The pivotal role of CTP synthetase in the metabolism of (deoxy)nucleosides in neuroblastoma
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"Nucleotides are water-soluble components which naturally occur, in larger or smaller portions, in both animal and vegetable foods. Combined with other components, they are the elements to bring the flavor in food."

from the Ajinomoto Europe website
Chapter 1

Introduction

1.1 General introduction to neuroblastoma

Incidence
Neuroblastoma is the most common extra cranial solid cancer of childhood. It is the most frequently occurring form of cancer in children after brain tumours, acute lymphatic leukaemia (ALL) and lymphoma. About 7% of all malignancies in children under 15 years of age are neuroblastomas. The reported incidence of neuroblastoma varies between 9 and 35 per million children, with slight variations between different parts of the world. Boys appear to have a 1.2-fold higher risk of developing neuroblastoma compared to girls (National Cancer Institute, 2002; Brodeur and Castleberry, 1997).

Symptoms
Neuroblastoma is a tumour of the neural crest and originates in the adrenal medulla or the paraspinal sites where sympathetic nervous tissue is present. The most common symptoms are a large tumour mass or pain in the bones caused by metastasis. Other common symptoms are bulging eyes (proptosis) and bruises around the eye (periorbital ecchymosis) and are caused by retrobulbar metastasis. Neuroblastomas can invade through the neural foramina and cause paralysis by compressing the spinal cord. Other, less frequently occurring symptoms include fever, anaemia, hypertension, diarrhoea, cerebellar ataxia, opsoclonus and myoclonus (National Cancer Institute, 2002; Azizkhan and Haase, 1993; Connolly et al., 1997; Rudnick et al., 2001).

Diagnosis
Beside the brain, cells derived from the neural crest are the primary source of neurotransmitters, such as dopamine, epinephrine and norepinephrine in the body. These catecholamines are synthesised from L-3,4-dihydroxyphenylalanine (L-DOPA), which in turn is synthesised from tyrosine. Normal, mature neural crest-derived cells store their neurotransmitters in secretory vesicles until release. After excretion, neurotransmitters exert their activity on other cells that are sensitive to neurotransmitters. The neurotransmitters are deactivated by presynaptic uptake, conjugation to sulphate or glucoronic acid and finally by excretion in the urine. In patients suffering from neuroblastoma, an increased production and subsequent secretion of catecholamines in urine is observed. Determination of metabolites of L-DOPA in urine is routinely used in the diagnosis of neuroblastoma.

The biosynthesis and degradation of the metabolites of L-DOPA is catalysed by a large number of enzymes. In mature, differentiated, neuronal cells, all the enzymes of the catecholamine metabolic pathway are expressed and thus the end-products of this pathway are synthesised. In less differentiated cells, such as neuroblastoma cells, not all enzymes involved in catecholamine biosynthesis are expressed. Undifferentiated neuroblastoma cells synthesise an increased amount of dopamine and DOPA (early metabolites in the DOPA-metabolism) compared to norepinephrine. The pattern of the degradation metabolites of L-DOPA in urine usually reflects the relative activities of the enzymes involved in the metabolism of L-DOPA. Thus, the pattern of L-DOPA metabolites excreted in urine serves as a marker for the detection of neuroblastoma and in addition provides information on the degree of differentiation of the tumour (Abeling et al., 1986).

Alternatively, neuroblastomas can also be detected by using \[^{131}\text{I}\] or \[^{125}\text{I}\] radio-labelled meta-iodobenzylguanidine (MIBG). MIBG is a structural analogue of the neurotransmitter norepinephrine. MIBG is recognised by the norepinephrine transport protein and accumulates in neuroendocrine tissue after its administration to the patient (Wieland et al., 1980).
Neuroblastoma cells abundantly express the norepinephrine transport protein, and therefore accumulate the radio-labelled MIBG thus allowing the detection of the neuroblastoma by scintigraphy. In rare cases, neuroblastoma can be discovered prenatally by foetal ultrasonography (National Cancer Institute, 2002) (Jennings et al., 1993).

### INTERNATIONAL NEUROBLASTOMA STAGING SYSTEM (INSS)

<table>
<thead>
<tr>
<th>INSS Stage</th>
<th>Description</th>
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<tbody>
<tr>
<td>Stage 1</td>
<td>Localised tumour with complete gross excision, with or without microscopic residual disease. Representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive. (ipsilateral: situated or appearing on or affecting the same side of the body)</td>
</tr>
<tr>
<td>Stage 2A</td>
<td>Localised tumour with incomplete gross excision. Representative ipsilateral non-adherent lymph nodes negative for tumour microscopically.</td>
</tr>
<tr>
<td>Stage 2B</td>
<td>Localised tumour with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically. (contralateral: occurring on or acting in conjunction with a part on the opposite side of the body)</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; localised unilateral tumour with contralateral regional lymph node involvement; midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column. Tumours originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S).</td>
</tr>
<tr>
<td>Stage 4S</td>
<td>Localised primary tumour (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver, and/or bone marrow (limited to infants less than 1 year age). Marrow involvement should be minimal (i.e. &lt; 10% of total nucleated cells identified as malignant by bone biopsy or by bone marrow aspirate). More extensive bone marrow involvement would be considered to be stage 4 disease. The results of a MIBG scan should be negative for disease in the bone marrow.</td>
</tr>
</tbody>
</table>

Table 1: International Neuroblastoma Staging System (Brodeur et al., 1993; Brodeur et al., 1988; Castleberry et al., 1997)
**Staging and prognosis**

The prognosis for children suffering from neuroblastoma is highly dependent on the age at diagnosis and on the stage of the disease. The disease is characterised by 5 distinct prognostic stages. The staging of the disease according to the International Neuroblastoma Staging System (INSS) is outlined in table 1. Approximately 75% of the children with neuroblastoma have metastasised disease at the time of diagnosis. About 20% of the children with neuroblastoma have stage I or II disease. Regardless of age, these children with stage I or II disease have a cure rate of over 90%. Approximately 20% of the children suffering from neuroblastoma present with stage III disease and ± 55% present with stage IV disease. The prognosis for patients with stage III/IV disease is poor with a survival rate of 10-25%, but strongly depends on the age of the patient. Children younger than 1 year of age, with favourable disease characteristics at diagnosis, have a 50-80% chance of 5-year event free survival, regardless of the stage of the disease. The long-term survival of children older than one year of age ranges from 10-40%. Stage IVs neuroblastomas comprise approximately 5% of all neuroblastomas and present mostly in very young infants. These tumours have a high rate of spontaneous regression and the 5-year survival is greater than 90% (Cotterill et al., 2000; National Cancer Institute, 2002).

**1.2 Genetic prognostic markers**

The clinical diversity of neuroblastoma correlates with several characteristic molecular biological features observed in neuroblastoma. The most significant prognostic factors are amplification of the MYCN oncogene, tumour cell ploidy, deletions of the short arm of chromosome 1 and gain of chromosome 17q.

Amplification of the MYCN oncogene has been demonstrated in about 25% of primary, predominantly stage 4, neuroblastomas, and its amplification is associated with rapid tumour progression an poor outcome (Brodeur et al., 1984; Seeger et al., 1985). The MYCN gene was cloned in 1983 (Schwab et al., 1983), by identifying an amplified DNA sequence with partial homology (38% amino acid identity) to the c-my c proto-oncogene in neuroblastoma cell lines with double minute chromatin bodies (DMs) and homogeneously staining regions (HSRs). Both DMs and HSRs represent DNA amplification and deletions. In neuroblastoma, HSR regions have been identified containing 50-400 copies of the MYCN gene per cell (Seeger et al., 1985).

Myc proteins are important regulators of proliferation in vivo. The MYCN gene product, MycN, is a nuclear phosphoprotein with a short half-life of approximately 35 minutes. MycN contains an N-terminal transactivation domain and a C-terminal region containing a basic helix-loop-helix/leucine zipper (bHLH-LZ) motif. The latter motif mediates DNA binding as well as interactions with other bHLH-LZ proteins, like Max (Wenzel et al., 1991) and Mad (Ayer et al., 1993). In order to activate transcription, MycN has to heterodimerise with Max. Max is a ubiquitously expressed protein with a long half-life (> 4 hr). Normally, Max expression levels are high and favour the formation of Max/Max homodimers that repress transcription. In addition, heterodimerisation of Max with other nuclear protein such as Mad and Mxi1 also function to repress transcription by competing with MycN for Max binding (Zervos et al., 1993). When cells enter the cell cycle, or when multiple MYCN copies are present, as in aggressive neuroblastoma cells, MYCN expression increases and heterodimerisation of Max and MycN occurs. This results in the transcriptional activation of a large set of genes that function in ribosome biogenesis and protein synthesis (Boon et al., 2001). MYCN functions as a classic dominant oncogene, which is demonstrated by the following observations. In vitro enhanced MYCN gene expression in human primary fibroblasts induced increased growth potential and tumorigenicity (Lutz et al., 1996), while
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Antisense mediated down-regulation in cancer cells of MYCN expression correlates with decreased proliferation and induction of differentiation (Negroni et al., 1991). In addition, targeted expression of the MYCN oncogene in neuroectodermal cells of transgenic mice results in the development of neuroblastoma (Weiss et al., 1997).

The DNA content of tumour cells is also a prognostic factor in neuroblastoma, with hyperdiploidy being associated with a favourable prognosis while diploidy is indicative of poor prognosis. Hyperdiploid tumours typically have whole chromosome gains with few structural rearrangements, indicating that these tumours have a basic defect in the machinery of mitosis and chromosome segregation (Look et al., 1991). The majority of neuroblastoma cell lines and advanced primary tumours either have a near-diploid or near tetraploid DNA content, as a result of genomic instability.

Deletion of chromosome 1p is observed in 19% to 36% of primary tumours (White et al., 1995; Maris et al., 1995; Caron et al., 1996). The importance of genes located on the short arm of chromosome 1, with respect to prognosis in neuroblastoma was first recognised by Brodeur and colleagues in 1977 (Brodeur et al., 1977). In general, deletion of part of chromosome 1p (1p LOH) is associated with an aggressive clinical course and poor outcome of the disease (Maris et al., 1995). The majority of tumours with 1p LOH have amplification of the MYCN oncogene, (White et al., 1997), which complicates the appreciation of the significance of 1p LOH as a sole prognostic marker (Gehring et al., 1995).

To date, the tumour suppressor genes located on the often deleted part of chromosome 1p have not yet been identified (Caron et al., 1995). There appear to be two distinct neuroblastoma tumour suppressor loci on chromosome 1p35-36. One of these loci is associated with MYCN amplification, which causes loss of alleles of apparent random parental origin. Another locus, however, is associated with MYCN single copy tumours in which the lost alleles on chromosome 1p are preferentially of maternal origin. Recently, however, evidence was obtained that no imprinted tumour suppressor gene is located on chromosome band 1p (Hogarty et al., 2002)(H.N. Caron, personal communication).

Gain of chromosome 17q may be the most common genetic abnormality in primary neuroblastomas, and it is often associated with amplification of MYCN and with deletions of the short arm of chromosome 1 (Bown, 2001; Savelyeva et al., 1994). Gain of 17q proved to be significantly associated with unfavourable outcome (Caron et al., 1996). The genes nm23-H1 and nm23-H1, which are located in chromosome 17q, were shown to be upregulated in MycN-expressing cells, suggesting a major role of these genes in the tumourigenesis of unfavourable neuroblastomas (Godfried et al., 2002).

1.3 Current treatment options

The treatment protocol for patients suffering from neuroblastoma is strongly dependent on the age of the patient, the stage of the disease and genetic prognostic factors like MYCN-amplification and ploidy. Localised tumours are cured by surgery followed by chemotherapy, depending on the molecular markers of the tumour. The treatment of inoperable or stage 4 metastatised neuroblastoma requires aggressive chemotherapeutic regimens, which are sometimes combined with radiotherapy. Chemotherapy is based on combined treatment with two or more cytostatic drugs. The chemotherapeutic strategies currently used for the treatment of neuroblastoma are often combinations of the following drugs: the alkylating agents cyclophosphamide, ifosfamide and dacarbazine; the anthracyclins doxorubicin and daunorubicin; the platinum compounds cisplatin and carboplatin; the epidophyllotoxins etiposide and tenoposide; and the vincaalkaloid vincristine. These currently used cytostatic drugs all serve to prevent the proliferation of the tumour cells, either
by causing irreparable damage to DNA or by preventing the cell from completing the cell cycle, in both cases inducing programmed cell death.

The Emma Kinderziekenhuis protocol for treatment of inoperable neuroblastoma patients includes first line treatment with $^{[131]}\text{I}^{\text{MIBG}}$, followed by chemotherapy and/or surgery. Treatment of neuroblastoma patients with $^{[131]}\text{I}^{\text{MIBG}}$ in most cases leads to a decrease of tumour burden, reducing the need for extensive chemotherapy, making surgery possible, and in some exceptional cases unnecessary (de Kraker et al., 1995). The specific uptake of $^{[131]}\text{I}^{\text{MIBG}}$ by neurendocrine tissue allows both diagnostic and therapeutic application of this agent in the treatment of neural crest derived tumours (Wieland et al., 1980). The mode of action of $^{[131]}\text{I}^{\text{MIBG}}$ is twofold: $^{[131]}\text{I}$ is both a $\beta$-emitting and a $\gamma$-emitting isotope, making $^{[131]}\text{I}^{\text{MIBG}}$ suitable for radio-therapeutic application. Due to the tissue specificity of $^{[131]}\text{I}^{\text{MIBG}}$ radio-therapy, side effects of the treatment are remarkably low (Voute et al., 1988). The most common side-effects of treatment with $^{[131]}\text{I}^{\text{MIBG}}$ are reversible thrombocytopenia and reduced thyroid reserve.

New and still experimental, protocols include maintenance treatment with 13-cis retinoic acid after the last cycle of chemotherapy. 13-cis Retinoic acid and its isomers all-trans retinoic acid 9-cis retinoic acid are strong inducers of differentiation in neuroblastoma cell lines. The rationale behind the retinoic acid treatment is two-fold: firstly, fully differentiated cells do not proliferate and thus the disease is kept in remission. Secondly, the apoptotic machinery is thought to be highly expressed in differentiated cells. DNA damage induced by chemotherapy followed by induction of differentiation by retinoic acid may increase the apoptotic response of the tumour cells.

Despite aggressive therapeutic strategies and intensive research, the likelihood for survival has not increased significantly for patients suffering from neuroblastoma. Therefore, new and effective strategies need to be developed and evaluated in an effort to increase the survival of patients suffering from high-risk neuroblastoma.

1.4 Nucleotide metabolism

Introduction to bases, nucleosides and nucleotides

Nucleotides are the building blocks from which the cell synthesises its DNA and RNA. A ribonucleotide is made up of a ribose ring in which the carbon atoms are numbered 1' to 5' (figure 1). In case of a deoxyribonucleotide, the 2' hydroxyl group is reduced to a hydrogen atom. At the 1' position a nitrogenous cyclic base is covalently bound. At the 5' position of the ribose, an inorganic mono-, di, or triphosphate ester is bound. The phosphate ions are designated $\alpha$, $\beta$ and $\gamma$, the $\alpha$ phosphate group being the one adjacent to the ribose. A nucleotide without the 5' phosphate ester is called a nucleoside. There are two families of nitrogenous bases: the purines and the pyrimidines. The most abundant purine bases are adenine and guanine, their respective (deoxy)ribonucleosides being (deoxy)adenosine and (deoxy)guanosine. Both DNA and RNA contain adenine and guanine. The pyrimidine bases are cytosine, uracil and thymine. The corresponding ribonucleosides of cytosine and uracil are cytidine and uridine, respectively. Both cytidine and uridine are incorporated into RNA. The pyrimidine ribonucleotides incorporated into DNA are deoxyctydine and thymidine, the corresponding deoxyribonucleoside of thymine.
examples of a nucleoside and its nucleotides

adenosine  adenosine-5'-monophosphate  adenosine-5'-diphosphate  adenosine-5'-triphosphate

components of natural occurring nucleosides

purine

pyrimidine

natural occurring bases

adenine

guanine

cytosine

thymine

uracil

natural deoxynucleosides

deoxyadenosine  deoxyguanosine  deoxycytidine  thymidine

Figure 1: Chemical structures of nucleosides and nucleotides.
Chapter 1

Pyrimidine metabolism

Most of this thesis is focused on the metabolism of pyrimidine nucleotides. Therefore, pyrimidine metabolism, which is a complex network of biochemical reactions (figure 2), will be described in detail. Besides their function in DNA and RNA synthesis, the pyrimidines are also required for other cellular processes such as phospholipid metabolism and glycosylation of proteins. The first three steps in the de novo synthesis of the pyrimidines are catalysed by CAD, a trifunctional enzyme cluster that contains the Carbamylphosphate synthetase, Aspartate carbamyltransferase and Dihydro-orotase activities (Ito et al., 1970; Jones, 1980). Dihydro-orotate is reduced to yield orotate by dihydro-orotate dehydrogenase (Chen and Jones, 1976). UMP is subsequently synthesised from orotate by the bifunctional enzyme UMP synthetase, which contains the orotate phosphoribosyl transferase and orotidine-5′-phosphate decarboxylase activities. Subsequent phosphorylation of UMP by nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK), respectively, yields UTP.

CTP synthetase (CTPs) catalyses the conversion of UTP into CTP and is considered to be the rate-limiting enzyme in the de novo synthesis of CTP from UTP. Mammalian CTP synthetase is a 66 kDa protein (Thomas et al., 1988). The enzyme may be present in dimeric and a more active tetrameric form, the latter being favoured at high enzyme concentrations and in the presence of ATP and UTP. To date, two isoforms of human CTP synthetase have been described. The gene encoding the most abundantly expressed isoform has been located on chromosome lp34.1 and the low expression isoform has been located on chromosome Xp22 (Takahashi et al., 1991; van Kuilenburg et al., 2000).

From CTP, the cytosine ribonucleotides CDP and CMP are synthesised as well as precursors for phospholipid biosynthesis. CDP is a substrate for ribonucleotide reductase (RR), which catalyses the reduction of all ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates (dNDP's). dNDP's are subsequently phosphorylated to dNTP's by NDPK and incorporated into DNA, with the exception of dUTP. Although UDP is a substrate for RR, dUTP is only erroneously incorporated into DNA and directly excised by uracil-DNA glycosylase (Verri et al., 1992; Focher et al., 1990). Both dUDP and dUTP are a source of dUMP, from which TMP is synthesised by thymidylate synthase (TS). dUMP is synthesised by hydrolysis of dUTP to dUMP via dUTPase and by deamination of dCMP via dCMP deaminase. TMP is the precursor of TTP, which is ultimately incorporated into the DNA.

There is also an alternative route for the cell to synthesise the pyrimidine nucleotides and that is by salvage of uridine and cytidine. Salvage is the phosphorylation of a nucleoside by a nucleoside kinase and the subsequent phosphorylation to its nucleoside triphosphate by NMPK and NDPK, respectively. While NMPK and NDPK are universal, nucleoside kinases are not. Uridine and cytidine are phosphorylated by uridine/cytidine kinase (UK), deoxycytidine is phosphorylated by deoxycytidine kinase (dCK) and by mitochondrial thymidine kinase 2 (TK-2), while thymidine and deoxyuridine are phosphorylated by thymidine kinase 1 (TK-1) and TK-2. The reverse reaction is catalysed by the enzyme 5'-nucleotidase, which has a broad substrate specificity.
The pyrimidines are degraded via a common pathway. First, cytidine and deoxycytidine are converted into uridine and deoxyuridine, respectively, by (deoxy)cytidine deaminase. Uridine phosphorylase and thymidine phosphorylase catabolise (deoxy)uridine and thymidine into uracil and thymine, respectively. Uracil and thymine are degraded in three steps to β-alanine and β-aminoisobutyrate, respectively, by subsequently dihydropyrimidine dehydrogenase (DPD), dihydropyrimidase (DHP) and β-ureidopropionase. The amino groups of the pyrimidine degradation end products are removed by transamination to yield malonyl semialdehyde and methylmalonyl semialdehyde, respectively, which are converted into malonyl CoA and methylmalonyl CoA. Via conversion of malonyl CoA and methylmalonyl CoA into propionyl CoA and succinyl CoA, respectively, the pyrimidine degradation enters the citric acid cycle (TCA cycle) (Hatse, 1999; Slingerland, 1996).

**Purine metabolism**

One of the cytostatic drugs described in this thesis is 2-Chloro-deoxyadenosine, an analogue of deoxyAdenosine. For that reason, a short overview of the purine biosynthesis will be given. The precursor of all nucleotides is phosphoribosylpyrophosphate (PRPP). PRPP is synthesised from ribose-5-phosphate and ATP by PRPP synthetase. Via a series of ten reactions the central purine nucleotide IMP is synthesised (figure 3). IMP can be phosphorylated further to ITP by NMPK and NDPK, respectively. ITP is, however, actively hydrolysed back to IMP by ITPase. From the branch-point nucleotide IMP, the adenine nucleotides are synthesised from the intermediate succinyl-AMP, resulting in AMP. AMP is subsequently phosphorylated to ADP and ATP by AMP kinase and NDPK, respectively. The other purine nucleotides, the guanine nucleotides, are synthesised via xanthine monophosphate (XMP), formed from IMP by IMP dehydrogenase (IMPDH). GMP is subsequently synthesised from XMP by GMP synthetase. GDP and GTP are synthesised from GMP by subsequent phosphorylation by GMP kinase and NDPK. IMPDH is the rate-limiting enzyme in the synthesis of the guanosine nucleotides. Like the pyrimidine deoxyribonucleotides, the purine deoxyribonucleotides are synthesised by reduction of ADP and GDP by RR.

The purine salvage pathway of the (deoxy)nucleosides is analogous to the pyrimidine salvage pathway and share NMPK and NDPK. The nucleoside kinases, however, are different. Adenosine is phosphorylated by adenosine kinase (ADK) and dAdo is phosphorylated by ADK, dCK and deoxyguanosine kinase (dGK), which also catalyses the phosphorylation of dGuo. To date, no existence of a human guanosine, inosine or xanthosine kinase has been reported (Slingerland, 1996; Hatse, 1999). In addition to salvage of the (deoxy)nucleosides, the purine bases adenine, hypoxanthine and guanine are salvaged to their respective nucleotides AMP, IMP and GMP by the phosphorybosyltransferases APRT and HGPRT, respectively. This is in contrast to the pyrimidine bases, which can only be converted to their corresponding nucleosides and, subsequently, to their nucleotides. The conversion of the purine nucleosides to the corresponding bases is catalysed by purine nucleoside phosphorylase.
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Fig. 2: Simplified scheme of pyrimidine metabolism.

DNA → dTTP → dUTP → dTDP → dTMP → Thymidine
Thymine → UMP → dUMP → dUDP → dUTP → dTTP
Dihydrothymine → N-Carbamyl-β-aminoisobutyrate → β-Aminoisobutyrate → TCA-cycle

RNA → UTP → CTP → DNA

CTPs

RR: Ribonucleotide reductase
dCK: deoxycytidine kinase

1. UMP/CMP kinase (NMPK)
2. Nucleoside diphosphate kinase (NDPK)
3. dCMP deaminase
4. TMP kinase
5. dUTPase
6. 5'-Nucleotidase
7. Urd/Cyd kinase
8. dCyd deaminase
9. Thymidylate synthase
10. dThd kinase (cytosolic TK-1)
11. dThd kinase (mitochondrial TK-2)
12. Urd phosphorlyase
13. dThd/dUrd phosphorlyase
14. Dihydropyrimidine dehydrogenase
15. Dihydropyrimidase
16. β-Ureidopropionase
17C: CAD Carboxylphosphate synthetase
17A: CAD Aspartate carboxyltransferase
17D: CAD Dihydroorotate
18: Dihydroorotate dehydrogenase
19: UMP synthetase
Figure 3: simplified scheme of purine metabolism.

Enzymes:
IMPDH: IMP dehydrogenase
RR: Ribonucleotide reductase

1. GMP synthetase
2. (d)GMP kinase
3. Nucleoside diphosphate kinase
4. GMP reductase
5. Adenylosuccinate synthetase
6. Adenylosuccinate lyase
7. AMP kinase (myokinase)
8. AMP deaminase
9. 5'-Nucleotidase
10. dGuo kinase
11. Purine nucleoside phosphorylase
12. Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)
13. Guanine deaminase
14. Xanthine oxidase
15. Ado kinase
16. Ado deaminase
17. Adenine phosphoribosyl transferase (APRT)
18. dCyd kinase
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1.5 Nucleotide metabolism as a target for chemotherapy

CTP synthetase as a target for chemotherapy

In resting cells, the purine nucleotides are more abundantly present than the pyrimidine nucleotides. In proliferating cells, however, the purine and pyrimidine nucleotides need to be present in equal amounts as they are incorporated into the newly synthesised DNA in equal amounts. A prominent characteristic of neuroblastoma cells, as in most tumour cells, is their excessive drive to proliferate, which requires vast amounts of nucleotides for energy and for RNA and DNA synthesis. To sustain this high turnover of nucleotides and the balance between purine and pyrimidine nucleotides, key enzymes of the purine and pyrimidine biosynthesis pathways are up-regulated. In malignant cells, including tumour cells derived from the neural crest, CTP synthetase is the main source of CTP, while salvage of cytidine is the predominant source of CTP in normal cells (Maehara et al., 1989; Slingerland et al., 1996). In some malignant cell types the expression of CTP synthetase is up-regulated and the intracellular CTP concentrations are higher in malignant cells than in their non-transformed counterparts (Verschuur et al., 1998; van den Berg et al., 1993; de Korte et al., 1986; van den Berg et al., 1993). Chemotherapeutic strategies based on inhibition of CTP synthetase are attractive to explore for two reasons. Firstly, as mentioned above, malignant cells predominantly synthesise CTP via CTP synthetase. Secondly, CTP is the precursor for the synthesis of cytidine deoxyribonucleotides, which are synthesised via reduction of CDP to dCDP by RR. dCTP is the only dNTP of which the pool size is not regulated by feedback inhibition of RR (Nicander and Reichard, 1985; Walters and Ratliff, 1975). dCTP is, however, a regulator of dCK activity (Heinemann et al., 1998). dCK plays a pivotal role in the activation of cytostatic drugs that are deoxynucleoside analogues. Therefore, synergistic interaction between inhibitors of CTP synthetase and deoxynucleoside analogues can be expected. The interplay between dCK and CTP synthetase will be discussed further below.

CTP synthetase inhibitors: 3-deazauridine and cyclopentenyl cytosine

To date, three specific inhibitors of CTP synthetase have been synthesised. The uridine analogue 3-deazauridine (DAU) was the first inhibitor of CTP synthetase and was first synthesised and described in the 1960's and 1970's. DAU is a competitive inhibitor of CTP synthetase and causes depletion of the cytidine ribo- and deoxyribonucleotides (Lockshin et al., 1984; Karle and Cysyk, 1984; Brockman et al., 1975). Although the pre-clinical data were promising, the results from phase I and phase I/II clinical trials involving patients suffering from solid and haematological malignancies showed hardly any anti-tumour effect and toxicity was severe (Yap et al., 1981; Creaven et al., 1982; Stewart et al., 1980).

In 1988, the glutamine antagonist copper:S-(methy1thio)-L-homocysteine was described as being a specific inhibitor of CTP synthetase (Rabinovitz and Fisher, 1988). However, no further reports describing this compound were published.

Cyclopentenyl cytosine (CPEC) was developed in the 1980's by the National Cancer Institute. CPEC is an analogue of cytidine in which the ribose moiety of Cyd has been replaced by a cyclopentenyl ring (Driscoll and Marquez, 1994) (figure 5). The cell readily takes up CPEC
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by nitrobenzylthioinosine-sensitive facilitated transport system (Zhang et al., 1993). Subsequently, CPEC is phosphorylated to its nucleoside monophosphate by uridine/cytidine kinase and by two successive phosphorylation steps the triphosphate, CPECTP, is formed (Kang et al., 1989). CPECTP quickly accumulates intracellularly and reaches concentrations that can be up to 100 times higher than the extracellular concentration (Ford, Jr. et al., 1991). CPECTP has a very long intracellular retention; the intra-cellular half-life was reported to be 24-30 hr in human colorectal cell lines (Ford, Jr. et al., 1991; Agbaria et al., 1997). CPECTP is the active compound that exerts the main cytotoxic effects of CPEC, namely inhibition of CTP synthetase (Kang et al., 1989). Thus, treatment of cells with CPEC leads to the depletion of the intracellular CTP pool, and also of the CDP and CMP pools (Moyer et al., 1986). Since CDP is a substrate for RR and the precursor of the cytidine deoxyribonucleotides, CPEC also causes depletion of the dCTP pool (Bouffard et al., 1994; Grem et al., 1991; Verschuur et al., 2002). The CPEC induced depletion of dCTP is reflected by inhibition of DNA synthesis and accumulation of the cells in the S-phase of the cell-cycle (Slingerland et al., 1995; Agbaria et al., 1997). Furthermore, CPECTP is incorporated into low molecular weight RNA, but not into DNA (Yee et al., 1992). The cytotoxic effects of CPEC can partially be reversed by administration of Cyd and Urd, which are competitive inhibitors for the uptake and phosphorylation of CPEC by the pyrimidine salvage enzyme system. dCyd can also reduce the cytotoxic effects of CPEC by providing dCTP after phosphorylation via dCK (Slingerland et al., 1995; Ford, Jr. et al., 1991; Ford, Jr. et al., 1995; Glazer et al., 1985). The reversal of CPEC induced toxicity has been reported to be specific for non-malignant tissue, as rescue of mice from treatment with potentially lethal doses of CPEC using Cyd did not compromise the anti-tumour activity of the compound (Ford, Jr. et al., 1995). In humans, the primary route of elimination of CPEC is by renal clearance (Politi et al., 1995; Zaharko et al., 1991). The major route of inactivation of CPEC is deamination to cyclopentenyl uracil (CPEU) by cytidine deaminase. The gene coding for cytidine deaminase has been located to chromosome 1p35-36, which is often deleted in neuroblastomas with LOH of chromosome 1. This may be a therapeutic benefit, as the cytidine deaminase activity might be lower in neuroblastoma cells with 1p LOH when compared to neuroblastoma cells without (Slingerland et al., 1997). CPEU is a non-toxic competitive inhibitor of uridine/cytidine kinase and thus, of the salvage pathway of uridine and cytidine.

CPEC was found to possess potent anti-tumour activity in vitro and in vivo in mice and rats (Gharehbaghi et al., 1999; Viola et al., 1995). A phase I clinical trial has been conducted in a limited number of adult patients suffering from refractory colorectal cancer (Politi et al., 1995). The dose limiting steady-state plasma concentration (C_{ss}) was 2.5 µM, above which haematological toxicity occurred, which was mild or absent at C_{ss} below 1.5 µM CPEC. However, unexpected cardiovascular toxicity was observed in 3 out of 26 patients, which occurred at C_{ss} above 1.5 µM CPEC.
1.6 Manipulation of nucleotide metabolism by CPEC

As described, CPEC causes depletion of the cytidine ribonucleotides and deoxyribonucleotides with concomitant accumulation of the cells in the S-phase of the cell cycle. Reasoning along this line, replenishing of the depleted cytidine nucleotide pools with S-phase active cytotoxic cytidine analogues is an attractive chemotherapeutic strategy to explore. Examples of therapeutically active deoxynucleoside analogues are the cytidine analogues 1-β-D-arabinofuranosyl cytosine (cytarabine, AraC) and 2',2'-difluorodeoxycytidine (gemcitabine, dFdC), and the deoxyadenosine analogue 2-chlorodeoxyadenosine ( cladribine, CdA). The observation that a brief pre-exposure to a non-toxic, but metabolically active concentration of CPEC indeed enhanced the metabolism and cytotoxicity of AraC in a human colon carcinoma cell line indicates that administration of CPEC at low doses may have potential clinical use as a biochemical modulator of deoxynucleoside analogues (Grem and Allegra, 1990).

Cytotoxic deoxynucleoside analogues are pro-drugs that are activated by phosphorylation to the corresponding di-and triphosphates. The rate-limiting enzyme in this process is dCK, both for the deoxycytidine analogues as well as CdA (Liliemark and Plunkett, 1986). dCK activity is under feedback control of dCTP and decreasing the intracellular concentration of dCTP relieves the inhibition of dCK and results in enhanced anabolism of deoxynucleoside analogues. The mechanistic representation of this concept is depicted in figure 4.

**Figure 4:** Mechanism of interaction between CPEC and gemcitabine (dFdC). The biochemical pathway indicated with bold black text is stimulated by CPEC, while the pathways indicated with italic text are inhibited by CPEC.
Figure: 5: Chemical structures of (deoxy)nucleoside analogues
1.7 Deoxynucleoside analogues

Gemcitabine

Gemcitabine, 2',2'-difluorodeoxycytidine, (Gemzar®, dFdC) is a relatively new agent with potential anti-tumour activity, as a single drug, as well as in combination with other drugs (figure 5). Gemcitabine is active in vivo against solid tumours and is a registered drug for the treatment of patients suffering from lung cancer and pancreas cancer. The maximum tolerated dose of gemcitabine is 780-2400 mg/m²/week, resulting plasma concentrations of dFdC > 20 μM (Abbruzzese et al., 1991; Fossella et al., 1997). Side effects of dFdF are thrombocytopenia and anemia. Clinical trials investigating the effectiveness and toxicity of gemcitabine in paediatric patients suffering from haematological malignancies and solid tumours are ongoing (National Cancer Institute information Internet site, http://www.nci.nih.gov/clinical_trials). The maximum tolerated dose in children suffering from relapsed or refractory leukaemia proved to be 3600 mg/m²/week, with hepatotoxicity being the dose limiting toxicity (Steinherz et al., 2002).

Gemcitabine is a pro-drug that needs to be activated by phosphorylation to its nucleotide-diphosphate and nucleotide-triphosphate forms to be therapeutically effective. The first and rate-limiting enzyme in the activation of gemcitabine and other deoxynucleoside analogues, like cytarabine and 2-chloro-deoxyadenosine (cladribine), is deoxycytidin e kinase (dCK) (Liliemark and Plunkett, 1986). Gemcitabine-monophosphate is subsequently phosphorylated to gemcitabine-diphosphate and gemcitabine-triphosphate by nucleoside monophosphate kinases and diphosphate kinases, respectively. Gemcitabine interferes with nucleotide metabolism in a number of ways (figure 6). Gemcitabine-diphosphate inhibits ribonucleotide reductase (RR), causing depletion of the deoxyribonucleotide pools, including dCTP. dCTP is a feedback inhibitor of dCK, thus inhibition of RR by gemcitabine-diphosphate stimulates the activation of gemcitabine by dCK (Heinemann et al., 1990). Gemcitabine-triphosphate is an inhibitor of CTP synthetase, RNA synthesis, DNA synthesis, DNA repair and is incorporated into the DNA, causing DNA damage (Heinemann et al., 1992; Ruiz van Haperen et al., 1993). After a gemcitabine moiety is incorporated into DNA, one more nucleotide is added, after which chain elongation stops (masked DNA chain termination), rendering the gemcitabine moiety resistant to excision by DNA exonuclease activity (Huang et al., 1991).

Furthermore, gemcitabine is a radio-sensitising agent, i.e. it increases the sensitivity of cells for (ionising) irradiation. The mechanism of the radio-sensitising effect of gemcitabine is still unclear, but has been associated with depletion of the intra-cellular dATP pools and redistribution of the cells into the S-phase of the cell cycle, where they are most sensitive for radiation induced DNA damage (Shewach et al., 1994; Lawrence et al., 1996).
Figure 6: simplified scheme of action of gemcitabine (dFdC, dFdCDP, dFdCTP: gemcitabine, 5'-diphosphate, 5'-triphosphate). The biochemical pathway indicated with bold black text is stimulated by dFdC, while the biochemical pathways indicated with italic text is inhibited by dFdC.

AraC
1-β-D-arabinofuranosyl cytosine (cytarabine, AraC) is a widely used cytostatic drug that has been used in the treatment of haematological malignancies for over 30 years. Low-dose (75-100 mg/m²) and high-dose (1000-3000 mg/m²) regimens are distinguished.

AraC is an analogue of deoxycytidine (figure 5) and is metabolised by the enzymes deoxycytidine salvage pathway. AraC is a pro-drug that is active in its nucleoside triphosphate form, AraCTP. As is the case in gemcitabine metabolism, the rate-limiting step in the activation of AraC is dCK. AraCTP is incorporated into DNA and causes cessation of chain elongation. Furthermore, AraCTP is an inhibitor of DNA polymerase and thus also causes inhibition of DNA synthesis (Zittoun et al., 1989; Morita et al., 1982; Wist, 1979).

A problem often encountered with AraC therapy is that leukaemic blasts of patients treated with AraC can acquire resistance against AraC. The cell can acquire resistance against AraC by increasing the (deoxy)cytidine deaminase activity (Momparler et al., 1996). Decreased dCK activity has also been reported to cause resistance against AraC (Riva and Rustum, 1985; Chiba et al., 1990; Owens et al., 1992), as were decreased formation and retention of AraCTP (Riva and Rustum, 1985) (Rustum and Raymakers, 1992; Schiller et al., 1996) and decreased incorporation of AraCTP into DNA (Kufe et al., 1980). Recently, it was demonstrated that resistance of acute myeloid leukaemia towards AraC is caused by alternative splicing of dCK mRNA, which leads to expression of inactive dCK proteins (Veuger et al., 2000; Veuger et al., 2002).
Chapter 1

Cladribine

Cladribine (CdA), or 2-Chloro-2'-deoxyadenosine, is an analogue of deoxyadenosine (figure 5). It is thought to be resistant to degradation by adenosine deaminase and is highly toxic to proliferating and non-proliferating lymphocytes. Cladribine is successfully used in the treatment of several haematological malignancies, such as hairy cell leukaemia and chronic lymphatic leukaemia. The maximum tolerated dose of CdA is 7 mg/kg/week, the dose-limiting toxicities being myelosuppression and neurologic events (Saven et al., 1993).

Like araC and gemcitabine, cladribine is taken up by facilitated transport and phosphorylated primarily by dCK, but also by deoxyguanosine kinase, to CdAMP. Subsequently, CdAMP is phosphorylated by two successive steps to CdATP, which is the toxic metabolite of cladribine. CdATP inhibits several enzymes involved in DNA replication. CdATP directly inhibits DNA synthesis by inhibition of DNA polymerase, but it is also incorporated into DNA. CdA has been reported to cause its own misincorporation into DNA as TTP, dCTP and dGTP (Hentosh et al., 1991). Furthermore, CdATP is an inhibitor of RR, which causes the drug to be self-potentiating as it decreases the dCTP pool and, therefore, increases dCK activity. CdA is also an inhibitor of DNA repair, which provides an explanation for the fact that CdA is also toxic to non-proliferating cells (Kuwabara et al., 1991; Seto et al., 1986).

1.8 Aim and scope

The main objective of this study is to explore whether inhibition of CTP synthetase would facilitate the metabolism of cytotoxic deoxynucleoside analogues in neuroblastoma, and if so, to what extent.

As stated in the general introduction, the main source of CTP in malignant cells is CTP synthetase. CTP synthetase as a target in neuroblastoma is an especially interesting topic of study, because the gene coding for CTP synthetase is located on chromosome 1p34, which occasionally shows loss of heterozygosity in neuroblastoma. Furthermore, CPEC, the CTP synthetase inhibitor studied in this thesis can be deactivated by the enzyme cytidine deaminase, which coding gene is located on chromosome 1p36, a frequently deleted region of chromosome 1 in neuroblastoma. The first step of the study described in this thesis is to investigate the effect and metabolism of CPEC as a single drug on neuroblastoma cell lines. Chapter 2 describes the metabolism and cytostatic effect of CPEC on a MYCN-amplified and 1p-deleted neuroblastoma cell line and a cell line without MYCN-amplification or 1p-deletion. Furthermore, the ability of these cell lines to recover from treatment with CPEC is described. The second step was to study and evaluate the role of CPEC as a modulator of the metabolism and cytotoxicity of deoxynucleoside analogues in neuroblastoma cell lines. In chapter 3, the stimulating effect of CPEC on the anabolism and cytotoxicity of AraC is described. Chapter 4 describes the interaction between CPEC and the deoxyadenosine analogue 2-Chlorodeoxyadenosine, which is metabolised in a different way than AraC. The effect of gemcitabine as a single drug and in combination with CPEC is the subject of chapter 5. In this chapter, also the regulation of dCK expression and activity in the presence of CPEC is described. Differentiation is thought to up-regulate the apoptotic machinery in the cell and
thus increase the apoptotic response to DNA damage. In chapter 6, however, it is demonstrated that differentiation of neuroblastoma cells, induced by retinoic acid, decreases the contribution of CTP synthetase to CTP biosynthesis and thus, causes resistance to CPEC. Chapter 7 describes a modification of a standard way of measuring dCK activity in cell homogenates. By choosing an alternative method of analysis, a novel metabolite of CdA was identified. Finally, in chapter 8 the studies described in this thesis are discussed and recommendations for future studies are made.

References


Chapter 1


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Chapter 1


Introduction


"So put me on a highway
And show me a sign
And take it to the limit one more time"

Eagles