evaluation of its potential use against pancreatic cancer. The hypothesis of the present study is that a combination of various (potentially synergistic) active compounds in an herbal extract, including prodrugs such as glycosides, may have more beneficial effects and a better toxicity profile (because the active metabolite is gradually released from the
prodrug) than pure compounds. Colchicine and related aglycones are known to have a good oral availability because of their lipophilic nature.\textsuperscript{14} Glycosides on the other hand act as prodrugs. Since they are hydrophilic they cannot passively be absorbed in the gastro-intestinal tract, unless the glycosidic moiety is removed by enzymatic action, mainly by glycosidases produced by colonic bacteria.\textsuperscript{15}

Therefore, preliminary \textit{in vitro} cytotoxicity studies of a crude extract of \textit{G. superba} and a colchicine-poor / colchicoside-rich extract were carried out on a human and a murine pancreatic cancer cell line (PANC-1 and PANC02, respectively). Subsequently, both extracts were tested \textit{in vivo} on established subcutaneous PANC02 murine pancreatic tumors.

Non-invasive techniques able to predict response early in the treatment regime are highly valuable looking at the malignant character of pancreatic cancer. In this way, personalized (patient-specific) treatment can be offered and treatment outcome can be improved. Positron Emission Tomography / Computed Tomography (PET/CT) using 2-deoxy-2-[\textsuperscript{18}F]fluoro-D-glucose (\textsuperscript{18}F-FDG) has been demonstrated to detect early treatment response to low-dose short-term cyclopamine therapy in pancreatic cancer preclinical models, before changes in tumor size occurred.\textsuperscript{16} In addition, in pancreatic cancer patients, \textsuperscript{18}F-FDG PET was able to differentiate tumor response to therapy in the postoperative setting, and could potentially serve to monitor recurrence patterns in the setting of neoadjuvant or adjuvant chemoradiotherapy.\textsuperscript{17} Cancer cells rely on aerobic glycolysis, also known as the Warburg effect, characterized by increased metabolism of glucose to lactate in the presence of sufficient oxygen. \textsuperscript{18}F-FDG accumulates in the cell in relation to the rate of glucose metabolism which is higher in cancer cells than in normal cells, leading to high signal regions on the PET images.\textsuperscript{18}. \textsuperscript{18}F-FDG PET imaging may provide evidence of biological responses of novel
anticancer compounds, which may facilitate the transition of these novel compounds from the preclinical to the clinical phase. The aim of this study is to determine the therapeutic efficacy of two different *G. superba* extracts in a murine pancreatic cancer model and to evaluate early treatment response by $^{18}$F-FDG PET/CT.

**Material and methods**

1. **Extracts, cell lines and animals**

Dried seeds of *Gloriosa superba* L. were kindly provided by Indena® (Milano, Italy) (batch n° C140020, certificate of analysis n° 11/0208/LSP). Seeds were dried an extra two weeks in an oven at a temperature of 45 °C, ground and sieved through a 2 mm grid. The ground seeds (5.3 kg) were extracted exhaustively and consecutively by percolation and maceration with 95 L of 80% ethanol (Fisher Scientitfic) at room temperature. The ethanol was removed under reduced pressure at 40 °C and the aqueous extract was lyophilized, yielding the crude extract (GS, 846.7 g).

A colchicine-poor / colchicoside-rich extract (GS2B) was prepared by means of liquid-liquid partition, removing most of the colchicine content (Fig. 2.). About 50 g of GS was dissolved in 300 ml 5% acetic acid (pH 2.37) and consecutive extraction steps with diethyl ether (3 x 300 ml) and methylene chloride (3 x 300 ml) were performed. After those extraction steps most of the colchicine was removed, yielding fraction GS2B, an extract from *G. superba* seeds containing a higher amount of colchicine derivatives other than colchicine, especially colchicoside. All solvents used were purchased from Acros Organics or from Fisher Scientitfic.

In both extracts the amount of colchicine, colchicoside and 3-O-demethylcolchicine was determined using a validated HPLC method, and established as 3.22% (m/m), 2.52% (m/m) and 1.52% (m/m) for GS, and 0.07% (m/m), 2.26% (m/m) and 0.46%
This corresponded to a total content of colchicine and derivatives of 5.79% (m/m) and 1.61% (m/m) expressed as colchicine in GS and GS2B, respectively. The amount of colchicine in GS2B was less than 0.1% (m/m). In view of the good oral bioavailability of colchicine and related aglycones, as well as the need for metabolic activation of the glycoside colchicoside by colonic bacteria, oral administration of the plant extracts was preferred for the in vivo experiments.

Human pancreatic epithelioid carcinoma cells (PANC-1) were purchased from ATCC and murine pancreatic adenocarcinoma cells (PANC02) were kindly provided by Prof. Dr. C. Gravekamp (Albert Einstein College of Medicine, New York, USA). The cells were cultured in DMEM medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin and RPMI 1640 medium supplemented with the same supplements as well as 1% sodium pyruvate, respectively, at a temperature of 37 °C in a cell incubator containing 5% CO\textsubscript{2} / 95% O\textsubscript{2}.

Six-to-eight weeks old female C57BL/6JRccHsd mice (n = 56, 16 - 20 g body weight) were purchased from Harlan Laboratories. The mice were housed in individually ventilated cages, under a 10/14 h dark/light cycle at 20 - 24 °C and 40 - 70% relative humidity. Bedding material and refuges were provided and all animals had access to tap water and food ad libitum. All animals were treated in accordance to the guidelines and regulations for use and care of animals. Animals were treated in accordance with the European Ethics Committee (decree 86/609/CEE). All mice experiments were approved by the local Ethical Committee of the University of Antwerp, approved study number: 2013-03 (18-11-2013).

2. Characterization of the compounds
2.1 *In vitro* cytotoxicity

The sulforhodamine B (SRB) assay was performed according to the method of Papazisis et al., with minor modification.\(^{20}\) Seeding densities of 3000 cells/well (PANC-1) and 500 cells/well (PANC02) were used. In each experiment, 7 different concentrations of plant extract were tested on the different cell lines and 6 replicate wells per concentration were used. The effect on PANC-1 was evaluated using concentrations of GS ranging from 0.0001 – 100 µg/ml at two different incubation times, *i.e.* 24 h and 72 h. The cytotoxicity on the PANC02 cell line was evaluated in a concentration range of GS from 0.001 – 10 µg/ml with the same incubation times. In view of the planned *in vivo* experiment with a PANC02 allograft, GS2B (i.e. GS without colchicine (COL)) as well as pure COL (as a positive control) were tested on the PANC02 cells, with an incubation time of 24 h (since for GS no important difference in IC\(_{50}\) was observed after 24 h or 72 h, see below). The concentration ranged from 0.1 – 100 µg/ml for GS2B and from 0.0001 – 10 µg/ml for COL. The stock solution was prepared by dissolving the plant extracts in sterile water and by passing the solution through a 0.22 µm filter. After incubation, culture media was removed prior to fixation, which was done by adding 200 µl of 10% cold trichloroacetic acid onto the cells. After one hour at 4 °C, the cells were washed five times with deionized water. The cells were then stained with 200 µl 0.1% SRB in 1% acetic acid for 15 min at room temperature. To remove the unbound stain the cells were washed with 1% acetic acid four times. The plates were left to dry at room temperature. The protein-bound stain was re-dissolved with 200 µl 10 mM unbuffered tris and transferred to 96-well plates for the optical density (OD) reading at 540 nm (iMark Microplate Absorbance Reader). Colchicine (97%, Sigma Aldrich) was used as a positive control. The survival percentages of the cells were calculated from the
optical density readings as: \((\text{mean OD of treated cells}/\text{mean OD of control cells}) \times 100\%\). These survival percentages were then fitted in a curve and the IC\(_{50}\) was then determined by non-linear regression analysis of the data with the WinNonlin\® software (Pharsight).

### 2.2 Acute toxicology

To evaluate the possible toxicological effects of the herbal extracts, 3 mg/kg of GS was administered daily for 5 days by oral gavage in 3 non-tumor-bearing C57BL/6 mice and body weight was determined during therapy and in follow-up for another 3 days. Animals were daily inspected for clinical signs of toxicity (Table S1).

### 2.3 Treatment efficacy

The experimental set-up is shown in Fig. 3a. Cultures of PANC02 cells were harvested using a 0.05% trypsin solution, washed twice in sterile PBS and resuspended in sterile PBS at a concentration of \(30 \times 10^6\) cells per ml. The viable cells were counted by using a Muse\® Cell Analyzer (Merck Millipore). The mice were inoculated with 100 µl of the cell suspension in the right hind limb. Each mouse was randomly assigned to one of 8 groups (\(n = 6 - 8\) mice per group) receiving a different treatment. Tumor growth of the subcutaneous model was evaluated with caliper measurements from the moment the tumors became palpable and after this three times a week before treatment and daily during treatment. The tumor volume was calculated as \(V = 0.5 \times ab^2\), with \(a\) and \(b\) being the long and short axes of the tumor, respectively. Treatment was administered during 10 days, starting 19 days after tumor inoculation when tumors had reached a volume of approximately 300 - 400 mm\(^3\). The mice of the negative control group received 200 µl water by oral gavage (p.o.) daily. The positive
control group was given 3 times/week gemcitabine (Actavis, 38 mg/ml), administered intraperitoneally at a dose of 60 mg/kg body weight (BW). GS and GS2B were administered p.o. daily at a dose of 0.3, 1 and 3 mg/kg BW total content of colchicine and derivatives expressed as colchicine. These doses corresponded to 5.2, 17.2 and 51.7 mg/kg BW GS and 18.8, 62.5 and 187.5 mg/kg BW GS2B, respectively. The animals were sacrificed at day 11. The percentage of tumor growth inhibition (%TGI) was calculated as:

\[
\%TG = 1 - \frac{(RTV_x - 1)}{RTV_c - 1}
\]

with RTV\textsubscript{X} being the relative tumor volume from the treated group and RTV\textsubscript{C} the relative tumor volume from the control group. \textit{In vivo} response was defined as described by Plowman et al.\textsuperscript{21}: %TGI > 85\% = high response; > 55\% and < 85\% = intermediate response; < 55\% = low response. Body weights were determined three times weekly before treatment, and daily during treatment. From each of the eight treatment cohorts, a subgroup (n = 3) underwent a baseline \textsuperscript{18}F-FDG PET/CT scan (before start of the treatment, D0) and one follow-up scan at 7 days into the treatment.

3. Data acquisition and analysis

3.1 PET/CT Imaging

\textsuperscript{18}F-FDG was produced at the Antwerp University Hospital's Department of Radiopharmacy in an automated module using a FASTlab FDG Cassette (GE Healthcare, Diegen, Belgium). Small animal PET imaging was performed using two Siemens Inveon PET-CT scanners (Siemens Preclinical Solution, Knoxville, TN). The acquired PET data was reconstructed using 4 iterations with 16 subsets of the 2D ordered subset expectation maximization (OSEM 2D) algorithm following Fourier rebinning. Normalization, dead time, random, CT-based attenuation and single-scatter
stimulation (SSS) scatter corrections are applied. CT voltage and amperage are set to 80 keV and 500 µA, respectively. Mice were fasted overnight (12 - 18 hours) and weighed before the start of each scan. Animals first received awake an intravenous (iv.) bolus injection of $^{18}$F-FDG (37 MBq) in the left lateral tail vein. This is immediately followed by a double measurement (Easy-Touch, Lifescan, France) of pre-scan whole blood glucose levels from a drop of blood from the contralateral tail vein. The average value was taken into account to correct for blood glucose levels. Twenty-five minutes after the $^{18}$F-FDG injection (= uptake period), mice were anesthetized with isoflurane (5% for induction and 2% for maintenance) and five minutes after the start of the anesthesia mice were prepared and positioned onto the PET scanner resulting in a total uptake period of 30 min. An overview of the scanning protocol is given in Fig. 3b. Sequentially to the PET scan, a 7 min CT scan was performed for attenuation as well as scatter correction purposes and for anatomical localization of the tumors. Regions of interest (ROIs) were drawn on the CT images manually by qualitative assessment covering the whole tumors slice-by-slice and subsequently tumor volume and tracer uptake was generated by summation of voxels within the tomographic planes using PMOD Software 3.3 (PMOD technologies, Zurich, Switzerland) and expressed by mean standardized uptake values (SUV). SUV was thereby defined as $(TC*BW)/D_{inj}$, where TC is radioactivity in the tissue (kBq/cc), BW is body weight of the animal (g) and $D_{inj}$ is injected dose (kBq). SUV$_{mean}$ represents the mean concentration of the tracer and was calculated with correction for the glucose (Glc) level: $(TC*BW*Glc)/D_{inj}$. SUV$_{mean}$ ratio was calculated as SUV$_{mean}$ at D7 over SUV$_{mean}$ at baseline (D0) for each animal.

### 3.2 Ex vivo validation by histology
Tumors were harvested on day 11 and fixed in formaldehyde. Tumors were paraffin-embedded and immunohistochemical (IHC) staining was performed on adjacent 3 - 4 µm tumor slices that were mounted on SuperFrost® microscope slides (Menzel-Glaser). A detailed description of the IHC protocol is given in Supporting Information (S1 IHC protocol). For the quantification of degree of apoptosis and proliferation, ten regions of interest (ROI) were then chosen at random per slice at a magnification of 200x. High necrotic regions were excluded for analysis. Per ROI the percentage of Ki-67 positive cells was determined and the mean of ten ROIs was the overall percentage of that slice. This percentage indicated the degree of proliferation. For the caspase-3 staining, the number of apoptotic bodies, which was scored between 1 to 5, with 1 being no to almost no presence of apoptotic bodies, was determined per ROI. Again the mean of ten ROIs was used to determine the degree of apoptosis. Per treatment group a mean score was calculated and this value was used for further analysis. Percentage of positively stained Ki-67 nuclei of tumor cells was counted for each tumor slide (containing the whole slide) by two independent observers (including one certified pathologist). To evaluate the number of caspase-3 positive (apoptotic) bodies present in each tumor specimen the following scoring system was used: score 0 (no caspase-3 staining), score 1 (1 - 5% caspase-3 staining), score 2 (6 - 10%), score 3 (11 - 30%), score 4 (31 - 50%) and score 5 (60% or higher).

3.3 Statistical analysis

Results were expressed as mean values of parameters ± standard error of the mean (SEM). To control if data were normally distributed, a Shapiro-Wilk normality test was applied. The parametric ANOVA test was used to determine statistical significance, followed by a post hoc Tukey analysis to establish the statistical
difference between the treatment groups. Non-parametric Kruskal-Wallis tests were used to determine statistical difference in degree of proliferation and apoptosis between groups, followed by a post hoc Dunn’s test, for multiple comparisons. Differences in SUVmean ratio were evaluated by one-way ANOVA followed by an uncorrected Fishers’ LSD test. To evaluate the correlation between the relative tumor volumes and the IHC scores, a non-parametric Spearman’s correlation assay was used. A p-value ≤ 0.05 was considered significant and statistical analysis was performed using GraphPad Prism 6 (Version 6.01).

Results

1. In vitro cytotoxicity

Fig. 4a shows that the calculated IC_{50} values for GS on the PANC-1 cells were 0.45 µg/ml (24 h) and 0.59 µg/ml (72 h), and on the PANC02 cells 0.17 µg/ml (24 h) and 0.19 µg/ml (72 h), respectively. The *G. superba* extract showed cytotoxic activity in a dose-dependent manner. Thus, it could be concluded that GS was highly cytotoxic on the two pancreatic cell lines. Fig. 4b demonstrates an IC_{50} value of 9.49 µg/ml for GS2B when incubated for 24 h on PANC02 cells, which is hundred times larger than the value observed for colchicine itself, *i.e.* 0.098 µg/ml and fifty-six times larger as for GS.

2. Acute toxicity test and effect of therapy on body weight of mice

In an acute toxicity study in healthy non-tumor-bearing animals prior to the experiment, no symptoms and no significant decreases in body weight were observed during the treatment. Also, three days after the treatment, body weights were still stable (Fig. S1.) and still none of the mice showed any clinical symptoms of toxicity.
3. Effect of therapy on PANC02 tumor growth and $^{18}$F-FDG uptake

In none of the different treatment groups significant ($p = 0.15$) differences in body weight could be observed after 10 days of therapy or placebo administration (Fig. S2), confirming that there is a tolerable toxicity on the normal tissue. No clinical signs of toxicity were observed during the entire experiment. One mouse of the control group was sacrificed due to too large tumor burden.

By eighteen days after PANC02 cells inoculation, all mice developed subcutaneous tumors in the right hind limb with a mean tumor volume of 300 - 400 mm³ on the day before treatment (day 0) and no statistical difference ($p = 0.31$) between the groups was observed at the start of treatment. The evolution of the mean relative tumor volumes (RTVs) for each group and for each day are shown in Fig. 5, while the percentages of tumor growth inhibition (%TGI) on day 7 and 10 are summarized in Table 1. After 7 days of treatment a TGI of 90% ($p = 0.0019$) was achieved by gemcitabine treatment. A dose-effect relationship was observed for GS and GS2B. Treatment with low, medium and high dose of GS resulted in 78% (intermediate response, $p = 0.0199$), 86% (high response, $p = 0.0040$) and 101% (high response, $p \leq 0.0001$) TGI, respectively. Treatment with low, medium and high GS2B led to TGI of 74% (intermediate response, $p = 0.0369$), 81% (intermediate response, $p = 0.0107$) and 93% (high response, $p = 0.0006$), respectively. Fig. 5 shows that on day 7, the RTVs of all treatment groups were significantly smaller compared to the control group. On day 10, significantly smaller RTVs were still observed for two out of three doses of both extracts compared to the control group. Table 1 also shows that on day 10 the gemcitabine-treated PANC02-bearing mice still had a high response with a TGI of 99% ($p \leq 0.0001$), while mice treated with high GS and GS2B showed TGI of...
74% (p ≤ 0.01) and 63% (p ≤ 0.05) respectively, indicating an intermediate response.

An intermediate response was also observed in the low GS and GS2B–treated groups with 58% (p ≤ 0.05) and 60% TGI (p ≤ 0.05), respectively. TGI values above 50% are considered relevant.\textsuperscript{22,23}

All treatment groups showed a clear $^{18}$F-FDG uptake (SUV\text{mean} = 0.88 ± 0.06) with no significant difference between groups at baseline before treatment (p = 0.78). After 7 days of treatment, overall $^{18}$F-FDG SUV\text{mean} ratios were decreased in the treated groups compared to the control group, demonstrating a decrease in metabolic activity (Fig. 6). A significant decrease in metabolic activity was observed for the gemcitabine group (p = 0.0018), the low, medium and high dosed GS group (p = 0.018, p = 0.030 and p = 0.0067, respectively). For the GS2B group, only the medium dosed group reached statistical significance (p = 0.0035). No correlation was found between SUV\text{mean} changes and RTV after 7 days of treatment ($r = 0.02476$, p = 0.2476).

Representative PET/CT images are shown in Fig. 7.

4. \textit{Ex vivo} histological analysis and correlation with tumor growth

H&E staining end-of-life (EOL) after 10 days of treatment revealed a highly proliferative, undifferentiated tumor, which in some mice infiltrated into the striated muscle tissue of the hind leg. In some larger tumors, necrotic regions were clearly visible. Tumors were well vascularized and nests of tumor cells were embedded in a desmoplastic stroma. Ki-67 IHC staining of the control tumors confirmed the highly proliferating character observed with H&E staining, more than 70% of the tumor cells were stained positively for Ki-67. The result of the Ki-67 staining for the different treatment groups is shown in Fig. 8a. A significant decrease in the number of Ki-67 positive tumor cells was observed in the gemcitabine, high GS and high GS2B-treated
group compared to control (p = 0.0217, p= 0.0006, p = 0.0034, respectively). A positive, high and significant correlation (r = 0.7381, p = 0.0458) was found between mean Ki-67 IHC scores and mean RTV (Fig. 8b). In addition, a significant correlation was found between SUVmean changes at D7 and Ki-67 scores EOL (r = 0.4691, p = 0.0319).

The result of the cleaved caspase-3 staining is shown in Fig. 8c. The control tumors have a low basal tumor cell apoptosis (< 5%), while a significantly increased caspase-3 staining was present in the group treated with high GS (p = 0.0072). Treatment with gemcitabine (p = 0.08) and high GS2B (p = 0.18) trended to higher values and induced caspase-3 in the tumors. A negative, high and significant correlation (r = -0.8073, p = 0.0181) was found between mean caspase-3 IHC scores and mean RTV (Fig. 8d). Representative images of Ki-67 and caspase-3 IHC performed on PANC02 tumors are illustrated in Fig. 9.

**Discussion**

The *in vitro* cytotoxicity of GS is mainly due to its main constituent colchicine. The extract also contains potential prodrugs such as colchicoside. Colchicoside can be converted by β-glucosidase activity of the microflora in the gastrointestinal tract to the cytotoxic aglycone 3-O-demethylcolchicine, one of the genuine minor constituents of GS. Although not expected to show a pronounced *in vitro* cytotoxicity, colchicoside may contribute to the desired effect *in vivo* after oral administration. An *in vitro* IC₅₀ value for GS2B was observed which is hundred times larger than the value observed for colchicine itself and fifty-six times larger as for GS. The remaining cytotoxicity of GS2B is mainly due to residual colchicine and 3-O-demethylcolchicine. To verify the hypothesis that a combination of various
(potentially synergistic) active compounds in an herbal extract, including prodrugs such as glycosides, may have more beneficial effects and a better toxicity profile (because of a slow-release effect) than pure compounds. An in vivo study using a PANC02 murine model of pancreatic cancer was performed. These extracts were evaluated against pancreatic cancer in this study but it may also be usable against other types of cancers due to the non-specific mechanism of action of both extracts.

No toxic side effects or manifest weight loss were noticed during the daily-treatment regime of 10 days; therefore the hypothesis of a potentially reduced systemic toxicity could not be verified. Both GS and GS2B were tested for their in vivo efficacy in a murine pancreatic cancer model, on established PANC02 tumors. A clear dose-response (low < medium < high) reduction in tumor growth inhibition was observed after 7 days of treatment with both extracts. The highest in vivo response to treatment (high GS and GS2B) was comparable with the tumor growth inhibition caused by gemcitabine treatment being the conventional therapy and thus the positive control group in this study. However, after ten days of treatment, only a low (medium dose) to intermediate tumor response (high and low doses) was observed for both herbal extracts. A clear dose-response could therefore no longer be concluded as the tumor volumes in some of these groups treated with the extracts slightly increased towards the end of the experiment. In contrast, gemcitabine treated mice maintained a high and statistically significant response to the treatment after ten days of therapy. A possible explanation may be that the highest dose of herbal extracts included in this study (3 mg/kg) is potentially suboptimal. Nevertheless these data clearly indicate an anticancer effect of the two herbal extracts. Because the high need for new, less toxic
treatments for pancreatic cancer patients, it would also be worthwhile to investigate the combination of the herbal extracts with gemcitabine for synergistic effects.

In the present study, all tumors were microscopically examined to investigate which cellular processes are responsible for tumor growth inhibition. IHC demonstrated clear effects on tumor cell proliferation and tumor cell apoptosis of both extracts. The highest inhibition of tumor cell proliferation was observed with gemcitabine, high GS and high GS2B-treated animals. Inhibition of tumor cell proliferation by gemcitabine has been demonstrated in pancreatic tumor mouse models\textsuperscript{24, 25}, however the main mechanism of action of gemcitabine remains induction of cell death. In the gemcitabine-treated tumors, indeed an increase in caspase-3 positive fragments was observed indicating an increase in apoptotic tumor cells. Also the highest dose of the two tested herbal extracts induced apoptosis, for GS even highly significant. Indeed colchicine and its analogs inhibit microtubule polymerization by binding to tubulin and thus inhibiting cell mitosis and inducing cell death. The main effects were clearly visible in the two groups receiving the highest dose of herbal extract (3 mg/kg), suggesting that further experiments with higher doses of the extracts are recommended.

A large reduction in \textsuperscript{18}F-FDG uptake was observed after 7 days for all treated groups, compared to control animals. These decreases in tumoral metabolic activity were correlated with Ki-67 scores (measured at the end of the experiment), which demonstrate the feasibility of using \textsuperscript{18}F-FDG PET imaging as an early biomarker for monitoring the applied treatments. However, after 7 days of treatment no clear correlation was found between these SUVmean changes and the reduction in tumor size for the different treatment groups. Firstly, there is extensive necrosis present in
some tumors due to the rapid growth and thus large size of the tumors at the end of the experiments. For response evaluation by caliper measurements tumors need to be measured as a whole, including necrotic regions, while only viable tumor cells take up $^{18}$F-FDG by facilitated transport. Secondly, it is not known which effect these herbal extracts exert on the complex glucose regulation in tumor cells and perhaps these herbal extracts may not have a homogenous impact on the metabolic rate of the PANC02 tumors. Thirdly, as this tumor model is an allograft model, an intact immune system is present in the host mouse. It is well known that administration of chemotherapeutic agents leads to apoptosis and/or necrosis of tumor cells, provoking an inflammatory response of the host that also aspecifically leads to an increase in $^{18}$F-FDG uptake.$^{26-28}$

Molecular imaging techniques like PET/CT may provide evidence of biological responses of novel anticancer compounds including herbal extracts, which can facilitate the transition of compounds from the preclinical to a clinical phase. An alternative to $^{18}$F-FDG as early response imaging biomarker could be $^{18}$F-fluorothymide ($^{18}$F-FLT). FLT is used for imaging tumor cell proliferation with PET and is trapped intracellular where its phosphorylation is mediated through thymidine kinase 1.$^{29,30}$ In our model, high basal proliferation and inhibition of tumor cell proliferation by the two herbal extracts was observed, making our model suitable for the early response detection with $^{18}$F-FLT PET. $^{18}$F-FLT PET has been demonstrated to be superior for early response prediction in some tumor models.$^{31}$

**Conclusion**

All of these results led to the conclusion that GS and GS2B in a 3 mg/kg dose of total content of colchicine and derivatives expressed as colchicine showed promising
anticancer activities in a murine pancreatic adenocarcinoma model, with relevant
tumor growth inhibition, a decrease in proliferation and increase in apoptosis. $^{18}$F-
FDG PET was able to non-invasively and longitudinally detect and monitor this
response. No toxic side effects or extreme weight loss were noticed during the daily-
treatment regime of 10 days. Since the colchicoside-rich / colchicine-poor extract
GS2B was also found to be active in vivo, in spite of the lack of in vitro cytotoxicity,
our results seem to support the hypothesis that colchicoside acts as a prodrug that is
activated after oral administration.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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fellowship for An Wouters and by the Agentschap voor Innovatie door Wetenschap en Techniek (IWT) through a PhD position for Rica Capistrano. Part of the work was funded by AntiCancer Fund (Belgium).

References


## Tables

Table 1. Treatment response in PANC02 allograft (No / Intermediate (Int.) / High)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%TGI</th>
<th>p-value</th>
<th>Response</th>
<th>%TGI</th>
<th>p-value</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>x</td>
<td>No</td>
<td>0</td>
<td>x</td>
<td>No</td>
</tr>
<tr>
<td>Gemcitabine (60 mg/kg)</td>
<td>90</td>
<td>0.0019</td>
<td>High</td>
<td>99</td>
<td>0.0001</td>
<td>High</td>
</tr>
<tr>
<td>GS (0.3 mg/kg)</td>
<td>78</td>
<td>0.0199</td>
<td>Int.</td>
<td>58</td>
<td>0.0419</td>
<td>Int.</td>
</tr>
<tr>
<td>GS (1.0 mg/kg)</td>
<td>86</td>
<td>0.0040</td>
<td>High</td>
<td>46</td>
<td>0.1430</td>
<td>Low</td>
</tr>
<tr>
<td>GS (3.0 mg/kg)</td>
<td>101</td>
<td>&lt;0.0001</td>
<td>High</td>
<td>74</td>
<td>0.0025</td>
<td>Int.</td>
</tr>
<tr>
<td>GS2B (0.3 mg/kg)</td>
<td>74</td>
<td>0.0369</td>
<td>Int.</td>
<td>60</td>
<td>0.0333</td>
<td>Int.</td>
</tr>
<tr>
<td>GS2B (1.0 mg/kg)</td>
<td>81</td>
<td>0.0107</td>
<td>Int.</td>
<td>30</td>
<td>0.5347</td>
<td>Low</td>
</tr>
<tr>
<td>GS2B (3.0 mg/kg)</td>
<td>93</td>
<td>0.0006</td>
<td>High</td>
<td>63</td>
<td>0.0160</td>
<td>Int.</td>
</tr>
</tbody>
</table>
Figure Legends

Fig. 1. Structure of colchicoside, 3-O-demethylcolchicine and colchicine.

Fig. 2. Preparation of the colchicine-poor extract. Liquid-liquid extraction scheme for the preparation of the colchicine-poor extract (GS2B).

Fig. 3. Study design: Experimental schedule. An overview of (a) general study design with low = dose of 0.3 mg/kg, medium = dose of 1 mg/kg and high = dose of 3 mg/kg. GS = G. superba crude extract and GS2B = colchicine-poor / colchicoside-rich extract. (b) The imaging time schedule. PreWBglc = pre-scan whole blood glucose levels.

Fig 4. a) In vitro dose-response curves for GS. Dose-response curves of PANC-1 and PANC02 cell lines treated with G. superba crude extract (GS) at two different incubation times, i.e. 24 h and 72 h as determined by the SRB assay. b) In vitro dose-response curves for GS2B and colchicine. Dose-response curves of PANC02 cell line treated with colchicine-poor extract (GS2B) and colchicine (COL) with an incubation time of 24 h as determined by the SRB assay.

Fig. 5. Mean relative tumor volume during the whole experiment. Effect of the treatments on established PANC02 tumor growth. Data are presented as mean ± SEM. GS = G. superba crude extract; GS2B = colchicine-poor extract.

Fig. 6. Uptake of $^{18}$F-FDG (SUVmean) in PANC02 tumors after treatment relative to baseline. Statistical significant differences from control (ANOVA,
followed by an uncorrected Fishers’s LSD) are indicated with an asterisk; * p ≤ 0.05 and ** p ≤ 0.01. Data are presented as mean ± SEM.

Fig. 7. Representative $^{18}\text{F}$-FDG PET/CT images at baseline and after 1 week of treatment. Transversal, sagittal and coronal slices are shown, with white arrows indicating the tumor. (a) One mouse of the control group, (b) one mouse of the gemcitabine group (60 mg/kg), (c) one mouse of the GS (3 mg/kg) group and (d) one mouse of the GS2B (1 mg/kg) group. All images scaled to the same color scale (SUV scale from 0 – 4.)

Fig. 8. Tumor cell proliferation determined by Ki-67-staining and tumor cell apoptosis determined by cleaved caspase-3 staining after 10 days of treatment. (a) The percentage of Ki-67 positive stained cells compared to control group. (b) Correlation between Ki-67 IHC scores and RTV. Statistical significant differences from control (Kruskal-Wallis, followed by post hoc Dunn) are indicated with an asterisk; * p ≤ 0.05, ** p ≤ 0.01. A Spearman’s correlation assay was used to determine correlation between Ki-67 IHC scores and RTV. Data are presented as mean ± SEM. (c) Cleaved caspase-3 scoring with score 0 (no caspase-3 staining), score 1 (1 - 5% caspase-3 staining), score 2 (6 - 10%), score 3 (11 - 30%), score 4 (31 - 50%) and score 5 (60% or higher). (d) Correlation between cleaved caspase-3 IHC scores and RTV. Statistical significant difference from control (Kruskal-Wallis, followed by post hoc Dunn) is indicated with an asterisk; ** p ≤ 0.01. A Spearman’s correlation assay was used to determine correlation between Ki-67 IHC scores and RTV. Data are presented as mean ± SEM.
Fig. 9. Representative images of Ki-67 and cleaved caspase-3 IHC performed on PANC02 tumors. (a - c) show Ki-67 staining, with brown nuclei being Ki-67 positive. a) represents a control (= untreated) PANC02 tumor, (b) medium GS-treated PANC02 tumor and (c) a high GS-treated PANC02 tumor. (d - f) show cleaved-caspase-3 IHC of the PANC02 tumors, with white arrows indicating brown-stained apoptotic fragments. (d) represents a control PANC02 tumor, (e) represent gemcitabine-treated PANC02 tumor with diffuse apoptotic bodies present in the tumor and (f) represents a high GS-treated PANC02 tumor with a mix of necrotic and apoptotic cells.
Fig. 1. Structure of colchicoside, 3-O-demethylcolchicine and colchicine.

99x25mm (300 x 300 DPI)
Fig. 2. Preparation of the colchicine-poor extract. Liquid-liquid extraction scheme for the preparation of the colchicine-poor extract (GS2B).

80x43mm (300 x 300 DPI)
Fig. 3. Study design: Experimental schedule. An overview of (a) general study design with low = dose of 0.3 mg/kg, medium = dose of 1 mg/kg and high = dose of 3 mg/kg. GS = G. superba crude extract and GS2B = colchicine-poor / colchicoside-rich extract. (b) The imaging time schedule. PreWBglc = pre-scan whole blood glucose levels.

99x118mm (300 x 300 DPI)
Fig 4. a) In vitro dose-response curves for GS. Dose-response curves of PANC-1 and PANC02 cell lines treated with G. superba crude extract (GS) at two different incubation times, i.e. 24 h and 72 h as determined by the SRB assay. b) In vitro dose-response curves for GS2B and colchicine. Dose-response curves of PANC02 cell line treated with colchicine-poor extract (GS2B) and colchicine (COL) with an incubation time of 24 h as determined by the SRB assay.
Fig. 5. Mean relative tumor volume during the whole experiment. Effect of the treatments on established PANC02 tumor growth. Data are presented as mean ± SEM. GS = G. superba crude extract; GS2B = colchicine-poor extract.

119x119mm (300 x 300 DPI)
Fig. 6. Uptake of 18F-FDG (SUVmean) in PANC02 tumors after treatment relative to baseline. Statistical significant differences from control (ANOVA, followed by an uncorrected Fishers’s LSD) are indicated with an asterisk; * p ≤ 0.05 and ** p ≤ 0.01. Data are presented as mean ± SEM.

94x108mm (300 x 300 DPI)
Fig. 7. Representative 18F-FDG PET/CT images at baseline and after 1 week of treatment. Transversal, sagittal and coronal slices are shown, with white arrows indicating the tumor. (a) One mouse of the control group, (b) one mouse of the gemcitabine group (60 mg/kg), (c) one mouse of the GS (3 mg/kg) group and (d) one mouse of the GS2B (1 mg/kg) group. All images scaled to the same color scale (SUV scale from 0 – 4.)

199x161mm (300 x 300 DPI)
Fig. 8. Tumor cell proliferation determined by Ki-67-staining and tumor cell apoptosis determined by cleaved caspase-3 staining after 10 days of treatment. (a) The percentage of Ki-67 positive stained cells compared to control group. (b) Correlation between Ki-67 IHC scores and RTV. Statistical significant differences from control (Kruskal-Wallis, followed by post hoc Dunn) are indicated with an asterisk; * p ≤ 0.05, ** p ≤ 0.01. A Spearman’s correlation assay was used to determine correlation between Ki-67 IHC scores and RTV. Data are presented as mean ± SEM. (c) Cleaved caspase-3 scoring with score 0 (no caspase-3 staining), score 1 (1 - 5% caspase-3 staining), score 2 (6 - 10%), score 3 (11 - 30%), score 4 (31 - 50%) and score 5 (60% or higher). (d) Correlation between cleaved caspase-3 IHC scores and RTV. Statistical significant difference from control (Kruskal-Wallis, followed by post hoc Dunn) is indicated with an asterisk; ** p ≤ 0.01. A Spearman’s correlation assay was used to determine correlation between Ki-67 IHC scores and RTV. Data are presented as mean ± SEM.

181x190mm (300 x 300 DPI)
Fig. 9. Representative images of Ki-67 and cleaved caspase-3 IHC performed on PANC02 tumors. (a - c) show Ki-67 staining, with brown nuclei being Ki-67 positive.  a) represents a control (= untreated) PANC02 tumor, (b) medium GS-treated PANC02 tumor and (c) a high GS-treated PANC02 tumor. (d - f) show cleaved-caspase-3 IHC of the PANC02 tumors, with white arrows indicating brown-stained apoptotic fragments. (d) represents a control PANC02 tumor, (e) represent gemcitabine-treated PANC02 tumor with diffuse apoptotic bodies present in the tumor and (f) represents a high GS-treated PANC02 tumor with a mix of necrotic and apoptotic cells.

199x150mm (300 x 300 DPI)
Supporting Information

**Fig S1. Body weights of three healthy mice during the acute toxicity experiment.**

Three healthy C57BL/6 mice were treated with 3 mg/kg of the crude herbal extract (GS) by oral gavage daily for 5 days, starting at day 1. Body weight was measured daily during treatment and for another 3 days thereafter.

**Fig S2. Mean body weight during the entire experiment.**

Fifty-five C57BL/6 mice were inoculated with PANC02 and body weight was measured 3/week. Mice were then treated for 10 days with their respective treatments and body weight was measured daily.

**S1 IHC protocol**

Before staining, the slices were deparaffinized by washing 3 times with xylene for 5 min, two times with 100% ethanol for 10 min, and finally two times with 95% ethanol for 10 min. For general morphology and presence of necrosis, Mayer hematoxylin and eosin (H&E) staining was performed. Additionally, immunostaining of caspase-3 (rabbit polyclonal antibody targeting human/mouse Asp175 of activated caspase-3, Cell Signaling Technology, 9661S) and Ki-67 D3B5 (rabbit monoclonal antibody targeting murine Ki-67, Cell Signaling Technology, 12202) were performed according to the manufacturer’s instruction. For cleaved caspase-3 and Ki-67 staining, heat induced antigen unmasking was performed for 10 or 20 min in citrate buffer (pH 6.0), respectively, after which the tissue slices were cooled down for 30 min. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. The slices were then washed two times in deionized water and one time in washing buffer, which is a tris buffered saline with Tween® 20 solution (TBST) for 5 min.
each. After the washing steps, caspase-3 slices were blocked with blocking solution (TBST/5% normal goat serum) for 1 h at room temperature. The blocking solution was then removed, cleaved caspase-3 primary antibody or mouse specific Ki-67 primary antibody (1/400 dilution for Ki-67 and 1/300 for caspase-3 in TBST/5% normal goat serum) was added and the slices were incubated overnight at 4 °C. After washing the slices with the buffer three times for 5 min, the slices were incubated for 30 min at room temperature with SignalStain® Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology). Color was developed using the chromogen 3,3’-diaminobenzidine (DAB) (DakoCytomation) for 10 min. After washing, the tissue slices were counterstained with Mayer’s hematoxylin. TBST/5% normal goat serum instead of the primary antibody was used as negative control in order to exclude false positive responses from non-specific binding of the secondary antibody.
**S1 Table.** Selected clinical observations used in cancer research and toxicological studies.

<table>
<thead>
<tr>
<th>Parameter:</th>
<th>What to look for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>General appearance</td>
<td>Dehydration, decreased body weight, missing anatomy, abnormal posture, hypothermia, fractured appendage, swelling, tissue masses, prolapse, paraphimosis</td>
</tr>
<tr>
<td>Skin and fur</td>
<td>Discoloration, urine stain, pallor, redness, cyanosis, icterus, wound, sore, abscess, ulcer, alopecia, ruffled fur</td>
</tr>
<tr>
<td>Eyes</td>
<td>Exophthalmos, microphthalmia, ptosis, reddened eye, lacrimation, discharge, opacity</td>
</tr>
<tr>
<td>Nose, mouth, and head</td>
<td>Head tilted, nasal discharge, malocclusion, salivation</td>
</tr>
<tr>
<td>Respiration</td>
<td>Sneezing, dyspnea, tachypnea, rales</td>
</tr>
<tr>
<td>Urine</td>
<td>Discoloration, blood in urine, polyuria, anuria</td>
</tr>
<tr>
<td>Feces</td>
<td>Discoloration, blood in the feces, softness/diarrhea</td>
</tr>
<tr>
<td>Locomotor</td>
<td>Hyperactivity, coma, ataxia, circling, muscle tremors</td>
</tr>
</tbody>
</table>
Body weight

- C57BL/6 1
- C57BL/6 2
- C57BL/6 3

Weight (g)

Day

1 2 3 4 5 8

22 23 24 25 26

105x72mm (300 x 300 DPI)