8. DISCUSSION

8.1 Effect of hMLH1 or p53 status on the mechanism of cytotoxicity of UCN-01 in colorectal cancer cell lines

8.1.1 Mechanism of cytotoxicity of UCN-01 in colon carcinoma cells: Effect of hMLH1 status

The observed reactions to UCN-01, G1-phase arrest and apoptosis, appeared to be dependent on the presence of the hMLH1 protein: The hMLH1$^+$ cell line was completely arrested in G1-phase and was not undergoing apoptosis, while the G1-phase arrest of hMLH1$^-$ cells was less complete and they displayed a higher extent of apoptosis. In hMLH1$^+$ cells, arrest in the G1-phase of the cell cycle was associated with binding of the p27^{KIP1} protein to cdk2, concomitant with inhibition of cdk2 kinase activity, and with hypophosphorylation of the Rb protein (**Fig. 14**). In hMLH1$^-$ cells, p27^{KIP1} was not bound to cdk2, resulting in maintenance of cdk2 kinase activity and hyperphosphorylation of the Rb protein: G1-phase arrest was therefore partially bypassed. By trapping cells in different phases of the cell cycle, we could show that the capacity of the hMLH1$^-$ cells to escape partially the UCN-01-induced G1-phase arrest and to enter the S- and G2/M-phases of the cell cycle was committing them to undergo cell death (**Fig. 16, 17**). Cell death in cells lacking the hMLH1 molecule was occurring in the first 1-2 days after the start of treatment with UCN-01 and was associated with activation of the caspase pathway (**Fig. 9**). A delayed cell death not linked to the activation of the caspase pathway was occurring in hMLH1$^+$ cells 8-10 days after the start of treatment (**Fig. 9, 10, 11**).

Activation of the G1-phase checkpoint has been shown to correlate with prevention from apoptosis induced by UCN-01 [106]. Analogously, cells susceptible to UCN-01-induced apoptosis were not capable to arrest in G1-phase [97, 110]. This has led to the hypothesis, proposed by Akinaga *et al.* [97], that the G1-phase checkpoint function might be a critical determinant of sensitivity of cells to UCN-01. This hypothesis has not yet been experimentally confirmed. UCN-01-induced apoptosis in human leukaemia cells was indeed accompanied by activation of the kinase activities of cdk2

and cdc2 [101], and in Wi-38 VA13 cells apoptosis also correlated with activation of cdk2 kinase [110]. On the other hand, it was shown that gastric cancer and pancreatic cancer cells arrested in G1-phase after UCN-01 treatment were nevertheless capable of undergoing apoptosis [105, 111], and that human osteosarcoma and pancreatic carcinoma cells undergoing apoptosis after UCN-01 treatment showed a reduced activity of cdk2 [97, 110]. In lung cancer cells, UCN-01-induced apoptosis did not correlated with any change of cdk2 activity [106].

The present data support the hypothesis of Akinaga [97]. Furthermore, they indicate the association between the G1-phase checkpoint and the mismatch repair system. hMLH1^{+} cells were strongly arrested in G1-phase (90%), while hMLH1^{-} cells were less arrested in G1-phase (70%) (**Fig. 12**). Moreover, trapping cells in G2/M-phase with nocodazole showed that 10% of hMLH1^{-} cells were leaving the UCN-01-induced G1-phase arrest and accumulated in G2/M-phase due to the presence of the mitotic inhibitor (**Fig. 15**). By contrast, hMLH1^{+} cells were not able to leave the G1-phase arrest induced by UCN-01. To determine the molecular determinant of this different G1-phase arrest response to UCN-01, we evaluated systematically expression levels of different protein known to be involved in the G1-checkpoint (**Fig. 13**). The p53 protein was downregulated in a hMLH1-independent manner. Downregulation of p53 protein was previously observed after UCN-01 treatment in breast epithelial cells [129] and in B-cell chronic lymphocytic leukaemia cells [102]. Along p53, we found p21^{CIP1} protein suppression after UCN-01 treatment, as previously reported by others [110, 129, 176]. Reduced protein levels of p53 and p21^{CIP1} proteins suggested that these molecules are not always linked to G1-phase accumulation after treatment with UCN-01. We could detect no difference in degradation of cyclin D1 between hMLH1^{+} and hMLH1^{-} cells after UCN-01 treatment (**Fig. 13**). Recently, it has been observed that MMR-proficient cells, unlike MMR-deficient cells, were able to degrade cyclin D1 after DNA damage induced by cisplatin, etoposide, mitomycin C and doxorubicin [70]. After cisplatin treatment, this MMR-dependent degradation of cyclin D1 was conferring MMR-proficient cells, but not MMR-deficient cells, a functional G1-phase checkpoint. In the present work, expression levels of the cdk2 and cdk4 kinases were found to be downregulated in both cell lines (**Fig. 13**), as previously reported in breast epithelial

cells [129]. Although the expression of the p27^{KIP1} protein was upregulated to the same extent in hMLH1$^+$ and in hMLH1$^-$ cells, this protein bound differently to cdk2 protein (**Fig. 14C**). While it was present in the cdk2 complex precipitated from hMLH1$^+$ cells, it was only barely detectable in the one obtained from hMLH1$^-$ cells. This difference was indicating a different binding of p27^{KIP1} to cdk2, and was concomitant with different inhibition of the cdk2 kinase activity: This was inhibited after UCN-01 treatment to 30% in hMLH1$^+$ cells and only to 70% in hMLH1$^-$ cells, as demonstrated by histone H1 assay (**Fig. 14C**). p27^{KIP1} protein has been found to be upregulated after UCN-01 treatment by several authors [97, 110, 177]. Interestingly, it was observed in breast epithelial cells after UCN-01 treatment that, although the levels of p27^{KIP1} were not changed, p27^{KIP1} was redistributed from cdk4 to cdk2 complexes. This was responsible for the inhibition of cdk2 kinase activity and correlated to the observed G1-phase arrest [129]. Although a redistribution of p27^{KIP1} from cdk4 to cdk2 was not checked in this work, it is possible that a similar mechanism, dependent on the presence of the hMLH1 protein, could be responsible for the observed inhibition of cdk2 after UCN-01 treatment. Rb hypophosphorylation has been frequently observed after UCN-01 treatment in concomitance with G1-phase arrest [110, 121, 123, 124, 126, 127, 129, 178, 179]. Regulation of phosphorylation of Rb protein is a central step leading to progression from G1- to S-phase. In the cascade controlling G1-checkpoint, Rb phosphorylation is upstream to cdk2/cyclin E, since the first event of phosphorylation of Rb is induced by cdk4/cyclin D1 and cdk6/cyclin D1 kinases; however, Rb phosphorylation is in part under the control of the activity of the kinase complex cdk2/cyclin E. This is an autoregulatory loop, since hyperphosphorylation of Rb, catalyzed by cdk2/cyclin E complex, leads to complete liberation of E2F1 transcription factor. One of the gene under the control of the E2F1 transcription factor is *cyclin E*. Indeed, Rb was found to be hypophosphorylated in hMLH1$^+$ cells, in which cdk2 activity was strongly inhibited, but not, or to a lesser extent, in hMLH1$^-$ cells, in which cdk2 activity was less inhibited.

In conclusion, we could show here for the first time that in the response to UCN-01 cdk2 activation, and thus ability to undergo G1-phase arrest, was dependent on a functional hMLH1 protein (**Fig. 33**).
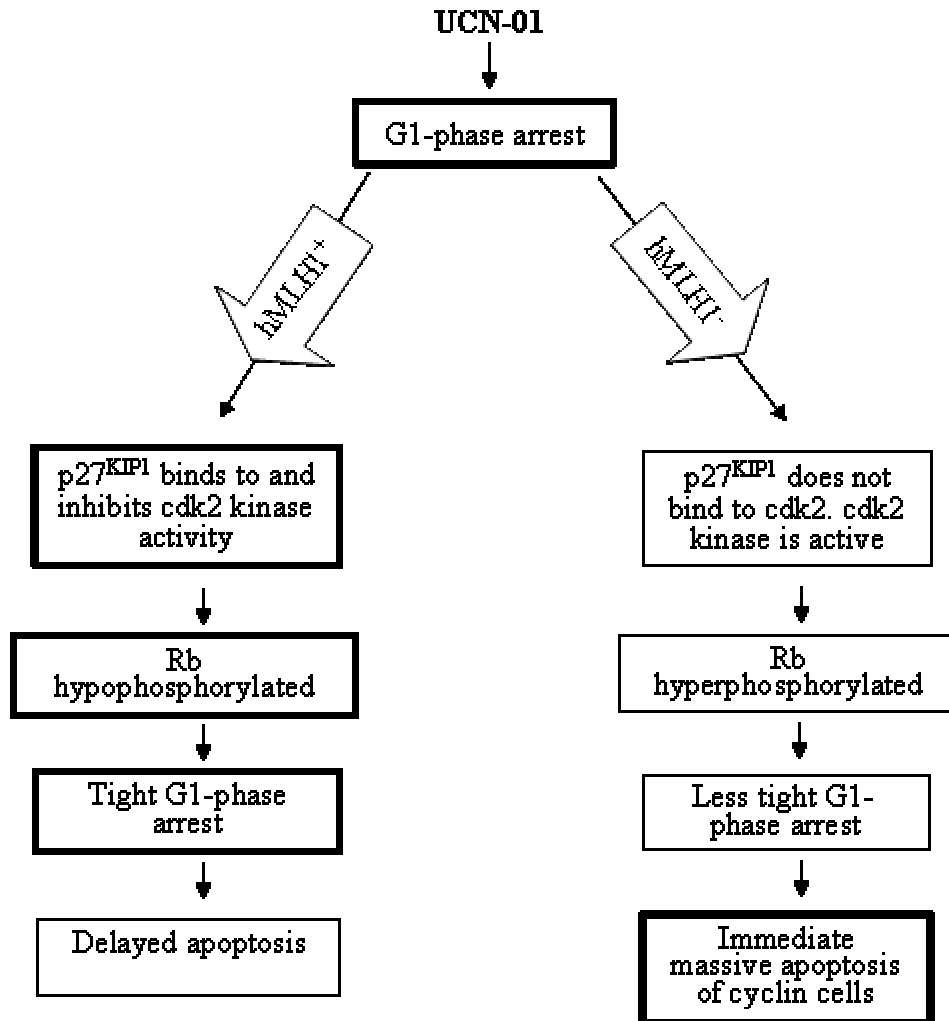
Fig. 33: The cellular effects of UCN-01 in HCT116 colon carcinoma cell lines are dependent on hMLH1 status.

The second response to UCN-01 was represented by apoptosis induction, apparently induced in cells not arrested in G1-phase: The hMLH1$^+$ cell line, initially strongly arrested in G1-phase, was undergoing a slow, caspase-independent cell death, while hMLH1$^-$ cells displayed a faster, caspase-dependent apoptosis (**Fig. 9, 10, 11**). This dual mechanism in the response of tumor cells to UCN-01 was observed recently in a leukemic cell line (HL60) and in a human prostate cancer cell line (PC3) [173]. Both cell lines underwent G1-phase arrest after UCN-01 treatment, but HL60 cells were carrying out apoptosis in the successive 20 hr, while PC3 cells changed in morphology and died within 6 days in a process that did not required caspase activation [173]. In the

present work, the different kinetics of responses to UCN-01 treatment were reflected in the results of the MTT assay (**Fig. 7**). At 2 days after start of treatment the hMLH1$^-$ cell line appeared more susceptible, as a consequence of the observed burst of apoptosis, while at 6 days after the start of treatment the situation was reversed: The hMLH1$^+$ cells appeared more susceptible to UCN-01 than the hMLH1$^-$ cells. Clonogenic assay, which integrates the long-term results of growth arrest- and apoptosis- induction, confirmed the MTT assay carried out at day 6 after the start of treatment (**Fig. 7**). As can be concluded from the clonogenic assay result, in the response to UCN-01 the cytostatic effect, more pronounced in hMLH1$^+$ cells, is likely to inhibit more effectively cell growth than the cytotoxic effect, characterizing hMLH1$^-$ cells.

In the present work, it was also demonstrated that, after UCN-01 treatment, hMLH1$^-$ cells arrested in or passing through S- and G2/M-phases were undergoing apoptosis. This was demonstrated by experiments performed with aphidicolin, nocodazole, and colcemid (**Fig. 16, 17**). These agents were used to arrest cells at the S- or G2/M-boundary. After pretreatment with these agents and addition of UCN-01 for the successive 24 hr, in hMLH1$^-$ cells apoptosis induced by UCN-01 was strongly enhanced, while no effect was observed in hMLH1$^+$ cells subjected to the same experimental conditions. This suggested that the hMLH1$^-$ cells, but not the hMLH1$^+$ cells, when arrested in S- or G2/M-phases, were particularly sensitive to the cytotoxic effects of UCN-01. These results are in accordance with a recent finding in human myeloblastic ML-1 cells showing that UCN-01 was able to initiate an apoptotic cascade in S-phase arrested cells [174].

We could show that the apoptosis-related Bax and Bcl-2 proteins were not affected by UCN-01 treatment neither in hMLH1$^+$ nor in hMLH1$^-$ cells, thus excluding a role of these proteins in UCN-01-induced apoptosis. Different authors showed that UCN-01-induced apoptosis was independent from expression of Bax or Bcl-2-related proteins in leukemic cells [102-104, 180] and in breast carcinoma cells [107]. On the other hand, overexpression of Bcl-2 in thyroid carcinoma cells was capable of conferring resistance to UCN-01 [119]. Further, in HCT116 cells, Bax and Bcl-X$_L$ proteins have been shown to influence UCN-01-induced apoptosis [181]. The role of members of the Bcl-2 family of proteins with respect to UCN-01-induced apoptosis is still not clarified. However, it

is possible to speculate on a possible involvement of the pro-apoptotic protein Bad in the response to UCN-01. This protein is inactivated by phosphorylation through at least two independent pathways: The Akt [182, 183] and the MAP kinase pathway [184, 185]. Since UCN-01 inhibits Akt kinase [120] and activates MEK1/2 kinases (**Fig. 20** and ref. [180]), UCN-01-induced apoptosis may be regulated by modulation of these two counteracting effects. It could be hypothesized that if the inhibitory effect of UCN-01 on the Akt pathway is stronger than the activation of MEK1/2 kinases, Bad will be not phosphorylated and apoptosis will take place, and viceversa. This hypothesis is undergoing verification in our laboratory.

In the present work, the inhibition of the MAP kinase pathway, achieved by two MEK1/2 inhibitors (PD98059 and U0126), interacted synergistically with UCN-01 to enhance apoptosis in the hMLH1[-] cell line (**Fig. 21**). It is important to note that disruption of the MAP kinase pathway was not, by itself, inducing cell death, although MEK1/2 activation has generally been associated with cytoprotective functions [186]. This finding is consistent with previous studies demonstrating that interruption of the MAP kinase signal cascade by pharmacological or other means potentiates apoptosis in cells exposed to other environmental stress, e.g. growth factor deprivation [187], or exposure to DNA damaging agents [180, 188-190]. The role of the MAP kinase pathway in UCN-01-induced apoptosis was demonstrated recently by Dai *et al*. [180]. Apoptosis was strongly enhanced in multiple myeloid and lymphoid leukaemia cells treated with a not cytotoxic, low concentration of UCN-01 (0.15 μM) in the presence of specific inhibitors of MEK1/2.

We have shown that treatment with U0126 alone was causing a strong G1-phase arrest in hMLH1[+] and hMLH1^{-} cells (**Fig. 22**). This confirms previous results [190] and indicates a role of the MAP kinase pathway in inducing cell cycle progression [23, 175]. In particular, ERK induces the expression of cyclin D1 [175]. This is primarily mediated by ERK-dependent AP-1 and ETS transcription factors that bind to the *cyclin D1* promoter [191]. Inhibition of this regulation by pharmaceutical inhibitors of the ERK pathway is inducing G1-phase arrest. In this work, the combination of U0126 and UCN-01 was shown not only to enhance apoptosis in hMLH1^{-} cells but also to reduce further G1-phase arrest (**Fig. 22**). By contrast, the G1-phase arrest in hMLH1^{+} cells was

maintained after treatment with combination of the two agents. This result further reinforced the connection here demonstrated between G1-phase arrest and protection from apoptosis after UCN-01 treatment.

We could therefore hypothesize that UCN-01 is causing activation of two pathways with counteracting effects. On one side, UCN-01 induces apoptosis; the absence of the hMLH1 protein and a defective G1-phase checkpoint are decisive for this pathway. On the other side, UCN-01 is inducing activation of the MAP kinase pathway that inhibits apoptosis. In fact, inhibition of the MAP kinase pathway induces apoptosis, at least in hMLH1⁻ cells. Thus, the decision between cell cycle arrest or apoptosis, after treatment with UCN-01, is dependent on the relative activation of one of the two pathways.

8.1.2 Mechanism of cytotoxicity of UCN-01 in colon carcinoma cells: Effect of p53 status

The effects of UCN-01 on isogenic colon carcinoma HCT116 cell lines (hMLH1⁻) differing by presence/absence of the *p53* gene were investigated. It was shown that the presence of a functional p53 protein affects UCN-01-induced cytotoxicity and apoptosis *in vitro*. The p53$^{+/+}$ cell line reacted to UCN-01 with induction of apoptosis, while p53$^{-/-}$ cells were not affected (**Fig. 24**). This different response was reflected by the long-term clonogenic assay, showing that p53$^{+/+}$ cells were more sensitive than p53$^{-/-}$ (**Fig. 23**).

The role of the p53 protein in response to UCN-01 treatment has been addressed by several authors, given the importance of p53 in the response to chemotherapeutic agents and in induction of apoptosis. A functional p53 protein was found to be not necessary for UCN-01 induced apoptosis in different types of cell lines [81, 87, 127]. p53 status did not affect the susceptibility of thyroid carcinoma- [119], human ovarian cancer- [81], NSCLC- cell lines [127] and of splenocytes [102] to UCN-01. By contrast, Jones *et al.* [131] demonstrated that the susceptibility was enhanced by the presence of a wild-type p53 protein. The role of p53 towards UCN-01 cytotoxicity seems to depend, therefore, on the cell types investigated. The contradictory results on this subject indicate that further studies are needed to determine if p53 status is the true determinant for direct UCN-01 cytotoxicity [97].

8.2 Effect of p53 or hMLH1 status on the mechanism of cytotoxicity of CPT-11 in colorectal cancer cell lines

In the present work, the effects of CPT-11 on colon carcinoma HCT116 cell lines differing by presence/absence of the *p53-* or of the *hMLH1-* genes were investigated. By following the details of the cellular reaction for over twelve days post-treatment, we could show for the first time that both a loss of the *p53* gene as well as a defect in the mismatch repair system affect the mechanism of CPT-11 induced cytotoxicity *in vitro*. The immediate reaction to CPT-11 was a p53- and hMLH1- independent G2/M-phase arrest. In $p53^{+/+}$,$hMLH1^{+}$ cell line this arrest lasts for at least 12 days. This cell line showed very little apoptosis whereas in the $p53^{+/+}$,$hMLH1^{-}$ cells the extent of apoptosis was higher and was accompanied by a shorter G2/M-phase arrest. By contrast, $p53^{-/-}$ cells were unable to maintain the G2/M-phase arrest. In these cells a massive, p53-independent apoptosis-induction ensues soon after a short G2/M-phase arrest (**Fig. 34**).
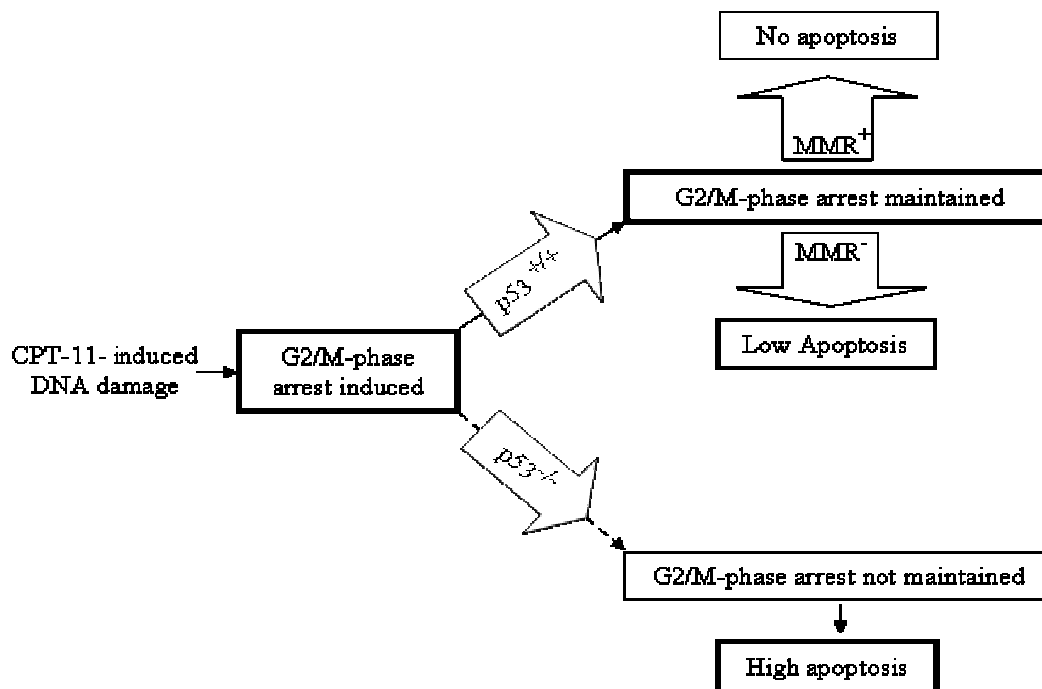

Fig. 34: The cellular effects of CPT-11 in colon carcinoma cell lines are dependent on p53 status as well as on hMLH1 status.

These different molecular reactions to treatment were reflected in the results of the MTT- but not in clonogenic survival- assay (**Fig. 25**), in agreement with previous reports [144, 145]. It was previously shown that SN-38 cytotoxicity is p53-independent in clonogenic assay [145]. A similar result was found in fibroblast cell lines after treatment with SN-38 [144]. On the other hand, in mice bearing human CRC cells LoVo and X17Lovo, a subclone of LoVo transfected with a dominant p53^{mut} protein, as well as in mice with HT 29 and HT 29A3 (a subclone transfected with a dominantly expressed p53^{wt} protein) tumors, it was shown that the presence of a p53^{wt} protein confers sensitivity to CPT-11 [147]. The same authors, however, could recently not confirm these data [139], reaching the conclusion that the CRC response to CPT-11 is dependent on several parameters including p53 status, but not exclusively [139].

Recent data obtained in our laboratory have clarified the role of p53 in the response to SN-38. Comparing a larger panel of p53^{wt} and p53^{mut} CRC cell lines, we could confirm that p53^{wt} cells underwent a prolonged G2/M-phase arrest and no apoptosis, while p53^{mut} cells underwent only a transient G2/M-phase arrest accompanied by high levels of apoptosis. However, the two groups of cell lines showed in clonogenic assay a different sensitivity towards SN-38; namely, the p53^{mut} group of cells were more resistant than the p53^{wt} group, suggesting that cell cycle arrest is more effective than apoptosis in long-term cytotoxicity to SN-38 (M. Bhonde and D. Moorthy, unpublished results).

Treatment of lung cancer- [192], colon carcinoma- [193], and ovarian carcinoma- cells [145] or fibroblasts [144] with CPT-11 or SN-38 was previously shown to induce G2/M-phase arrest. In the present work, we show that after CPT-11 treatment a long-term G2/M-phase arrest is maintained only in the p53$^{+/+}$ cell lines, while p53$^{-/-}$ cells are only transiently arrested (**Fig. 27**). Simultaneously with long-term G2/M-phase arrest in p53$^{+/+}$ cells, giant polyploid cells emerge (**Fig. 28**), indicating that the inhibition of mitosis does not prevent about 10-15% of cells from DNA synthesis and endoreduplication. This was observed also after treatment of several established colorectal p53-intact cell lines with SN-38 (M. Bhonde and D. Moorthy, unpublished results), thus indicating that it is not a peculiarity of the HCT116 cell line. The detection of polyploid cells in the p53^{wt} cell lines might be related to the observed

overexpression of the $p21^{CIP1}$ protein in these cells (**Fig. 31**), since $p21^{CIP1}$ overexpression can induce endoreduplication [194, 195]. Polyploidy could also be indicative of senescence: Indeed, a recent report indicates that the SN-38-induced long-term arrest of $p53^{+/+}$ cells is associated with a senescence process [196]. The polyploid cells were much less numerous and underwent apoptosis within 5 days in the $p53^{-/-}$ cell line (not shown), but survived for a prolonged period in the $p53^{+/+}$ cell lines (**Fig. 28** and data not shown).

It was observed in this work that after CPT-11 treatment the triggering of G2/M-phase arrest was p53-independent, while maintenance of it was a p53-dependent process (**Fig. 27**). Previous reports indicated also that p53 and $p21^{CIP1}$ proteins are essential for the sustaining but not for triggering of the G2/M-phase arrest after DNA-damage induced by ionizing radiation or temozolomide treatment [159, 195].

In the present work, after treatment with CPT-11 we found in all three cell lines a similar brief increase of phosphorylation of cdc2 at Tyr-15, which peaked at 24-48 hr and coincided with the initial phase of the G2/M-phase arrest (**Fig. 30**). It was followed in $p53^{+/+}$ cells by the expected strong overexpression of p53 and $p21^{CIP1}$ proteins and, simultaneously, by the inhibition of cdc2 kinase activity (**Fig. 31**). The $p21^{CIP1}$ protein was found to coprecipitate with the cdc2 kinase which was inhibited to 30%. These results suggest that the triggering of the G2/M-phase arrest is related to a brief p53-independent phosphorylation of cdc2 at Tyr-15, common to all three cell lines, while the long-term inhibition of cdc2 kinase activity is most probably due to $p21^{CIP1}$ binding and could be the main mechanism of the maintenance of G2/M-phase arrest in $p53^{+/+}$ cells. The p53 protein regulates G2/M-phase cell cycle arrest by a number of p53-dependent pathways. p53 transcriptionally activates the cyclin dependent kinase inhibitor $p21^{CIP1}$, the protein Gadd45 which prevents formation of the active cdc2/cyclin B1 complex, and 14-3-3σ protein which anchors cdc2/cyclin B1 in the cytoplasm. Transcriptional repression of *cyclin B1* and of *cdc2* genes by p53 additionally enhances the G2/M-phase arrest [48]. As regards the role of the hMLH1 protein in G2/M-phase arrest, we observed that the G2/M-phase arrest after CPT-11 treatment was maintained for a longer time in the $hMLH1^+$ than in the $hMLH1^-$ cell line (**Fig. 27**), indicating that the hMLH1 molecule may affect indirectly or directly the

maintenance of the arrest. The contribution of intact hMLH1 to the G2/M-phase arrest after DNA damage resulting from treatment with mismatch-inducing agents (6TG or MNNG) has been observed previously [71, 72].

Apoptosis induction after treatment of tumor cells with SN-38 *in vitro* was reported previously [145]. The authors pointed out the discrepancy between the apoptosis results – which were p53-dependent – and the clonogenic assay results, which appeared to be independent from the p53 status and concluded that a p53-independent process, not immediately detectable as apoptosis, must have taken place in p53-inactivated cells [145]. Gupta *et al.* [146] have shown that two p53^{mut} cell lines were more sensitive to CPT when compared to isogenic parental p53^{wt} cell lines, due to loss of the CPT-induced G1-phase checkpoint function in the p53^{mut} cells. Our results indicate that CPT-11 treatment of p53$^{-/-}$ colon carcinoma cells leads to the mitotic catastrophe and eventually to apoptosis, while p53$^{+/+}$ cells remain arrested (**Fig. 27**). Recently, it has been shown in glioma cells [195] that if p53 is not functional the arrest after treatment with the mismatch-inducing drug temozolomide is bypassed, and cells die within several days in a manner consistent with mitotic catastrophe. Similarly, mitotic catastrophe is followed by delayed cell death after gamma irradiation [197] or treatment with etoposide [51, 198] of cells lacking downstream effector molecules of p53, like 14-3-3σ [50] or p21^{CIP1} [51]. In conclusion, given the role of p53 as a master regulator of cell cycle arrest in response to DNA damage, its absence could lead, as it was the case in our experimental model system, to death of cells undergoing mitosis in the presence of damaged DNA.

The clear dichotomy between long-term arrest in p53$^{+/+}$ *versus* apoptosis in p53$^{-/-}$ cells after CPT-11 treatment suggests that the decision between arrest and apoptosis after CPT-11 treatment is strongly dependent on the p53 status. Our results indicate that apoptosis, at least partially, was the result of the lack of arrest maintenance: The partial release from arrest by caffeine led to an increase of apoptosis of p53$^{+/+}$ cells while the induction of arrest by nocodazole reduced the extent of apoptosis in p53$^{-/-}$ cells (**Fig. 29**). It was previously observed in two established cell lines that intact p53 was associated with a prolonged arrest and p53-absence with a short arrest followed by apoptosis after DNA damage [199]. Furthermore, it was recently reported that the

different reaction to SN-38 treatment (arrest *versus* apoptosis) is strictly dependent on p21^{CIP1} protein [200].

In the HCT116 p53$^{-/-}$ cells used in the present study, Bcl-2 and Bax showed no change in expression after treatment with CPT-11 (**Fig. 32**). p53–independent apoptosis, previously observed in colon- [201] or lung-carcinoma [202] after camptothecin or irinotecan treatment, was associated with alterations of Bax, Bcl-X$_L$ or Bcl-2 expression. In this work, the expression of Bcl-X$_L$, one of the proteins inactivating Bax, was not altered in both p53$^{+/+}$ cell lines but was decreasing in p53$^{-/-}$ cells (**Fig. 32**). This p53-independent, CPT-11-induced downregulation of Bcl-X$_L$ may have contributed to the observed apoptosis, since the ratio of Bax to Bcl-X$_L$ amounts was increasing, suggesting that the amount of free Bax was increasing.

The percentage of apoptotic cells after treatment with CPT-11 was higher in p53$^{+/+}$,hMLH1$^-$ cells than in p53$^{+/+}$,hMLH1$^+$ cells, suggesting that an intact hMLH1 molecule may prevent CPT-11-induced apoptosis (**Fig. 26**). This effect may be due to different efficiency of repair of the CPT-11-induced DNA damage; indeed, recent data indicate that MMR-deficient cells are able to repair the double-strand breaks by homologous recombination more efficiently but are more error-prone than MMR-proficient cells [203]. This notion is supported by the observation that in short-term MTT assays the established cell lines MMR-deficient are more susceptible to topo I inhibitors than MMR-proficient cells (**Fig. 25** and ref. [150]). The repair of double-strand breaks by homologous recombination may however not be the major repair pathway in the case of CPT-11-induced damage.

Although the MTT test appears to reflect better the fraction of dying cells after treatment with CPT-11, the present study does not allow predicting the sensitivity of tumors to this drug. The data on the effects of p53- or MMR- status on sensitivity to CPT-11 in mouse models are also inconsistent [139, 147], while a retrospective study of patients treated with CPT-11 suggests that MMR-defect increases susceptibility to the drug [149]. Since our data show that both p53- or MMR- status affect the mechanism of cytotoxicity, the genotype-based predictions may require that both p53-

or hMLH1- status of the patients are taken into account. Further studies *in vivo* are necessary to assess additional determinants of the *in vivo* response to CPT-11.