The pivotal role of CTP synthetase in the metabolism of (deoxy)nucleosides in neuroblastoma
Bierau, J.

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Neuroblastoma is the most common extra cranial tumour of childhood and originates from the sympathetic nervous tissue. Poor prognosis for patients suffering from neuroblastoma is associated with deletion of part of chromosome 1p (1p LOH) and amplification of the MYCN oncogene. Exponentially growing neuroblastoma cells primarily biosynthesise CTP via CTP synthetase, whereas their differentiated counterparts biosynthesise CTP by the salvage of cytidine. Cyclopentenyl cytosine (CPEC) is a compound that in its nucleotide triphosphate form is a potent inhibitor of CTP synthetase. In chapter 2 it is shown that CPEC depleted the cytidine nucleotide pools, causing profound and long lasting cytostasis in MYCN-amplified and MYCN-single copy cell lines. While MYCN-single copy cells did not resume proliferation after treatment with CPEC, the ability of MYCN-amplified cells to recover from treatment was dependent on the duration of exposure to CPEC and the concentration of CPEC used. In both types of neuroblastoma, CPEC caused retardation in the S-phase of the cell cycle, potentially increasing the sensitivity to cytotoxic deoxynucleoside analogues.

The first and rate-limiting step in the anabolism of deoxynucleoside analogues is catalysed by deoxycytidine kinase (dCK), which is regulated via feedback inhibition by dCTP. Thus, depletion of dCTP by incubation with CPEC should increase the dCK activity. In chapters 3-5, we demonstrated that pre-treatment of SK-N-BE(2)c neuroblastoma cells with CPEC increased the anabolism of cytarabine (AraC) (chapter 3), cladribine (CdA) (chapter 4) and gemcitabine (dFdC) (chapter 5). As single drugs, the order of toxicity was dFdC >> AraC >> CdA, with SK-N-BE(2)c being resistant to CdA. Pre-incubation of SK-N-BE(2)c cells with CPEC greatly enhanced the cytotoxicity of AraC and dFdC and rendered them highly sensitive to CdA. The increase in toxicity appeared to be caused by increased accumulation of cytotoxic metabolites. In case of AraC, the increase in AraCTP caused profound inhibition of DNA synthesis, while the CdA metabolite CdAMP appeared to be the major metabolite causing cytotoxicity in neuroblastoma cells. After pre-treatment with CPEC, a relative accumulation of AraCMP and CdAMP was observed, indicating that after release of the feedback inhibition of dCK, nucleoside monophosphate kinase was the rate limiting enzyme in the anabolism of AraC and CdA. Pre-treatment with CPEC increased the intracellular concentration of all dFdC metabolites proportionally. The rate-limiting step in the CPEC-stimulated anabolism of dFdC, is the incorporation of dFdC into DNA. An increased expression and activity of dCK paralleled the increased metabolism of deoxynucleotide analogues during incubation with CPEC, as described in chapter 5. This indicates that CPEC causes increased expression of dCK due to depletion of (d)CTP and inhibition of DNA synthesis.

dFdC proved to have potent anti-tumour activity against panel of neuroblastoma cell lines, consisting of MYCN-amplified and MYCN-single copy cell lines. As discussed in chapter 5, there was no difference between the ED50 values for dFdC of MYCN-amplified and MYCN-single copy cell lines. However, in all MYCN-single copy cell lines tested, dFdC induced differentiation without inducing apparent cell death, while in 6 out of 9 MYCN-amplified cell
lines dFdC did induce cell death. In Shep21N cells, dFdC induced cell death only when MYCN was expressed. The specific dCK activity in MYCN-amplified cell lines was 60% higher than in MYCN-single copy cell lines, possibly causing an increased intracellular concentration of dFdC metabolites in MYCN-amplified cell lines when compared to MYCN-single copy cell lines.

In chapter 7 it was shown that SK-N-BE(2)c cells were rendered resistant to CPEC after pre-treatment with retinoic acid (all-trans, 9-cis and 13-cis). This was caused by a decreased proliferation rate, combined with a shift of CTP biosynthesis towards salvage of cytidine instead of CTP synthetase, thus bypassing the inhibitory effect of CPECTP on CTP synthetase.

The dCK activity of the neuroblastoma cell lines tested was measured using a newly developed, non-radioactive, reversed-phase HPLC method with UV detection, described in chapter 8. This method is sensitive, cheaper and less elaborate than the traditional methods to measure dCK activity, which use TLC and paper chromatography to separate the radioactively labelled substrate and product. By using reversed-phase HPLC with UV detection, a novel metabolite of CdA was identified: 2-Chlorodeoxyinosine. This demonstrates that CdA is a substrate for adenosine deaminase.

In conclusion, the studies presented in this thesis clearly demonstrate that CPEC is an excellent drug to increase the cytotoxic effect of deoxunucleoside analogues in neuroblastoma. Furthermore, dFdC proved to be promising drug for the treatment neuroblastoma in vitro and, therefore, deserves further pre-clinical development towards clinical application of the drug in the treatment of patients suffering from neuroblastoma.
"The more I know, the less I understand
All the things I thought I knew, I'm learning again"

Don Henley