SUMMARIZING DISCUSSION AND CONCLUDING REMARKS

TAS-102 is a novel anticancer formulation, which combines trifluorothymidine (TFT) with the thymidine phosphorylase inhibitor, TPI. TAS-102 is currently being evaluated in phase II clinical trials against gastric and colorectal cancer and has already shown prolonged stable disease in this heavily pretreated 5-FU refractory population[1]. TAS-102 was developed as an oral formulation, in which TPI increases the bioavailability of TFT by preventing TFT breakdown by thymidine phosphorylase (TP). TFT is the active metabolite, which can be incorporated into the DNA and inhibit the DNA synthesis enzyme thymidylate synthase (TS)[2,3]. These events will result in the induction of cell death and/ or cell cycle arrest in cancer cells. An additional advantage of the addition of TPI to TFT is that TPI may inhibit the angiogenesis promoting effect of TP and subsequently the development of metastasis[4]. TP has been reported to be overexpressed in various cancer types, and has been related to a high microvessel density, the induction of metastasis, a higher invasive potential and a poor prognosis for the patient[4,5,6]. The exact molecular role of TP in these events, however, remains to be elucidated. The main goal of the research described in this thesis was to investigate the dual potential of TAS-102. First, the mechanism underlying TFT-dependent cytotoxicity and drug combinations that may enhance its activity in colon cancer cells. Secondly, the anti-angiogenic potential of TPI was examined.

TFT RESISTANCE

Drug resistance is an important limitation in cancer therapy. Resistance can be intrinsic or acquired during treatment. TS overexpression was reported to be involved in resistance to 5-FU and also to 5-FU-based formulations. TS is also an important target of TFT, and thus will play a role in resistance[7]. Other factors can also play a role in TFT-resistance, such as the key activating enzyme thymidine kinase (TK)[8]. In chapter 2, resistance to TFT was examined by generating resistant H630 colorectal cancer cells making use of two different TFT-exposure schedules. In the first schedule, cells were continuously exposed to increasing concentrations in time, up to cells growing continuously in medium with 20 μM TFT. In the second schedule, cells were intermittently exposed for 4 h every week up to 250 μM TFT. These two resistant cell lines, named H630-cTFT and H630-4TFT respectively, were found to have different mechanisms underlying their resistance. Both cell lines were highly resistant to TFT, with IC_{50} values over 200 fold higher than the parental cell line. TS or TP did not play a role in the resistance to TFT in both cell lines. H630-4TFT expressed the

TK mRNA and protein to a lower extent, and almost no intracellular accumulation of active TFT-metabolites was detected. A decreased level of TK expression has been reported previously to be involved in TFT resistance[8]. In addition, the equilibrative nucleoside transporter (ENT) was expressed (mRNA) at lower level compared to the parental cells. This may also result in a decreased intracellular accumulation. In H630-cTFT cells, alterations in the expression or activity of TFT related metabolic enzymes were not found. Therefore, mRNA microarray expression analysis was performed, revealing a 47 fold upregulation of the enzyme secretory phospholipase A2 (sPLA2). The key role of sPLA2 in TFT resistance was further demonstrated, since inhibition of this enzyme almost completely reversed resistance. The exact mechanism of sPLA2 in TFT resistance remains unclear. This cell line had many alterations in chromosomal regions, as determined by array CGH. Taken together, it can be concluded that the different exposure schedules resulted in two different TFT-resistant variants in which TK was important in H630-4TFT and sPLA2 in H630-cTFT.

MECHANISM OF TFT-INDUCED CELL DEATH

Cell death is one important end-point of anti-cancer treatment with cytotoxic agents. Cell death can be induced via the activation of various pathways, which may also cross-react[9,10]. Apoptotic cell death is mainly induced via the intrinsic and extrinsic apoptotic pathways that can also cross-activate each other[9]. Since TFT has shown activity in 5-FU resistant cell lines[2,11], and has demonstrated to be active in 5-FU pretreated patients[1], we determined whether these drugs may activate the apoptotic core machinery in different ways. We found that TFT induced growth inhibition and cell death that was independent of p53[12] (Chapter 3). P53 is absent or mutated in the majority of colorectal cancers[13]. 5-FU has been reported to be more dependent on p53 to exert its activity, although reports about the role of p53 in 5-FU mediated cell death are contradictory[14,15,16]. Comparison of the effects of TFT and 5-FU on the survival of colorectal cancer cells showed TFT to be more active[17] (Chapter 4). However, this could not be attributed to a differential activation of cell death mediators, but rather by the level of cell death induction. TFT induced higher levels of cell death and activated both caspase-dependent and caspase-independent cell death, involving the lysosomal protease cathepsin B, to a higher extent than equitoxic concentrations of 5-FU. Moreover, 5-FU activated an autophagy response known to have survival rescue function, which was not activated by TFT. Especially the induction of autophagy can be a limitation for 5-FU as anticancer therapy[18]. Since TFT does not elicit an autophagic response, this is an advantage over 5-FU. However, it must be noted that the exact role of autophagy in cancer

and cancer therapy remains somewhat controversial[19]. Autophagy was described as a tumor suppressor mechanism by killing cells undergoing transformation, but also enables cell survival after the induction of stress. Some anticancer agents induce autophagy that contributes to the decay of tumor cells, such as the mTOR inhibitor rapamycin[20]. Other studies focus on the development of autophagy inhibitors to prevent a survival rescue response[19]. In case of 5-FU, autophagy appears to be an unwanted side-effect and inhibition of autophagy resulted in a much higher level of cytotoxicity[17,18]. Therefore, a combination of 5-FU with autophagy inhibitors might increase its cytotoxic action on the long term[21]. Another difference in the actions of drugs is that TFT induces cell cycle arrest at the G_2/M-phase and causes the appearance of polyploidy cells[22] (Chapter 5), whereas 5-FU was reported to induce a G_1- and S-phase arrest and polypoloidy has not been reported to be induced[23]. The differences in response to TFT and 5-FU-induced damage are likely related to differences in their mechanisms of action. Although TFT and 5-FU have overlapping actions, e.g. inhibition of TS and the induction of DNA damage, 5-FU can also interfere with RNA synthesis. Moreover, the extent of DNA damage and TS inhibition may be different between the two agents, or TFT may have other actions that remain to be identified.

ENHANCEMENT OF CYTOTOXICITY

Novel anticancer agents are often administered in combinations with more standard treatments to increase the cytotoxic potential. For example, 5-FU is combined with oxaliplatin and irinotecan in the regimens FOLFOX and FOLFIRI, respectively, which are currently being extended with other agents, mostly anti-angiogenic agents. TAS-102 is potentially an excellent candidate to be combined with other agents. In this thesis we describe the combination of TFT with docetaxel (Chapter 5) and erlotinib (Chapter 6).

Docetaxel is a taxane that inhibits microtubule function[24], resulting in cell cycle arrest in the S/G_2/M-phase and the induction of polyploidy[25]. TFT also induces an arrest in the G_2/M-phase and causes polyploidy. The induction of polyploidy was an unexpected effect for a nucleoside analogue, which are known to predominantly cause a S-phase arrest because of interference with DNA synthesis[22]. When cells were exposed to docetaxel, cell cycle arrest was already detectable after 24 h exposure. The combination of TFT with docetaxel was synergistic, but only when cells were 24 h pre-exposed to docetaxel (Chapter 5). Interestingly, administration of TFT followed by docetaxel displayed strong antagonistic activity, and was accompanied by less polynucleation and cell death induction than seen under the synergistic combinations. Synergy was related to a higher level of cell death,

polyploidy, the formation of aberrant mitotic spindles, and an G_2/M-phase arrest that was accompanied by phosphorylation of the cell cycle kinase Chk2 and dephosphorylation of cdc25c. Thus, synergistic activity is associated with the ability of docetaxel to initiate mitotic failure prior to TFT-dependent cytotoxicity.

The epidermal growth factor receptor (EGFR) is a relatively novel target in cancer therapy. EGFR is often overexpressed in colorectal cancer, making it a potential target for therapy. To examine whether EGFR inhibition in combination with TFT could enhance the cytotoxicity in colon cancer cells with moderate sensitivity to EGFR-tyrosine kinase inhibitors (TKI), erlotinib was used[26]. The evaluated combinations displayed synergistic activity that was independent of the exposure-schedule (Chapter 6). TFT activated the pro-survival signaling pathways Akt and MAPK, that has also been described for other anticancer compounds such as cisplatin[27]. The addition of erlotinib resulted in inhibition of these signaling pathways, which was accompanied by an increased cell cycle arrest and DNA damage induction. The increase in TFT-induced DNA damage might be related to inhibition of DNA repair enzymes, which has previously been reported to be an indirect target of erlotinib[28,29]. The combination was synergistic in cells that were sensitive or moderately sensitive to erlotinib. Since the combination between TFT and erlotinib was synergistic in cells that were moderately sensitive to erlotinib, it potentates the use of TFT in such combinations. Although erlotinib has failed as a therapy in colorectal cancer, this study provides a basis for molecules targeting EGFR in combination with TFT. Future studies should focus on the new generation TKIs and the multi-targeted TKIs as combination strategy in colorectal cancer[30,31].

ANTI-ANGIOGENIC POTENTIAL OF TPI

Thymidine phosphorylase (TP) is also known as the platelet derived-endothelial cell growth factor (PD-ECGF). Numerous studies, mostly with immunohistochemical staining, revealed a relationship between TP expression and angiogenesis[6]. However, the exact role of TP in the induction of angiogenesis is still unclear. TP converts thymidine to thymine and deoxyribose-1-phosphate (dR-1-P). dR is the first metabolite from this reaction that can be secreted by the cells. Many studies focus on dR as angiogenic mediator of TP, however whether dR is a valid and only angiogenic mediator of TP is unclear[4,5]. dR itself has angiogenic activity at high concentrations in various *in vitro* and *in vivo* studies[32,49]. It remains to be elucidated whether TP itself, a product such as a sugar, or a more downstream event such as ones involving angiogenic factors VEGF, IL-8, bFGF and TNF-α contribute to the angiogenic

activity of TP[33]. In Chapter 8, the metabolic conversion of the sugars by TP was investigated. Although the conversion of deoxyribose-sugars has been demonstrated in bacteria, it has never been described in human eukaryotic cells. We demonstrated that dR-1-P and dR-5-P accumulated intracellularly to a high extent, however the level conversion was cell type dependent. Moreover, the balance between the (measured) sugars was highly regulated, since the conversion to other sugars was rapid. Previously it was already reported that dR-1-P was converted rapidly, possibly to dR-5-P[34]. The exact metabolic pathway that is further activated, e.g. pentose phosphate pathway, glycolysis or the formation of advanced glycation endproducts (AGE), remains to be identified. Since the recently gained interest in the Warburg effect[35], and the role of TP in this same pathway[36], further studies should focus on molecular mechanisms of TP in sustaining cell viability. Since it is unknown what the fate of the sugars in the cell is, radioactive labeled thymidine (labeled in the sugar moiety) was used and subsequently it was determined in which cellular fractions the thymidine-sugar-metabolites accumulated. This revealed that a large fraction of the formed sugars accumulated in the cytoskeleton and in the membrane fraction of the cells. The membrane fraction could be related to an increase in the expression of migration-proteins that can be glycosylated to increase the motility[37]. Many proteins in the membrane are involved in cell-cell communications and cell migration. Especially the sugars on the proteins (glycosylated proteins) play important roles in these contacts. It should be further examined whether the formed sugars by TP can be used for glycosylation of proteins.

Angiogenic activity can be detected in blood samples by monitoring endothelial progenitor cells (EPCs). These cells are currently evaluated for the use as a possible biomarker, and as a diagnostic tool and for their therapeutic potential and gene therapy[38,39,40]. EPCs are highly proliferative and they have typical endothelial characteristics *in vitro*. EPCs are not derived from CD133+ cells or CD45+ haematopoietic precursors, but they do have the surface markers; VEGF2, CB34, CD31, CD146 and CD105[41]. One proteomics report revealed that TP plays an important role in angiogenesis of EPCs[42]. Therefore, we compared the TP activity of EPCs (endothelial colony forming cells, ECFC) with that of human umbilical vein endothelial cells (HUVECs), and whether the migration and invasion of these cells could be stimulated by stimulating TP (Chapter 9). ECFCs and HUVECs had a low TP activity, which is in agreement with previously published data[42]. The migration of ECFCs and HUVECs could be stimulated by dR or dR and TdR, respectively. TPI did not inhibit the migration. Moreover, the invasion was neither stimulated nor inhibited by these agents. Possibly, intrinsic TP in endothelial cells (ECFCs or HUVECs) is not important for their angiogenic potential.

To examine whether TP expressing cells can secrete products that simulate angiogenesis, conditioned medium was used and the angiogenic potential of endothelial cells (HUVECs) was determined (Chapter 10). Cells with high TP expression secreted the angiogenic factors IL-8, bFGF and TNF-α to a higher extent than non-TP expressing cells. This correlated with an increased migration and invasion of endothelial cells towards medium derived from TP expressing cells. This was mediated by the activation (phosphorylation) of p70/S6k, but not the focal adhesion kinase (FAK). Interestingly, TP activity was upregulated by conditioned medium from Colo320 TP1 cells, but not by that of RT112/TP cells, indicating that Colo320 TP1 cells secrete molecules that increases TP expression, which is possibly TNF-α[43]. Inhibition of TP with TPI reduced the migration by that was induced by the conditioned medium of both cancer cell lines. L-dR reduced the migration to some extent, indicating that both TP and dR can modulate angiogenesis. Taken together, both TP and dR can stimulate angiogenesis, while TP also activates various angiogenic factors (IL-8, bFGF and TNF-α) increasing the angiogenic potential. Since all inhibitors completely reversed the induced migration and invasion, it is likely that these factors are linked with each other. The exact molecular mechanism between these aspects should be determined in future studies.

Since TP expression in cancer cells has also been reported to play a role in their invasive characteristic[44,45], the invasion was also determined in the cancer cells with a high and no TP expression (Chapter 10). However, there was no relation between cells with or without TP expression in the invasion. Interestingly, cells with a high TP expression had lower phosphorylation levels of the kinases FAK and p70/S6k. These kinases are involved in migration, cell-cell contacts and proliferation[46,47], and have previously been reported to play a role in TP mediated cell migration in endothelial cells[48,49]. Probably, these kinases do not play a role in the tested cancer cells with a high TP expression.

CONCLUSIONS AND FUTURE PERSPECTIVES

The formulation TAS-102 combines the anticancer agent TFT with the anti-angiogenic agent TPI. The dual targeting of this formulation provides perspective for the treatment of colorectal and gastric cancer. TFT has advantages over current therapies using 5-FU, since we found that TFT is more potent in killing tumor cells and does not induce autophagy that can counteract tumor cell kill. Moreover, TFT was shown to act in synergy with various different anticancer agents. These aspects should be further investigated and evaluated, in order to design clinical combination studies in patients with solid tumors. TP also has an

important role in the induction of angiogenesis that TPI can potently inhibit, in addition to causing an increase in TFT bioavailability. The anti-angiogenic effect of TPI should be further determined in *in vivo* models, which can also help to find out the molecular mechanism underlying its angiogenic effect. In conclusion, the novel formulation TAS-102 provides promising perspectives as an anti-cancer agent for the treatment of colorectal and gastric cancer.

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