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Coadministration of a *Gloriosa superba* extract improves the *in vivo* antitumoural activity of gemcitabine in a murine pancreatic tumour model

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Abstract

Background: Gloriosa superba L. (glory lily, Colchicaceae) contains colchicine, and related alkaloids such as 3-*O*-demethylcolchicine and its glycoside colchicoside. Previously the *in vivo* efficacy of a crude extract and a colchicine-poor / colchicoside-rich extract of *G. superba* seeds was shown in a murine model of pancreatic adenocarcinoma.

Hypothesis/Purpose: The efficacy can be improved without obvious signs of toxicity by increasing the treatment dose; the efficacy of gemcitabine can be improved by coadministration of a *Gloriosa superba* extract.

Study Design: A survival experiment was carried out in a murine model of pancreatic adenocarcinoma and the semi-long-term toxicity of both *G. superba* extracts was determined; a combination therapy with gemcitabine was evaluated.

Methods: A crude ethanolic extract (GS) and a colchicine-poor / colchicoside-rich extract (GS2B) were prepared, containing 3.22% colchicine, 2.52% colchicoside and 1.52% 3-*O*-demethylcolchicine (GS), and 0.07%, 2.26% and 0.46% (m/m) (GS2B). They were evaluated in a murine model of pancreatic adenocarcinoma at a dose of 4.5 mg/kg (p.o., daily) total content of colchicine and derivatives during 3 weeks, or at 3.0 mg/kg (p.o., daily) combined with gemcitabine (60 mg/kg, i.p., 3x/week) during 54 days.

Results: A significant effect in tumour growth over time was observed for gemcitabine and the combination therapy compared to the control group. No significant difference was observed for the groups treated with colchicine and both extracts. However, combination therapy was significantly better than the monotherapy with gemcitabine. Moreover, survival analysis showed a significant prolongation of the survival of the groups treated with gemcitabine and the combination therapy. A slight difference in survival was observed between gemcitabine and the combination therapy, the latter one being slightly better. No significant prolongation of survival was observed for the extracts and colchicine compared to the control group.

Conclusion: Although a relevant tumour growth inhibition and a difference of relative tumour volume compared to the control group were observed on day 11, and a slightly longer survival was noticed for GS2B, the most important conclusion from this study is that the crude *G. superba* extract (GS) might have an added value combined with gemcitabine in the treatment of pancreatic tumours.

Keywords

Gloriosa superba; Colchicaceae; pancreatic tumours; gemcitabine; combination therapy

Abbreviations

BW	Body weight
GS	Crude ethanolic extract of *Gloriosa superba*
GS2B	Colchicine-poor / colchicoside-rich extract of *Gloriosa superba*
RTV	Relative tumour volume
TC	Total content of colchicine and derivatives (expressed as colchicine)
TGD	Tumour growth delay
TGI	Tumour growth inhibition

Introduction

Gloriosa superba L. (glory lily), belonging to the Colchicaceae, has traditionally been used for various diseases and is widely cultivated as an ornamental plant. It contains colchicine, which is an inhibitor of tubulin polymerisation used against gout, and related alkaloids such as 3-*O*-demethylcolchicine and its glycoside colchicoside (Jana et al., 2011; Maroyi et al., 2011; Risinger et al., 2009). The use of pure colchicine as an anticancer agent has been excluded for toxicity reasons (Stanton et al., 2011; Yue et al., 2010), although more recently this has been questioned again (Slobodnick et al., 2015; Solak et al., 2015). However, it is known that plant extracts may display a better activity profile than the individual constituents (Wagner et al., 2009). *Gloriosa superba* was selected for the present study because it is rich in colchicoside, the glucosidic derivative of 3-O-demethyl-colchicine, being the active aglycone. In a previous study the *in vivo* efficacy of a crude extract and a colchicine-poor / colchicoside-rich extract of *G. superba* seeds was shown in a murine model of pancreatic adenocarcinoma, supporting the hypothesis that colchicoside indeed can be considered as a prodrug that is activated after oral administration (Capistrano et al., 2016). Among all cancers, pancreatic cancer has an extremely poor prognosis and is one of the most deadly types. Most pancreatic cancer patients die within the first year of diagnosis and only 6% will survive five years. The lack of progress in primary prevention, early diagnosis and treatment, underscores the need for new approaches in pancreatic cancer research (American Cancer Society, 2013; Cascinu et al., 2009; Ducreux et al., 2015; Li et al., 2004). Mice having a fully competent immune response were used, since the immune system can play a potentially beneficial role during therapy. This implies the use of mouse-strain specific tumour models (syngeneic tumours), i.e. the application of murine PANC02 cancer cells rather than human pancreatic cancer (PANC-1) cells (Campbell et al., 2014). In the present study a survival experiment was carried out and the semi-long-term toxicity of both the crude extract and a colchicine-poor / colchicoside-rich extract of *G. superba* seeds was determined. A combination therapy with gemcitabine was also evaluated to establish whether this combination had an added value to monotherapy with gemcitabine, a nucleoside analogue that is the first-line treatment in patients with locally advanced pancreatic cancer (Ducreux et al., 2015). However, even gemcitabine alone or in combination with other chemotherapeutics such as cisplatin or 5-fluorouracil or radiotherapy only has a low objective response and a low survival benefit (Arslan et al., 2014).

Materials and methods

Plant material and preparation of the extracts

Dried seeds of *Gloriosa superba* L. were kindly provided by Indena® (Milano, Italy) (batch n° C140020, certificate of analysis n° 11/0208/LSP). A crude ethanolic extract (GS) and a colchicine-

poor / colchicoside-rich extract (GS2B) was prepared as reported before, and in both extracts the amount of colchicine, colchicoside and 3-*O*-demethylcolchicine was determined using a validated HPLC method (Capistrano et al., 2015). GS contained 3.22% colchicine, 1.30% colchicoside expressed as colchicine and 1.27% 3-*O*-demethylcolchicine (all m/m) expressed as colchicine, corresponding to a total content of colchicine equivalents (TC) of 5.79%. Similarly, GS2B contained 0.07%, 1.16% and 0.38% (m/m), respectively, corresponding to a total content of colchicine equivalents (TC) (expressed as colchicine) of 1.61%. The amount of colchicine in GS2B was less than 0.1% (m/m).

Cell line

Murine pancreatic adenocarcinoma cells (PANC02) were kindly provided by Prof. Dr. C. Gravekamp (Albert Einstein College of Medicine, New York, USA) and cultured as previously described (Capistrano et al., 2016).

Animals

Six-to-eight weeks old female C57BL/6 mice (16 - 20 g body weight (BW)) were purchased from Harlan laboratories. The mice were housed in individually ventilated cages, under a 10/14 h dark/light cycle at constant temperature and humidity and 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. All mice experiments were approved by the local Ethical Committee of the University of Antwerp, approved study number: 2014-34.

Subcutaneous tumour model

Cultures of PANC02 cells were harvested using a 0.05% trypsin-EDTA solution, washed twice in sterile PBS and resuspended in sterile PBS at a concentration of 30 x 10^6 viable cells per ml. The viable cells were counted by using the Muse® Cell Analyzer. Mice (n = 66) were inoculated with 100 µL of the cell suspension in the right hind limb. Tumour growth of the subcutaneous model was evaluated by means of calliper measurements from the moment the tumours became palpable and this three times a week. The tumour volume was calculated as in the previous study (Capistrano et al., 2016). Each mouse was randomly assigned to one of 6 groups (n = 11 mice per group) and assigned to a different treatment (Table 1).

Preliminary acute toxicity study

Before the actual survival experiment an acute toxicity study was carried out to evaluate the acute toxic effects of a dose of 4.5 mg/kg BW total colchicine and derivatives (TC). Non-tumour-bearing C57BL/6 mice (n = 3 per treatment group) were given 4.5 mg/kg BW colchicine, or the equivalent

dose of GS or the colchicine-poor / colchicoside-rich extract, during 5 consecutive days. The body weight was determined daily, and the mice were also daily inspected for clinical signs of toxicity as reported before during the experiment and 5 additional days of follow-up (Montgomery, 1990; Capistrano et al., 2016).

Treatments

Treatment was started 12 days after tumour inoculation when tumours had reached a volume of approximately 100 mm^3. The first day of treatment was assigned "day 1" (Fig. 1). Mice of the negative control group received 200 µl water p.o. (gavage) daily (group 1) (Table 1). The positive control group was given 3 times/week gemcitabine (Actavis, 38 mg/ml) (group 2). It was administered at a dose of 60 mg/kg BW i.p. Group 3 was treated with colchicine at a dose of 4.5 mg/kg BW (daily, p.o.). GS and GS2B were administered p.o. daily at a dose of 4.5 mg/kg BW TC during 3 weeks, i.e. group 4 was given 77.6 mg/kg BW GS and group 5 was given 281.3 mg/kg BW GS2B. Group 6 was treated with a combination of extract GS at a dose of 3.0 mg/kg BW TC (p.o., daily) and gemcitabine (60 mg/kg, i.p., 3x/week) during 54 days. Tumour growth was evaluated three times per week and the mice were sacrificed when the tumour had reached a volume of more than 1500 mm^3 or a tumour weight of more than 10% of the total body weight, and/or a weight loss of more than 20% was observed.

Statistics

Results were expressed as mean values of parameters ± standard error (SE). 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. When only two groups were compared, as on day 51, a Mann-Whitney test was used to determine statistical significance. A piecewise linear regression model, in this case a so called linear mixed model was also fitted. This is a type of regression that accounts for the dependence between observations within the same mouse. "Normal" regression assumes that all observations are independent, which is not the case in this study since multiple measurements were made per mouse. A mixed model accounts for the repeated measurements by including random effect terms into the regression equation. These terms model the effect of each individual mouse on the outcome, but were not the real interest of this study. The interesting terms, time and treatment, are referred to as the fixed effects. The construction of the fixed effects part of a linear mixed model is performed in an analogous way as model building in ordinary linear regression. So a mixed model is a statistical model containing random effects in addition to the usual fixed effects. A Kaplan–Meier survival curve was constructed and the logrank test was used to determine statistical difference between the survivals of the different treatment groups. A p-value $\leq$ 0.05 was considered significant and statistical analyses were performed using GraphPad Prism 6 (Version 6.01) and SPPS (version 22.0).

Results and Discussion

As shown in Fig. 2 the actual treatment doses did not cause any weight loss greater than 10% of the initial body weight. None of the observation criteria were met by any animal during this acute toxicity study. In addition, 5 days after treatment the body weights were still stable and still none of the mice showed any clinical symptoms of toxicity. It could be concluded that the given dose of the different treatments did not cause any acute toxicity.

All mice developed subcutaneous tumours in the right hind limb (100% successful tumour growth). Eleven days after inoculation, the tumours had reached a mean volume of 100 mm^3, the mice were randomised and treatment started. The starting mean tumour volume per group was evaluated to check whether there was no significant difference between the groups (Fig. 3) and the tumour volumes were normalised at this time point. The mean relative tumour volumes were then calculated for each group for each day. The mean relative tumour volumes on day 11, 21 and 51 were graphed (Fig. 4) and the difference between the groups was statistically evaluated determining the significance of the effect of each treatment. The %TGI was calculated for day 11 and 21. The tumour growth delay (TGD), which is the difference or delay in days for treated versus control groups to reach a specified volume (in this case twice the starting volume) was also determined. The %TGI and TGD are summarised in Table 2.**Fout! Verwijzingsbron niet gevonden.** All animals were weighed daily during treatment to evaluate toxicity and the mean body weight per group was graphed (Fig. 5). Table 3 summarises the mean body weight of the different treatments and the relative mean body weight at day 21. This table shows a clear gain of body weight for all groups after 21 days of treatment.

The individual scatter of each mouse was plotted (Fig. 6 left). This graph shows the relative tumour volume of all individual mice. Each dot represents one measurement in one mouse at a given time point, with separate panels for each mouse. The strip text shows the mouse identification and the treatment. The different colours represent the different treatments. The individual scatter was also graphed per treatment group and is shown in Fig. 6 (right). A plot of the mean relative volume (on the log scale) versus time is shown in Fig. 7. Each dot represents the mean of the ln(relative tumour volume) on one time point. The different lines represent the different treatments. These data were then fitted into a piecewise linear regression model (Fig. 8) and in this case a linear mixed model.

A Kaplan-Meier survival curve was graphed and shown in Fig. 9. The survival fraction is the fraction of mice that was still alive at a certain time point, so for each time point, the graph shows the fraction

of mice still in de study per group. A drop of the line of a group indicated an event, which was in this case a removal of a mouse from the study due to one of the defined study endpoints.

The mean RTV on day 11 and 21 showed a significant difference between the control group and the gemcitabine group. A difference between the control group and the combination therapy was also observed on both days. On day 11 a significant difference was observed for GS2B, and although no significant difference was observed for colchicine and GS, a slight decrease in RTV was present. A significant lower RTV was observed for the combination therapy at day 51 compared to the gemcitabine group. The tumour growth inhibition was also calculated for the different groups on day 11 and 21. A relevant %TGI (> 50%) was observed for gemcitabine and the combination therapy on both days with %TGI of 86% and 82% for gemcitabine and 94% and 92% for the combination therapy. A relevant %TGI (57%) was also observed for GS2B on day 11. A tumour growth delay of 12 and 21 days was observed for gemcitabine and the combination group, respectively, as well as a TGD of 5 days for GS2B.

Although the treatment dose was 1.5 times higher compared to our previous study (Capistrano et al., 2016), it was still below the median lethal dose (LD_{50}) of colchicine, which is 6 mg/kg p.o. for mice. No important body weight loss was observed throughout the experiment, and no severe side effects were observed. Therefore, it can be concluded that no extreme toxicity, even after daily treatment of more than 23 days at relatively high concentrations, was caused by the extracts or colchicine, indicating the relatively moderate toxicity of colchicine and the extracts.

A piecewise linear regression model, in this case the linear mixed model was also fitted. This is a type of regression that accounts for the dependence between observations within the same mouse. "Normal" regression assumes that all observations are independent, which was not the case in this study since multiple measurements were made per mouse. So a mixed model accounts for the repeated measurements by including random effect terms into the regression equation, such as missing value due to the euthanisation of mice when the tumour volume had reach 1500 mm^3. The individual scatter plots and the plot of the mean relative tumour volume suggested that in the control, colchicine, GS and GS2B groups, the tumour growth was linear on the log scale from the start on. In the gemcitabine and combination group, there was a phase of slow growth until day 25, which is followed by a phase of increased growth at a pace comparable to the other 4 treatments. Due to this observation, the evolution of tumour growth with time was modelled using piecewise linear regression, accounting for the change in the slope ("knot") around day 25. This regression model included the change in slope at a given point in time. In all treatments, a knot (slope change) is modelled at day 25. However, only in the combination therapy and gemcitabine group the change in slope was substantial. The hypothesis was then tested whether the change in slope was different between the groups. So, overall comparisons

between groups showed that the null hypothesis, which is that the tumour growth over time was the same in all treatment groups, was rejected with $p < 2 \times 10^{-16}$. By rejecting the null hypothesis of an equal slope before the knot, and an equal change in slope beyond day 25, it could be concluded that there was a statistical difference in tumour growth over time between treatment groups.

For the pairwise comparisons, no significant differences were observed between the control group and the groups treated with colchicine, GS and GS2B. There was however a significant difference between the control group and the combination therapy and gemcitabine. Moreover, the combination therapy was significantly better than the gemcitabine group.

The Kaplan-Meier survival curve shows for each time point the fraction of mice still alive per group. The calculations performed in this study take censored observations into account. If the data of a certain mouse was censored, it was either because the mouse was removed from the study for reasons not related to the endpoints of the study, or because the study has ended and that mouse was still alive and no information beyond the time of censoring was available. While it seems intuitive that the curve ought to end at a survival fraction computed as the total number of subjects who died divided by the total number of mice, this is only correct if there are no censored data. A strong decline in survival fraction at a certain time point indicated that a great number of mice has reached the endpoint of 1500 mm^3 and were euthanised. So, for the survival analysis, a significant longer survival time compared to the control group was observed for the group treated with gemcitabine ($p \leq 0.001$) and the combination therapy ($p \leq 0.0001$). All other treatment groups did not show a significant longer survival compared to the control group. The survival of the combination therapy compared to the gemcitabine group did not show a significant difference, however, after 58 days of treatment fewer mice treated with the combination group had died compared to the gemcitabine group due to the defined study endpoints. This result was observed on the Kaplan-Meier curve where the survival fraction at the end of the study was lower for gemcitabine compared to the combination therapy.

There have been a few studies on the combination of gemcitabine with an herbal extract before, more in particular a *Rauwolfia vomitoria* extract (Yu et al., 2014) and Pao Pereira (*Geissospermum vellosii*) (Yu et al., 2013). Gamma-tocotrienol potentiated the antitumour activity of gemcitabine in an orthotopic pancreatic tumour model in nude mice (Kunnumakkara et al., 2010). However, this is the first report on the coadminsitration of a *Gloriosa superba* extract with gemcitabine.

Conclusion

In summary, statistical analysis of the tumour growth over time between the treatment groups was assessed by using a mixed linear model and showed a significant difference for gemcitabine and the combination therapy compared to the control group. No significant difference in growth over time was observed for the groups treated with colchicine and both extracts. The combination therapy was significantly better than the monotherapy with gemcitabine. Moreover, survival analysis showed a significant prolongation of the survival of the groups treated with gemcitabine and the combination therapy. A slight difference in survival was observed between the gemcitabine and the combination therapy, the latter one being slightly better. No significant prolongation of survival was observed for the extracts and colchicine compared to the control group. Although a relevant tumour growth inhibition for the colchicine-poor extract and a difference of RTV compared to the control group were observed on day 11 and a slightly longer survival was noticed for the colchicine-poor extract, the most important conclusion from this study is that the *G. superba* extract might have an added value combined with gemcitabine in the treatment of pancreatic tumours. For future research it seems worthwhile also to evaluate the coadministration of the colchicoside-enriched extract with gemcitabine.

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Conflicts of interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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