20 patients had a statistically significant worse prognosis (median survival of 68 versus 113 months, p = 0.037). All other investigated proteins were not of prognostic significance in this subgroup of patients. Downregulation of the p27 protein in the human neuroendocrine cell line BON resulted in an increased phosphorylation of the RB protein as well as an increase of cells in the S-Phase and G2/M Phase of the cell cycle.
Discussion: The loss of p27 seems to play a critical role in the progression of gastro-enteropancreatic neuroendocrine tumors. The analysis of p27 expression identifies subgroups in metastatic disease with less favorable prognosis (p27 low expression). The underlying mechanism may be due to increased cell cycle progression in those tumors. We propose that the determination of p27 expression could be used to individualize therapeutic strategies in this tumor entity in the future.

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Lenalidomide and CC-4047 inhibit the proliferation of Namalwa cancer cells while expanding CD34+ progenitor cells. New insights on the combination therapy with HDAC inhibitors for hematological cancers

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Clinical studies involving patients with Myelodysplastic Syndrome and Multiple Myeloma have demonstrated the efficacy of lenalidomide (CC-5013) by reducing and often eliminating malignant cells while restoring bone marrow function. To better understand these clinical observations, we investigated and compared the effects of lenalidomide and its analog CC-4047, on the proliferation of two different hematopoietic cell models: the Namalwa cancer cell line and CD34+ progenitor cells. We found that both compounds have anti-proliferative effect on Namalwa cells and pro-proliferative effect on CD34+ cells, while p21WAF-1 expression was upregulated in both cell models. In Namalwa cells, we determined that the upregulation of p21WAF-1 correlates well with the inhibition of CDK2, CDK4 and CDK6 activity leading to pRb hypophosphorylation and cell cycle arrest. In contrast, in normal CD34+ progenitor cells, despite upregulated p21WAF-1 expression, we observed an increase of the cell division rate, leading to the enhancement of CD34+ expansion. Finally, we found that CC-4047 and lenalidomide have synergistic effects with two different HDAC inhibitors (Valproic acid and Trichostatin A) in both increasing the apoptosis of Namalwa cells and enhancing CD34+ cell expansion. Taken together, our results indicate that lenalidomide and CC-4047 have opposite effects in tumor cells versus normal progenitor cells and could explain, at least in part, the reduction of malignant cells and the restoration of the bone marrow observed in patients undergoing lenalidomide treatment. Moreover, this study provides new insights on the cellular pathways affected by lenalidomide and CC-4047, and proposes new potential clinical uses such as bone marrow regeneration. Finally, our in vitro experiments showing the efficacy of the combination of CC-4047 and lenalidomide with Valproic acid and Trichostatin A suggest that HDAC inhibitors might be ideal candidates for combination therapy by elevating the therapeutic index to treat hematological malignancies.

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AT7519, a selective small molecule inhibitor of cyclin dependent kinases: pharmacodynamic biomarker activity in a Phase I study

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A series of Cyclin Dependent Kinase (CDK) inhibitors was developed using Astex's fragment based medicinal chemistry approach, linked to high throughput X-ray Crystallography. A compound from this series, designated AT7519, is currently in early phase clinical development. The use of pharmacodynamic biomarkers of compound activity has become increasingly important with the advent of novel, molecularly targeted therapies, to aid determination of the minimum biologically effective dose. To this end a series of pre-clinical studies was performed to validate the biomarker assays for application in the clinical development of AT7519. We describe here the biomarker studies that are being utilised as exploratory end points in a Phase I solid tumour trial with AT7519. Pre- and post-dose skin punch biopsies were taken and the activity of the compound monitored by assessing inhibition of the proliferation markers Ki67 and Proliferating Cell Nuclear Antigen (PCNA) and the CDK substrates phospho-nucleophosmin (pNPM) and phospho-retinoblastoma (pRb). In addition the induction of tumour apoptosis was monitored in patient serum samples using a cytokeratin cleavage ELISA.
Data generated from the early cohorts on study are presented here, demonstrating that the assays developed are applicable to the clinical setting.

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Human papilloma virus integration begins in the tonsillar crypt and leads to the alteration of p16, EGFR, and c-myc during tumor formation

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Purpose: The prevalence of human papillomavirus (HPV) infection is high in the oropharyngeal mucosal regions, of which the tonsil is the most commonly affected. There may be a link between HPV and the pathogenesis of TC, because of common anatomical characteristics between cervical and tonsillar cancer (TC).
Experimental Design: We aimed to clarify whether HPV directly affects the oncogenesis and biologic behavior of TC by making a comparison between infection prevalence, physical status and viral loading numbers, and clinicopathologic prognostic factors. To compare HPV-related molecules between TC and tonsillitis (CFT), p16, survivin, HIF-1 overexpression (p = 0.022).
Conclusions: HPV-16 integration could be directly related to tonsillar carcinogenesis initially in tonsillar crypts followed by cell cycle aberration, such as p16 overexpression related to the G1-S phase and amplification of c-myc oncogene.

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Characterization of alvocidib (flavopiridol)-mediated inhibition of CDK enzyme activity and the down-regulation of gene transcription

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Drugs directed against cyclin-dependent kinases (CDKs) have been proposed as anti-cancer agents. The sanofi-aventis compound alvocidib (flavopiridol) was the first CDK inhibitor administered in man and has shown promise in Phase I against fludarabine-refractory chronic lymphocytic leukemia (CLL) [1]. The leading hypothesis for the molecular mechanism of alvocidib in CLL is that alvocidib inhibits CDK9-mediated transcription of pro-survival factors such as *MCL1* [2], resulting in apoptosis of the target B–cells. Here, we present a detailed *in vitro* characterization of alvocidib-mediated inhibition of CDK enzymatic activity and the down-regulation of gene transcription.
Alvocidib potency against several CDKs was evaluated using an enzymatic end-point assay based on ^{33}P incorporation. We show that alvocidib is a pan-CDK inhibitor with nM activity on all CDKs tested (Table 1). By far the strongest effect was observed on CDK9/T1 (IC50=2 nM), which promotes transcript elongation by phosphorylating RNA polymerase II. To further characterize CDK9/T1 inhibition by alvocidib, we used a continuous *in vitro* kinase assay, which allows the measurement of initial reaction velocities. We found that CDK9/T1 catalysis proceeds by a sequential random mechanism. The Km and Kd values for substrate and ATP will be reported and contrasted with published values for other CDK/cyclin complexes. Alvocidib inhibits CDK9/T1 in an ATP competitive manner and acts as a tight binding inhibitor (Ki=1.3 nM).

Table 1:

	IC50, nM	Ki, nM
CDK1/B1	10	
CDK2/A2	20	
CDK2/E	220	100
CDK4/D1	35	
CDK5/p25	430	
CDK7/H	150	
CDK9/T1	2	1.3

In order to investigate the consequences of alvocidib-mediated CDK9 inhibition on transcription, we monitored *de novo* mRNA synthesis in HCT116 cells by ^{3}H-uridine incorporation. We found that alvocidib abolished *de novo* mRNA synthesis within 3 hrs with an IC50 of 69 nM. These results were further supported by microarray and RT-PCR expression analysis. Six hours of 190 nM alvocidib treatment of HCT116 cells down-regulated 3,275 genes (98.7% of all drug responsive transcripts, including *MCL1*), and up-regulated only 44 genes. RT-PCR of *MCL1* mRNA levels 6 hours after drug exposure demonstrated an IC50 for

alvocidib-mediated mRNA down-regulation of 64 nM, consistent with the IC50 determined for inhibition of global mRNA synthesis. Replacement of fetal calf by human serum did not significantly change the outcome. Results presented here on alvocidib-mediated down-regulation of *MCL1* in HCT116 cells are in agreement with previous data published for CLL.

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DNA-interactive agents

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Selective targeting of critical components of DNA replication by HKH40A (RTA 502)

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Mammalian replication is tightly regulated because it must occur at the right times and only once per cell cycle. The timing is carefully choreographed during the G1 and S phases of the cell cycle. Kinases that play important roles include the licensing kinase Cdc6, and Cdc7, that is required for replication inititation. Disruption in the timing of replication leads to premature entry into S phase. Consequently mammalian cells have checkpoint controls that prevent premature progression through the cell cycle. In contrast, cancer cells frequently exhibit mutated controls hence disruptions in the assembly of the replication complex can be lethal to these cells. Disruption in timing of replication in wt p53 tumor cells causes initiation of the p53-dependent apoptotic cascade. In absence of functional p53, tumors with disrupted timing of the replication enter into S phase that cannot be completed, which results in genomic instability, mitotic crisis and death. HKH40A is a synthetic anti-tumor agent with excellent in vitro and in vivo activity against a number of GI tumors. It binds to DNA but its biological activity is manifested only after the initial complex hijacks a protein that is involved in repair, transcription or replication. Sensitive wt p53 cancer cell lines such as colon HCT116 and RKO or hepatoma HepG2 are killed by p53-mediated apoptosis (IC_{50} < 1 nM; LC_{50} ~ 30 nM). Cancer cell lines with mutated p53 such as the colon HT29 and pancreas such as ASPC1 or 10.05 are also killed by the drug (IC_{50}~2 nM; LC_{50}~90 nM), but die as a result of a G2-M block and grossly dysregulated S-phase. The compound showed potent activity in orthotopic liver cancer in rats, as well as in HCT116 and pancreatic cancer BxPC3 and MIA Paca-2 xenografts in mice. It is a candidate for Phase 1 trials. The most downregulated genes in DNA micorarrays of drug-treated HCT116 cells are ribonucleotide reductase M2 (RRM2) and the kinase Cdc7. RRM2 is responsible for maintaining dNTP pools and is frequently up-regulated in cancer where it is a factor in chemoresistance of tumors to drugs. Cdc7 (with Dbf4) phosphorylates MCM2–7 proteins, which are obligatory components of the replication complex. RRM2 and Cdc7 are also downregulated at the protein level (Western analysis), maximally at 48 hrs. Cdc6 is not affected by the drug treatment at the RNA level, but is dramatically downregulated at the protein level after 3 hrs. Western analysis of mutated p53 lines revealed similar changes in these three critical components of replication, suggesting that the effect of the drug on the expression of RRM2, Cdc6 and Cdc7 is p53-independent. FACS analysis of treated cells is consistent with replicative arrest and premature entry into S-phase, that tumor cells unlike normal cells are not able to survive. Thus, agents that target critical components of replication control should be selectively toxic toward cancer cells whose checkpoint functions are impaired.

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Poly(ADP-ribose) polymerase-1 mediated transcriptional responses to genotoxic stress: a possible target for anticancer therapy

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PARP-1 plays a critical role in various cellular responses to genotoxic stress, albeit its function has been attributed primarily to DNA damage repair. We have previously demonstrated that (i) PARP-1 binds to undamaged DNA where it recognizes non-B DNA structures such as hairpins, cruciforms, and unwound regions that can form in the transcriptional regulatory elements [1−3], (ii) PARP-1 can bind to the promoter of its own gene both in vitro and in vivo [1,3], and (iii) PARP-1 interactions with non-B DNA structures lead to its catalytic activation [2]. Together, these data implicate PARP-1 in coordinating structural transitions in genomic DNA and chromatin during transcription. Here we investigated the PARP-1 function in activation of nuclear transcription factors, such as activator protein-1 (AP-1), which participates in adaptive responses of the genome to various oxidant and toxic stimuli. Genetic inhibition of PARP-1 resulted in a delayed activation of Jun transcription factors, and significantly diminished DNA-binding activity of the Fos family proteins. We show that PARP-1 proficient cells exposed to oxidative stress exhibited an early and marked induction of individual components of the AP-1 and that stimulation of the AP-1 expression by DNA damage was dramatically attenuated in cells with PARP-1 null genetic background. Thus, PARP-1 is a dynamic sensor of cellular stress playing important role not only in DNA damage repair but also in DNA damage-induced transcription. Therefore, strategies directed at targeting the DNA-binding properties of PARP-1 may alleviate cellular resistance to genotoxic stress because non-covalent contacts of the DNA-binding domain of PARP-1 with DNA, either containing strand breaks or undamaged, underlie the molecular basis for PARP-1 function. We tested the feasibility of this approach using an enforced expression of the DNA-binding domain of PARP-1. We show that the expression of a dominant-negative mutant of PARP-1 sensitized prostate carcinoma cells to DNA-damage in vitro, and significantly enhanced the radiation-induced inhibition of tumor growth in vivo. It is conceivable that the shared components of the DNA repair and transcription systems, such as PARP-1, allow both systems to control cellular survival in response to genotoxic stress.
This work was supported in part by grants from the U.S. Army and the National Cancer Institute (to VS).

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The cytotoxic activity of the monofunctional alkylator S23906 is mediated by generation of DNA double strand breaks that are repaired by homologous recombination in mammalian cells

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Background: S23906, a new monofunctional DNA alkylating agent is under evaluation in phase I clinical trials. We have recently shown (*Léonce et al., Cancer Res*, in press) that cellular exposure to S23906 is accompanied by the formation of DNA double strand breaks (DSBs) as detected by single cell electrophoresis and gamma-H2AX formation. In this work we wished to further characterize the DSBs and establish their role in the antiproliferative activities of S23906.
Results: Exposure of tumor cells to S23906 is accompanied by a dose-dependent formation of DSBs that can be detected within minutes by single cell electrophoresis. Incubation with S23906 for 1 h followed by post-incubation in drug-free media revealed that the even short exposure to S23906 is accompanied by long-lasting DNA damage that is difficult to repair. Co-incubation with aphidicolin, a specific inhibitor of DNA synthesis was accompanied by decreased DSBs formation suggesting that the DSBs may be a result of collision between the DNA-S23906 adducts and the advancing replication fork. Importantly, co-exposure to aphidicolin and S23906 followed by post-incubation in drug-free media was accompanied by a gradual formation of DSBs, that after 4 h post-incubation had reached the same levels as after 1 h in the presence of S23906 alone. Therefore, inhibition of ongoing DNA synthesis does not interfere with the initial formation of the S23906 adducts which retain their ability to induce DNA damage for hours after the drug has been removed. Cells deficient in homologous recombination repair showed up to 16-fold increased sensitivity to S23906 compared to parental cell lines whereas no differences were observed for cells deficient in end-joining. The sensitivity of recombination-deficient cells was associated with an inability to repair the S23906-induced DSBs. In contrast, the formation of DSBs was not affected.
Conclusions: Our results indicate that the cytotoxic activity of S23906 is mediated by the formation of DSBs that, at least in part, are replication dependent. They further suggest that expression levels of proteins involved in recombination repair may serve to identify patients particularly likely to respond to this interesting agent.