The pivotal role of CTP synthetase in the metabolism of (deoxy)nucleosides in neuroblastoma
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General discussion and recommendations
Introduction

Cytotoxic nucleoside analogues are a class of compounds that is widely used in anti-cancer therapy and in anti-viral therapy. Although synthesised over 2-3 decades ago, drugs like AraC, 5-Fluoro-uracil and 6-Mercaptopurine are still being used in the treatment of a variety of haematological and solid malignancies. The development of new nucleoside analogues is still ongoing. Detailed knowledge of nucleotide metabolism is of pivotal importance for both the design and elucidation of the mechanism of action of novel nucleoside analogues. New nucleoside analogues that in recent years have found their way to the clinic, as potent anti-cancer agents, are 2-Chlorodeoxyadenosine (cladribine, CdA), 1-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine, FAra-A) and 2',2'-difluorodeoxycytidine (gemcitabine, dFdC). In addition, many more cytotoxic nucleoside analogues have been developed and are being tested with the aim to improve the treatment of patients suffering from cancer or viral diseases.

As becomes clear from chapter 1, cytotoxic nucleoside analogues are currently not being used in the treatment of patients suffering from neuroblastoma. Because the prognosis for patients suffering from high-risk neuroblastoma has not increased significantly over the last decade, novel approaches towards the treatment of neuroblastoma have to be explored. One approach is to target the nucleotide metabolism of neuroblastoma cells (and other neural crest-derived tumours). The studies described in this thesis demonstrate that the nucleotide metabolism of neuroblastoma is a promising target for chemotherapy. Slingerland and colleagues were the first to demonstrate that CPEC has cytotstatic activity against human neuroblastoma in vitro (Slingerland et al., 1995a; Slingerland et al., 1995b). These results were part of a series of detailed measurements of the ribonucleotide metabolism of neural crest-derived cells that showed that an imbalance exists in the pyrimidine metabolism of these cells (Slingerland et al., 1995c; Slingerland et al., 1994). Furthermore, it was demonstrated that exponentially growing SK-N-SH cells use relatively more uridine than cytidine and that the contribution of CTP synthetase to the cytidine nucleotide pools in exponentially growing cells is more substantial than their differentiated counterparts (Slingerland et al., 1996).

In the present thesis, the effects of CPEC on neuroblastoma cells and mechanism of action are studied in more detail. The studies described in this thesis clearly demonstrate that inhibition of CTP synthetase by CPEC is an attractive strategy for chemotherapy in human neuroblastoma cells.

Combination therapy

The main effect of CPEC as single agent proved to be cytostasis (chapter 2). However, when CPEC was combined with AraC, dFdC or CdA cell death became a much more important effect. Although pre-incubation with CPEC increased the phosphorylation of all three deoxynucleoside analogues tested in SK-N-BE(2)c cells, there were significant differences between the different analogues. While SK-N-BE(2)c cells were sensitive to AraC and dFdC, they were resistant to CdA. dFdC was, by far the most potent, followed by AraC. In contrast to AraC, dFdC possesses self-potentiating characteristics as it is an inhibitor of RR and hence stimulates its own phosphorylation via dCK by virtue of dCTP depletion. Despite the fact that CdA is also a self-potentiating agent, no cytotoxic effects were observed in SK-N-BE(2)c cells, even though these cells did take-up CdA. CdA has a complex mechanism of action and also its metabolism is more complex than that of AraC and dFdC. An explanation for the resistance to CdA observed in SK-N-BE(2)c cells, may be competition between CdA
and the abundant dAdo nucleotide pools. Furthermore, CdA is metabolised to 2-Chloroadenine (CAde) (Bontemps et al., 2000) which is a substrate for APRT. From CAde, 2-Chloroadenosine-monophosphate is synthesised, which is subsequently phosphorylated to the di- and triphosphates. 2-Chloro-Adenosine is considerably less toxic than CdA (Bontemps et al., 2000), likely due to competition with the adenosine nucleotides.

A 24-hr pre-incubation with 100 nM CPEC, increased the sensitivity of SK-N-BE(2)c cells towards AraC and dFdC and, surprisingly, sensitised SK-N-BE(2)c for CdA as well. A relative accumulation of AraCMP and CdAMP was observed, which indicates that the rate-limiting factor in the anabolism of AraC and CdA in cells treated with CPEC is nucleoside monophosphate kinase activity. In the case of dFdC, the pool sizes of all dFdC metabolites were increased proportionally. This is in line with the findings of van Rompay and colleagues who have found that dFdCMP is a better substrate for UMP/CMP kinase than AraCMP (Van Rompay et al., 1999).

Proof of principle that pre-incubation with CPEC increases the sensitivity of neuroblastoma towards cytotoxic deoxycytidine analogues was obtained in chapter 5 in which sensitisation of neuroblastoma cells towards dFdC was demonstrated in a panel of cell lines. These results strongly suggest that CPEC would be a suitable drug to increase the efficacy of dFdC or AraC. The combination of CPEC and dFdC deserves further pre-clinical investigation in an in vivo model to develop this combination strategy towards a possible future clinical setting.

**Differentiation**

In chapter 6 it was demonstrated that retinoic acid-induced differentiation rendered SK-N-BE(2)c cells resistant to CPEC. The probable causes of this phenomenon were decreased proliferation and increased salvage of cytidine, which bypasses the inhibition of CTPs. The combination of CPEC and retinoic acid is, therefore, not an attractive combination for chemotherapy. Our observation is in line with those of others who have found that upon differentiation with retinoic acid, neuroblastoma cells became resistant to DNA damaging agents (Biedler et al., 1991;Lasorella et al., 1995;Ronca et al., 1999). A possible explanation might be that upon differentiation the expression of MYCN decreases (Han et al., 1995;Spengler et al., 1997). MYCN has an important function in the induction of cell death, as it sensitises neuroblastoma cells for drug-induced apoptosis (Fulda et al., 1999). With respect to combination therapy involving retinoids and genotoxic agents, encouraging results have been obtained using the synthetic retinoid fenretinide. Fenretinide is thought to induce apoptosis by induction of oxidative stress via the induction of free radicals (Shen et al., 1999;Sun et al., 1999). Combination of fenretinide with cisplatin and carboplatin led to a synergistic increase in the degree of apoptosis induced (Lovat et al., 2000).

Induction of differentiation, instead of cell death, by CPEC and dFdC, as we observed in chapters 2 and 5, may be regarded as useful characteristic of these drugs. A commonly encountered problem with chemotherapy is the tumour-lysis syndrome. Because many tumour cells become necrotic at the same time, their contents are spilled into the patients' bloodstream, which leads to immune responses and fever. If differentiation induced by CPEC and dFdC proofs to be irreversible, which still has to be investigated, this would be very useful in the treatment of neuroblastoma. After having reached a fully differentiated state, cells are thought to be removed from the body by means of apoptosis.

Why all tested MYCN-single copy cell lines and some MYCN-amplified cell lines differentiate when they are incubated with CPEC or dFdC is unclear. When cells are induced to differentiate by natural agents, they loose, as a consequence, their self-renewal capacity. It may be that in the cells, in which differentiation was observed after treatment with CPEC or
dFdC, the process of differentiation is blocked, thus favouring proliferation over differentiation. However, when proliferation is inhibited by treatment with CPEC or dFdC, the process of differentiation may, as a consequence, be allowed to proceed. Previously, it has been suggested that, during replication of the cellular genome in the S-phase of the cell cycle, the cell is highly susceptible agents that induce differentiation by disturbance of the tight regulation of DNA precursor synthesis and utilisation (Hatse et al., 1999). Further characterisation of the changes that are induced in these cell lines upon incubation with cytotoxic nucleoside analogues would enhance the knowledge on the differentiation inducing properties of cytotoxic nucleoside analogues. It would be interesting to study the expression of genes that are associated with differentiation, cell-cycle control and apoptosis, such as p53, pRb, bcl-2 and Id2 upon treatment with CPEC and dFdC. The expression/activity profiles of enzymes involved in the catecholamine biosynthesis could also provide information regarding the degree of differentiation of cells obtained after treatment with CPEC or dFdC.

Regulation of dCK activity and expression

dCK is the rate-limiting enzyme in the anabolism of deoxynucleoside analogues. We observed that CPEC caused a depletion of the intracellular (d)CTP pool and concomitantly increased the phosphorylation of AraC, dFdC and CdA. The decreased CTP pool results in depletion of the dCTP pool, which in turn causes inhibition of DNA synthesis and increases the activity of dCK. The activity and/or the expression of dCK is reported to be dependent on four factors:
1. dCK activity is regulated via feedback inhibition by dCTP. Thus, low intra-cellular levels of dCTP increase the activity of dCK (Ives and Durham, 1970).
2. The activity of dCK may be cell-cycle dependent, while the mRNA expression is not. Cell-cycle dependent regulation of dCK activity is a much-debated subject, but appears to depend on the type of cell line studied (Arner and Eriksson, 1995).
3. Inhibition of DNA synthesis and DNA damage causes an increase in the expression and activity of dCK (Sasvari-Szekely et al., 1998) (Csapo et al., 2001).
4. The dCK activity is regulated by post-translational modification. Our results suggest that in SK-N-BE(2)c cells all four factors mentioned influence the dCK activity. The fact that the phosphorylation of AraC and dFdC correlates with the cell-cycle distribution is in line with increased dCK activity during the S-phase of the cell-cycle. However, after 4 days of incubation with 100 nM CPEC, the intracellular levels of UTP, ATP and GTP are reduced by 50% when compared to non-CPEC treated controls, while the in vitro dCK activity remained elevated. Since UTP and ATP are the phosphate donors utilised by dCK, this may be responsible for the observed decline of the phosphorylation of AraC and dFdC after 4 days of incubation with 100 nM CPEC. The effect observed might still, in part, be cell-cycle dependent. Up to 70% of the cells were arrested in the G0/G1-phase of the cycle, after 4 days of incubation with 100 nM CPEC. The intracellular concentration of ribonucleotides of cells in the G0/G1-phase of the cycle is 30-70% less than that of cells in the S-phase of the cell cycle (van den Berg et al., 1995; McCormick et al., 1983). The observed depletion of UTP, ATP and GTP after 4 days of incubation with CPEC might thus be a combined effect of cell-cycle distribution and prolonged exposure to CPEC.

Upon incubation with CPEC, the phosphorylation of AraC and dFdC remains elevated when compared to non-CPEC treated controls, even after 4 days of incubation with 100 nM CPEC. This suggests that dCK activity is regulated in part by the dCTP pool. The mRNA expression increased upon incubation with CPEC and remained elevated, as was the case for the amount of dCK protein and activity measured in cell lysates. These results suggest that the expression of dCK is increased as a response to inhibition of DNA synthesis or to depletion of (d)CTP.
In chapter 7, a non-radioactive procedure to measure the dCK activity in cell lysates is described. In contrast to the elaborate standard procedures using substrates and thin layer chromatography (TLC) or weak-ion exchange paper chromatography, we developed a reversed-phase HPLC procedure with UV detection. By doing so, we observed a novel metabolite of CDA in cell lysates of MOLT-3 cells, the deamination product 2-Chloro-deoxinosine (CdI), which we probably would not have detected using TLC or paper chromatography. This finding demonstrates that CDA is not resistant to deamination by ADA. CdI is a very interesting topic of study, because knowledge of its biochemical characteristics may increase the knowledge and understanding of the metabolism and cytotoxicity of CDA. In this respect, it would be interesting to investigate whether or not CdI is a substrate for the human (deoxy)nucleoside kinases dCK, dGK and Adenosine kinase. It would also be very interesting to find out if CdIMP is a substrate for adenylosuccinate synthetase. If so, adenylosuccinate synthetase may catalyse the conversion of CdIMP to succinyl-2-Cl-dAMP, which in turn would be converted back to CdAMP by adenylosuccinate lyase. Conversion of CdIMP to CdAMP via the proposed pathway would salvage the therapeutically valuable metabolites of CdA.

Theoretically, CdIMP may inhibit the synthesis of GMP from IMP via XMP, leading to an accumulation of IMP. Considering the chlorine atom at the 2-position of the purine ring of CdI, CdIMP may be a substrate for IMP dehydrogenase. However, the potential reaction product, 2-Chloro-dXMP, cannot be converted to GMP by GMP synthetase as the chlorine at the 2-position blocks transfer of the amino-group to this position.

Suggested studies for the future

Our studies show that administration of low, but biochemically active, concentrations of CPEC is a powerful tool to modulate the cytotoxicity of AraC, dFdC and Cda. As a single drug, CPEC had a profound cytostatic effect on neuroblastoma cells. However, for a possible future clinical application of CPEC in the treatment of neuroblastoma, or any other solid tumour, its' potential must be sought in combination therapy. Because CPEC caused cytostasis rather than cell death, from which MYCN-amplified neuroblastoma cells are capable to recover, it would be better to induce cell death in vivo by combining CPEC with e.g. dFdC.

As of September 2002, a phase I/II clinical trial has been started investigating the pharmacokinetic properties and therapeutic effect of CPEC in patients suffering from leukaemia. Given our results obtained neuroblastoma cell lines and pending the results of the clinical trial, we feel that the possibilities of the use of CPEC for the treatment of neuroblastoma should be further developed. Moreover, from our results, it appeared that dFdC is a very promising agent for the treatment of neuroblastoma as well. In addition to the suggestions above, the following studies are suggested.

- In chapter 5 we showed that dFdC is a promising drug for the treatment of neuroblastoma. In neuroblastoma cells that express MYCN, cell death is induced after exposure to dFdC, while this is not the case in cells that do not express MYCN. Further investigation of the effect of MYCN-expression on the expression and activity of enzymes from the pyrimidine pathway that are either targets or detoxifying enzymes is, therefore, warranted. The uptake and metabolism of gemcitabine and CPEC should be studied in detail in a panel of neuroblastoma cell lines consisting of both MYCN-amplified and MYCN-single copy cell lines. These experiments should also be performed in the Shep21N cell line, in which MYCN expression can be turned off after addition of tetracycline to the culture medium. Measurement of the flux of radioactive gemcitabine metabolites combined with in vitro enzyme activity assays will give detailed information on the expression of the enzyme
systems involved in both anabolism and degradation of gemcitabine and CPEC in neuroblastoma. Primary target-enzymes to be studied are CTP synthetase, deoxycytidine kinase, ribonucleotide reductase, (deoxy)cytidine deaminase and uridine/cytidine kinase.

- In this thesis, it is demonstrated that inhibition of CTP synthetase by CPEC stimulates the anabolism and increases the cytotoxicity of the cytotoxic nucleoside analogues AraC, dFdC and CdA. An elegant path to follow would be to target CTP synthetase at the level of mRNA expression, preferentially by stable, inducible, expression of short interfering RNA (siRNA) with subsequent administration of AraC or dFdC and measurement of dCK activity. This way, interference in metabolic pathways other than the synthesis of CTP is excluded, hence providing proof of principle that inhibition of CTP synthetase stimulates the anabolism of deoxycytidine analogues.

- Isolation of mammalian, preferentially human, CTP synthetase and subsequent kinetic experiments to determine the number of nucleotide binding sites and their possible catalytic and regulatory function will provide a better understanding of the function of the enzyme. It may also contribute to the design and synthesis of new inhibitory small molecules with different toxicity profiles than CPEC.

- A characteristic of dFdC, which certainly deserves attention, is its ability to increase the sensitivity of cells towards ionising radiation (radio-sensitisation or radio enhancement). The mechanism by which dFdC increases radio-sensitivity is still largely unclear. In some cell lines, radio-sensitisation has been associated with redistribution of the cells to S-phase of the cell cycle, in which they are most sensitive towards ionising irradiation (Latz et al., 1998). Radio-sensitisation by dFdC has also been associated with depletion of the intracellular dATP pool (Shewach and Lawrence, 1995). Because of its radio-sensitising potential, phase I/II clinical trials have been performed combining gemcitabine with external beam irradiation radiotherapy in adult patients. Although the results are promising, severe toxicity in the field of irradiation due to sensitisation of normal tissue has been reported (Wolff et al., 2001; Eisbruch et al., 2001). Studies in mice, however, suggest that normal tissues recover more quickly from gemcitabine treatment than tumour tissue (Milas et al., 1999). Targeting of radioactivity to gemcitabine radio-sensitised tumour cells would serve a dual purpose. The efficacy of combined chemo- and radiotherapy may be increased, and the injuries inflicted on normal tissue may be reduced to a minimum. With respect to the use of radio-sensitising agents, the biology of neuroblastoma cells provides a potentially effective and elegant possibility for combined chemo- and radiotherapy. As mentioned in chapter 1, neuroblastoma cells selectively accumulate the norepinephrine analogue $^{[131]}\text{I}\text{MIBG}$. Pre-treatment with dFdC will radiosensitise both normal and neuroblastoma tissue, while radioactivity is selectively targeted to neuroblastoma tissue using $^{[131]}\text{I}\text{MIBG}$, thus sparing normal tissue. As mentioned, a major side effect of radio-sensitising agents when combined with external beam irradiation is severe injury to healthy tissue in the field of irradiation. This would theoretically be circumvented by the proposed combination of a radio-sensitising agent with addressed radiotherapy.

- Continuing this line of thought of combined chemo-radiotherapy, it would be interesting to study the role of mitochondria in gemcitabine- and ionising irradiation-mediated cell death. The gene coding for the initiator caspase, caspase-8 has been reported to be silenced or deleted in most MYCN-amplified, poor prognosis neuroblastomas (Teitz et al., 2000). However, other apoptotic routes can bypass induction of apoptosis by caspase-8.
For instance, irradiation of mitochondria is a primary event that initiates ionising irradiation-induced apoptosis (Taneja et al.). Furthermore, Rebbaa and co-workers provided evidence that in neuroblastoma cells apoptosis, induced by genotoxic agents, is mediated by mitochondria and downstream apoptotic signalling intermediates (Rebbaa et al., 2001). Gemcitabine is primarily phosphorylated by dCK, but is also a substrate for deoxyguanosine kinase, which is located in the mitochondria, and may therefore be metabolised in mitochondria (Sjoberg et al., 1998). Incorporation of gemcitabine into mitochondrial DNA might enhance the effect of ionising irradiation on mitochondria and thus increase the induction of apoptosis. To verify this hypothesis, mitochondria from gemcitabine treated neuroblastoma cells need to be isolated, and mitochondrial dFdC metabolites as well as dFdC incorporated into mtDNA need to be measured. The role of mitochondria in the induction of cell death could be measured by adding of irradiated mitochondria and mitochondria isolated from irradiated neuroblastoma cells, with and without prior incubation with gemcitabine, into naive cell-free extracts. The increase in the activity of effector caspases (caspase-3), compared to untreated controls, would provide a measure for the cell death induced by the added mitochondria.

Summary of recommendations:

Recommendations for future research:
- The combination of CPEC and AraC or dFdC should be developed further in a pre-clinical setting.
- Proof of principle that inhibition of CTPs is responsible for the increased anabolism of deoxynucleoside analogues should be obtained.
- Detailed studies of the metabolism of dFdC in a panel of neuroblastoma cell lines are recommended.
- The effect of the (over)expression of MYCN on the purine and pyrimidine metabolism in neuroblastoma is highly recommended.

Recommendations for a possible clinical setting:
- Retinoic acid should be used with great caution when it is to be combined with an inhibitor of CTP synthetase or inhibitor of CTP de novo synthesis.
- The combination of CPEC with AraC or dFdC is likely to work best when AraC or dFdC are administered after CPEC rather than simultaneously.
- Ideally, the dCK activity should be measured in a tumour biopsy when treatment with a deoxynucleoside analogue is intended.
- The use of dFdC for the treatment of neuroblastoma may be attractive to explore.

References


General discussion and recommendations


"It may be raining, but there's a rainbow above you"

_Eagles_