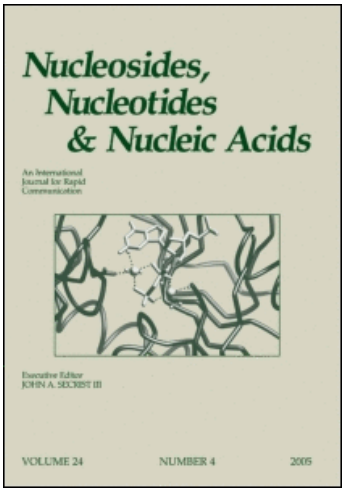

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Pyrimidine and Purine Analogues, Effects on Cell Cycle Regulation and the Role of Cell Cycle Inhibitors to Enhance Their Cytotoxicity

Jennifer Sigmond[a]; Godefridus J. Peters[a]
[a] Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

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PYRIMIDINE AND PURINE ANALOGUES, EFFECTS ON CELL CYCLE REGULATION AND THE ROLE OF CELL CYCLE INHIBITORS TO ENHANCE THEIR CYTOTOXICITY

Jennifer Sigmond □ *Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*

Godefridus J. Peters □ *Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*

□ *In anti-cancer treatment, deoxynucleoside analogues are widely used in combination chemotherapy. Improvement can be achieved by rational design of novel combinations with cell cycle inhibitors. These compounds inhibit protein kinases, preventing the cell cycle from continuing when affected by deoxynucleoside analogs. The efficacy is dependent on the site of cell cycle inhibition, whether multiple cyclin-dependent kinases are inhibited and whether the inhibitors should be given before or after the deoxynucleoside analogs. The action of cell cycle inhibition in vivo may be limited by unfavorable pharmacokinetics. Preclinical and clinical studies will be discussed, aiming to design improved future strategies.*

Keywords Pyrimidine analogues; Purine analogues; Deoxynucleoside analogues; Cell cycle inhibitors

INTRODUCTION

Defects in cell cycle regulation can lead to uncontrolled proliferation of cells; e.g., cancer. The tumor suppressor gene p53 is involved in cell cycle progression and is frequently mutated in neoplasms. In fact, in more than 50% of the tumors, the p53 gene is inactivated. Normally, activation of p53 by DNA damage results in cell cycle arrest, allowing DNA repair or induction of apoptosis when DNA repair fails to complete its action. There are many downstream targets of p53 and p53 interacting proteins that regulate the cell cycle. Mutation or inactivation of these cell cycle proteins such as p53 will allow cells with damaged DNA to continue to grow and thus are important in oncogenesis.

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Address correspondence to Godefridus J. Peters, Department of Medical Oncology, VU University Medical Center, POB 7057, 1007 MB, Amsterdam, The Netherlands. E-mail: gj.peters@vumc.nl

Progression through the cell cycle is controlled by various cytoplasmatic proteins: cyclins, cyclin-dependent kinases (cdks), and the anaphase promoting complex (APC). APC is also known as the cyclosome that triggers sister chromatids to separate and is responsible for degradation of mitotic cyclin B. Sequential expression of different cyclins and cdks regulate progression of cells to G1 (cyclin D, cdk4 and 6), S (cyclin E, cdk2), G2 phases (cyclin A, cdk1 and 2), or mitosis (cyclin B, cdk1). Their levels in the cell rise and fall with the stages of the cell cycle, establishing a one-way direction through the cell cycle (Figure 1). Cdks are protein kinases that cannot be active without binding to their corresponding cyclins. Association of cyclins with cdks leads to partial activation of the complex. However, cyclin activating kinases (caks) that are constitutively active through the cell cycle complete the cyclin-cdk complex activation by phosphorylating a threonine residue in the kinase.[1] Phosphorylation is not only able to activate cdks, but phosphorylation may also be inhibitory when it occurs at sites near the amino terminus in the ATP binding cleft of the kinase subunit.[2,3] The kinases Wee1 and Myt1 inactivate the Cdk1-cyclin B complex by this inhibitory phosphorylation.

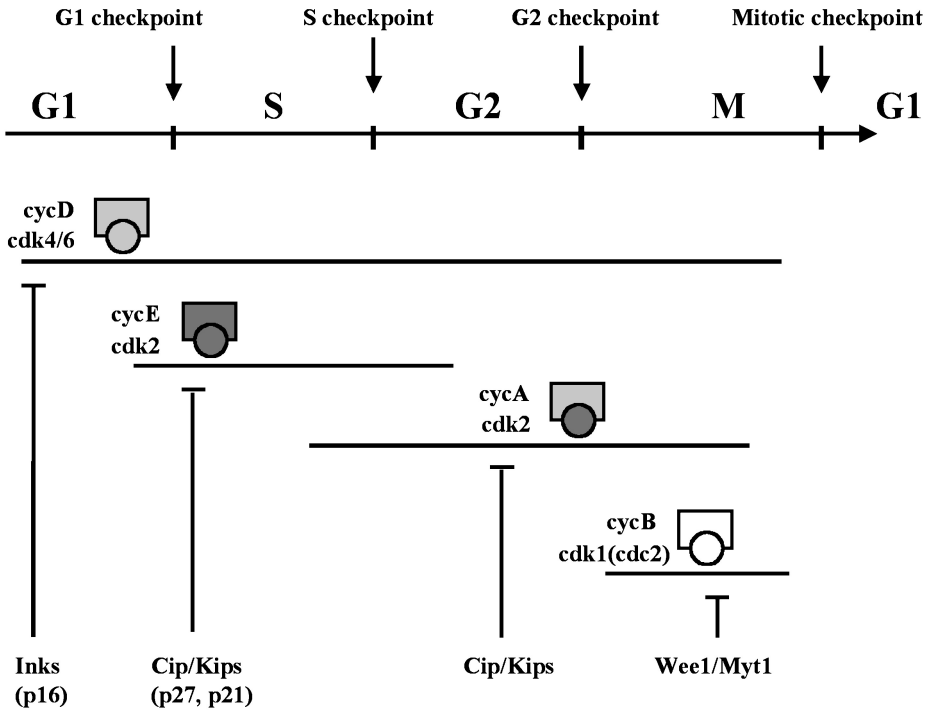

FIGURE 1 Cyclin-cdk expression during the cell cycle. Requirement of favorable conditions for the expression of cyclin D is necessary. Inks include p16, Cip/Kips include p27 and p21. (Source: modified from review by McGowan.[4])

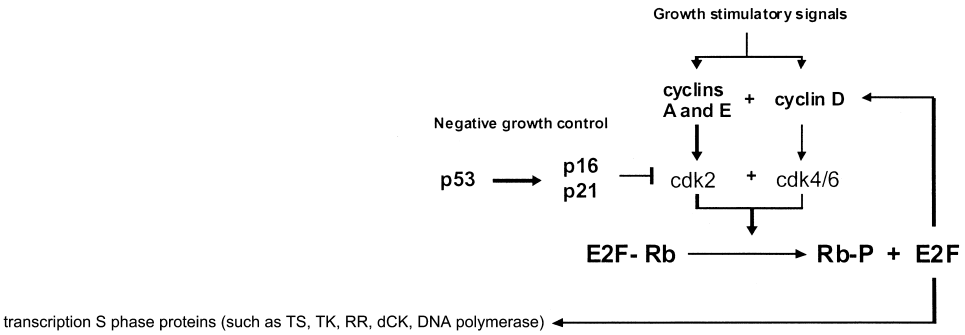

FIGURE 2 Simplified scheme of cell cycle proteins regulating the transition from G1 to S phase or inducing a G1 arrest. Cdk2 and cdk4/6 catalyze hyperphosphorylation of Rb in the E2F-Rb complex leading to the release of E2F, which can activate transcription of S-phase enzymes. Wt p53 through activation of p21 can inhibit this process by inhibition of cdk2.

When cyclin D-cdk4/6 complexes are formed, they enter the nucleus and phosphorylate the retinoblastoma (Rb) protein, releasing the transcription factor E2F from the Rb-E2F complex. Transcription targets of the E2F family include S-phase related proteins such as various salvage and de novo enzymes in deoxynucleotide synthesis. These include thymidine kinase and thymidylate synthase (TS) responsible for formation of 2′-deoxythymidine-5′-triphosphate (dTTP), an essential precursor during DNA synthesis.[5] In addition, next to the cyclin D-cdk4/6 complex the cyclin E-cdk2 complex also phosphorylates Rb, leading to transition of the cell from the G1 to the S phase. Activated cyclin-cdk complexes can also be counteracted by cyclin-dependent kinase inhibitors (CDKIs) such as the Ink4 family, including p16, which is able to inhibit the cyclin D-cdk4/6 complex and the CIP/KIP family, including p27 and p21, that inhibit the cyclin E-cdk2 complex as shown in Figure 2. Levels of p21 are increased by activation of wt p53 after DNA damage leading to a G1 cell cycle arrest.

To secure the quality of the cell cycle progression, DNA damage and spindle checkpoints are present in different cell cycle phases. The G1 and G2 checkpoints sense DNA damage before entering the next phase, while the spindle assembly checkpoint blocks cytokinesis when improper alignment of the spindle fibers occur. Apoptosis is triggered if DNA damage is irrepairable. Checkpoints are control mechanisms that enforce entering late cell cycle phases after completing the early phases perfectly, guarding the integrity of cells and preventing induction of early step mutagenesis that can lead to cancer predisposition.

CELL CYCLE INHIBITORS

In cancer patients, uncontrolled proliferation of cancer cells causes growth of tumors that eventually causes failure in the function of healthy

tissues. Efforts are taken to inhibit the neoplastic proliferation. A decrease in growth rate can be established by inhibition of the cell cycle. Therefore, different cell cycle inhibitors have been developed that act on different levels: 1) direct inhibition of cdks, 2) indirect inhibition of cell cycle via various signaling transduction pathways including by depletion of essential substrates for DNA synthesis, and 3) interference with cytoskeletal processes.

Direct inhibitors of cdks include staurosporine, UCN-01, flavopiridol, and roscovitine (Table 1), which bind to the ATP binding site of their cdk targets. Staurosporine and its derative UCN-01 (7-hydroxystaurosporine) are able to inhibit cdk2, but also have other pleiotropic effects such as protein kinase C inhibition (see next part). Flavopiridol inhibits phosphokinases; its activity is strongest on cyclin-dependent kinases (cdk-1, -2, -4, -6, -7) and less on receptor tyrosine kinases (EGFR), receptor associated tyrosine kinases (pp60 Src), and signal transducing kinases (PKC and Erk-1 inducing apoptotic and anti-angiogenesis.[6] Roscovitine, a purine analogue, has cdk2 inhibiting properties and is currently investigated in clinical trials.

Inhibition of the cell cycle can be established on different levels (Figure 3). Complex signaling transduction pathways require involvement of various protein kinases. Protein tyrosine kinases (PTKs) are a large and diverse multigene family that is involved in intracellular signaling pathways and regulates key cell functions such as proliferation, differentiation, and antiapoptotic signaling. The main classes of PTKs are receptor protein-tyrosine kinases (RPTKs) and the smaller group of cytoplasmic protein kinases, which are both divided in 20 and 10 subfamilies, respectively.[7,8] PTKs are associated with oncogenic activation and are highly regulated. Several antibody and small molecule PTK inhibitors are investigated both preclinically and

TABLE 1 Targets and Effects on Different Cellular Processes and Proteins of Cell Cycle Inhibitors Staurosporine,[17–19] UCN-01,[20–26] Flavopiridol,[6,22,27–30] and the Purine Analogue Roscovitine[31–37]

Cell cycle inhibitors	Staurosporine	UCN-01	Flavopiridol	Roscovitine
Cell cycle proteins	cdk 2(−)	cdk 2, 4, 6(−) cyclin D(−) pRb(−)	cdk 2, 4, 6, 7 (−) cyclin D(−) pRb(−)	cdk1,2,5,7(−) cyclin D(−) pRB(−)
PKC	(−)	(−)	(−)	
Proapoptotic proteins				cyt C, bci-xs (+)
Survival/growth	MAPK(−)	akt(−)	mcl1(−), XIAP(−), BAG-1 (−)	MAPK(+) mcl1 (−), NF-kB(−), Traf-5(−), mdm(−)
Angiogenesis	(+)	(−)	(−)	
DNA repair		(−)		

(−): inhibition, (+) stimulation.

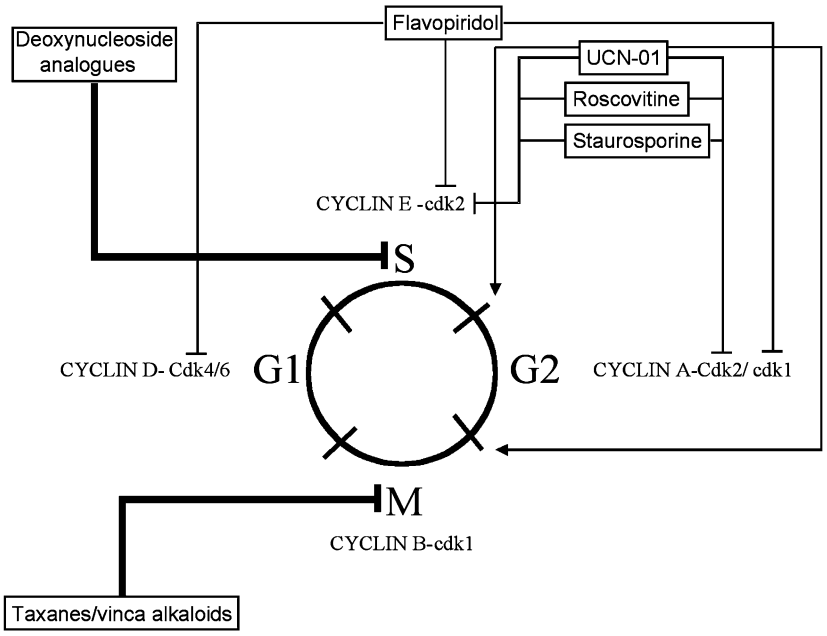

FIGURE 3 Targets of cell cycle inhibitors: nucleoside analogues, taxanes/vinca alkaloids, and cdk inhibitors. Deoxynucleoside analogues induce an S-phase arrest via DNA damage, taxanes/vinca alkaloids inhibit mitosis via destabilization or stabilization of the cytoskeleton, cdk inhibitors have different targets, while UCN-01 also abrogates the S/G2 and G2/M checkpoint.

clinically. The ones that have entered the market include Herceptin, Imatinib (Gleevec/Glivec), and Gefitinib (Iressa, ZD1839).

Protein kinase C (PKC) is an enzyme involved in a variety of pathways leading to cell division, differentiation, and survival. PKC phosphorylates proteins at serine/threonine residues and thereby activates other proteins. The PKC family can be divided into three groups, classical (cPKC-α, -βI, -βII, and -γ), novel (nPKC-δ, -ε, -η, -θ, and -μ), and atypical (aPKC-ξ and -ι). Classical and novel PKCs are activated by diacylglycerol (or phorbolesters); the atypical PKCs are activated by lipid cofactors such as phosphatidylinositol 3,4,5-P_3 (PI-3,4,5-P_3) and ceramide. Although earlier experiments show various neoplastic or tumor suppressive effects of different PKC isoforms, several PKC inhibitors (such as staurosporine or its analogue UCN-01) show anti-tumor effects in vitro and in vivo.

Proteosome-mediated degradation of p27 and cyclin A is required to enforce the cell cycle progression through the G1/S checkpoint. Enhanced p27 degradation is associated with uncontrolled proliferation.[9–13] Other cyclins (cyclin D and E) can also be ubiquinated and thereby targeted for proteosome degradation. Proteosome-ubiquitination inhibitors are becoming a focus in development of anti-tumor agents. However, besides

"protein degradation" targeted cell cycle inhibition, "protein synthesis" directed developments are also currently in progress. Histone deacetylase (HDAC) activity plays a key role in the regulation of gene expression or epigenetics. Deacetylation of histones induces conformational changes in the associated DNA segment leading to silencing of genes. Development of HDAC inhibitors that selectively switch on tumor suppressor genes promise desirable anti-cancer effects. The HDAC inhibitor trichostatin A (TSA) stimulates expression of p21 and cyclin A,[14] thereby inhibiting cell cycle progression. Suberoylanilide hydroxamic acid (SAHA), another HDAC inhibitor, is currently in clinical trial and shows antitumor activity. Preliminary results from clinical trials suggest that these agents are promising. Several HDAC inhibitors that exhibit impressive antitumor activity in vivo and remarkably little toxicity are now in clinical trials.[15,16]

Cells can also be arrested in the M phase by interference with cytoskeletal processes that are manifested by the taxanes (paclitaxel, docetaxel) and vinca alkaloids (vincristine, vinblastine, vinorelbine) that either destabilize or stabilize microtubules during the M phase, blocking cell cycle progression (Figure 3).

BASIS FOR EFFECTIVE COMBINATION OF CELL CYCLE INHIBITORS WITH DNA DAMAGING AGENTS

In anti-cancer treatment, suitable combination therapies are rationally designed in order to increase efficacy and circumvent development of resistance. Increasing efficacy may result from the use of agents that work on different levels, inducing additive effects or, ideally, synergistic interactions. Many combinations in clinical use consist of antimetabolites together with other anti-cancer agents. Ribonucleoside and deoxynucleoside analogues are antimetabolites that interfere with DNA synthesis. They are widely used for treatment of both solid tumors and hematological malignancies. They compete with natural physiological nucleosides and interact with various intracellular targets to induce cytotoxicity. A generally shared mechanism of action is inhibition of nucleotides precursor synthesis, which results in the incorporation of nucleoside analogue metabolites into the DNA and/or RNA, resulting in arrest of the cells in the S phase (Figure 3). When damage is irreversible, apoptosis is induced. In the next section, different types of nucleoside analogues are discussed.

DNA damaging agents such as nucleoside analogues that arrest cells in the S phase are suitable agents to be combined with cell cycle inhibitors that act on different cell cycle phases, thereby enhancing the cytotoxicity of both drugs. When cells are inefficiently blocked in one cell cycle phase, the cells that nevertheless manage to pass through the cell cycle can be arrested in the next upcoming targeted cell cycle phase, thereby increasing the therapeutic response. Combination treatment using targeting of different

cell cycle phases can focus on drugs that target the G0/G1, G1/S, and G2/M transitions.

Various cdk inhibitors have been developed such as UCN-01 and the trisubstituted amino purines (R)-roscovitine (ROSC, CYC202), olomoucine (OLO), purvalanol, and bohemine (Figure 4). Roscovitine has high specifity to cdk2, but also cdk1, cdk5, and cdk7 can be inhibited.[31,32] It has antitumor activity in numerous cancer cell lines and xenografts.[38–40] Roscovitine inhibits Rb protein phosphorylation, causes loss of cyclin D1, and activates the mitogen-activated protein kinase (MAPK) pathway.[33] In vitro, roscovitine arrests cells in the G2/M and G1/S transition[32,41–43] and is able to induce apoptosis in all cell cycle phases.[40] In addition, upregulation of the proapoptotic factor bcl-xs is observed in HNCC cell lines.[34] p53-independent and -dependent induction of apoptosis have been suggested; roscovitine can activate p53 through suppression of MDM2[37] and can induce apoptosis by release of cytochrome c via p53AIP1,[35] while p53-independent apoptosis can be induced by downregulation of the survival stimulating proteins mcl1, NF-kB, and Traf5 that were presumably due to inhibition of phosphorylation of RNA polymerase II.[36]

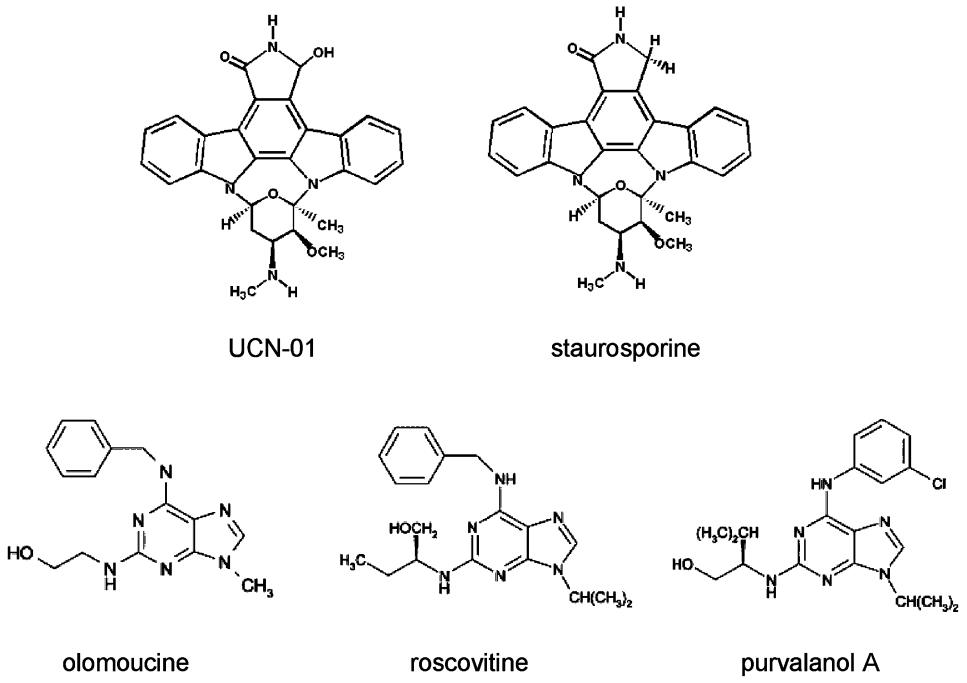

FIGURE 4 Staurosporine, UCN-01, and purine analogue inhibitors of cdks. These purine analogues cannot be transformed to their nucleotides due to the substitution of the N9 position. In contrast to other purine analogues they act as the parent compound. Roscovitine is a good cdk2 and cdk5 inhibitor, but a poor cdk6 and cdk4 inhibitor. Next to roscovitine, the other purine analogue inhibitors olomoucine and purvalanol also demonstrate selectivity for cdks 1, 2, 5, 7, and 9.

As earlier described, UCN-01 is able to induce a G1 arrest that results from cdk2 inhibition leading to Rb hypophosphorylation. UCN-01 treatment of cancer cells is also associated with a decline in cyclin D expression, DNA repair, and an inhibition of the growth stimulating Akt.[23,26] However, UCN-01 also has the potential to abrogate the G2/M checkpoint via "checkpoint kinase" (chk) 1 and possibly chk2.[21] Chk1 is also involved in regulation of the S-phase checkpoint.[44] In DNA damaged cells, low concentrations of UCN-01 cause S-phase arrested cells first to progress into the G2 phase before going into apoptosis while high concentrations cause immediate S → M transition.[45]

The G2/M checkpoint involves two upstream key players: the protein kinases ataxia telangiectasia (ATM) and ATM-Rad3-related (ATR), which upon activation prevent cells from entering mitosis to repair the damage, and, when irreversible, apoptosis is induced (reviewed in several papers).[46–50]

As shown in Figure 5, downstream of these protein kinases are chk1 and chk2 that are phosphorylated and activated by ATM and ATR. Activated chk1 and chk2 can phosphorylate the protein phosphatase cdc25c on serine 216,[51,52] thereby inactivating cdc25c. Normally, cdc25c dephosphorylates and activates the cdk1-cyclin B complex that is leading to entry into the mitosis phase. When the ATR or ATM is activated, inactivation of cdc25c

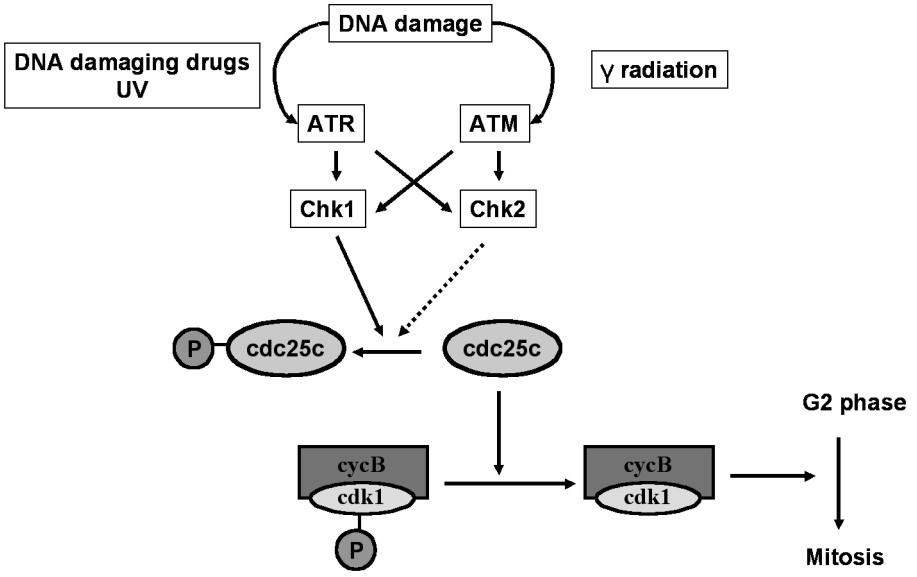

FIGURE 5 Role of checkpoint kinases in cell cycle control. After DNA damage, chk1 and chk2 can be activated by ATR and ATM (depending on the source of DNA damage). Activated checkpoint kinases inactivate the phosphatase cdc25c by phosphorylation. Depletion of active cdc25c prevents cyclineB-cdk1 from being dephosphorylated, leading to inactivation of the cyclineB-cdk1 complex, and thereby preventing G2-M transition of cells and enabling cells to repair the induced DNA damage.

prevents the transition of cells from the G2 to the M phase, allowing cells to repair the damage. ATM appears to be activated by irradiations, causing double-strand breaks (DSBs). It is generally accepted that ATR activation is also stimulated by DNA damaging chemotherapeutics such as alkylating agents. While most cancer cells have mutations in the G1/S checkpoint (such as the tumor suppressor genes p53 and Rb), only exceptional cancer cells have defects in the G2/M checkpoint.[53] Cancer cells treated with checkpoint inhibitors that possessed an inactive p53 and a partially active G2/M checkpoint were more enhanced in their sensitivity to DNA damaging agents compared to their equivalents with functional p53.[54–57] This supports the statement that abrogation of the G2/M checkpoint is an attractive target when it is combined with DNA damage. The use of checkpoint inhibitors combined with DNA damaging agents has been proposed to have a greater therapeutical window.[58–63] Since deoxynucleoside analogues possess DNA damaging properties, they would be an excellent combination together with checkpoint G2/M abrogators.

DEOXYNUCLEOSIDE ANALOGUES

Deoxynucleoside analogues (Figure 6) are antimetabolites that compete with natural deoxynucleosides that are used for DNA synthesis. Bases

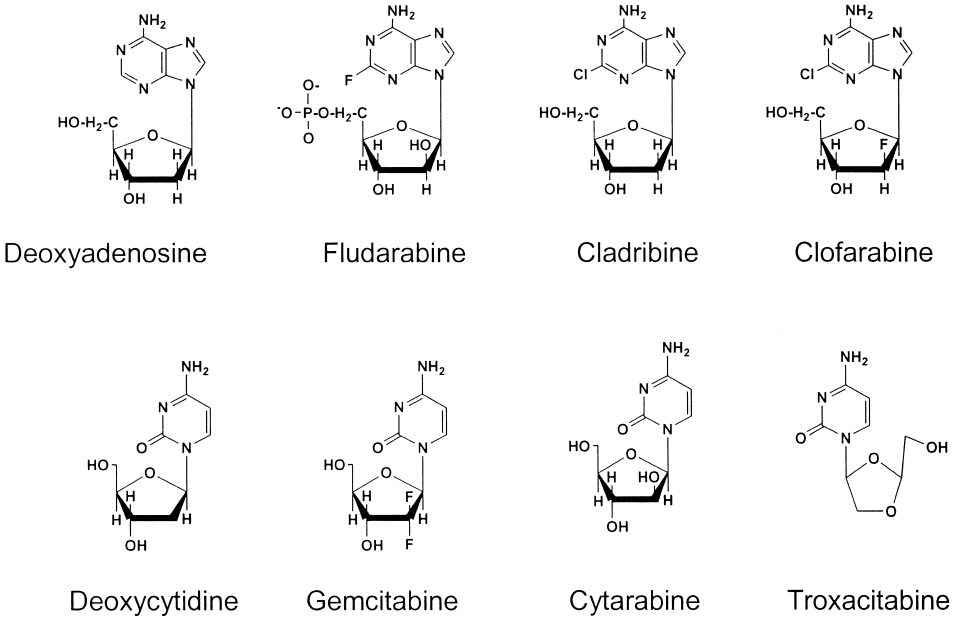

FIGURE 6 Chemical structures of purine analogues fludarabine, cladribine, clofarabine, and the pyrimidine analogues compared to their natural deoxynucleosides.

and ribonucleosides can also have similar effects. The analogues can be divided into 1) purine analogues: e.g., fludarabine, cladribine, and clofarabine, which are deoxyadenosine deratives, 2) pyrimidine analogues: e.g., cytarabine, gemcitabine, troxacitabine, tezacitabine, 1-(2-*C*-cyano-2-deoxy-1-β-D-*arabino*-pentofuranosyl) cytosine (CNDAC) and 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl) cytosine (DMDC), which compete with the natural deoxynucleoside deoxycytidine, and 1-(3-*C*-ethynyl-β-D-ribo-pentofuranosyl) cytosine (EcyD, TAS-106), which competes with cytidine for phosphorylation by uridine/cytidine kinase, and 3) fluoropyrimidines: uracil/tegafur (UFT), 5-fluorouracil (5-FU), its prodrug capecitabine (Xeloda), and trifluorothymidine (TFT), which inhibits deoxythymidine triphosphate de novo synthesis and can be incorporated into DNA.[64] All compounds need to be metabolized to exert their cytotoxic effect. Several of the purine and pyrimidine enzymes required for these conversions (TK1, dCK) or which serve as a target (TS, RR) are regulated by cdks (see, e.g., Figure 2). However, another group of purine analogues (see Figure 4), the trisubstituted aminopurines, do not need to be activated and can inhibit various cdks directly.

The deoxyadenosine deratives fludarabine (fluoroadenine-β-D-arabinoside, Fludara® administered as Fludara-phosphate) and cladribine (2-chlorodeoxyadenosine, CdA, Leustatin®) are used alone or in combination for treatment of refractory chronic lymphocytic leukaemia and hairy-cell leukaemias, respectively,[65,66] and show activity to other haematological malignant disorders such as non-Hodgkin's lymphomas. Both compounds are incorporated into the DNA preventing DNA synthesis, inducing cell death. In addition, fludarabine can be incorporated into both DNA and RNA. Moreover, fludarabine is able to inhibit DNA primase, DNA polymerases, 3′-5′ exonuclease activity of DNA polymerases delta and epsilon, and DNA ligase I, involved in DNA synthesis and repair.[67,68] Fludarabine and cladribine are both transported by specialized nucleoside membrane transporters and are phosphorylated to their active corresponding nucleotide F-Ara-ATP and CdATP. The initial step in this activation is catalyzed by deoxycytidine kinase (dCK). Besides DNA incorporation, both diphosphates are also able to inhibit ribonucleotide reductase (RR), which is responsible for generating deoxynucleoside triphosphates (dNTPs) required for DNA synthesis reviewed in Kolberg et al.[69] A decrease in dNTP pools results in a self-potentiating effect, increasing F-Ara-ATP/CdATP incorporation into DNA.[67] DNA damage induced by fludarabine and cladribine induces p53-dependent and p53-independent apoptotic pathways.[70] They both interact with the proapoptotic factor Apaf-1, while cladribine may induce apoptosis by disrupting the integrity of mitochondria, thereby releasing cytochrome C, inducing the caspase cascade.[71] Similar toxicity profiles were found that include moderate myelosuppression and profound and prolonged immunosuppression. Clofarabine (2-chloro-2′-arabino-fluoro-2′-

deoxyadenosine, CAFdA, Clofarex™) is the latest developed analog of deoxyadenosine that shows not only antitumor activity in hematological malignancies in vitro[72–75] and in vivo,[76,77] but also has the potential to be used in treatment of solid tumors, particularly in colon tumors.[78,79] Clofarabine was synthesized as a rational extension of the deoxyadenosine analogues experiences to overcome some of the limitations and incorporate the best qualities of both fludarabine (inhibition of DNA polymerases) and cladribine (inhibition of RR). The metabolism is similar to that of fludarabine and cladribine, which are all resistant to deamination by adenosine deaminase (ADA).

The group of pyrimidine analogues includes cytarabine (cytosine arabinoside, arabinosylcytosine, Ara-C, Cytosar®-U, Tarabine® PFS), which is frequently used in hematological malignancies, particularly in acute myeloid leukemia. In contrast to cytarabine, the other pyrimidine analogue 2′,2′-difluorodeoxycytidine (dFdC, Gemcitabine, Gemzar) is active in solid tumors such as non-small-cell lung cancer (NSCLC), pancreatic cancer, bladder cancer, and ovarian and breast cancers. Cytarabine is administered as a single agent or in combination with an anthracyline such as daunorubicin or doxorubicin, while gemcitabine is administered in combination with cisplatin, carboplatin, 5-FU, and paclitaxel. They both require dCK to be activated to their metabolites Ara-CTP and dFdCTP, which are incorporated into RNA (only gemcitabine) and DNA (both gemcitabine and cytarabine), which is the main mechanism of action. However, gemcitabine is also able to inhibit RR that leads to a self-potentiating effect by inducing a decrease of dNTPs pools, enhancing its own incorporation into DNA. Low levels of the natural metabolite dCTP also stimulate the rate-limiting step of its activation by dCK, of which the levels have shown to be a predictive factor of in vivo gemcitabine sensitivity.[80]

Cytarabine and gemcitabine are transported into cells by nucleoside transporters. Cytarabine is transported by equilibrative nucleoside transporters (ENT),[13,33,63] while gemcitabine can be transported by both ENTs and concentrative nucleoside transporters (CNT).[81–86] For gemcitabine, it has been shown in vitro that increased hENT1 expression is a determinant of gemcitabine sensitivity.[87] In vivo, Spratlin et al. showed that the absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma.[88] Gemcitabine and cytarabine can both be inactivated by deoxycytidine deaminase (dCdA). Troxacitabine (BCH-4556, Troxatyl, L-OddC), is an L-pyrimidine nucleoside analogue that is resistant to inactivation by dCdA. In addition, its transport into the cells requires no nucleoside transporters, but mainly enters by passive diffusion.[89] After activation by dCK, its main mechanism of action is DNA incorporation and inhibition of repair DNA polymerases.[90] In vitro and in vivo results show that it has potential in treatment of

acute myeloid leukemia (AML), pancreatic cancer, and solid tumors.[91–96] Currently, troxacitabine is investigated in different stages in clinical trials that show promising activity in treatment of haematological malignancies[97,98] while a dose-finding study in advanced solid tumors[99] and an efficacy trial in metastatic renal cell carcinoma showed modest activity.[100] The metabolism and mechanism of action of Tezacitabine [(E)-2′-deoxy-2′-(fluoromethylene)cytidine (FMdC)] is similar to that of gemcitabine; however, antiangiogenic effects were also observed in mice with human tumor xenografts.[101] Tezacitabine-diphosphate is a more potent inhibitor of RR than gemcitabine-diphosphate.

5-FU and capecitabine belong to the group of fluoropyrimidines. 5-FU is widely used for treatment of various solid tumors, such as colorectal, breast, stomach, pancreatic, ovarian, cervical, and bladder cancers. 5-FU has multiple mechanisms of action; its metabolite 5-fluoro-2′deoxyuridine-5′monophosphate (FdUMP) mediates inhibition of thymidylate synthase, which catalyzes 2′-deoxythymidine-5′-monophosphate (dTMP) synthesis, a precursor for DNA synthesis. 5-FU can also be incorporated into RNA and DNA.[102] 5-FU is clinically administered with Leucovorin (LV) to increase its antitumor activity. Next to single agent administration, several 5-FU combinations are approved in the treatment of cancer. Capecitabine is a prodrug of 5-FU that is selectively tumor activated because the activating enzyme thymidine phosporylase (TP) is highly overexpressed in tumor cells compared to normal cells.[103] Trials are currently in progress to investigate capecitabine in combination with other drugs.

PRECLINICAL STUDIES

Staurosporine Deratives and Nucleoside Analogues

Staurosporine potentiated cytotoxic effects of deoxynucleosides.[104,105] However, staurosporine is too toxic to be used in the clinic. Its analogue UCN-01 is being investigated clinically. The combination with the DNA damaging agent 5-FU was enhanced through down-regulation of thymidylate synthase messenger RNA, which is involved in DNA synthesis.[106] Since 5-FU resistance is associated with elevated TS expression levels, the proposed mechanism is that UCN-01 inhibits phosphorylation of Rb, leading to a decrease in free E2F protein. Downregulation of free E2F levels will prevent transcriptional activation of S-phase proteins and thus synthesis of TS[107] resulting in increased sensitivity to 5-FU. Sequential treatment appears to have more effect on TS activity than simultaneous exposure, but data are controversial. While Abe et al. suggest that pretreatment with UCN-01 is more beneficial for the decrease in TS activity, Hsueh et al. show that 5-FU exposure prior to UCN-01 has the largest inhibiting effect on TS inhibition.

Our results also show that, based on induction of apoptosis, 5-FU addition prior to the PKC inhibitors seemed preferable.[108]

Other synergistic combinations with UCN-01 have also been observed, such as coadministration with cisplatin, gemcitabine, ara-C, fludarabine, or 5-FU. Combinations with antiproliferative agents such as topisomerase II inhibitors resulted in additive toxicity.[109–111] Cells with non-functional p53 were more susceptible to the supraadditive effects of certain DNA damaging agents compared to cells with functional p53 as proposed by Wang et al.[112] which was also observed by us (data not shown). UCN-01 inhibits chk1, which seems to be the mechanism of synergy (Figure 7). UCN-01 inhibits cdk2, leading to a G1 arrest. When cells are arrested in the S phase, inhibition of chk1 abrogates the G2/M checkpoint, allowing cells with damaged DNA to continue through the cell cycle, inducing apoptosis. Shi et al. also showed that S-phase arrested cells are sensitized by UCN-01.[113] However, they imply that apoptosis is induced in S-phase arrested cells without cell cycle progression, suggesting direct activation signaling for cell death or inhibition of survival pathways in cells with S phase DNA content.

4′-N-Benzoyl-staurosporine (PKC412, CGP 41251) is a staurosporine analogue that can be orally administered and is more selective for protein kinase C but with lower potency than staurosporine itself. PKC412 is

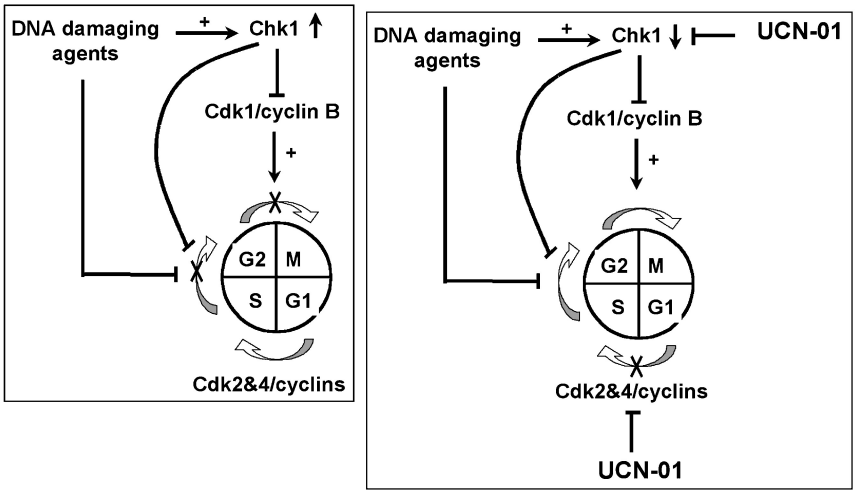

FIGURE 7 A) Proposed mechanism of action combining checkpoint abrogators with DNA damaging agents such as purine and pyrimidine analogues; A: DNA incorporation of drug metabolites induces an S-phase arrest. Activation of Chk-1 deactivates the cdk1-cylin B complex, inhibiting G2/M transition. B) When DNA damage is combined with checkpoint abrogators such as UCN-01, cells are also arrested in the G1 phase since UCN-01 is able to inhibit cdk2. Because UCN-01 deactivates chk1, this represses the stop in both S/G2 and G2/M phase transition induced by the DNA damaging agents, leading to progression in the cell cycle with damaged DNA inducing apoptosis.

capable of cell cycle inhibition and is associated with anti-angiogenic effects. PKC412 inhibits the phosphatidylinositol 3′-kinase (PI3K)/Akt survival pathway,[114] it appears to interrupt the MAPK pathway[115] and inhibits ligand-induced autophosphorylation of the vascular endothelial growth factor (VEGF) receptor tyrosine kinases involved in angiogenesis.[116] In various human tumor cell lines and xenografts it showed antiproliferative effects[117,118] and potentiates antitumor activity of other cytostatica such as paclitaxel, docetaxel, and 5-FU.[119] PKC412 also has radiosensitizing properties.[120]

Flavopiridol + Nucleoside Analogues

Flavopiridol was the first "pure" cdk inhibitor (cdk-1, -2, -4, -6, -7) to reach a clinical trial and displays a sequence dependent cytotoxic synergy with chemotherapeutical agents.[121] For DNA damaging agents such as 5-FU, gemcitabine, or Ara-C, the best schedule for synergistic interactions is their administration prior to exposure of flavopiridol. The observed sensitization of S-phase arrested cells to cytotoxic effects of flavopirodol remains to be elucidated. Two mechanisms of action were speculated. The flavopiridol-associated cdk inhibition can lead to reduced phosphorylation status of Rb that can result in decreased transcriptional activation of S phase proteins by E2F-1. S-phase proteins include ribonucleotide reductase, including the M2 subunit of RR that is also a target of gemcitabine. Inhibition of RR could lead to enhanced sensitivity to gemcitabine. In addition, Jung et al. showed that flavopirodol increased sensitization to gemcitabine in gastrointestinal cancer cell lines that correlated with downregulation of RR M2 subunit that appears to be related to a decrease in E2F-1 expression.[122] Another proposed explanation of flavopirodol sensitization of S-phase arrested cells is the inappropriately persistent E2F-1 activity as a consequence of cdk inhibition by flavopiridol, which may lead to induction of apoptosis. The levels of E2F-1 change during the S phase of the cell cycle; from low (entering), to high (during), to low when leaving the S phase. For appropriate processing through the cell cycle, timed deactivation of E2F-1 is necessary that is partly regulated by cdk2-cyclin A complex.[123–126] When flavopiridol inhibits cdk2, deactivation of E2F-1 fails, leading to persistent E2F-1 activity that may be high enough to surpass the threshold required to induce apoptosis. Earlier results show that transformed cells are more sensitive to cdk2-cyclin A antagonists compared with normal cells, presumably because the E2F-1 levels are elevated.[127] In contrast to the proposed schedule of DNA damaging agents prior to flavopiridol, Karp et al.[128] investigated a timed sequential therapy with flavopiridol in vitro using bone marrow cells from relapse and refractory acute leukaemia patients. They showed that pretreatment with flavopiridol primes both AML and ALL cells

for enhanced sensitivity to Ara-C. The opposite sequence of administration was not investigated.

CLINICAL STUDIES

In various clinical trials the combinations with flavopiridol and UCN-01 have been investigated.[129] Several of the initial clinical studies of UCN-01 were hampered by the fact that it binds avidly to alpha 1 acid glycoprotein in the plasma.[130] This problem is specific for clinical studies since it was not observed in animal models.[131] The problem was partly solved by adjusting the administration protocol in various clinical studies. Another approach involves the development of analogues with less affinity for this plasma protein. One improved candidate could be Gö6976,[132] but other new compounds are also being investigated.

At MD Anderson, a clinical study[133] was initiated with AML and myelodysplastic syndrome (MDS) in which Ara-C was given at 1 and 1.5 g/m^2/day on days 1–4 as a continuous infusion followed by UCN-01 at 45 mg/m^2/day at days 2–4. From 10 initial patients, one complete remission and two partial responders were observed. No further update on clinical data and cellular pharmacology are available as yet. A phase I and pharmacokinetic study of UCN-01 and fludarabine in relapsed or refractory low-grade lymphoid malignancies is currently recruiting patients.[134] Treatment will consist of up to six courses, or cycles, of UCN-01 and fludarabine given in combination. UCN-01 will be given alone to determine the safety profile for that patient. Thereafter, UCN-01 and fludarabine will be given together. UCN-01 will be infused over a period of 36 h (except for the first course, which will take 72 h), and fludarabine will be infused over 30 min. Fludarabine will be given from one to five days, starting the same day the UCN-01 infusion begins. A Phase I trial of UCN-01 in combination with 5-FU was completed in patients with advanced solid tumors.[135] 5-FU was administered as a weekly 24-h infusion (2600 mg/m^2) while UCN-01 was administered once every 4 weeks immediately after 5-FU at a dose of 135 mg/m^2 over 72 h in cycle 1 and 67.5 mg/m^2 over 36 h in subsequent cycles with relatively safe toxicity profiles that warrants further study in phase II trials. Of the 32 patients that were accessable for response, 8 patients (6 colon, one small bowel, one unknown primary) developed a stable disease. However, 7 patients had previously received 5-FU as a standard regimen. Considering these results in pretreated patients, these data suggest that cell cycle modulation is a worthwhile approach to enhance the efficacy of 5-FU.

Two Phase I trials with the staurosporine derative PKC412 have been performed: one in combination with protracted continuous infusion of 5-FU in patients with advanced solid malignancies[136] and one combined with gemcitabine and cisplatin in NSCLC patients.[137] In patients coadministered

with 5FU, the recommended Phase II dose of PKC412 was 150 mg/day when combined with a continuous infusion of 200 mg/m^2/day 5-FU (21 days every 4 weeks) with a dose limiting toxicity of grade 2. The regimen showed indications of activity. In advanced NSCLC patients, the estimated dose of 50 mg/day (4-week cycle) had a relatively safe toxicity profile that can be safely added to cisplatin (100 mg/m^2 on day 2) and gemcitabine (1000 mg/m^2 on days 1, 8, and 15) treated patients. In a few patients, a partial response was observed. Phase II studies have not yet been reported.

According to the National Cancer Institute (NCI) clinical trial database (http://www.cancer.gov/search/clinicaltrials/), several clinical Phase I and II studies have been initiated, but for most of these studies no final or intermediate reports have been published. In a clinical Phase I trial, flavopiridol is combined with gemcitabine in patients of solid tumors.[138] Flavopiridol is given 1–7 h on day 1 (course 0), with gemcitabine one week later over 60–150 min on days 1 and 8 and flavopiridol again over 1–7 h on days 2 and 9. No clinical data update is available as yet. A Phase I study of flavopiridol, gemcitabine, and the topoisomerase-I inhibitor irinotecan is also ongoing in patients with unresectable or metastatic solid tumors.[139] Patients receive gemcitabine over 30 min followed by irinotecan over 30 min on days 1 and 15. On days 2 and 16, patients will also be given flavopiridol over 60 min. This trial is a dose-escalation study of flavopiridol.

Furthermore, a Phase I/IIa study of seliciclib (CYC202 or *R*-roscovitine) in combination with gemcitabine/cisplatin in patients with advanced NSCLC was performed.[140] Since *R*-roscovitine showed antitumor activity in in vivo NSCLC xenografts and showed synergic effects when given in combination with gemcitabine, a Phase I trial was set up. The first-line treatment gemcitabine/cisplatin did not appear to affect the pharmacokinetics of *R*-roscovitine. Fourteen out of 27 patients were evaluable for response; 6 patients showed a partial response, 7 of them were reported to have a stable disease, and 1 patient developed a progressive disease, resulting in a response rate of 42.9%. The maximal tolerated dose was determined as *R*-Roscovitine 800 mg b.i.d. and gemcitabine 1000 mg/m^2/cisplatin 75 mg/m^2. These promising results will be the basis for further clinical development.

After promising Phase I and II studies[141,142] with the antisense drug Aprinocarsen (LY900003, Affinitak, ISIS 3521), which is a 20-length phosphorothioate oligodeoxynucleotide specific for PKC-alpha, the combination with cisplatin and gemcitabine was investigated in advanced NSCLC patients. This Phase I/II study[143] was extended to a randomized Phase III trial of gemcitabine/cisplatin and aprinocarsen in advanced NSCLC patients. However, this study showed no significant differences in response rates and did not improve survival.[144] It has not yet been elucidated whether this is due to a direct (negative?) interaction of Aprinocarsen with activation of

gemcitabine, or activation of DNA repair enzymes, which would abrogate the action of cisplatin.

Caution should be taken when combining protein or tyrosine kinase inhibitors with drug combinations, since each separate drug might interact different from the combination.

CONCLUSIONS AND FUTURE DIRECTIONS

The use of nucleoside analogues in combination with cell cycle inhibiting agents should be investigated further in detail. Targeting different and similar cell cycle phases and abrogration of cell cycle checkpoints are both attractive strategies. The sequential combination of DNA damaging agents prior to checkpoint abrogators such as UCN-01 showed promising results in vitro. However, it is not clear whether the effects of UCN-01 on the cell cycle are more important than other pleiotropic effects such as PKC inhibition. Since UCN-01 sensitizes p53 defective cells, this combination could work in the clinic, especially since the majority of the tumors show defects in the G/S checkpoint (p53/Rb). However, new UCN-01 analogues should be developed since it binds avidly to alpha 1 acid glycoprotein in the plasma. To determine whether the cell cycle effects or the PKC inhibition is of importance in combination treatment, the use of oligonucleotides specifically directed to targets can be included in this investigation. For the development of the substituted purine analogues, analysis of potential metabolism should be included in preclinical development.[145]

In addition, it should be determined whether combinations with targeted agents should focus on different targets in the same signaling pathway or in different pathways since pathways are connected. Another strategy is to develop or select agents that have multiple targets that act on different or similar signaling pathways. In addition, for each tumor type, there should be different approaches of antitumor treatment since different tumor types respond better to specific kinds of chemotherapies and have altered signaling pathways. To enhance the clinical response, patients should also be selected based on tumor specific properties. The expression of proteins that are involved in the drug metabolism should be measured in the tumor since mutations or gene methylation can decrease or elevate its expression. For drug activating enzymes, the expression levels should be high enough to establish a good profile for antitumor response.

Mechanistic studies of chemotherapeutic agents will help to develop new effective combination chemotherapies for treatment of cancer.

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ABBREVIATIONS

ADA	Adenosine deaminase
AML	Acute myeloid leukemia
APC	Anaphase promoting complex
ATM	Ataxia telangiectasia
ATR	ATM-Rad3-related
Cak	Cyclin activating kinase
Capecitabine	Xeloda
cdk	Cyclin-dependent kinase
CDKI	cdk inhibitor
Cladribine	2-Chlorodeoxyadenosine, CdA, Leustatin®
Clofarabine	2-Chloro-2′-arabino-fluoro-2′-deoxyadenosine, CAFdA, Clofarex™
CLL	Chronic lymphocytic leukaemia
CNDAC	1-(2-*C*-Cyano-2-deoxy-1-β-D-*arabino*-pentofuranosyl)cytosine
CNT	Concentrative nucleoside transporter
Cytarabine	Cytosine arabinoside, arabinosylcytosine, Ara-C, Cytosar®-U, Tarabine® PFS
dCK	Deoxycytidine kinase
dFdC	2′,2′-Difluorodeoxycytidine, gemcitabine, Gemzar
DMDC	1-(2-Deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine
dNTP	Deoxynucleoside triphosphate
DSB	Double-strand break

dTMP	2′-Deoxythymidine-5′-monophosphate
dTTP	2′-Deoxythymidine-5′-triphosphate
EcyD	1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)cytosine, TAS-106
ENT	Equilibrative nucleoside transporter
FdUMP	5-Fluoro-2′deoxyuridine-5′monophosphate
Fludarabine	Fluoroadenine-β-D-arabinoside, Fludara®
FMdC	(E)-2′-deoxy-2′-(fluoromethylene)cytidine, tezacitabine
5-FU	5-Fluorouracil
HDAC	Histone deacetylase
LV	Leucovorin
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
NSCLC	Non-small-cell lung cancer
OLO	Olomoucine
PKC	Protein kinase C
PKC412	4′-N-Benzoyl-staurosporine, CGP 41251
PTK	Protein tyrosine kinase
Rb	Retinoblastoma
ROSC	(R)-Roscovitine, CYC202
RPTK	Receptor protein-tyrosine kinase
RR	Ribonucleotide reductase
SAHA	Suberoylanilide hydroxamic acid
TFT	Trifluorothymidine
TP	Thymidine phosphorylase
Troxacitabine	BCH-4556, Troxatyl, L-OddC
TS	Thymidylate synthase
TSA	Trichostatin A
UCN-01	7-Hydroxystaurosporine
UFT	Uracil/tegafur
VEGF	Vascular endothelial growth factor