Role of thymidine phosphorylase/platelet-derived endothelial cell growth factor inhibition in the cytotoxicity of fluoropyrimidines

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Summarizing discussion

& concluding remarks

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In this thesis we aimed to investigate the mechanisms of action of the novel oral anti-cancer drug formulation TAS-102, which consists of TFT and the potent specific inhibitor of thymidine phosphorylase TPI. Initial clinical studies in the 1970's with TFT as a single antitumor agent were discontinued due to the rapid degradation of TFT by TP *in vivo*. In addition, TP is often overexpressed in colorectal tumors, and numerous studies (mostly using immunohistochemical stainings) showed that high TP is an independent prognostic factor for poor clinical outcome of malignant diseases. TP is associated with angiogenesis (e.g. increased microvessel density) and subsequently might stimulate the development of metastases. TAS-102 was specifically developed to increase the antitumor activity of TFT, in which TPI serves an important dual role: it enhances the bioavailability of TFT *in vivo* and has anti-angiogenic properties. For that purpose the role of both TFT and TPI in the antitumor activity of TAS-102 was investigated, thereby focusing on the various enzymes involved in TFT metabolism. TAS-102 is currently tested in the clinic against several gastrointestinal malignancies (summarized in **Chapter 2**), and therefore colorectal cancer cell lines were mostly used in the studies described in this thesis.

TFT metabolism

The role of the enzymes directly involved in TFT metabolism and the effect of TFT on its various targets are mainly discussed in **Chapters 3, 4** and **11**. To determine the possible limiting factor(s) in the cytotoxicity of TFT cancer cell lines with different cellular characteristics were used. Cell lines with either an overexpression or deficiency of one of the enzymes involved in TFT metabolism were included. Cells were exposed in different treatment schedules *in vitro* to TFT and 5FU, which in contrast to TFT can be activated by TP, and sensitivity was assessed in the presence and absence of TPI. We surprisingly found that despite the high rate of TFT degradation, Colo320TP1 cells with high TP expression are not more resistant to TFT, while TPI did not increase TFT sensitivity at long-term exposure (see also results De Bruin *et al.* [1]). It was expected that addition of TPI would result in decreased IC_{50} values, because it was previously shown that TFT is an excellent

substrate for TP. To explain this we aimed to prevent activation of TFT to a maximum extent and shortened the exposure time to 4 hr followed by drug free culturing of the cells. In this situation TFT sensitivity still remained unaffected for TP overexpressing cells or when TPI was added to the medium. This was also an unexpected finding compared to *in vivo* experiments using different animal xenograft models [2],[3]. It was demonstrated previously that TPI enhanced the bioavailability of TFT due to prevention of degradation by e.g. high TP expressing liver cells, subsequentially leading to increased antitumor activity of TFT. In conclusion, *in vivo* TPI is required for TFT to exert any significant antitumor activity, whereas *in vitro* addition of TPI is not necessary to increase TFT cytotoxicity (**Chapter 2**).

Rapid uptake and subsequent phosphorylation of TFT were observed, but despite a high rate of degradation, TFT activation is sufficient to inhibit TS and to enable incorporation into DNA, although the contribution of each effect is exposure time dependent. TPI was only able to affect TFT cytotoxicity in cells with high TP expression at very short exposure (<4 hrs). Furthermore, inhibition of TP by TPI increased formation of active TFT metabolites (almost 2-fold) in high TP expressing Colo320TP1 cells, but this was not directly related to an increase in TFT incorporation into DNA. Emura *et al.* [4] previously determined that more TFT is incorporated into DNA of tumor cells after repeated short-term administration of TFT, which is enhanced in the presence of TPI. TFT has high affinity for TK like most TdR analogs, and possibly even more compared to TP resulting in rapid conversion rates of TFT into its active metabolites, which in turn might diminish TFT degradation by TP and therefore explaining the minor effect of TPI *in vitro*. Emura *et al.* [5] previously found that *in vivo* the tumor response to TAS-102 is independent of TP expression itself, while the ratio TK/TP did correlate with tumor growth inhibition, suggesting that next to the presence of systemic TPI, both activation and degradation of TFT in tumor cells play a significant role in TFT-induced antitumor activity.

Although incorporation of TFT into DNA is a main determinant in the *in vitro* cytotoxicity of TFT to colon cancer cells, TFT is able to inhibit TS activity in high TS expressing cancer cells. These cells were more sensitive to TFT than 5FU (upto 4-fold) and were shown to be cross-resistant to TFT compared to 5FU, which was more pronounced at short-term exposure (>3-fold or more at 4 hr exposure). The

conversion of TFT to its active form by TK is essential, and because it only involves one activation step TFT exerts cytotoxicity within a few hours, in contrast to 5FU, which requires more conversion steps (**Chapter 4**). TS *in situ* activity recovers quickly after removal of the TFT influx, indicating that continuous exposure is necessary to inhibit TS. Therefore longer repeated exposure to TFT might bypass resistance due to increased TS expression, which together with increased incorporation of TFT into DNA results in the highest antitumor effects.

Resistance to TFT

Besides the role of TS as a proliferation factor in tumorigenesis [6],[7], TS expression is considered to be an important prognostic factor in 5FU-based chemotherapy for colorectal cancer patients, although its relation with other prognostic factors (e.g. p53, Rb, Bcl-2; [8]) is not well-known. It was determined earlier that patients with tumor cells with low TS levels (either in mRNA expression, protein levels or catalytic activity), respond better to TS inhibition-based chemotherapy [9],[10],[11],[12]. Our results (**Chapter 3**) showed that 5FU-resistant H630R10 colon cancer cells expressing high TS [13] [14] exhibited an 30 to 40-fold increase in IC_{50}s for both 5FU and TFT compared to the parental cell line, indicating the important role of TS levels in the efficacy of these TS inhibitors, while they may convey cross-resistance to other TS-inhibiting agents, such as the antifolate pemetrexed (ALIMTA) [15]. Besides increased TS levels, other factors might play a role as well in the resistance to TFT. For that reason we made H630 human colon cancer cells resistant to TFT *in vitro* by increasing the TFT concentration stepwisely. The results of this study are discussed in **Chapter 11**. H630 cells were made resistant using two exposure schedules, either by continuous exposure (final 20 µM TFT) or short-term repetitive exposure (final 4 hr 250 µM TFT/week) to TFT, resulting in the H630 variants H630-cTFT and H630-4TFT, respectively, which are >200-fold resistant compared to H630. Both cell lines were cross-resistant to FdUrd, a metabolite in 5FU metabolism which is activated by TK, but not to 5FU or 5'DFUR, which are activated by TP. Further analysis revealed that TP activity remained unchanged in these cells (as expected), while no increase in TS levels (protein, mRNA, enzyme activity) was observed as well. Total TK activity was significantly

lower in H630-4TFT (>90%) due to both decreased TK mRNA and protein expression. Murakami *et al.* [16] showed that 5FU acquired-resistant DLD-1 colorectal cancer cells were not cross-resistant to TFT, but had decreased activity of the important 5FU-activating enzyme orotate phosphoribosyltransferase (OPRT), indicating that 5FU-resistant colon cancer cells are not necessarily cross-resistant to TFT. These data therefore explain that TFT can exert antitumor activity against 5FU-resistant cancer cells (see also [17]). Overall it can be concluded that, next to increased TS levels, decreased activation of TFT by TK is likely to be another important mechanism for resistance to TFT.

Surprisingly, no decreased TK activity was seen in H630-cTFT, meaning that the expression or activity of enzymes directly involved in TFT metabolism do not necessarily have to be associated with TFT resistance, and therefore may be dependent on that the manner of resistance induction that was used. Using whole human genome microarray analysis we observed a strong increase in secretory phospholipase A2 (sPLA2) mRNA levels (> 40-fold) in H630-cTFT, which was an unexpected finding. sPLA2 plays a role in phospholipid metabolism and prostaglandin synthesis, but also FAS-mediated apoptosis, which indicates a strong disturbance in signal transduction in these cells. This was confirmed by the increased production of arachidonic acid in H630-cTFT cells, which are preferentially released from phospholipids by PLA2 enzymes. Other groups previously determined that elevation of sPLA2-IIa expression might be related to the development or progression of prostatic adenocarcinoma [18],[19], which supports the hypothesis that dysregulation of sPLA2 expression may play a role in colorectal carcinogenesis as well. Although our data suggest a relationship between changed phospholipid metabolism and flouropyrimidine resistance, the exact mechanism of resistance to TFT (and FdUrd) in these H630-cTFT cells with respect to downstream events after arachidonic acid production (e.g. prostaglandin synthesis by cyclooxygenases) needs further investigation.

Role of TP expression in fluoropyrimidine sensitivity

TP is able to cleave several fluoropyrimidines, while it has a broad substrate specificity and is able to phosphorolyze a wide variety of pyrimidine nucleosides [20].

Therefore it can (in)activate several different anti-cancer drugs that are currently used in the clinic (e.g. 5'DFUR) or are still under investigation, such as TFT. TP can be upregulated by several cytokines (e.g. TNF-α, IFN-α [21],[22]), but chemotherapeutic drugs also often upregulate TP expression [23],[24],[25]. However, others describe that upregulation of TP is dependent on its intrinsic expression of cells [23], which can be influenced by the presence of chemotherapeutic compounds. This is less relevant for TAS-102, because the enzyme is directly inactivated by TPI, but for other fluoropyrimidines this could be of influence on the treatment of tumors with these drugs. Our group previously investigated whether exposure of colon cancer cells to TFT or 5'DFUR would influence TP expression [26], and results showed that different effects on TP mRNA and protein expression can be induced by these drugs, which were not uniform for the panel of cell lines used, but TP activity remained unchanged in these cell lines. The presence of TPI inhibited TP activity in all cell lines, while after cleaning up samples from potential residual TPI no recovery of TP activity was found, leading to the conclusion that TPI is bound to the enzyme and therefore being effective in prolonging inhibition of TP activity, which is potentially an additional beneficial effect of TPI in the TAS-102 formulation.

These results demonstrate that regulation of TP is drug and cell line dependent, and is (in part) indeed related to the intrinsic metabolic properties of cells, which might be affected in tumor cells. In addition, low TP expression might confer resistance to several fluoropyrimidine anti-cancer agents [20], such as the oral 5FU prodrug capecitabine, which is activated by TP and therefore dependent on sufficient TP levels at the tumor site. This does not count for TAS-102, since we determined using the Hollow Fiber Assay (HFA) [27] that it is effective against both tumors with either low or high TP levels, which is discussed in **Chapter 5**. We demonstrated that, next to being a cost-effective drug screening method, this assay is also an excellent way to study several short-term pharmacodynamic end points *in vivo* after fluoropyrimidine treatment. We demonstrated the importance of TP in the activity or resistance to various oral fluoropyrimidine formulations, by using hollow fibers filled with human TP-deficient Colo320 or high TP expressing Colo320TP1 colorectal cancer cells, which were implanted subcutaneously in BALB/c mice. These mice were treated orally with TAS-102, TFT alone, 5'DFUR +/- TPI or capecitabine at their maximum tolerated dose

(MTD). Analysis revealed that TAS-102 induced considerable growth inhibition against both Colo320 cells, and omission of TPI from the formulation neutralized these effects. This result was reflected in the pharmacodynamic evaluations where TAS-102 induced clear a cell cycle arrest and significant apoptosis (>8-fold) as a result of DNA damage, which were more pronounced in Colo320 than in Colo320TP1 cells. However, addition of TPI reduced the effects of 5'DFUR-induced cytotoxicity and apoptosis of Colo320TP1 cells, although not completely. In conclusion, for the 5FU prodrug 5'DFUR (and capecitabine) sufficient TP levels are necessary to induce significant antitumor effects, while the mechanisms of action of TAS-102 are not directly related to TP levels at the tumor site, but only exerts cytotoxicity when systemic TPI is present. Thus, in the TAS-102 formulation the main action of TPI seems to consist of the increase in the bioavailability of TFT enabling to enhance TFT activation in tumor cells. In addition, TPI can also increase TdR concentrations in plasma [5], which prevents breakdown of TFT, because TdR can compete with TFT in TP-mediated phosphorolysis. There was no evidence that TdR accumulation would negatively affect TFT-induced cytotoxicity *in vitro*, while lowered TdR pools can prevent rescue of thymine-less induced cell death.

TP vs UP in fluoropyrimidine sensitivity and angiogenesis

Although high tumoral TP expression might theoretically inactivate TFT (+/- TPI), our data showed that this was not the case. This means that for *in vivo* and clinical application high TP levels in the tumor are not necessarily a negative parameter, while TAS-102 is also active against low TP expressing cells. On the other hand, 5'DFUR needs high TP for selectivity, and because TP is involved in the activation of 5'DFUR and other pyrimidine nucleoside analogs the regulatory mechanisms of this enzyme are still of interest. TPI protected the (tumor) cells from 5'DFUR, but this was only partial as can clearly be seen from the apoptotic effect. This indicated that 5'DFUR activation is not only dependent on TP, as previously anticipated [28], but that another phosphorylase, presumably uridine phosphorylase (UP), also plays a role. An interesting observation by De Bruin *et al.* was that in SW1398 and SW948 colon cancer cells TdR phosphorolysis could not be completely inhibited or was partially inhibited bij TPI (40%), despite the fact that these cells had comparable IC_{50}-values and phosphorolytic activity compared to other cells where

TPI could inhibit TP activity by 100%, thereby blocking TdR phosphorolysis completely. We therefore suggested UP might be responsible for the remaining activity.

It is general knowledge that TP and UP have a broad and overlapping substrate specificity [29],[30], but in many studies the role of UP in fluoropyrimidine sensitivity is generally underestimated. In order to elucidate this controversy, the relative contribution of both phosphorylases in the sensititvity to 5FU and 5'DFUR is discussed in **Chapters 6 and 7**. To modulate the enzyme activities TPI and the specific potent inhibitor of UP 5-benzylacyclouridine (BAU) [31] were used. Results showed that in normal HaCaT keratinocytes and peripheral blood mononuclear cells TPI and BAU inhibited most TdR and Urd phosphorolysis (>80%), respectively, while both TP and UP contributed to the phosphorolysis of 5'DFUR. In the colon cancer cell lines overlapping substrate specificity was found for TP and UP, while both enzymes were responsible for converting the natural substrates TdR and Urd and the fluoropyrimidines 5'DFUR and 5FU, although cell line dependent. E.g. it was remarkable that in SW1398 UP is almost completely responsible for phosphorolysis of TdR (in contrast to SW948) and 5'DFUR, indicating that UP might have a more significant role in the activation of 5'FUR in some gastrointestinal tumors. A second observation was that after inhibiting both phosphorylases still conversion of 5FU (>40%) was detected in the tumor and normal cell lines, suggesting the relative minor role of TP in 5FU sensitivity. This can be explained by the fact that 5FU can also be activated to FUMP by OPRT [32],[33]. A more interesting observation was that in SW1398 still phosphorolysis of TdR, Urd and 5'DFUR (up to 20%) was detected after inhibition of both phosphorylases, and in addition, UP was not completely responsible for Urd conversion in the low UP expressing SW948 cells. For these substrates probably other pyrimidine phosphorylases are responsible for their phosphorolysis, which was previously postulated [34],[32]. However, significant 5'DFUR resistance in SW1398 cells may be observed when all pyrimidine phosphorylase activity is inhibited. Furthermore, the growth inhibition experiments showed that Colo320 is relatively sensitive to 5'DFUR compared to e.g. SW948 and SW1398, which was a surprising result, while no TP activity by TdR cleavage was detected in this cell line. For its high TP expressing variant Colo320TP1 we observed

that inhibition of TP by TPI can increase sensitivity (IC_{50}) to 5FU and 5'DFUR 11-fold and 50-fold, respectively, indicating the relative minor role of TP in 5FU activation, which is partly due to the low availability of the co-substrate d-R-1-P [35]. Although it is common knowledge that TP and UP expression varies between different organs and animals [36],[20],[30],[37], these results clearly indicate the possible variations in substrate specificity. Overall it can be concluded that in addition to TP, UP plays a significant role in the activation of fluoropyrimidines (e.g. 5FU prodrugs) at the tumor site, which will be enhanced because TP and UP levels are often elevated and should therefore receive more attention. Future studies should include the contribution of UP in studying intracellular metabolism of 5'DFUR.

Next to TP and UP being important enzyme intermediates in (fluoro)pyrimidine metabolism, TP has also been shown to be involved in angiogenesis, while it is identical to the pro-angiogenic platelet-derived endothelial cell growth factor (PD-ECGF) [38],[39]. Numerous (immunohistochemical) studies (summarized in [28]) showed that TP (and UP) are often upregulated in tumor cells and increased TP levels in tumor stroma, resulting in increased microvessel density (MVD), which in turn is associated with poor outcome of diseases [40],[41]. Takahashi *et al.* [42] previously reported that high MVD is well correlated with increased TP expression and is associated with metastasis formation in human colorectal cancer [43],[44] and gastric cancer [45]. Inhibition of TP might therefore improve prognosis for some colorectal cancer patients [46]. The exact mechanisms behind the pro-angiogenic activity of TP are unknown, but most research is focused on the metabolites involved in the enzymatic reaction TdR > thymine + dR-1-P. Several studies focused specifically on dR, which is a secondary product in the reaction by dephosphorylation of dR-1-P (see also [47],[48]). Since it was previously shown to have angiogenic [41] and anti-apoptotic [49] properties, research shifted to this molecule in how it might induce neo-vascularisation as a chemotactic compound, while it is also supposed to form concentration gradients, which stimulates the migration of endothelial cells towards tumors. However, controlling the balance of angiogenesis (in tumors) is likely to be controlled by the presence or absence of a variety of pro- and anti-angiogenic factors, which might indicate that TP has a non-dominant role in this balance. In addition, no uniform consistency exists between studies that

investigated the association TP with angiogenesis in xenografting experiments, while high TP expressing tumors did not always result in increased neo-vascularisation or overall tumor growth [47].

TP levels are high in several human tissues (e.g. colon, bladder, liver), but TP is predominantly expressed by macrophages, platelets and endothelial cells. Our results showed that peripheral mononuclear cells were able to convert significant amounts of TdR by TP within a few hours, and TP and UP preferentially converted TdR and Urd, respectively, while both enzymes were responsible for converting 5'DFUR into 5FU (**Chapter 7**). For the first time it was demonstrated that a much higher basal TP activity (>4-fold) was seen for macrophages (and dendritic cells) compared to undifferentiated monocytes. In addition, several studies determined that tumor infiltrating macrophages frequently show overexpression of TP, which, next to tumor cells themselves, may contribute to the high TP levels in colorectal tumor stroma [50],[44]. This contribution might directly be related to increased angiogenesis in these tumors, thereby possibly influencing fluoropyrimidine sensitivity and/or beneficial tumor survival effects around necrotic areas. Therefore, more attention should be given with respect to the potential tumor promoting effect of macrophages. To provide more insight in the efficacy of TFT (or even capecitabine), it might therefore be important to monitor TP levels in circulating monocytes and/or platelets before and during treatment of cancer patients with TAS-102, e.g. to generate information on the duration of TP inhibition by TPI.

Targeting angiogenesis in addition to current chemotherapy is an important new development [51],[52]. We observed after 10-days treatment of mice with TAS-102, TPI is already able to reduce blood vessel formation, which is likely to be related to the suppression of the invasive capacity and metastasis. The potential of TPI to block these processes *in vivo* was also investigated by other groups [53],[54],[55],[3],[56]. TPI significantly inhibited TP-induced neovascularization in a dose-dependent manner in different mice models. Matsushita *et al.* [56] determined, using the mouse dorsal air sac assay, that blood vessel formation (partly) induced by high TP expressing epidermoid carcinoma KB/TP cells could be suppressed by TPI, and that TPI alone is able to decrease the growth rates of these cells and to increase the apoptotic index significantly. Furthermore, Takao *et al.* [3] showed that

chemotactic motility and basement membrane invasion of KB/TP cells, and the number of macroscopically observed liver metastases can also be decreased by TPI alone. Comparable anti-invasive and anti-metastatic activities by TPI alone were also observed against human lung adenocarcinoma cells [55]. The antitumor activity of TPI as a single agent and its important role in tumor growth clearly indicate that TP is an important determinant in the invasiveness and induction of metastasis of high TP expressing solid tumors [57]. Because of these properties of TPI, TAS-102 is a promising candidate for clinical use. With respect to these findings it might be interesting to combine TAS-102 with other (novel) agents counteracting blood vessel formation in the future.

Drug combinations

Several (pre)clinical studies have already shown enhanced tumor regression by combining an anti-angiogenic agent with a cytotoxic agent [58],[59], which makes TAS-102 potentially an excellent candidate to be combined with other cytotoxic agents. In this thesis we described the combination of TAS-102 (as TFT) with other anticancer agents. TFT was combined *in vitro* with other TS inhibitors (**Chapter 8**), oxaliplatin (OHP; **Chapter 9**) or irinotecan (CPT-11; **Chapter 10**) in different schedules in order to determine their interactions. Combining TFT with FdUrd is no option, since the effects were purely additive, probably due to overlapping mechanisms of action [60]. At low folate conditions TFT and folate-based TS inhibitors (AG337, ZD1694, GW1843) showed moderate synergism in growth inhibition to colon cancer cells, as a result of two-sided TS inhibition leading to enhanced DNA damage induction. At high folate conditions only additive effects were seen. The low-folate status probably increases the binding of antifolates to the TS co-factor binding site, while folate levels in medium and cells is an important determinant in the efficacy of antifolates [61]. More pronounced synergism in colon cancer cells was observed when TFT was combined with the DNA synthesis inhibitors OHP and SN38, the active metabolite of CPT-11, which resulted in increased DNA strand break formation and cell death. These drug combinations were dose- and schedule-dependent, since TFT pre-incubation can decrease OHP-induced cytotoxicity, and for the TFT-SN38 combinations most pronounced synergistic

interactions were found when cells were pre-incubated with TFT, in contrast to 5FU, where the most cytotoxic schedule was SN38 followed by 5FU exposure, both *in vitro* [62],[63] and *in vivo* [64], [65]. This was an unexpected result, since TFT and 5FU are fluoroprymidines with overlapping mechanisms of action, and both increase synergism using sequential dose-schedules compared to simultaneous drug exposure of TFT and SN38. This difference is possibly at the level of inducing DNA alterations with respect to trapping topo-I cleavable complexes, which can be induced by certain nucleoside analogs, including 5FU [66],[67],[68], but it is unknown whether TFT is also able to form cleavable complexes or enhances the formation of CPT-11-induced cleavable complexes. Our data clearly indicate that TFT acts different compared to combinations of 5FU with SN38 or OHP, illustrating the importance of dose-scheduling and integration of the pharmacokinetic drug profiles. These combinations have potential for further investigation *in vivo*, because synergism was observed in the whole panel of cell lines used for these studies, although the metabolic and biological interactions of the drugs must be positively assessed prior obtaining potential clinical efficacy.

Conclusions

In conclusion, TP plays an important role in various areas and on different levels in (colorectal) cancer, and therefore will remain an interesting target with respect to its consideration into future clinical oncological trials including TAS-102, fluoropyrimidines, or even folate-based TS inhibitors. Although further studies are warranted in order to elucidate the role of TP in oral fluoropyrimidine sensitivity and angiogenesis, thereby including the role of UP in fluoropyrimidine activation or degradation, and the mechanism of the angiogenic effect mediated by downstream effector molecules after phosphorolysis by TP. These aspects of TP should be further characterized in order to help designing therapies (TAS-102) or modifying current therapies (capecitabine) in which TP is involved. In addition, the dual-targeted properties of the TAS-102 formulation is a promising candidate in the treatment of solid tumors, while current strategies favor attacking multiple targets. For this reason the potential anti-angiogenic effect of TPI *in vivo* should be given more attention, preferably in the current phase I/II trials. To improve the treatment of

gastrointestinal malignancies, it might be interesting to combine TAS-102 with other angiostatic or cytotoxic agents in future clinical drug combination studies, such as with the VEGF inhibitor bevacizumab, the DNA synthesis inhibitors OHP or CPT-11, or even with EGFR inhibitors.

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