The cytotoxic drug cyclo-pentenyl cytosine: from manufacturing to anti-tumor activity and (cardio)toxicity

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SUMMARY

In this thesis pharmaceutical aspects as well as anti-tumor activity and cardiotoxicity of the experimental cytostatic drug cyclopentenyl cytosine (CPEC) are explored. CPEC is derived from the nucleoside neplanocin A and structurally related to the clinically used cytostatic drugs cytarabine and gemcitabine.

By inhibiting the enzyme CTP-synthetase (cytidine triphosphate synthetase), CPEC decreases the 'de novo' synthesis of CTP from UTP (uridine triphosphate), resulting in depleted CTP pools. CTP can also be generated from the so called 'salvage pathway' out of cytidine. However, several malignancies have been shown to predominantly use the 'de novo synthesis' involving CTP synthetase. A depletion of CTP pools may ultimately lead to death as a result of impaired RNA and DNA synthesis and S-phase accumulation, resulting in cell death.

In **chapter 2** an overview is given of the studies undertaken with CPEC. Originally the drug was selected out of several analogues of neplanocin A based on its antiviral properties and indeed *in vitro* studies show activity against a wide range of viruses. However, its anti-tumor activity was considered more interesting for further development and no animal or human studies have been undertaken to investigate its antiviral properties.

CPEC has been most extensively studied in hematological malignancies and several *in vitro* and *in vivo* studies show activity of CPEC against leukemia. Other promising results were achieved in models for colorectal carcinoma and neuroblastoma.

There is one clinical trial published using CPEC in a phase I study in patients with solid tumors. Besides dose limiting hematological side effects, the most severe toxicity observed was cardiovascular. This side effect was seen in the higher dose groups.

As new trials with low dose CPEC were planned and only the raw substance was available, there was a need for a pharmaceutical formulation of CPEC. In **chapter 3** we describe the development of a stable sterile infusion concentrate that can be easily administered.

Because the cardiotoxicity as observed in the phase I trial was thought to be related to high CPEC plasma levels, therapeutic drug monitoring was considered to be necessary in future trials. In **chapter 4** a HPLC-MSMS method is described which enabled us to quantitatively determine low levels of CPEC. Although not at levels as low as CPEC, its metabolite CPEU (cyclopentenyl uridine), could also be detected with this method. CPEU is not thought to have anti-tumor activity and its plasma levels did not seem to be related with cardiotoxicity in the phase I study. Therefore, the higher limit of detection for CPEU was not considered as a problem for the use of the method in clinical trials.

Several *in vivo* studies showed promising activity of CPEC in leukemia. However, these studies used murine leukemic cells and no data were available with human leukemic cells. In **chapter 5** we analyzed the activity of CPEC on human acute lymphoblastic leukemia (ALL) cell lines *in vitro,* as well as on corresponding human primary ALL cells in a xenogeneic *in vivo* model using NOD/scid mice. Our *in vitro* results on five different human cell lines show activity of CPEC in the nanomolar range (IC50 6-15nM).

Based on their mechanisms of action and earlier *in vitro* results, combination therapy of CPEC with the cytotoxic drug cytarabine was suggested to have a synergistic effect. However, we detected no such effect on our leukemic cell lines after coincubation with CPEC and cytarabine. Therefore, single agent therapy with CPEC was studied in the NOD/scid mice inoculated with primary human ALL cells. Whereas no activity nor toxicity was seen in the lower dose ranges (0.5 mg/kg for 2 or 5 days per week) a marginal anti-leukemic activity was observed at 1.5 mg/kg (5 days per week) and 5 mg/kg (2 days per week), however, this activity was associated with severe systemic toxicity.

In the phase I study with CPEC the most severe side effect was cardiovascular. Cardiotoxicity induced by cytotoxic drugs is unfortunately not uncommon and in **chapter 6** several classes of cytotoxic drugs that have been associated with cardiotoxicity are reviewed. Anthracyclines are well known for this toxicity and its mechanism has been extensively studied. Oxidative stress seems to play an important role, however, apoptosis, genetic causes and influence on calcium homeostasis might also be involved. Prevention mainly consists of restricting the maximum cumulative dose and avoiding peak levels. Alternatively administration of a liposomal formulation or addition of a protective agent can be applied. However, none of these measures offer full protection.

Although not as frequently as anthracyclines, other cytotoxic drugs associated with cardiotoxicity are 5-fluorouracil, cyclophosphamide, cisplatin and more recently trastuzumab, imatinib and sunitinib.

The mechanism behind the severe hypotension associated with CPEC in the phase I trial had not been clarified. Before initiating clinical studies with CPEC it was necessary to investigate if the cardiotoxicity could be reproduced and what might have been the mechanism. **In chapter 7** we first studied the effects of CPEC on contraction force and frequency in a model using isolated atria of male Wistar rats. No changes in frequency were detected and although a trend in decrease of contraction force was observed, the differences were not significant. Our second hypothesis focused on the possible induction of apoptosis in the heart by CPEC. In an *in vivo* model we administrated CPEC to male Wistar rats and evaluated the presence or absence of apoptosis by 99mTc-AnnexinV scintigraphy, followed by postmortem determination of radioactivity in tissues, and histological confirmation. This model had been used before to demonstrate the apoptosis inducing potential of doxorubicin. We detected no increase in cardiac uptake of 99mTc-AnnexinV after treatment with CPEC, thereby having no indications for increased apoptosis in the heart.

With the models described in chapter 7 we only studied a few aspects that might have an association with cardiotoxicity. In **chapter 8** we investigated whether cardiotoxicity induced by cytotoxic drugs might have a genetic origin. Even in the case of anthracycline induced cardiotoxicity little is known about the role of genetics. We first attempted to reproduce the results of a previous study reporting an association between single nucleotide polymorphisms (SNPs) and doxorubicin induced cardiotoxicity. SNPs in genes related to the NAD(P)H oxidase enzyme complex (p22phox, p40phox and Rac2) and the drug efflux pumps MRP1 and MRP2 were investigated. We were able to reproduce the association between a SNP in Rac2 and anthracycline induced cardiotoxicity. No other associations were observed, which might have been caused by the relatively small size of our group of patients with anthracycline induced cardiotoxicity.

The role of the candidate genes was further explored by studying changes in expression after exposing rat cardiomyocytes to doxorubicin and CPEC. No changes in expression were seen for the genes involved in the NAD(P)H oxidase enzyme complex. However, after 24 hr of incubation doxorubicin decreased the expression of the MRP1 gene and CPEC seemed to induce a small decrease in the expression of MRP2.

Based on the association with the SNP in Rac2 and the decrease in expression in MRP1 and MRP2, we concluded that genetic aspects of the NAD(P)H oxidase enzyme complex and the efflux pumps may be involved in cytostatic drug induced cardiotoxicity.

In **chapter 9** the results from the studies in this thesis are discussed and future aspects are indicated. Although CPEC as a single agent in our leukemic *in vivo* model did not fulfill its promises, we believe that this agent might still have potential in combination with other agents in several malignancies. Its possible cardiotoxic side effects could not be reproduced in an animal model.

As demonstrated by the results of our last study cardiotoxicity of cytotoxic drugs might also have genetic aspects. However, we only studied expression profiles of a few genes and it is not unlikely that a vast amount of other genes might be involved as well. Moreover, the frequency of clinically overt cardiotoxicity is relatively low, indicating that a high number of patients would have to be genotyped in order to identify patients at risk. Therefore it may be concluded that pharmacogenetics will not provide an absolute answer but may be a valuable addition to the existing tools for the management of chemotherapy induced cardiotoxicity.