Chapter 1

General introduction
COLORECTAL CANCER

Cancer is the number one cause of death worldwide, replacing ischemic heart disease\(^1\). Cancer includes all types of malignant tumors, in which cells display an abnormal growth. When a malignant tumor grows, it can invade into nearby tissues and metastasize to distant organs via the blood or the lymph vessels. Almost all tumors are initially caused by mutations in the DNA. These mutations can be induced by carcinogens (tobacco smoke, chemicals). Other cancer-promoting genetic abnormalities can be randomly acquired by errors in DNA replication (aging), or are genetically inherited (the cancer genes, such as APC in colon cancer).

The genetic abnormalities affect two classes of genes, the oncogenes (genes promoting cancer growth) and the tumor suppressor genes (genes suppressing cancer growth). Six hallmarks of cancer were described and are widely used as a basis for cancer investigations\(^2\). These hallmarks include (1) self sufficiency in growth signals, (2) insensitivity to growth inhibitory signals, (3) evasion of programmed cell death (apoptosis), (4), limitless replicative potential, (5) sustained angiogenesis, (6) tissue invasion and metastasis. In addition, this list can be extended with (7) evasion of the immune system\(^3\) and because of recent renewed interest with (8) autophagy\(^4\) and with (9) metabolic (glycolytic) abnormalities (e.g. the Warburg effect)\(^5\).

Of all cancers, colorectal cancer (CRC) is the second most common cancer leading to death in the Western world\(^6\). The incidence of CRC in the Western world is in general higher than in the Asian countries, although the incidence in Japan is increasing. In contrast, gastric cancer is more abundant in Japan than in European countries. In the Western world, it is estimated that over 207400 persons die each year from CRC, but these numbers are increasing, possibly due to the Western diet. In the Netherlands, approximately 11000 new cases are diagnosed and 4500 patients die of this disease each year. About 75% of the CRC patients have sporadic disease, the remaining 25% have a familial history of CRC, where about 5% of the latter is due to genetic mutations as cause of inherited cancer risk.

CELL DEATH AND SURVIVAL

The growth of cancer cells can result from enhanced proliferation and/or diminished cell death induction. Inefficient elimination of cancer cells might be a consequence of alterations in the cell death pathways. This means that cancers can grow because of these mutations
or alterations in the cell death pathways, preventing cells from dying. Treatment of cancer cells with anticancer agents or radiation therapy will result in the induction of cell death.

Numerous cell death pathways have been described. Apoptosis is one of the most important programmed cell death mechanisms, in which caspases play key-roles. Two classical apoptotic cell death pathways have been described that can interact (Figure 1). The intrinsic apoptotic pathway involves activation of the initiator caspase 9. This pathway is activated upon cellular damage, such as DNA damage, that leads to modulation of the expression of members of the Bcl-2 family. The balance between proapoptotic and antiapoptotic members determines whether mitochondria are disrupted.

When the mitochondrial is disrupted, cytochrome C is released into the cytoplasm, after which the apoptosome is formed, consisting of procaspase 9, dATP and Apaf-1. This will result in cleavage of the procaspase 9, which is then active. Active caspase 9 on its turn cleaves and activates executioner caspases, such as caspase 3. The extrinsic apoptotic pathway is induced after activation of caspase 8. Upon binding of death ligands, such as tumor necrosis factor (TNF), TNF-related apoptosis inducing ligands (TRAIL) and Fas ligand to specific death receptors (DRs) at the cell surface (TRAIL receptors (DR4, DR5), CD95/Fas and TNF-α receptor-1), the adaptor molecules Fas-associated death domain (FADD) or TNF Receptor-associated death domain (TRADD) are recruited to the receptor that subsequently bind procaspase 8. This binding results in cleavage and activation of caspase 8. Caspase 8 will subsequently activate the executioner caspase 3, that cleave substrate proteins, including poly ADP ribose polymerase (PARP), leading to the orderly destruction of cells. Additionally, caspase 8 can cross-activate the intrinsic apoptotic pathway via cleavage of the proapoptotic Bcl2 members, Bax and Bid, causing the mitochondrial release of cytochrome c. Apoptotic cell death is characterized by specific changes in cellular and nuclear morphology, including DNA fragmentation, cell shrinkage and fragmentation into apoptotic bodies, facilitating a non-inflammatory disposal of cells.

Besides caspase dependent cell death, caspase independent cell death also occurs. Alternative pathways of cell death can be mediated by lysosomal cathepsins and/or calpains. Cathepsins are proteases that induce cell death after leakage from the lysosomes, such as the release of cathepsin B into the cytoplasm. When released into the cytoplasm, they cleave substrate proteins, thereby inducing cell death ranging from apoptotic to necrotic types of death. Necrosis is considered a non-regulated form of death, associated with enlargement of the cell and subsequent cell lysis. Cathepsins were reported to be involved in the cell death induction after several anticancer agents, including that of taxanes. Additionally, cathepsins can also activate the caspase-dependent cell death, possibly via the mitochondria (Figure 1).
Autophagy is a mechanism which can act both as a survival and as a cell death pathway. Autophagy is a reversible cellular state, which is induced by amongst other metabolic stress. It maintains the cellular metabolism through recycling of cellular components when the availability of external nutrient sources is limited. Cell death is then prevented by removal of damaged proteins and organelles. After the induction of metabolic stress, autophagy is activated via the PI3K pathway. During autophagy, the autophagy marker light chain 3 (LC3B-I) is converted to LC3B-II through lipidation by a ubiquitin-like system involving autophagy related gene 7 (Atg7) and Atg3 that allows LC3B to become associated with autophagic vesicles. Indicators of autophagy are the conversion of LC3 to the lower migrating form LC3-II and the presence of LC3B in autophagosomes. Double membrane vesicles are formed and enfold proteins, cytoplasm, protein aggregates, and organelles that are then delivered to lysosomes where they are degraded. When the metabolic conditions are restored, cancer cells recover and continue to proliferate. Autophagy has recently been described to be induced by various anticancer agents, including 5-fluorouracil, rapamycin and tamoxifen. Since autophagy can prevent cell death it is an unwanted side-effect of cancer therapy allowing tumor cells to survive treatment. The exact role of autophagy in cancer remains somewhat controversial. On one
hand, autophagy enables tumor survival under stress conditions, but on the other hand it can also act as a tumor suppressor mechanism by killing cells that undergo tumorigenesis. This was proposed following the observation that cell death occurred concomitant with features of autophagy upon excessive stimulation of autophagy through overexpression of beclin1 during tumorigenesis\textsuperscript{4,14,15}. Excessive cellular damage may lead to cell death by overstimulating autophagy and cellular self-consumption. In this way, autophagy is not able to sustain and cell death will be induced. However, whether this type of autophagic cell death induction will also act in the clinic remains to be evaluated. \textit{In vivo} models to support these concepts are limited\textsuperscript{14}.

**ANGIOGENESIS**

Angiogenesis is the formation of new blood vessels from existing vessels (Figure 2)\textsuperscript{16}. When a tumor grows, the distance of the cancer cells from the blood vessel increases. When the distance becomes too large, cells do not get enough oxygen and nutrients, resulting in hypoxia. As a response to hypoxia, cells will secrete various factors that stimulate angiogenesis, such as Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8) and is usually induced by hypoxia inducible factor-1\textalpha{} (HIF-1\textalpha{}). Endothelial cells from nearby blood vessels subsequently start to migrate, proliferate and invade towards the hypoxic site and subsequently form an organized and functional structure. New treatment strategies aiming to inhibit this process and thereby preventing tumor growth and metastasis are under exploration.

![Figure 2 - Schematic overview of the induction of angiogenesis. When the tumor grows, angiogenic factors, such as VEGF, bFGF and IL-8 are secreted that induce the proliferation, migration and invasion of endothelial cells towards the tumor. This will lead to the formation of a new functional blood vessel, thus securing the supply of nutrients required for continued tumor growth.](image-url)
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Thymidine phosphorylase in angiogenesis

The platelet derived endothelial cell growth factor (PD-ECGF) is also known as thymidine phosphorylase (TP). Numerous immunohistochemical and TP-activity studies have shown that TP is upregulated in a wide-range of solid tumors compared to normal healthy tissues. A high TP expression in tumor sites has clearly been related to a high microvessel density, the induction of metastasis and a poor prognosis for the patient. The location of TP expression, however, varies between tumor type, is dependent on the tumor grade and has been reported to be highly expressed in tumor cells, in the invasive part of the tumor (bladder) or in tumor stromal cells (colon, breast). TP is also upregulated in other diseases in which angiogenesis plays a role in the pathology. One example is rheumatoid arthritis (RA).

Figure 3 - Schematic overview of the conversion of thymidine to thymidine-related sugars and possible pathways which can stimulate angiogenesis. TK: thymidine kinase; TP: thymidine phosphorylase; TdR: Thymidine; dR: deoxyribose; dR-1-P: deoxyribose-1-phosphate; dR-5-P: deoxyribose-5-phosphate; AGE: advanced glycation endproducts; G3P: glyceraldehyde-3-phosphate.

TP catalyzes the reaction of thymidine (TdR) to deoxyribose-1-phosphate (dR-1-P) and thymine. dR-1P can be converted to either deoxyribose (dR) or isomerized to deoxyribose-5-phosphate (dR-5-P) (Figure 3). dR can form advanced glycation endproducts (AGE), which may play a role in the induction of angiogenesis. dR-5-P can also be dephosphorylated to dR or split into glyceraldehyde-3-phosphate (G3P) and acetaldehyde. G3P can enter the glycolytic or pentose phosphate pathway. The exact product that is
formed by this reaction is cell type dependent. To date, several hypotheses have been postulated concerning the mechanism of the pro-angiogenic effect of TP, all involving the generation of dR-1-P and dR from the phosphorylytic breakdown of TdR. It is believed that dR plays a role in the angiogenic effects of TP, since this is the main product of the reaction that can be secreted from the cells. dR has shown angiogenic properties in various in vitro studies using endothelial cells. In these studies, addition of dR and TP could activate angiogenic properties of endothelial cells and activated the focal adhesion kinase (FAK) and p70S6k. FAK plays an important role in the invasion and migration of cells and in cell death. P70/S6k is the important downstream kinase of mTOR, regulating cell proliferation, metabolism and also angiogenesis. This pathway was activated by dR and TP stimulation in endothelial cells. In addition, TP is often co-expressed with the important angiogenic factor VEGF. TP has been related to the secretion of other angiogenic factors, such as IL-8.

Many studies have used cancer cell lines that express TP, showing that TP induces invasion in vitro and metastasis in vivo in mice. Moreover, TP plays a role in the prevention of apoptosis induction in both cancer and endothelial cells. To reduce the tumor aggressiveness and angiogenesis, TP inhibitors have been synthesized, including TPI. TPI alone decreased the invasion of KB/TP and A549/TP cancer cells and suppressed the number of liver metastasis in various mouse models. In endothelial cells, conditioned medium from cancer cells stimulated the angiogenic properties, e.g. migration and invasion. TPI significantly inhibited these properties. TPI inhibited (TP-induced) angiogenesis. In a KB/TP dorsal air sac assay, angiogenesis was highly induced, which was inhibited by TPI. Another possibility to inhibit the biological action of TP is by addition of L-deoxyribose (L-dR), a stereoisomer of dR. L-dR inhibits the actions of dR, but not directly the TP enzymatic activity. Although L-dR is often used to study the actions of TP, dR is not the only product that may be secreted as a pro-angiogenic factor. Moreover, it is not exactly known how cancer cells with a high TP expression influences the behavior of endothelial cells.

**CANCER THERAPY**

Once diagnosed, cancer can be treated with surgery, chemotherapy and/or radiotherapy, depending on the stage of disease. Usually, therapies are combined to improve the anti-cancer response. Types of chemotherapy are very diverse. Many patients with CRC have microscopic-metastatic disease at the time of diagnosis. About 50% of the patients with apparent early stage disease will eventually develop metastasis. The standard therapy in
CRC consists of a (wide) surgical resection and anastomosis to restore continuity. Surgery is feasible for about 75% of the tumors with primary CRC. However, after a complete resection of the primary tumor, recurrence is often observed (50%). The incidence of recurrence can be decreased by adjuvant therapy. Adjuvant chemotherapy is administered to eradicate micrometastatic disease, which may remain after surgery. 5-Fluorouracil (5-FU), alone or modulated with leucovorin (LV), oral fluoropyrimidines (such as UFT and capecitabine), raltitrexed, irinotecan and oxaliplatin are the classic cytostatic drugs studied alone or in combination as adjuvant therapy for colon cancer. Monoclonal antibodies directed against the epidermal growth factor receptor (EGFR) and VEGF (e.g. cetuximab and bevacizumab, respectively), have demonstrated activity in metastatic disease and are currently under investigation. Cetuximab is a G1 immunoglobulin (IgG1) which binds selectively to EGFR, with greater affinity than its natural ligands. Cetuximab reduces the expression of EGFR and interferes with the transmission of various signaling pathways. Bevacizumab is a humanized anti-VEGF monoclonal antibody that interferes with the process of tumor angiogenesis by sequestering VEGF and preventing receptor activation. Both monoclonal antibodies have demonstrated their effectiveness in advanced CRC. Newer combination strategies that are currently investigated are with EGFR and VEGF tyrosine kinase inhibitors. However, when these antibodies are combined in one therapy against metastatic CRC, the effect is detrimental as shown by CAIRO2 and PACCE phase III trials.

Adjuvant chemotherapy for CRC
For stage III patients, adjuvant chemotherapy consists of 5-FU-leucovorin (folinic acid; LV) regimens. Currently, 5-FU/LV is often combined with either oxaliplatin (FOLFOX) and irinotecan (FOLFIRI), cetuximab or bevacizumab. Adjuvant therapy may be curative in stage III-IV disease. However, about one-third of the treated patients will die because of metastatic disease. Treatment of patients with recurrent or advanced (stage IV) CRC depends on the location of the disease. The standard initial treatment for non-metastatic CRC is surgical excision of the primary tumor and the regional lymph nodes. For patients with metastatic CRC, systemic chemotherapy is effective to prolong survival and time to disease progression.

Systemic chemotherapy for metastatic CRC
For many decades, the first-line treatment option for metastatic CRC was 5-FU, to which LV was added. The addition of LV to 5-FU increased the response rate about 2-fold, but failed to significantly improve the overall survival. To further improve 5-FU therapy, various
combinations and treatment schedules were evaluated. Initially, 5-FU was evaluated as a bolus injection, which was subsequently compared with a continuous infusion. From several clinical trials, a continuous infusion resulted in better tolerance profile than the bolus 5-FU, although this was not accompanied by a significant benefit for the overall survival. Currently one of the most widely used schedules is a short (2 h) high dose infusion followed by a 22 or 46 h infusion at a lower dose, usually combined with irinotecan or oxaliplatin (FOLFIRI and FOLFOX) regimens.

To further improve therapy, oral formulations were developed. Oral treatment is generally preferred above the cumbersome continuous infusions, to improve the quality of life of the patients and it consumes less hospital resources. Therefore, oral (5-FU-based)-formulations were developed. These formulations have at least equal efficacy compared to continuous infusions with 5-FU. Ftorafur is used in several combinations to improve its bioavailability. UFT consists of ftorafur, combined with uracil (1:4 molar ratio). Uracil is the natural substrate for dihydropyrimidine dehydrogenase (DPD). Addition of uracil to ftorafur results in less degradation of 5-FU by DPD. UFT has been approved in most developed countries (excluding USA) for advanced CRC. S-1 was approved in Japan in 1999 for advanced and recurrent gastric cancer and has subsequently been approved for various other malignancies in Eastern-Asia, including CRC. S-1 consists of ftorafur with 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (OXO) (molar ratio ftorafur:CDHP:OXO 1:4:1). CDHP and OXO have no antitumor activity but modulate 5-FU metabolism. CDHP is an inhibitor of DPD, resulting in an increased 5-FU circulation time. CDHP is about 200-fold more potent than uracil in inhibiting DPD. OXO limits the gastrointestinal toxicity of ftorafur. This toxic effect is mediated by phosphoribosylation of 5-FU to FUMP by OPRT. OXO specifically accumulates in gastrointestinal tissues, compared to tumors, preventing 5-FU activation in normal mucosa but not in the tumor. Xeloda (capecitabine) was developed to decrease the systemic toxicity and to enhance 5-FU activation at the tumor site. However, the survival for metastatic CRC patients did not increase with the use of this new prodrug, but was at least as effective as 5-FU/LV.

**TAS-102**

TAS-102 consists of the combination of the fluoropyrimidine trifluorothymidine (TFT) with a thymidine phosphorylase inhibitor (TPI), to prevent metabolic break down. TFT is the active agent, targeting the DNA and inhibiting the enzyme thymidylate synthase (TS). TS is a rate-limiting enzyme in the pyrimidine de novo deoxynucleotide synthesis, therefore it is an excellent target for chemotherapeutic strategies. TS plays a key role in the de novo synthesis of thymidine-nucleotides, converting deoxyuridine monophosphate (dUMP) to
deoxythymidine monophosphate (dTMP). Upon inhibition of TS, DNA synthesis is halted and consequently dUMP accumulates in the DNA. The addition of TPI has two potential advantages: (1) it prevents inactivation of TFT by inhibiting TP-mediated breakdown and (2) it potentially may inhibit angiogenesis that is induced by TP. TAS-102 is currently studied in clinical trials phase II as an oral formulation.

**Difference between TFT and 5FU**

5-FU is activated to FUMP by the enzyme OPRT. FUMP will be converted to FUDP and subsequently to FdUDP. FdUDP can be activated to FdUTP and degraded to FdUMP (Figure 4). dUTP can directly degrade FdUTP to FdUMP, but it is not clear whether TF-TTP is a substrate. FdUMP irreversibly inhibits TS. Irreversible inhibition of TS is one of the major mechanism of action of 5-FU. When activated to FUMP, it can be incorporated into the RNA. When activated to FdUTP, it can be incorporated into the DNA. These events result in damage to both DNA and RNA.

![Figure 4](image_url) - Schematic overview of the metabolic pathways of 5-FU and TFT. TAS-102 is the combination of TFT with the thymidine phosphorylase inhibitor TPI. UFT combines ftorafur with Uracil and S-1 with CDHP. TP: thymidine phosphorylase; DPD: Dihydropyrimidine dehydrogenase; TK: thymidine kinase; TS: thymidylate synthase; OPRT: orotate phosphoribosyl transferase; UP: uridine phosphorylase.
TFT, when monophosphorylated (TFT-MP) by thymidine kinase (TK) also inhibits TS (Figure 4). The activation of TFT to TFT-MP has less activation steps than from 5-FU to FdUMP. However, TFT does not inhibit TS irreversibly. TFT, when tri-phosphorylated (TFT-TP), can be incorporated into the DNA as well. Upon DNA incorporation, DNA synthesis will stop and cells will die from the induced damage. Compared to 5-FU, TFT needs less activation steps to be activated for DNA incorporation. 5-FU can additionally be incorporated into the RNA, when activated to FUTP. Upon RNA incorporation, RNA synthesis will be inhibited, thereby inducing cell death. TFT can not be incorporated into the RNA.

Both TFT and 5-FU induce cell death that is partially mediated via the activation of caspases, however TFT activity seems to be less dependent on p53. Moreover, TFT induces an arrest in the G2/M-phase and polyploidy, while 5-FU induces an arrest in the S-phase of the cell cycle. Although the G2/M-phase arrest and polyploidy are known to be induced by microtubule inhibitors, such as taxanes, they are less expected to be associated with the activity of a nucleoside analogue that targets the S-phase of the cell cycle, e.g. by TS inhibition and DNA damage induction. In addition, TFT (as TAS-102) inhibits cell growth and induces cell death more potently than the 5-FU prodrug 5’-DFUR in mice. These differences between TFT and 5-FU indicate different cellular actions which might result in differences in resistance profiles.

TFT resistance
TFT has shown activity in 5-FU-resistant cells, both in in vitro and in vivo studies. Resistance to nucleoside analogues is in general conferred by direct alterations in (expression of) enzymes involved in fluoropyrimidine metabolism. This means that next to increased expression of the target enzyme TS, decreased activation by TK or increased degradation by thymidine phosphorylase (TP) are possible mechanisms responsible for (induced) resistance to TFT. In colon cancer cells TS is often overexpressed, resulting in possible drug resistance, which in turn is associated with poor response and/or survival rates in patients after treatment with TS inhibitors. Decreased cellular uptake through nucleoside transporters and increased export by multi-drug resistance proteins (MRP) might play a role as well. Data from previous reports indicate that 5-FU-resistant colon cancer cells are not necessarily cross-resistant to TFT, explaining that TFT exerts antitumor activity against 5-FU-resistant cancer cells. For TFT, a decreased TK expression and activity is an important mechanism of resistance. However, enzymes involved in TFT metabolism do not necessarily have to be related to the induction of TFT resistance. The method to induce TFT resistance may lead to different mechanisms of resistance. In this thesis we describe that a continuous exposure to increasing doses of TFT resulted in resistance in which secretory
phospholipase A2 and the equilibrative nucleoside transporter (hENT) were involved. When cells were exposed intermittently to increasing doses of TFT, resistance was mediated by decreased TK and hENT expression.

Combination studies with TFT

To improve the action of anticancer compounds drugs are usually given in combinations. Combinations can exist with other chemotherapeutic agents, with radiation therapy or immunotherapy. 5-FU is currently administered in combination with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX). Therefore, various combinations with TFT are currently under investigation.

TFT and antifolates inhibit the enzyme TS, although in a different manner. At low folate conditions, TFT and folate-based TS inhibitors have been reported to be synergistic, whereas at high folate conditions, the combination had only additive activity. When TFT was combined with oxaliplatin, an increase was found in platina-DNA adducts and subsequent increased DNA damage and cell death. The combination was schedule dependent. A combination of TFT with irinotecan resulted also in schedule dependent synergism (when cells were pre-treated with TFT) due to increased DNA strand breaks and cell death induction. A combination of TFT with the taxane docetaxel was synergistic, mainly when cells were pre-treated with docetaxel, leading to the induction of poly-nucleated cells and cell death. In another combination where the EGFR-TKI erlotinib was added, no schedule dependent cell death was reported. Synergism by this combination was related to inhibition of pro-survival signaling which was activated by TFT. This combination was synergistic in cells that were sensitive to TRAIL due to an increased induction of cell death, and the prevention of the induction of polynucleated cells by TFT.

Potential other combinations that can be tested in the future are a combination with the proteasome inhibitor bortezomib, the tyrosine kinase inhibitor sunitinib and the VEGF blocking antibody Avastin. Especially the combination with anti-angiogenic agents such as sunitinib and Avastin might have potential, because of the potential role of TPI in inhibiting angiogenesis. Moreover, many (metastatic) CRCs have mutations in these pathways. Additionally, a combination of TFT with irradiation can have potential, since TFT induces a G2/M-arrest and polyploid cells.

TAS-102 in clinical studies

TFT was initially developed as an antiviral agent and is registered as Viroptic® for the use against herpes simplex virus (HSV) infections. In addition, TFT has been approved by the
Food and Drug Administration (FDA) for the treatment of primary keratoconjunctivitis and epithelial keratitis\textsuperscript{91}.

The antitumor potential of TFT has been investigated in various clinical trials I and II phase, where TFT was administered intravenously (i.v.). The first report on antitumor effects of TFT in colon and breast cancer patients dates from 1971\textsuperscript{82}. This study showed that multiple administrations of TFT can reduce the tumor size. However, the systemic administration of TFT alone (2.5 mg/kg/day) in doses that were administered every 3 h for 8–13 days resulted in severe bone marrow suppression. Interestingly, TFT had less toxicity towards the gastrointestinal tract, when compared to 5-FU or FdUrd, although toxicities were found against the haematopoietic system\textsuperscript{83}. Although promising in the first trials, development was initially halted, because of the short half-life (only 12 minutes after injection) of TFT due to the rapid clearance and extensive degradation by thymidine phosphorylase (TP). Due to these disadvantages, the anti-tumor effect of TFT alone was only moderate with no objective responses\textsuperscript{82}.

Since the development of a specific TP-inhibitor, the thymidine phosphorylase inhibitor (TPI) by Taiho Pharmaceuticals (Tokushima, Japan), TFT regained its clinical interest. TFT re-entered clinical development in combination with TPI as TAS-102. Currently, three phase I clinical trials have been completed where different dosing schedules were used\textsuperscript{84,85,86}. In the first study, TAS-102 was administered orally once daily for 14 days, followed by a 1-week rest, repeated every 3 weeks. The maximum tolerable dose was set at 50 mg/m\textsuperscript{2}/day\textsuperscript{84}. In a second study, TAS-102 was administered once daily on either days 1-5 and 8-12 every 4 weeks or days 1-5 every 3 weeks. The recommended phase II doses for TAS-102 were 100 mg/m\textsuperscript{2}/day and 160 mg/m\textsuperscript{2}/day, respectively\textsuperscript{85}. An additional recommendation of this study was to evaluate multiple daily dosing schedules. This was also recommended by Emura et al. who showed in a preclinical study that multiple daily dosing may result in a better clinical benefit\textsuperscript{67}. In another study, a multiple-daily dosing schedule was evaluated, with a schedule of 3 times a day on days 1-5 and 8-12 every 4 weeks\textsuperscript{86}. In this study no responses were noted, but 9 of the 14 enrolled patients demonstrated prolonged stable disease in this heavily pretreated 5-FU refractory population. From this multiple daily dosing schedule study, TAS-102 was reported to be well tolerated with manageable hematological and non-hematological toxicities. TAS-102 is currently studied in clinical trials phase II against colorectal and gastric cancer.
AIMS AND OUTLINE OF THESIS

The aim of the research described in this thesis was to determine new mechanisms of action of the novel oral anticancer formulation TFT (TAS-102, containing TFT and TPI) in colorectal cancer models in order to identify possible mechanisms of resistance and to test combinatorial strategies to improve efficacy. Also the role of thymidine phosphorylase in angiogenesis and the possible benefits of using TPI to inhibit this process were explored.

Drug resistance is a major complication in cancer therapy. TFT has shown activity in 5-FU resistant cells \textit{in vitro}, but also in 5-FU refractory patients. Cells with a high TS expression can be cross-resistant to TFT. In Chapter 2, novel mechanisms of TFT resistance are described in H630 cells that were made resistant by exposing cells to two different treatment schedules. These two differently generated resistant cell lines were characterized for their expression levels of metabolic enzymes, gene expression profile and chromosomal alterations.

Cell death is an important result of treatment of cancer with anticancer agents (Chapter 3 and 4). Therefore we determined the mechanism of cell death induction by TFT and compared this with that of 5-FU to explain possible differences in response to TFT. For that purpose we determined the exact activation route by caspases, but also examined other cell death routes, including the role of p53 (Chapter 3), the lysosomal cathepsin B and the pro-survival route autophagy (Chapter 4).

Combinations of TFT with other agents might increase the cytotoxic effect. Since TFT increases the accumulation of cells in the G$_2$/M-phase of cell cycle and induces polyploidy, a combination with a taxane (such as docetaxel) that acts in these phases of cell cycle can be a rational combination. Chapter 5 focuses on the combination of TFT with docetaxel, using different combination schedules, since cell cycle effects are important for these types of combinations. Nucleoside analogues may increase the pro-survival signaling of cells. Erlotinib is a tyrosine kinase inhibitor of EGFR, inhibiting the pro-survival signaling. Since EGFR overexpression is often found in CRC patients, we tested the combination of TFT with a tyrosine kinase inhibitor (Chapter 6). These combination studies were performed to give sufficient preclinical support for initiating future clinical combination studies with TAS-102.

Many immunohistochemical studies show a variation in the location of TP expression in tumor sites. TP can be expressed in the tumor cells, but also in the macrophages or in other stromal cells that are present in the tumor surroundings. This location depends on the tumor type and varies between tumor grades. There has been a positive correlation between this TP expression and angiogenesis, and various previous studies indicate a role for TP in angiogenesis. A review describing the role of TP in angiogenesis is described in Chapter 7.
General introduction

Since it is still unknown by which pathway and also which sugars are formed by conversion of thymidine by TP, LC-MS/MS detection was performed to analyze sugars. In addition, it was also analyzed to which cellular compartment where the sugar-metabolites accumulated (Chapter 8).

Various models exist in which angiogenesis can be determined. Recently, endothelial colony forming cells (ECFC) have been used as a new model. ECFCs are circulating endothelial (progenitor) cells (EPC) that are derived from the bone marrow and circulate in the blood. These cells can differentiate into endothelial cells and contribute to the formation of new blood vessels at tumor sites. We examined the effect of TP in the ECFC model and the more conventional model of the human umbilical vein endothelial cells (HUVECs) (Chapter 9). Chapter 10 describes the role of TP expression in cancer cells on the stimulation of angiogenesis (HUVEC migration and invasion). In this study, the mechanism underlying TP-dependent induction of angiogenesis is studied in more detail. Finally, the results are summarized and discussed into perspective in Chapter 11.
REFERENCES


49 Comparison of fluorouracil with additional levamisole, higher-dose folinic acid, or both, as adjuvant chemotherapy for colorectal cancer: a randomised trial. QUASAR Collaborative Group. Lancet. 1999;355:1588-96


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69 Bijnsdorp IV, Temmink OH, Prins HJ, Losekoot N, Adema AD, Smid K, Honeywell RJ, Ylstra B, Eijk PP, Fukushima M, Peters GJ. Resistance induction to trifluorothymidine in colon cancer cells is associated with decreased thymidine kinase and equilibrative nucleoside transporter expression or secretory phospholipase A2 overexpression. Molecular Cancer Therapeutics, in press.


