Molecular aspects of 5-Fluorouracil and Oxaliplatin activity
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General Introduction
Colorectal cancer

General
Worldwide colorectal cancer is one of the most common cancers and recent data\(^1\) show an estimated number of one million cases and a half a million deaths every year. The incidence is highest in developed countries and might therefore be associated with differences in environmental circumstances, life style and consumption patterns. In the Netherlands about 6000 cases were diagnosed and 3400 deaths in the year 2000\(^2\). These numbers are estimated to increase to 9000 new cases and 4300 deaths in 2015. Mean survival after 5 years is around 55% for all stages but ranges form 95% for Stage I to 4% for Stage IV\(^3\). Although the improvement in the treatment of colorectal has led to a considerable increase in survival in the last decades the treatment options are still limited and can be improved.

Oncogenesis
Besides the role of environmental circumstances, life style and consumption patterns the carcinogenesis of colorectal cancer can also have a genetic background. Patients with familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) are predisposed to develop colorectal cancer\(^4\).

For the transformation of somatic cells to cancer cells a number of mutational changes have to occur that lead to genomic instability and subsequently the acquisition of limitless replicative potential, insensitiveness to growth inhibitory signals or evasion of programmed cell death or apoptosis\(^5\). In the gut at the bottom of the crypts slowly cycling stem cells are located. The stem cells have self-renewal capacity and upon division a new stem cell and a more differentiated cell are formed. These more differentiated and more rapidly cycling transit-amplifying cells end up at the lumen at a fully differentiated stage. Since the stem cells in the crypts are slowly cycling and have a longer life span compared to their more differentiated progeny it is likely that these cells are more vulnerable to accumulate multiple mutations in pathways that regulate self renewal and differentiation. After transformation the resulting tumors consist of a mixed population of cells. Within this mixed population of cells a small number of cells exist that maintain the tumor and are called tumor initiating cells or cancer stem cells. Isolation or enrichment of these cancer stem cells and transplantation results in tumor formation composed of a similar mixture of cells as the original tumor from which the cells were isolated. This supports the hypothesis that cancer stem cells have properties similar to normal stem cells\(^6\). Although cancer stem cells have similarities with normal stem cells it is also possible that they originate from more differentiated cells and acquired stem cell like properties.
during transformation(6). The self renewal properties of cancer stem cells indicate that pathways as Wnt, Notch, Sonic Hedgehoc and BMI-1 are involved as in normal stem cells.

Pathways

The Wnt signaling pathway (Figure 1) is a critical regulator of stem cells and is frequently associated with development of cancer(7, 8). Central component of the Wnt pathway is β-catenin. Without binding of Wnt proteins to their receptor complex, consisting of members of the Frizzled and Lrp family of proteins, β-catenin is phosphorylated by GSK3 in a complex with APC and Axin and thereby targeted for proteosomal degradation. Upon binding of Wnt-ligands, phosphorylation of β-catenin is inhibited and results in accumulation of the protein and subsequently in translocation to the nucleus. In the nucleus it binds to the transcriptional activators Tcf/Lef and Wnt target gene expression. β-Catenin also binds to cadherins and plays a role in cell-cell adhesion. In colorectal cancer mutations in APC or Axin are frequently observed and cause deregulation of β-catenin stability and continuous activation of the Wnt signaling pathway(8). The subsequent expression of Wnt target genes as c-Myc and Cyclin D results in increased proliferation and together with additional mutations such as in K-Ras and p53 in malignant transformation(7).

Figure 1. Wnt-signaling pathway

The Notch signaling pathway (Figure 2) is another pathway that is involved in fate determination and self renewal. Four Notch receptors (Notch 1-4) are identified together with five ligands (Delta-like 1, 3 & 4 and Jagged 1 & 2). Binding of Notch ligands, expressed on neighboring cells, to the Notch receptor is followed by proteolytic cleavage by γ-secretase of the intracellular domain (NICD). After translocation of NICD to the nucleus it binds to CSL/RBP-J which is thereby converted
from a transcriptional repressor into a transcriptional activator. The role of Notch in intestinal cell fate has been demonstrated by use of γ-secretase inhibitors. Inhibition of γ-secretase and subsequent deactivation of the Notch pathway resulted in conversion of proliferating crypt cells into post-mitotic goblet cells. Furthermore, it has been demonstrated that Notch signaling not only occurs in crypts as demonstrated by Hes1 expression, but also in adenomas in APCmin mice demonstrating that, besides the Wnt pathway, it might play a role in oncogenic transformation.

The third pathway that might be involved in colorectal cancer is the Sonic Hedgehog (Shh) pathway. Via binding of Shh to the transmembrane protein Patch (Ptch1) repression of the G-protein-coupled receptor component Smoothened (Smo) is relieved and downstream genes like Gli1 and 3 are activated. Overexpression of Gli1 in colorectal cancer is associated with proliferation and might be the result of inactivating mutations in Ptch1 or activating mutations in Smo.
Figure 4. BMI-1 signaling pathway

The role of BMI-1 signaling pathway (Figure 4) in self renewal and cancer proliferation has been demonstrated in leukemia (12) but also in colon cancer it is frequently overexpressed(13). Activation of the BMI-1 pathway results in the inhibition of CDKN2A which codes for two cyclin dependent kinase inhibitors INK4A (p16) and ARF (p14). Inhibition of INK4a results in phosphorylation and inactivation of Rb by the CDK4/Cyclin D complex and subsequently the activation of E2F dependent expression of cell-cycle genes. Inhibition of ARF allows the inactivation of p53 and the p53 dependent expression of pro-apoptotic genes by MDM2. Therefore inhibition of these pathways by BMI-1, promote cancer cell proliferation and escape of p53 dependent apoptosis.

Pathological staging

The development stages of colorectal cancers are described using the Astler-Coller modification of Dukes’ classification (14) or the TNM classification (15, 16) and are related to the site and penetration of the tumor as shown in Figure 5 and Table 1. Duke’s A stage (TNM Stage 0, I T1) tumors are restricted to the mucosa and sub mucosa layer and do not penetrate the muscle layers as found in the Duke’s B stage (Stage I T2, II A-B). In Duke’s C stage (Stage III A-C) the tumor has also metastasized to lymph nodes and when the tumor is further metastasized to other organs, predominantly to the liver, it will be classified as Duke’s D (Stage IV). Treatment and prognosis of colorectal cancer is depending on the stage at diagnosis.
### Table 1. TNM classification of colorectal cancer

<table>
<thead>
<tr>
<th>TNM definitions</th>
<th>AJCC stage grouping</th>
</tr>
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<tbody>
<tr>
<td>Primary tumor (T)</td>
<td></td>
</tr>
<tr>
<td>TX: Primary tumor cannot be assessed</td>
<td>Stage 0 Tis, N0, M0</td>
</tr>
<tr>
<td>T0: No evidence of primary tumor</td>
<td>Stage I T1, N0, M0</td>
</tr>
<tr>
<td></td>
<td>T2, N0, M0</td>
</tr>
<tr>
<td>Tis: Carcinoma in situ: intraepithelial or invasion of the lamina propria</td>
<td>Stage IIA T3, N0, M0</td>
</tr>
<tr>
<td>T1: Tumor invades submucosa</td>
<td>Stage IIB T4, N0, M0</td>
</tr>
<tr>
<td>T2: Tumor invades muscularis propria</td>
<td>StageIIIA T1, N1, M0</td>
</tr>
<tr>
<td></td>
<td>T2, N1, M0</td>
</tr>
<tr>
<td>T3: Tumor invades through the muscularis propia into the subserosa or into nonperitonealized pericolic or perirectal tissues</td>
<td>Stage IIIB T3, N1, M0</td>
</tr>
<tr>
<td></td>
<td>T4, N1, M0</td>
</tr>
<tr>
<td>T4: Tumor directly invades other organs or structures and/or perforates visceral peritoneum</td>
<td>Stage IIIC Any T, N2, M0</td>
</tr>
<tr>
<td>Regional lymph nodes (N)</td>
<td></td>
</tr>
<tr>
<td>NX: Regional nodes cannot be assessed</td>
<td>Stage IV Any T, Any N, M1</td>
</tr>
<tr>
<td>N0: No regional lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>N1: Metastasis in 1 to 3 regional lymph nodes</td>
<td></td>
</tr>
<tr>
<td>N2: Metastasis in 4 or more regional lymph nodes</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis (M)</td>
<td></td>
</tr>
<tr>
<td>MX: Distant metastasis cannot be assessed</td>
<td></td>
</tr>
<tr>
<td>M0: No distant metastasis</td>
<td></td>
</tr>
<tr>
<td>M1: Distant metastasis</td>
<td></td>
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</tbody>
</table>

**Figure 5. Pathological staging**
Treatment modalities

Primary treatment for colorectal cancer is surgical resection of the tumor. Dependent on the stage of the tumor the cure rate is about 95% for Duke’s A tumors but much less for Duke’s B, 60%, and Duke’s C tumors, 35% (17). To decrease the recurrence rate of about 50% after surgical resection patients are treated with adjuvant chemotherapy (18).

Since its development in 1957 5-FU has become the backbone of chemotherapy (19) and after 5 decades treatment of colorectal cancer still consist of 5-FU usually in combination with Irinotecan, Oxaliplatin or more recent in combinations with Cetuximab or Bevacizumab.

Development of 5-FU was based on the observation that cancer cells show increased incorporation of uracil into DNA as compared to normal cells (20, 21). This observation led to the first rational designed and targeted anticancer drugs and 5-FU was shown to be the most active compound (22). The mechanism of action of 5-FU as shown in Figure 6 is mainly based on inhibition, by its active metabolite FdUMP, of thymidylate synthase (TS) which results in depletion of thymidine triphosphate and subsequently in inhibition of DNA synthesis. Activation of 5-FU to FdUMP is mediated by the enzymes of the pyrimidine metabolic pathways but in contrast to uracil also orotate phosphoribosyltransferase was shown to be involved (23). Besides inhibition of TS, 5-FU can be incorporated into RNA and into DNA which might also contribute to the antitumor effect. Incorporation into DNA and subsequent excision by the uracil DNA glycosylases UNG2, Smug1 or TDG results in formation of AP-sites and DNA strandbreaks which were associated with both sensitivity and resistance to 5-FU depending on downstream processing of the DNA strandbreaks (24-26). Cytotoxicity mediated by RNA incorporation might be mediated by incorporation into snRNA that affects splicing of pre-mRNA and processing of pre-rRNA (27-30). The activity is also dependent on the catabolic enzyme dihydopyrimidine dehydrogenase (DPD) which is responsible for the degradation of about 80% of the drug that enters the cells. The response rate after treatment with 5-FU alone is very poor, but addition of leucovorin (LV), increasing the cofactor levels for TS and thereby enhancing the binding of FdUMP to TS, improved the response rates and overall survival (31). Today 5-FU can be replaced by the oral 5-FU pro-drug Capecitabine (Xeloda). Capecitabine is activated to 5-FU in three enzymatic steps including thymidine phosphorylase (TP) which is frequently overexpressed in colorectal cancer. Capecitabine shows comparable response rates and survival as 5-FU/LV (32, 33). Another oral prodrug of 5-FU is Tegafur which is used in combination with Uracil (UFT) and LV or in combination with CDHP and Oxonic acid (S-1) (34, 35).
Figure 6. Metabolism of 5-FU

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP: adenosine-5’-diphosphate;</td>
<td>1. orotate phosphoribosyltransferase, E.C. 2.4.10;</td>
</tr>
<tr>
<td>ATP: adenosine-5’-triphosphate;</td>
<td>2. uridine phosphorylase, E.C. 2.4.2.3;</td>
</tr>
<tr>
<td>dTMP: 2’-deoxythymidine-5’-monophosphate;</td>
<td>3. uridine kinase, E.C. 2.7.1.48;</td>
</tr>
<tr>
<td>dTDP: 2’-deoxythymidine-5’-diphosphate;</td>
<td>4. nucleoside monophosphate kinases, E.C. 2.7.4.4;</td>
</tr>
<tr>
<td>dTTP: 2’-deoxythymidine-5’-triphosphate;</td>
<td>5. nucleoside diphosphate kinases, E.C. 2.7.4.6;</td>
</tr>
<tr>
<td>dUMP: 2’-deoxyuridine-5’-monophosphate;</td>
<td>6. RNA polymerase E.C. 2.7.7.6;</td>
</tr>
<tr>
<td>FUMP: 5-fluorouridine-5’-monophosphate;</td>
<td>7. ribonucleotide reductase, E.C. 1.17.4.1;</td>
</tr>
<tr>
<td>FUDP: 5-fluorouridine-5’-diphosphate;</td>
<td>8. thymidine phosphorylase E.C. 2.4.2.4;</td>
</tr>
<tr>
<td>FUTP: 5-fluorouridine-5’-triphosphate;</td>
<td>9. thymidine kinase, E.C. 2.7.1.21;</td>
</tr>
<tr>
<td>FdUMP: 5-fluoro-2’-deoxyuridine-5’-monophosphate;</td>
<td>10. DNA polymerase, E.C. 2.7.7.7;</td>
</tr>
<tr>
<td>FdUDP: 5-fluoro-2’-deoxyuridine-5’-diphosphate;</td>
<td>11. thymidylate synthase, E.C. 2.1.1.45;</td>
</tr>
<tr>
<td>FdUTP: 5-fluoro-2’-deoxyuridine-5’-triphosphate;</td>
<td>12. dihydroptymidine dehydrogenase, E.C. 1.3.1.2;</td>
</tr>
</tbody>
</table>
Introduction of Irinotecan further improved 5-FU based regimens. Irinotecan is a camptothecin derivative that inhibits Topoisomerase I and results in DNA strandbreaks. In combination with 5-FU/LV it improved the overall survival from 14.1 months to 17.4 months as compared to 5-FU/LV (36).

Oxaliplatin is a third generation platinum compound and shows activity in colorectal cancer in contrast to cisplatin. Formation of platinum DNA adducts is thought to mediate the activity of Oxaliplatin. The DNA adducts formed by oxaliplatin are similar to cisplatin and carboplatin but at equitoxic concentrations oxaliplatin forms less adducts(37). Since repair of cisplatin and oxaliplatin DNA adducts by the nucleotide excision repair system (NER) is similar for both drugs, this indicate that the lower degree of DNA adducts formation by oxaliplatin is not a result of NER(38). In
contrast to cisplatin, the oxaliplatin DNA adducts are not recognized by the mismatch repair system and therefore activity is not hampered by defects in the mismatch repair system as shown for cisplatin(39). This might explain activity of oxaliplatin in colorectal cancer. Other factors that might explain the activity of oxaliplatin in colorectal cancer are the recently described organic cation transporters (OCTs) which are highly expressed in colorectal cancer and involved in uptake of oxaliplatin(40, 41).

Combinations of 5-FU and Oxaliplatin show comparable response rates and survival as 5-FU with Irinotecan (42, 43). In more recent trials the monoclonal antibodies cetuximab or bevacizumab have been combined with 5-FU. Cetuximab targets the epidermal growth factor receptor (EGFR) that is over expressed in 60-80% of colorectal cancers. Bevacizumab specifically binds to vascular endothelial growth factor (VEGF) and prevents VEGF signaling in endothelial cells resulting in inhibition of angiogenesis. Survival increased to around 24 month’s using these monoclonal antibodies in combination with standard therapy for colorectal cancer (44). However, in a recent CAIRO study it was shown that cetuximab should not be added to fluoropyrimidines, oxaliplatin and bevacizumab(45).

**Thesis outline**

In this thesis several (molecular) aspects of the anti cancer drugs 5-fluorouracil and Oxaliplatin are described. Today it is over 50 years ago that 5-fluorouracil was developed and it is still the main anti tumor agent for the treatment of colorectal cancer and is part of therapy for other cancers as well. Only in recent years novel compounds such as Oxaliplatin and Irinotecan were added to the spectrum of compounds that are now widely used for treatment of colorectal cancer but always in combination with a fluoropyrimidine (5-FU or a prodrug) and usually also with Leucovorin. Although 5-FU is used for decades there are still many questions on how the drug exerts its effects and on how it can be further improved. Especially knowledge about the incorporation of 5-FU into RNA and DNA of colorectal tumors during and after treatment is still limited. The role of the 5-FU incorporation in both RNA and DNA, extracted from tumor specimens, from patients in the overall anti tumor effect was further evaluated. Oxaliplatin is a novel third generation platinum compound which has many similarities with the classical platinum drug cisplatin. Since Oxaliplatin shows activity in the intrinsic cisplatin resistant colorectal cancer it is of great interest to study how its activity differs from cisplatin. It might be expected that resistance towards oxaliplatin has similarities with cisplatin but because of its activity in colorectal cancer different resistance mechanism as compared to cisplatin
may be expected.

After the general introduction (Chapter 1) this thesis has three chapters (Chapters 2-4) in which the development of methods to determine the incorporation of 5-fluorouracil into RNA (Chapter 2) and DNA (Chapter 3) is described and subsequently data on incorporation in tumor samples of colorectal cancer patients treated with different combinations of 5-fluorouracil with leucovorin is presented (Chapter 4). The role of 5-fluorouracil incorporation into RNA and DNA was put into perspective with the extensively studied mechanism of thymidylate synthase inhibition in the same samples.

In Chapter 5 the activity of oxaliplatin in a panel of selected and unselected cell lines is described together with some mechanisms that are involved in the activity of the drug. Cell lines were selected with different p53 status and different sensitivities towards cisplatin and studied for their sensitivity to Oxaliplatin compared to cisplatin. The sensitivity was related to formation of DNA adducts and the expression of transporters and of DNA repair genes.

Resistance to Oxaliplatin was studied and described in Chapter 6. Colorectal and ovarian cancer cell lines with different p53 status were made resistant to Oxaliplatin with 2 schedules after which the mechanism of resistance was studied. Differences in accumulation of the drug as well as the formation of DNA adducts were compared to the parental cell lines. Furthermore transporter and DNA repair gene expression were measured which could explain the resistance to Oxaliplatin. Finally the resistant cell lines were subjected to array Comparative Genomic Hybridization and gene expression array to get more insight on changes at the genomic level and pathways that lead to a resistant phenotype.

The results of the above studies are summarized and discussed in Chapter 7 and put in perspective with possible future directions.
References


General introduction