Antitumoral effect and reduced systemic toxicity in mice after intratumoral injection of an in vivo solidifying calcium sulfate formulation with docetaxel

Stefan Grudén, Martin Sandelin, Veera Rasanen, Patrick Micke, Mikael Hedeland, Niklas Axén, Marie Jeansson

1. Introduction

Lung cancer is the most frequently diagnosed malignancy worldwide as well as the most common cancer related cause of death [1]. During the course of the disease the vast majority of patients will develop metastases [2]. The median survival of patients with advanced disease has traditionally been less than a year. However, during recent years the progress with maintenance chemotherapy [3], targeted therapy and immune therapy has given new hope to lung cancer patients and the life expectancy is now many years in subgroups of advanced stage lung cancer [4].

Docetaxel is an effective treatment approved for non-small cell lung cancer (NSCLC) [5–7], breast- [8,9] and ovarian [10] carcinomas. The substance is also used for treatment of other malignancies. Docetaxel kills cells by disrupting the function of microtubules which are essential for cell survival [11,12]. Docetaxel prevents new cells from forming, causes existing cells to undergo apoptosis and stops other cells from maturing and replicating. As with all cytotoxic agents, none of these actions are specifically aimed at tumor cells, resulting in effects on normal tissue cells and thereby drug related side-effects. Thus, although
docetaxel is an effective cytostatic agent, its effectiveness in clinical practice can be compromised as systemic treatment leads to a variety of acute and long term side-effects [13-16]. Although patients initially often respond to systemic or local (i.e. radiotherapy) treatment there almost inevitably comes a point where the disease progresses due to selection of resistant clones [17] or dose limiting toxicity makes further treatment impossible. Due to tumor heterogeneity, patients sometimes have a disease progression in some lesions while other lesions still are sensitive to systemic treatment [18]. With an increasing life expectancy in lung cancer patients the need for novel local treatments of progressing lesions are much warranted.

In recent years, advancements in nanotechnology and biomaterials provide enormous opportunities for anti-cancer drug delivery. By the incorporation of cancer drugs in novel delivery systems, the systemic toxicity of free drugs can be reduced, while the drug can be maintained at a high concentration in tumor tissue. For decades, various calcium salts have been used as long term and resorbable implants, primarily as bone void fillers for orthopedic and dental applications [19]. Calcium sulfate is water soluble and its crystal structure can be controlled by the amount of bound crystal water. Incorporation of drug substances allows for a modified release of the drug. Calcium sulfate dihydrate salt contains the highest amount of crystal water. When calcium sulfate dihydrate is heat treated above 100 °C it may be transformed to a hemihydrate. This reaction is reversible if the right amount of water is added. When a fine grained powder of drug loaded calcium sulfate hemihydrate is mixed with water, an injectable paste is formed, which solidifies in vivo as the formulation recrystallizes to the dihydrate form, hence forming a local slow drug release implant in the injected area [20]. Calcium sulfate is therefore a reasonable choice as biomaterial for parenteral slow release formulations with docetaxel.

Altogether there are slow release depot products based on calcium sulfate such as implantable depots containing cisplatin [21] or tetraplatin [22], Calcium sulfate is also a well-known pharmaceutical excipient for oral use [23]. Furthermore, a calcium sulfate based product with an incorporated antiandrogen has recently been tested in a phase Ila clinical trial for local treatment of prostate cancer [20]. This product is an injectable in vivo solidifying formulation releasing the antiandrogen drug for approximately 6 months. This tested product is manufactured with the same calcium sulfate technology (NanoZolid) as the explorative formulations of the present work. In this study, mice inoculated with Lewis lung carcinoma cells were used as a model for evaluation of intra-tumoral injectable depots made with calcium sulfate as a drug carrier containing docetaxel. The local intra-tumoral formulations were compared with systemic treatment as well as with placebo controls for comparison of antitumoral effect, systemic side-effects and plasma concentrations of docetaxel.

2. Methods

2.1. Aim

The aim of the study was to generate a slow release formulation based on calcium sulfate with incorporated docetaxel. These formulations were tested with regards to antitumor efficacy and systemic side effects in mice with subcutaneous Lewis lung carcinoma tumors and compared to placebo and systemic administration of docetaxel.

2.2. Animals and tumor cell line

Sixty 6–12 weeks old C57bl/6j females (Charles River GmbH, Germany) were included in the study. The mice were housed 5 per cage under standardized light-dark cycle conditions with free access to food and water. Animal studies were reviewed by the local ethics committee of Uppsala University (Sweden) and approved (C109915/16).

Lewis lung carcinoma (LLC, ATCC, UK cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin) at 37 °C in a humidified 5% CO2 incubator. LLC cells (50 μL containing 1 x 106 cells) were mixed with 100 μL Matrigel (354263, Fisher Scientific) and injected subcutaneously on the back of mice at day 0, six days before docetaxel treatment.

2.3. Calcium sulfate (NanoZolid) formulations with docetaxel

Docetaxel (J60174 Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) was embedded into NanoZolid in two different doses (concentrations), a low dose (NZ DTX-L, 5% in the powder) and a higher dose (NZ DTX-H, 10% in the powder). A placebo group received unloaded NanoZolid (NZ). NanoZolid was prepared by heating/dehydrating calcium sulfate dihydrate (Ca4SO4·2H2O from Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to calcium sulfate hemihydrate. The heat treated powder was micronized by wet-milling in 2-propanol (VWR International GmbH, Darmstadt, Germany) to a grain size of 2.3 μm (DS50). The milled powder was dried at room temperature evaporating the 2-propanol. For the active formulations, the micronized calcium sulfate powder was first dispersed in a docetaxel methanol (Merck KGaA, Darmstadt, Germany) solution. The solvent was thereafter evaporated while the solution was agitation. This formed a dry powder with docetaxel finely distributed in the calcium sulfate powder. Powders with 5% and 10% by weight of docetaxel in calcium sulfate as well as a non-loaded placebo powder were prepared. Prior to administration to the animals the prepared docetaxel/calcium sulfate powder was mixed with a 0.25% sodium carboxymethyl cellulose aqueous solution (Apotek Produktion & Laboratorier AB, Umeå, Sweden) at proportions 1.0 mL of solution to 1.0 g of powder, forming a viscous but injectable paste. The paste solidified within approximately 10 min and formed a solid body in vivo. The carboxymethyl cellulose acts as retarder for the solidification. The hydration reaction is exothermal but, for the evaluated amounts of formulation and the relatively slow solidification necessary for practical handling, the temperature rise of the depot is negligible. The majority of the hydration occurs during the first 15 min after mixing, and is completed after one hour. Prior to the animal tests, the release characteristics of docetaxel from the formulations were evaluated in an in vitro method. Solidified 100 mg lumps of the formulations were individually immersed in 300 mL of 0.9% sodium chloride solution in a flat bottomed 500 mL clear glass flask, stored at 37 °C. Liquid samples of 1.0 mL per time point were withdrawn without compensational adding after 1, 2, 3, 6, 13 days.

NanoZolid treatments were started 6 days after tumor injection. The mice received a target amount of 50 μL of formulation paste. The dose was prepared just prior to injection. The formulations were injected into the tumor using a 1 mL syringe and a 21 G needle. Approximately 10% of the dose was expected to be released during the test period, equaling doses of 12.5 mg/kg and 25 mg/kg docetaxel per body weight. Docetaxel for systemic treatment (Hospira Nordic AB, Sweden) was diluted with saline and injected at a dose of 25 mg/kg intraperitoneally (i.p.). Systemic treatment was started at day 6 and was repeated every 48 h four times (day 6, 8, 10, 12).

2.4. Determination of antitumor effect

Six days after subcutaneous inoculation of LLC cells (1 x 106) into the back of C57bl/6j mice, they were divided into four groups.
many. The internal standard [2H5]-docetaxel (docetaxel-d5) was prepared in DMSO:acetonitrile (1:9 v/v) at 0.1–0.2 mg/ml. Docetaxel-docetaxel-d5 (collision energy 10 eV, cone voltage 35 V). The dwell time of a calibrator solution or 50 μL of water or 50 μL of a calibrator solution or 50 μL of a QC solution was added. The internal standard solution (20 μL of docetaxel-d5 80 ng/mL) was added, followed by 100 μL of acetonitrile to precipitate plasma proteins. The samples were then mixed for 10 min and centrifuged for 5 min (11500g). For analysis, 75 μL of the supernatants were transferred to vials and they were mixed with an equal volume of water prior to injection.

2.5. Measurement of systemic toxicities

Blood samples were withdrawn from a tail vein and collected in a tube with anticoagulant (K3 EDTA, Microvette, Sarstedt) on day 0, 7, 13, 15. Blood cell counts were measured in whole-blood using an automated hematology analyzer (XP-300, Sysmex, Sweden) after a 1:20 dilution in Cellpack buffer (Sysmex) according to the manufacturer’s instructions. Body weight was measured on day 0, 8, 10, 12, 13, 14, 15 and expressed as the relative body weight compared to day 0.

2.6. Plasma concentration of docetaxel

Blood samples were withdrawn from a tail vein and collected in a tube with anticoagulant (Li-Heparin, Microvette, Sarstedt) on day 0, 7, 13, 15. Plasma was collected after centrifugation at 2000g and stored at −80°C until analysis.

Plasma samples were prepared as follows. To 10 μL of plasma (study sample, calibrator or QC sample), 50 μL of water or 50 μL of a calibrator solution or 50 μL of a QC solution was added. The internal standard solution (20 μL of docetaxel-d5 80 ng/mL) was added, followed by 100 μL of acetonitrile to precipitate plasma proteins. The samples were then mixed for 10 min and centrifuged for 5 min (11500g). For analysis, 75 μL of the supernatants were transferred to vials and they were mixed with an equal volume of water prior to injection.

2.7. Histology

Tumors were dissected at endpoint and fixed in 4% formalin overnight. Tissue was paraffin embedded, sectioned at 3 μm, and stained with hematoxylin and eosin (HE). Morphology was evaluated for each tumor. The area of necrosis was estimated in relation to the total area (5% steps), and mitotic index was evaluated by counting mitosis in 10 high power fields. All analyses were performed by two histological trained observers (V.R. and P.M.).

2.8. Statistical analysis

Survival (endpoint) was plotted using the Kaplan-Meier method and compared by the log-rank test. Groups were compared by one-way analysis of variance (ANOVA) and differences between groups were determined by Holm-Sidak’s multiple comparisons test. Statistical analysis was done using GraphPad Prism. Data was unevenly distributed and transformed using the natural logarithm. Data are presented as geometric mean ± SEM or ± 95% confidence interval as stated in figures. A p < 0.05 was considered statistically significant.

3. Results

3.1. In vitro release of docetaxel from NanoZolid

The docetaxel in vitro release profiles from NanoZolid showed a typical slow release behavior comparable to that of the NanoZolid-based product candidate Liproca Depot [25], see Fig. 1. The two formulations in the present study showed similar release profiles although having a twofold difference in drug-load, indicating a drug-load release mechanism dependency. During the first 13 days about 5% of the drug in the low dose formulation and 2.5% of the drug from the high dose were released in the in vitro test.

3.2. In vivo antitumor efficacy

The in vivo antitumor efficacy was tested on xenograft LLC established on C57Bl/6J mice. Tumor volume was measured and body weight was recorded for 15 days. Treatment was started on day 6, at which time point tumor volume was similar in all groups (Fig. 2). All groups receiving docetaxel treatment had a significant antitumor effect compared to placebo treated mice already two days after treatment start. Mice euthanized due to tumor volume endpoint are illustrated in the survival graph in Fig. 3. All docetaxel treated groups showed a significant survival benefit compared to the placebo treated group. However, over the duration of the test, there was no significant difference in survival or tumor volume between the docetaxel treated groups.

3.3. Systemic toxicity

As shown in Fig. 4A, systemic treatment with docetaxel (IPDTX) significantly reduced body weight from day 11 compared to intra-tumoral treatment with NZDTX. Mice treated with intra-tumoral
NZDTX showed no body weight loss over the study period for either dose. Both white blood cells (Fig. 4B) and red blood cells (Fig. 4C) were significantly lower in mice receiving systemic treatment, compared to intra-tumoral treatment with NZDTX. WBC declined significantly already one day after treatment start (day 7) and RBC five days after (day 11) and both continued to stay low throughout the study period. WBC and RBC counts appear unaffected by intra-tumoral NZDTX treatment. Mice with systemic docetaxel (IPDTX) displayed a significantly elevated platelet count from day 11 not seen in groups with intra-tumoral NZDTX treatment (Fig. 4D). The placebo treatment did not show any significant changes over the evaluated seven days.

3.4. Histology of tumors

Histological evaluation showed solid tumor growth. The cellular changes were more pleomorphic including atypical mitosis, large cells, and enlarged irregular nuclei in the intra-tumoral treatment groups (NZDTX-L and NZDTX-H) compared to the groups receiving placebo or systemic treatment (Fig. 5A–D). Necrosis was frequent with a trend towards more necrosis in the high dose intra-tumoral treatment (NZDTX-H) compared to other groups (Fig. 5E). Cell proliferation based on mitotic counts did not show any significant difference between groups (Fig. 5F). All groups showed sparse infiltration of inflammatory cells in the peritumoral tissue, with a tendency to higher abundance in the groups with intra-tumoral treatment. The extracellular deposits of calcium sulfate, observed in all groups with intra-tumoral injections (NZ, NZDTX-L, NZDTX-H) did not lead to significant inflammatory changes.

3.5. Plasma concentration of docetaxel

Plasma samples were subjected to UHPLC-MS/MS analysis to determine the concentrations of docetaxel. On average, the higher dose formulation, NZDTX-H, gave a significantly higher plasma concentration compared to the lower dose preparation, NZDTX-L, by a factor of four to five. However, the plasma concentration ranges for the NZDTX-L and NZDTX-H groups overlap significantly. The high dose, NZDTX-H, formulation also gave a higher plasma concentration than the systemic IPDTX treatment, by a factor of two (Fig. 6). However, this concentration difference is not believed to be related to a corresponding difference in systemic exposure to docetaxel, since the peak concentration for the IPDTX probably occurs much earlier than the first sampling time. The concentration range (spread) for the IPDTX has less variation than that for the intra-tumoral docetaxel treatments. In the NZDTX-L group some mice did not show plasma concentrations above LLOQ. These mice were still included in the study as remains of the NZ formulation was still visible in the tumors, and docetaxel was detected below 2.5 ng/ml (the...
LLOQ) but not quantifiable. These concentrations are therefore set to 1.25 ng/ml, i.e. ½LLOQ [26]. Systemic treatment, IP DTX, showed an average plasma concentration of ~10 ng/ml at the 7 and 9 days measurements (in both cases 24 h after injection). However, in mice, docetaxel has a t1/2 of 8.6 h for drug elimination from plasma, thus the concentration in plasma collected 24 h after i.p. injection had passed the highest concentration [27]. In contrast, plasma concentrations from formulation released docetaxel suggest a more time-stable docetaxel concentration in plasma.

4. Discussion

Cytotoxic treatment represents the standard for most patients with advanced malignancies. In particular in lung cancer, although commonly applied, chemotherapy reveals only modest effects in relation to serious systemic side effects. New drug formulations may reduce this systemic toxicity and presents a promising treatment option to achieve local control of the disease. In the present study we demonstrate that a novel calcium sulfate formulation with docetaxel can be administered intra-tumoral with potent antitumor effects and more importantly, with significantly reduced systemic side effects compared to systemic treatment with docetaxel. The milder systemic toxicity profile was evident by significantly less weight loss as well as decreased bone marrow suppression, symptoms often limiting the dosing of systemic therapy in cancer patients.

The general challenge in developing depot formulations for local treatment is to combine a stable encapsulation of the drug substance with a controlled release, providing clinically relevant local drug concentrations over a sufficient period of time. In addition, depot compositions might pose a high risk for inflammatory responses and may lead to fibrous tissue encapsulation of the depot. For such compositions long-term toxicity studies may be necessary.

The calcium sulfate formulations tested in this study formed solid implants 10–20 min after mixing with the diluent. NanoZolid loaded with docetaxel released the cytotoxic agent in vitro in a controlled manner releasing about 5% of the embedded docetaxel from the low dose formulation (NZDTXL) and about 2.5% from the high dose formulation (NZDTXH) the first 13 days (Fig. 1). The release profile is similar to that of 2-hydroxyflutamide, another hydrophobic active substance, in a similar slow release system utilizing calcium sulfate [25]. As docetaxel has a log P value of 2.4 and is practically insoluble in water, (solubility 0.0127 mg/mL) [28], the doubled amount of docetaxel in the high dose formulation (NZDTXH), and thereby an increased hydrophobic character of the solid lump, may be an explanation to the observed relatively lower release from the high dose formulation in the in vitro water bath [27]. On the other hand, when NanoZolid is injected into the tumors of the mice, i.e. not only in a hydrophilic milieu but also amphiphilic and lipophilic, the release is shown to be dose related as seen in plasma concentrations (see Fig. 6). We therefore believe that the in vitro test used is of limited value to determine the release characteristics, since
the in vivo–in vitro correlation seems to be relatively low. An improved in vitro test should better simulate the combined hydrophobic and hydrophilic conditions of the tissue.

Several other technologies combining cytostatic drugs in biodegradable matrices are under development [29–31] but few vehicles combine biocompatibility and bioresorption with continuous and controlled release of cytostatic drugs over a period of weeks or months. Other implant formulations with docetaxel have used advanced lipophilic or solid nanoparticle systems which have the disadvantage to depend on initial dissolving, disintegration and distribution of the vehicle to release the active substance at the target site [29–31].

As mentioned above, depot compositions pose a high risk for inflammatory responses and may lead to fibrous tissue encapsulation. In the current study histology showed a minimal inflammatory response with neutrophils and lymphocytes only lining the solidified formula while surrounding tissue remained normal (Fig. 5). The favorable biocompatibility may be due to the formulation’s tissue friendly pH which remains stable during depot

Fig. 5. Histology of LLC tumors. Histological sections of representative Lewis cancer grafts stained with hematoxylin-eosin: NZ (placebo) group (A), intra-tumoral treatments NZDTX-L (B) and NZDTX-H (C), and systemic treatment with docetaxel IPDTX (D). Geometric means ±95% CI for necrosis showed a trend towards more necrosis in the NZDTX-H group (E) while the number of mitotic cells remained similar (F).

Fig. 6. Plasma concentration of docetaxel in tumor bearing mice. Plasma concentration of docetaxel with geometric mean ±95% CI. NZDTX-H had significantly higher plasma concentration compared to NZDTX-L. Six mice at day 7 and eight mice at day 9 in the NZDTX-L had plasma concentrations below the detection limit of 2.5 ng/ml and were set to ½LLOQ (1.25 ng/ml). IPDTX plasma concentrations were low for reasons explained in Results and Discussion.
breakdown [32]. This is in contrast to polymer based depot formulations that reduce pH as they are breaking down [33]. However, it should also be noted that the study was performed in a short time span and further studies are needed to evaluate long term effects.

In this work, both NanoZolid formulations with docetaxel had antitumor efficacy comparable to systemic delivery of docetaxel in LLC flank tumors. Treatment was started 6 days after inoculation of tumor cells on the right flank of female mice. At this time point tumors had reached a size of a little more than 100 mm³ (Fig. 2), making an intra-tumoral injection of 50 μl formula possible. As a 50 μl injection into the tumor may affect the ‘true’ tumor volume measured we also injected NanoZolid without docetaxel in a placebo group (NZ). The systemic treatment did not involve any NanoZolid. Although the formulation was injected intra-tumorally, some of the formulation may also have escaped the tumor and solidified peri-tumorally. At the day 8 tumor volume measurement some of the formulation was dissolved and the tumor tissue had presumably partly adapted to the injected volume. It was therefore decided not to compensate the tumor volume with the injected formulation volume. Also, such volume compensation would not have changed the conclusions on antitumor efficacy.

A significant benefit of docetaxel treatment on tumor growth was seen already 2 days after treatment start (i.e. day 8 in Fig. 2) in all treatment groups compared to placebo treated mice. As LLC tumors are growing fast, tumors started reaching endpoint (≥ 12 mm in any direction) at day 8, at which time all mice in the placebo group were euthanized and a few in the other groups (Fig. 3). At this point one option would have been to euthanize all mice to get the same time point for histology and tumor weight. However, as long term systemic side effects were also of major interest, we decided to follow the mice until endpoint or day 15. The placebo group showed an equal amount of injected drug carrier material, made from calcium sulfate, did not itself induce any initial (i.e. the first 7 days) effects on body weight or blood cell values.

Although it should be noted that LLC cells lack some of the phenotypes of human lung cancers [34]. In the current study we utilized subcutaneous tumors in mice that are much easier to reach than a typical non-small cell lung cancer tumor or other tumors. For the injection into such tumors other administration devices are likely better suited as well as exact guidance with ultrasound. However, we believe that the results can be in some degree transferred to the clinical situation, where docetaxel is an established treatment option to achieve local control of the disease.

As expected, systemic treatment with docetaxel resulted in significant weight loss, reduced white and red blood cell counts, and an increase in platelets, parameters not affected in mice receiving NZDTX treatment. Plasma levels of docetaxel were measured on day 7 and 9. These time-points were both 24 h after i.p. injection of docetaxel in the NZDTX-H group. For the other NZ groups, day 7 and 9 represent 24 h and 72 h after formulation injection, respectively. Docetaxel has a fast clearance in plasma and only low levels remained 24 h after i.p. injection (Fig. 6), something seen in both patients and animal studies [27,35]. We presume that the high plasma concentration spikes described in the literature after i.p. or i.v. injections are the reason for systemic adverse effects despite the rapid clearance of docetaxel [35]. Moreover, plasma levels of docetaxel were also seen in NZDTX treatments with significantly higher levels found in the NZDTX-H group. Importantly, the levels appear stable over time as plasma concentrations remained at the same level for both day 7 and 9 (Fig. 6). Despite the difference in plasma concentration from the two different intra-tumoral formulations, we could not detect a difference in tumor size or blood cell counts comparing these groups. Histology of tumors showed a trend towards more necrosis in the high dose depot formulation (NZDTX-H), however this did not reach statistical significance (Fig. 5). It should be noted that tumors come from different time points, see survival graph in Fig. 3, which could increase variation in data. Moreover, necrosis did not correlate with days of tumor growth or survival. Furthermore, there was no correlation between plasma levels of docetaxel and survival (endpoint) in the NZDTX groups. This lack of differences may have pharmacokinetic explanations including therapeutic threshold or drug distribution and availability. The therapeutic window for docetaxel seems to be in the range of 4–35 ng/ml as in vitro studies have shown this to reduce murine and human cell survival by 50% [36]. The range of docetaxel measured in plasma from NZDTX groups is in accordance with this and it can be presumed that the concentration is similar or higher locally in the tumors. One obvious limitation of this study was the lack of determination of intratumoral levels of docetaxel. Therefore, no definite conclusion could be made on how the two formulations differ intratumorally.

5. Conclusions

Cytotoxic treatment represents the standard for most patients with advanced malignancies. In particular in lung cancer, although commonly applied, chemotherapy reveals only modest effects in relation to serious systemic side effects. New drug formulations for intra-tumoral treatment may reduce systemic toxicity and presents a promising treatment option to achieve local control of the disease.

In the present study, intra-tumoral treatment of subcutaneous LLC tumors in mice with this new formulation showed similar antitumor efficacy as systemic administration of docetaxel. More importantly, while mice treated systemically with docetaxel displayed several signs of systemic toxicities, including weight loss and decreased levels of blood cells, mice with local intra-tumoral depot with docetaxel containing depots did not. Furthermore, intra-tumoral depots of the formulation were well tolerated and released docetaxel at amounts sufficient to be detectable in plasma. While the safety profile of both calcium sulfate and docetaxel are well established, further studies are needed to prove the efficacy of the studied drug formulation in a clinical setting. In summary, our study demonstrates a new drug formulation with docetaxel for intra-tumoral injection, with maintained antitumor efficiency and reduced systemic toxicity, thus a promising treatment option to achieve local control of the disease.

Declarations

Ethics approval and consent to participate

Mice were used in the study. The mice were housed 5 per cage under standardized light-dark cycle conditions with free access to food and water. Animal experiments were reviewed by the local ethics committee of Uppsala (Sweden) and approved (C109915/16).

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

SG and NA are consultants for LIDDS AB and have equity interests in LIDDS AB. The other authors declare that they have no competing interests.
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