Molecular aspects of 5-Fluorouracil and Oxaliplatin activity

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Chapter 7

Summarizing discussion and future perspectives

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In this thesis several mechanistic aspects that might determine the activity or resistance towards to 5-Fluorouracil and oxaliplatin are described. These compounds are two major components in the treatment of colorectal cancer together with Irinotecan and, more recently, the antibodies cetuximab and bevacizumab. The use of multiple agents in the treatment of colorectal cancer makes it necessary to fully understand the factors that determine the activity and/or resistance of the individual components of the treatment to be able to design rational treatment schedules and prevent or overcome resistance to therapy.

5-Fluorouracil

After its discovery in1957(1) 5-Fluorouracil has been used in the treatment of colorectal cancer for over 50 years and is still one of the main components of colorectal cancer therapy today.

Several enzymes are involved in the activation of 5-FU to its active metabolite FdUMP which is a potent inhibitor of Thymidylate Synthase (TS). Inhibition of TS leads to decreased levels of Thymidine triphosphate (TTP), which is necessary for DNA synthesis. This depletion leads to an inhibition of DNA synthesis and subsequently to decreased proliferation. The role of TS in anti tumor activity and resistance to 5-FU has been studied both in vitro (2-6) and in clinical studies (7-12)and showed that expression or induction of TS were related to outcome of treatment.

Besides inhibition of TS, 5-FU can be incorporated into RNA and DNA(2, 13-19). Lack of suitable methods limited the translation of these in vitro studies to clinical practice. Therefore we developed methods to determine the incorporation of 5-FU into RNA (Chapter 2) and into DNA (Chapter 3) without the use of radio-labeled 5-FU. The methods are based on isolation of RNA and DNA after 5-FU treatment and the complete degradation to bases after which 5-FU could be derivatised and measured with gas chromatography and mass spectrometry (20, 21). The developed methods were compared with incorporation with radio labeled 5-FU and showed similar results. Application of the methods (Chapter 4) in tumor biopsies of patients treated with 5-FU combined with leucovorin showed that 5-FU was incorporated into both RNA and DNA at detectable levels during treatment. Incorporation was detectable to at least 66 hour after treatment while the drug was cleared from circulation after treatment in hours.(21, 22) No significant differences were found between primary tumors, liver metastasis and normal mucosa from the same patient. Incorporation of 5-FU into RNA and DNA affects protein synthesis and leads to DNA strand breaks that might

lead to apoptosis. However, in this study no significant correlation was found between incorporation into RNA and DNA in a sub group of patients with the clinical response to 5-FU based therapy. This is in contrast to TS enzyme activity in same group of patients which showed a significantly lower TS activity in tumor samples of patients that responded to therapy(7).

Oxaliplatin

The third generation platinum compound oxaliplatin shows many similarities with cisplatin and its activity is thought to be mediated by formation of platinum DNA adducts. However, its activity in cisplatin resistant colon cancer indicates that different mechanisms play a role. The mismatch repair system, necessary for full activity, could be responsible for the intrinsic resistance of colorectal cancer to cisplatin(23). Since oxaliplatin forms less DNA adducts compared to cisplatin at equitoxic concentration(24, 25), this indicates that adduct recognition and down stream signaling might play a role in the activity of the drug.

In Chapter 5 we describe the activity of oxaliplatin in a panel of selected and unselected colon and ovarian cancer cell lines that differ in p53 status or sensitivity towards cisplatin. Mutated p53 did not affect sensitivity to oxaliplatin but in-active p53 showed a resistant phenotype which was associated with decreased accumulation of the drug and decreased formation of platinum DNA adducts. The whole panel of cell lines did not show a significant correlation between sensitivity and total platinum accumulation and DNA adduct formation. After exposure to oxaliplatin cells arrested in G2/M or S phase (wt-p53) while G1-phase arrest followed by S-phase arrest was observed in cell lines with mt-p53. Cisplatin resistant cell lines showed decreased expression of the copper transporter CTR1 or the organic transporter 1 (OCT1, SLC22A1) which could mediate resistance.

To get more insight in potential resistance mechanisms for oxaliplatin (Chapter 6) we induced resistance with 4 hour pulses of oxaliplatin to mimic the clinical situation or with 72 hour pulses to mimic continuous exposure. Resistant cell lines were established from the colorectal cancer cell line LoVo-92 and its variant with inactive p53 LoVo-Li and in the ovarian cancer cell line A2780. Sensitivity to oxaliplatin in parental and resistant cell lines was highly correlated with total platinum acccumulation. The total platinum accumulation was significantly correlated to mRNA expression of the organic cation transporters OCT1-3 while ATP7A was significantly correlated to formation of platinum DNA adducts. Resistance in LoVo-92 and A2780/cOHP could be explained by reduced total platinum accumulation and DNA adducts formation. However, in A2780/4OHP accumulation and adducts formation were unchanged. These results indicate various mechanisms of resistance in this cell line panel and therefore global

gene expression micro arrays and aCGH arrays were performed. Pathway analysis of gene expression data showed several pathways that were significantly enriched. Most significantly changed pathways were Aryl Hydro Carbon Receptor pathway, p53 Signaling pathway, Role of BRCA1 in DNA damage response and Xenobiotics Metabolism pathways. The most frequently altered genes in the AHR pathway were ALDH1A1 and ALDH1L2, which were increased and decreased, respectively in 5 out of the 6 resistant cell lines. The p53 signaling pathway showed decreased apoptotic signaling via decreased expression of pro-apoptotic BAX and BCC3 (PUMA) but increased expression of executionary caspases 6 and 7 was observed but this was accompanied by increased expression of inhibitors of apoptosis (IAP) genes like BIRC1 (NIAP), BIRC4 (XIAP) or BIRC5 (Survivin). Highly increased expression of aldo-keto reductases C1 and C3 was observed in the Xenobiotics Metabolism pathways and specifically for LoVo-Li/4OHP genes in the BRCA1 in DNA damage response pathway. These changes in gene expression illustrate that besides decreased accumulation of oxaliplatin and decreased formation of DNA adducts, apoptosis resistance, increased homologous DNA repair and increased xenobiotics metabolism may also play a role in oxaliplatin resistance. At the genomic level mainly small focal deletions and gains were observed. Common or overlapping aberrations were most frequently observed in resistant cell lines of the same parental cell line. The ovarian cancer cell lines showed the highest number of aberrations indicating a more vulnerable phenotype.

Conclusion and future perspectives

The studies described in this thesis show that many factors are involved in the activity and resistance to cytotoxic drugs. Especially in combinations with other multiple targeted novel drugs insight in the multiple cellular pathways that are involved or affected by standard cytotoxic drugs is very important to optimize the design of novel therapeutic regimens.

Incorporation of 5-FU into RNA and DNA after bolus injection of 5-FU in combination with leucovorin showed no significant correlation with treatment outcome and indicates that the contribution to the overall effect of 5-FU is less important than inhibition of TS. Recent data showed that addition of leucovorin to 5-FU might play a role in the lack of correlation between RNA incorporation of 5-FU and response(26). In rescue experiments with uridine it was shown that growth inhibition by 5-FU was reduced when uridine was added after 5-FU. However, rescue by uridine was much less when 5-FU was used in combination with leucovorin indicating that at maximal TS inhibition the effect of uridine on growth inhibition was reduced. Treatment response

of the 5-FU prodrug Capecitabine (Xeloda) which is used without leucovorin might therefore show a correlation with RNA incorporation. A similar mechanism could be involved in incorporation of 5-FU into DNA. To maximize the cytotoxic effects of 5-FU incorporation into RNA and DNA drug combinations should be developed that enhance the incorporation of 5-FU without affecting the inhibition of TS. Several options are posible to increase the effect of 5-FU incorporation into RNA and DNA on the overall effect of 5-FU. Recent studies showed that the enzyme UMP kinase, involved in the activation of 5-FU, was involved in resistance to 5-FU and was associated with decreased incorporation of 5-FU into RNA while no changes were observed in other relevant enzymes(27). Treatment with low-dose 5-aza-deoxycytidine (DAC), an inhibitor of DNA hypermethylation, could restore UMPK levels and activity(28) Inhibition of ribonucleotide reductase (RR) might also favor RNA incorporation of 5-FU by preventing the conversion of 5-FU-nucleotides to 5-FU-deoxynucleotides but might result in reduced formation of FdUMP and subsequently in reduced TS inhibition. Inhibition of TS by 5-FU or the specific TS inhibitors like ZD9331 leads to accumulation of dUTP. Previous studies showed that dUTP incorporation, depending of deoxyuridine triphosphate nucleotidohydrolase (dUTPase) activity, enhanced the effect of TS inhibition(29) and suppression of dUTPase by small interfering RNA sensitized several cancer cell lines to TS inhibition(30). Therefore combining 5-FU with treatments that reduces dUTPase levels or activity would be a rational approach to enhance the incorporation of both dUTP and dFdUTP into DNA and thereby increasing the response to 5-FU. A recent study showed that in response to oxaliplatin-induced DNA damage dUTPase levels were reduced and resulted in increased dUTP levels which was further enhanced by fluoropyrimidines(31).

Oxaliplatin is very active in combination with 5-FU which might be related with increased DNA damage induced by both oxaliplatin and 5-FU but also decreased DNA repair as result of decreased deoxynucleotide levels. Similar effects could be obtained by the combination of oxaliplatin with pemetrexed which also results in reduced deoxynucleotide levels and which has been shown to be synergistic in in-vitro experiments(32).

Synergistic interactions between 5-FU and pemetrexed with oxaliplatin are usually explained by increased DNA damage and decreased repair. However, our data (Chapter 5) showed no significant relation between sensitivity and platinum DNA adduct formation or total platinum accumulation in a panel of selected and unselected cell lines. This indicates that sensitivity is cell line dependent and that multiple targets might play a role(33). Also in oxaliplatin resistant cell lines no significant correlation was found between sensitivity and formation of platinum DNA adducts in contrast to total platinum accumulation (Chapter 6). Since the sensitivity to oxaliplatin in the

resistant cell lines is significantly correlated to total platinum accumulation it might be the result of direct effects of oxaliplatin on mitochondria while induction of apoptosis via DNA damage pathways is decreased. Reduced accumulation in oxaliplatin resistant cell lines might be caused by decreased expression of influx transporters and especially the organic cation transporters OCT1-3 (34) that were highly correlated with total platinum accumulation in the oxaliplatin resistant cell lines. Decreased apoptosis via DNA damage pathways is mainly the result of decreased expression of pro-apoptotic genes Bax and Puma. Furthermore protection of resistant cells to oxaliplatin-induced ROS by increased levels of aldo-keto reductases and aldehyde dehydrogenases might play a role in resistance towards oxaliplatin(35-38). In contrast to changes in gene expression oxaliplatin resistance could not be related to genomic aberrations.

To overcome oxaliplatin resistance new combination therapies should focus on restoration of the induction of apoptosis via targeting anti-apoptotic proteins such as Bcl-2. ABT-737, a potent inhibitor of Bcl-2 and Bcl-XL, showed synergistic interaction with cisplatin(39) and could restore the balance between Bax and Bcl-2 in the oxaliplatin resistant cell lines(40). Also targeting the Birc family of proteins (cIAP, XIAP, Survivin) might help to circumvent apoptosis resistance. Since decreased accumulation of platinum drugs is one of the most frequently observed resistance mechanisms strategies to increase uptake should also be one of the main goals in development of novel platinum containing regimens.

Recent data show that the novel platinum compound mitaplatin, a compound combining cisplatin and dichloro acetate (DCA), targets both DNA and the mitochondria associated Warburg effect(41). Inhibition of the Warburg effect by DCA in cancer cells is mediated by inhibition of pyruvate dehydrogenase kinase and promotes apoptosis by release of cytochrome c. Therefore this novel platinum compound may overcome platinum resistance mediated by altered DNA damage response.

A major challenge remains the development of personalized therapy using the repertoire of classical and novel targeted drugs that will become available with emphasis on eradication of tumor intiating cancer cells together with the increasing possibilities for the screening of tumor specimens of individual patients.

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