Investigation of efficacy and tolerability of innovative nonsurgical treatments for basal cell carcinoma

PhD thesis

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1. Introduction

Basal cell carcinoma (BCC) is the most common malignancy in the fair-skinned population, however associated mortality is very low, as metastasis formation is an extreme rarity. Nevoid basal cell carcinoma syndrome (NBCCS), with mutations of *PTCH1* or *SMO* genes, predispose to the formation of BCCs. The enhanced activity of Smoothened (Smo) protein has been shown to have a role as an oncogene in the development medulloblastoma and prostate cancer, besides BCCs. Surgical excision is the gold standard method to treat BCCs with a high curative rate. Locally advanced BCC (laBCC), that are incurable with surgery or radiotherapy, by way of severe local tissue destruction it can lead to death. The Smo-inhibitor vismodegib drug revolutionized the treatment of such cases. It provided remarkable response rates. The objective response rate (ORR) was 60.3%, including 31.5% complete response (CR) and a significant 28.4% partial response (PR) rate. Despite the impressive efficacy of vismodegib one cannot underestimate the high rate of the severe drug-related adverse events (AEs). These could severely impair the quality of life (QOL) and may result in the discontinuation of the therapy in a significant proportion of patients. Secondary drug resistance has a higher chance to develop if the therapy is discontinued for any time. Nowadays, off-label use of PD1 inhibitors are increasingly popular as alternatives for Smo-inhibitor resistant laBCCs. However, we must emphasize that frequently anti-PD1 drugs cannot be chosen due to comorbidities. Resistance to anti-PD1 drugs may also develop in certain BCCs. For such patients there is a need to find further therapeutic options, either alone or as an adjuvant

therapy with minimal side-effects. Ascorbic acid (ascorbate, vitamin C) is an essential vitamin most well-known for its potent antioxidant properties. In addition to its antioxidant effects, studies have also shown that high-dose ascorbate have a cytotoxic effect on cancer cells. In the last decades a growing body of evidence, based mainly on case series, has become available of the possible beneficial use of high dose intravenous ascorbic acid (IVA), however the exact level of the therapeutic effect is still ambiguous. It has been also shown that ascorbate boosted the cytotoxic effects of amphotericin B on *Candida albicans* (*C. albicans*) cells.

Aims

Project I.

Our aim was to evaluate the outcome of vismodegib therapy in our patients and to compare it with the literature. We assessed the efficacy of vismodegib therapy on patients with or without comorbidities, the latter included any malignancies. We reviewed the efficacy of vismodegib, classified and graded the adverse events. We evaluated the efficacy of management options that can be utilized to alleviate or eliminate side effects of vismodegib. We attempted to assess whether discontinuation of vismodegib, due to unbearable side effects, had an influence on the development of secondary resistance. Lastly, we tried to compare if there is any difference between non-NBCCS and NBCCS patients in terms of development of drug resistance.

Project II.

The high-dose IVA clinical trial was conducted at our department, the Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, as a single center trial. We set out to evaluate whether high-dose IVA therapy is effective either as a monotherapy or as an adjuvant therapy for BCC's which are otherwise uncurable. We assessed the efficacy of high-dose IVA and compared it to the results of the Smo-inhibitor vismodegib treatment. We examined whether high-dose IVA can be administered for long periods of time and in the presence of comorbidities. Furthermore, we assessed the mean and highest dose that could be administered via a peripheral vein or through a Port-A-Cath device. The side effect profile of high-dose IVA was also analysed and matched with that of Smo-inhibitor vismodegib.

Project III.

We aimed to test whether ascorbate, through its pro-oxidant properties can effectively kill C. albicans in vitro. We proposed that the electron transport chain (ETC) might play a crucial role when ascorbate exerts its cytotoxic effect. We evaluated the effect of the inhibition of ETC with the usage of antimycin A. We also aimed to assess the effect that the concentration of Fe^{2+} can exert on the killing potential of ascorbate.

Methods

Project I.

In this project clinical data of 11 laBCC patients were analysed retrospectively. Patients with or without NBCCS were included. They were all treated in our department with vismodegib. The therapeutic effect was evaluated by monitoring the following parameters: diameter of the largest tumor, sum of the diameter of the lesions and number of tumors. Side effects were registered and graded, and we also gathered information on data of the therapeutic options to relieve or alleviate them. We gathered all data for efficacy and side effects from the medical documentation. Clinical photographs have been also taken regularly.

Statistical analysis

We performed statistical analyses using Graphpad Prism version 7.00 (Graphpad Software Inc, CA, USA). Data was tested using the Student's t-test and Kaplan-Meier analysis as suitable. Graphs display arithmetic means and error bars represent standard deviations (SD). The statistically significant value of p was considered <0.05.

Project II.

Design, eligibility criteria and patient demographics

The high-dose IVA clinical trial was conducted at our department, the Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, as a single center trial. The Regional Committee of National Science and Research Ethics (TUKEB 80/2010) and the National Institute

for Quality- and Organizational Development in Healthcare and Medicines (39.798/56/09) approved the off-label use of ascorbate. Patients, who met the inclusion and exclusion criteria, were considered eligible and recruited for high-dose IVA therapy. All patients in the study group were diagnosed with laBCCs, they were not suitable for surgical excision or radiation, and at the time of the study no other treatment option, including vismodegib were available. The subjects could be selected for the study regardless their NBCCS status. All patients gave their informed consent to the study.

Treatment protocol

We administered 1.8 g/kg body weight IVA per each infusion , with doses ranging from 75 to 175 g, through a Port-A-Cath device three times a week. The high-dose IVA infusions were formulated from concentrated (500 mg/ml) ascorbate solutions. These were diluted in 1000 ml Ringer's lactate infusion and then carefully administered for 3 hours with full protection from light.

Evaluation of study outcome

For each patient at the baseline evaluation we chose one to six target lesions to monitor them monthly using digital photography and image analysis. Imaging techniques, as magnetic resonance imaging (MRI) and computer tomography (CT) scan were obtained, if needed, to give further data of changes in tumor volumes. So that to assess the response to the therapy, we adapted guidelines of the Response Evaluation Criteria in Solid Tumors (RECIST v.1.1). The disappearance of all lesions was declared as complete response. Partial response

was referred to the >30% value of decrease in sum of all longest diameters of lesions monitored. Development of a new tumor and/or an unequivocal escalation in overall disease status were defined as progressive disease (PD). At least 20% increase in sum of all longest diameter of target lesions was also specified as PD. Cases where the decrease in the sum of all longest diameters did not qualify for CR or PR, nor the criteria of PD is fulfilled we determined to stable disease (SD).

Histopathological examinations

Before the beginning and after IVA treatment tumor biopsy samples were collected in case of patient 1 and 2. In case of patient 3 and 4 an initial biopsy was obtained before treatment, but they did not consent to the collection of another, follow-up biopsy. The 10% formalin-fixed and paraffin-embedded biopsy samples were stained with HE from all patients.

Additional samples from patient 1 were used for immunohistochemistry evaluation. We attempted to assess the degree of vascularization in two different subtypes (micronodular, adenoid) of BCC in patient 1. In order to that monoclonal mouse anti-CD31 (PECAM-1, clone: 89C2, Cell Signaling, MA, USA) staining was performed.

Project III.

Materials

Depending on the experimental setup, the L-ascorbic acid was diluted and dissolved in phosphate buffered saline (PBS), and in another setup in YPD (BD-DifcoTM, yeast extract 10g/L; peptone 20g/L; dextrose 20g/L), glycerol (38g/L) media. In all

experiments the final concentration of ascorbate was set to 90 mM. ETC inhibitor antimycin A (extracted from streptomycin sp.) was dissolved in ethanol solution and further on diluted to 10 μM concentration. The iron chelator 2,2′-bipyridyl (bipyridyl) was diluted to 500 μM.

Cell culture

CEC 749 strain of *C. albicans* was used in this experiment. It was grown on YPD agar and subcultured in liquid YPD medium in a shaking incubator routinely at 30°C (New Brunswick Scientific, Edison, NJ). In the experiments we obtained log phase cultures acquired from reculturing stationary overnight cultures. All broth cultures were centrifuged at 3,200 rpm for 10 min (centrifuge 5417 C; Eppendorf, Hamburg, Germany) and then re-suspended in PBS solution. Use of optical density (OD570 of 0.65) we adjusted the concentrations to give an average cell density of 10^7 CFU/mL.

Experimental design

We used $3x10^7$ cells per each the growth media that was 3 mL. Two aliquots (10μL each) were withdrawn from the media containing L-ascorbic dissolved in PBS at each time point (0, 10, 20, 30, 60, and 90 minutes). The cell viability was evaluated by colony counting method in all experiments. At each time point aliquot was plated on PBS media. We have been incubating plates between 24-48h at 30°C. In another setup *C. albicans* cells were refreshed for 4h in YPD with antimycin A (10μM) added or not after 1h. The cells were washed, re-suspended and shaked in the incubator (157rpm, 37°C) with or without ascorbate in either PBS or YPD. The aliquot samples

were withdrawn at the same time points as mentioned before. To evaluate whether the killing potential is dependent on the presence of Fe^{2+}, we used an iron chelator, called bipyridyl (500μM) dissolved in PBS. We re-suspended cells and bipyridyl was added, and incubated with shaking (157rpm, 37°C) and the aliquots were withdrawn as before (0, 10, 20, 30, 60, and 90 minutes).

Statistics

Experiments were repeated at least 3 times. Data points are means and error bars are standard deviations. Means were compared for significance ($p<0.05$) by one-way ANOVA and Bonferroni post-hoc test.

Results

Project I.

Efficacy of vismodegib therapy

The mean age of all patients was 73±15 years, including 9 female and 2 male patients. 7 patients had laBCC without NBCCS and in 4 cases laBCC developed due to NBCCS. The Smo-inhibitor treatment was administered for an average of 16±15.69 months for the 11 patients who participated in this study. When the period from the time of initial response until documented tumor progression was measured, no difference was observed between the two patient groups (2±0.69 vs. 2±0.82 months; non-NBCCS vs. NBCCS respectively; p=0.76). A CR was achieved in 3 patients, and after the discontinuation of vismodegib no relapse could be observed. Additional 3 patients

showed excellent improvement, but afterward they were deceased due to conditions unrelated to laBCCs and drug-therapy. The treatment was temporarily suspended due to intolerable side effects in the case of two non-NBCCS patients. Both patients initially showed a good therapeutic response, but when vismodegib therapy was restarted the efficacy became gradually lower and subsequently lost, and their condition worsened. Other patients showed notable response to treatment, but not a CR. By the end of the follow-up period the average number of lesions (20±25.47) decreased to 5.5±16.52. The average of the diameter of the largest tumor was significantly reduced after treatment (2.7±2.7 cm; 3.1±2.2 cm vs 6.1±10.5 cm; non-NBCCS vs NBCCS. Almost all patients, except one, had a coexisting benign tumor or some form of malignancy other than the BCC, such as polycytemia vera, colon, prostate or bladder cancer. During vismodegib treatment no deterioration in the status of these cancers could be observed.

Assessment of side effects of vismodegib treatment

The most common vismodegib-related side-effects were weight loss, muscle cramps, dysgeusia and alopecia. They developed in both NBCCS and non-NBCCS groups. No grade 4 adverse event was recognized. We attempted to find a way to alleviate side effects and achieved moderate success in case of muscle crumps and weight loss. In order to prevent alopecia and dysgeusia therapeutic options provided only limited success.

Project II.

Efficacy of high-dose IVA

In this single centre clinical trial 6 patients were screened, from those, 4 patients could be enrolled, and the 2 patients were excluded during the screening period. Three out of the selected four patients had NBCCS. The high-dose IVA therapy was administered for a mean duration of 42 ± 23.6 weeks. In 83% of all target lesions we could detect some degree of a benefit (partial and stable response). 27% of all tumors, that responded to therapy, partial response was seen whereas the remaining 73% displayed stable response. No patients developed any detectable new lesions. The overall treatment response assessment showed stable disease for three patients and in case of one patient progressing disease was observed. Follow-up skin biopsy of patient 2 showed a tumor-free scar tissue. Despite all our efforts in patient 1 and patient 3 worsening of some lesions could not be completely prevented. Therefore, we have decided to switch to vismodegib, as it became available as in a phase III. clinical trial setting. Thereafter patient 1 died of sepsis caused by urinary tract infection, irrespective of vismodegib therapy. We also introduced vismodegib for patient 3, and later on he died of glioblastoma multiforme, which was also unrelated to Smo-inhibitor treatment. In case of patient 2, during IVA therapy we could measure the decrease of the longest diameter of the tumor on the skin surface by 13%. For that it could be rated as stable disease, but still an unequivocal intrasellar invasion was detected after 4 months of treatment. Palliative radiotherapy was initiated to manage the intrasellar invasion. However, the patient died soon after due to complications of

irradiation therapy. Patient 4 dropped out from the study and we could obtain further data about his condition.

Side-effects of high-dose IVA

We did not observe any significant adverse events, including nephrolithiasis, during IVA treatment. Only some well-tolerable and mild side-effects developed, such as an occasional nausea (Grade 1) in patient 1. During the administration of IVA into a peripheral vein, patient 3 and patient 4 indicated a mild burning sensation (Grade 1).

Assessment of microvessel density in different BCCs by CD31 staining

It has been studied that in aggressive subtypes of BCCs the microvessel density is greater when compared to lesions with slower rate of growth. In case of patient 1 with multiple lesions, most micronodular have shown improvement during IVA therapy, whereas adenoid type lesion has not responded to the therapy. In order to reveal the differences of microvessel density between the two type of lesions an immunostaining with CD31 on one adenoid and one micronodular biopsy specimen of patient 1 have been done. We recognized a remarkably lower level of microvessel density in the adenoid subtype compared to the micronodular type lesion.

Project III.

Ascorbate could exert its killing potential in PBS growth media, and this effect strongly depends on Fe^{2+} concentration

Ascorbate could eradicate *C. albicans* when the cells were incubated and shaken in PBS at 37°C (after 90 min. > 5 logs killing). The use of iron chelator bipyridyl (500 μM) could inhibit the killing effect of ascorbate (>2 logs). This may be explained by the reduced concentration of $HO^{\cdot}$, which is accumulated in the Fenton reaction catalysed by free Fe^{2+}.

The killing potential of ascorbate is increased with antimycin A

The inhibition of complex III in ETC in *C. albicans* cells with antimycin A increased the killing effect of ascorbate in PBS media.

2. Conclusions

Project I.

In our experiment 7 of the 11 patients treated with vismodegib achieved PR or CR, which is comparable that was reported in the literature (46-66%). In cases of patients with malignancies other than laBCCs we did not observe any tumor progression. We can state that in case of prostate, bladder cancer, polycytemia vera and meningioma vismodegib can be safely administered, however it would need further investigation with a larger sample size. We have demonstrated a relatively high proportion of patients, 5 of 11 patients discontinued the therapy due to severe side effects. In order of likeliness to occur, adverse events were the following: weight loss, dysgeusia, muscle

cramps and alopecia, no Grade 4 was observed. For alopecia, and dysgeusia none of the treatment modalities were found to be effective. In order to maintain weight during therapy, enhanced oral nutrition supplementation was given, and this led to only temporary success. Best-managed side effect was muscle crump, as combination of central muscle relaxants gave at least a moderate efficacy in terms of control of unwanted muscle contractions. Secondary resistance developed in 2 of our cases, both after a temporary suspension of the treatment; no primary resistance was observed. Interestingly, both of these secondary resistance cases occurred in the non-NBCCS group. On the other hand, although an earlier study observed development of secondary resistance in 60% of the NBCCS patients, in our study no secondary resistance could be detected in this patient group. This finding should be further examined, as NBCCS patients generally need the therapy drug for a longer period of time and a temporary suspension of drug at certain time intervals would enable them to tolerate and in turn continue the treatment for the required amount of time.

Project II.

In our pilot study with the use of high-dose IVA therapy for laBCC we could achieve stable disease 3 out of 4 cases, and in one case we observed progressive disease in the overall response. When compared to results achieved by the Smo-inhibitor vismodegib treatment, high-dose IVA is clearly inferior to vismodegib. Based on this experiment we concluded that the first-choice treatment for otherwise incurable laBCC should be vismodegib. As high-dose IVA showed beneficial effect in 83% of all lesions and a 4-16% change in those lesions

that showed reduction in size, high-dose IVA could be further investigated as a potential adjuvant treatment. It must be noted that when the dose exceeded 75 g per infusion, it had to be given through a Port-A-Cath device to prevent damage of peripheral vein. The potential use of high-dose IVA is further supported by the fact, that this therapy regimen, even in cases of 125 to 175 g per infusion, did not cause any severe side effects for a 42 ± 23.6 weeks period of time. Grade 1 nausea occasionally appeared in case of patient 1, and no other systemic side effects could be detected, aside from a mild burning sensation in the peripheral vein due to the administration of the IVA. Adenoid type BCC lesions, which are less vascularised as confirmed by CD31 staining, showed no reduction in size to IVA therapy. Some lesions of more aggressive micronodular type showed response clinically. In contrast to this, other lesions of the same histology type progressed despite high-dose IVA therapy. We could not reveal why tumors with the same histology type respond so differently; therefore, we could not explain why high-dose IVA could not exert anti-tumor effect on selected lesions. Further studies, taking the complex intra- and extracellular microenvironmental factors, level of vascularization of individual lesions, the subsequent oxygenation, metabolic activity and Fe^{2+} levels of the tumor cells, into consideration, are needed to reveal how the efficacy of IVA could be enhanced.

Project III.

Ascorbate could kill completely *C. albicans* in PBS media at 37°C, when adequate oxygenation is provided with shaking. In another experimental setup the inhibition of ETC with antimycin boosted the efficacy of ascorbate to kill *C. albicans*. We could

assume that this can be further explained with the increased oxidative stress and subsequently elevated H_2O_2 concentration. Our attempt to assess the effect that the concentration of Fe^{2+} can exert on the killing potential of ascorbate revealed that cell-permeable iron-chelator bipyridil could inhibit the killing effect of ascorbate, thus free Fe^{2+} is essential to ascorbate to exert its antifungal killing potential.

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