Minireview

Cell Cycle-mediated Drug Resistance: An Emerging Concept in Cancer Therapy[1]

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Abstract

The concept of combining chemotherapeutic agents to increase cytotoxic efficacy has evolved greatly over the past several years. The rationale for combination chemotherapy has centered, in the past, on attacking different biochemical targets, overcoming drug resistance in heterogeneous tumors, and by taking advantage of tumor growth kinetics with increasing the dose-density of combination chemotherapy. The overall goal was to improve clinical efficacy with acceptable clinical toxicity. With our increased understanding of the cell cycle and the impact chemotherapeutic agents have on the cell cycle, it is increasingly apparent that this physiology can create drug resistance, thereby reducing combination chemotherapeutic efficacy. This is particularly relevant with the advent of cell cycle-specific inhibitors but also has relevance for the action of standard chemotherapeutic agents currently in clinical practice. This cell cycle-mediated resistance may be overcome by a greater understanding of chemotherapeutic cell cycle effects and by appropriate sequencing and scheduling of agents in combination chemotherapy. In this review, we have elected to illustrate the evolving concept of cell cycle-mediated drug resistance with novel drug combinations that include the taxanes, camptothecins, and fluorouracil. This review indicates that as our understanding of the cell cycle grows, our ability to appropriately sequence chemotherapy to overcome cell cycle-mediated drug resistance can have a great impact on our therapeutic approach in the treatment of human cancers.

Introduction

Previous models for the use of combination chemotherapy have been based upon the concepts of fractional or log-kill, as proposed by Skipper *et al.* (1, 2), heterogeneous drug-resistant tumor clones, as proposed and refined by Goldie and Coldman (3), and drug synergy (4–6). The underlying rationale is the realization that, except for a few select cancers (*i.e.,* Burkitt's lymphoma and choriocarcinoma), individual chemotherapeutic agents for the majority of tumors have not increased cure rates in the treatment of cancer. The delivery of combination chemotherapy has been further refined by the application of Gompertzian mathematical modeling to chemotherapy, resulting in the concept of dose density, as proposed by Norton and Simon (6, 7).

However, with advancements in our understanding of the basic mechanisms of oncogenesis, cell cycle physiology, and apoptosis, we now have a better understanding of the effects of chemotherapy on normal and cancerous cells. With this knowledge, it is becoming increasingly apparent that the cell cycle plays a critical role in chemosensitivity for combination chemotherapy. This is particularly critical for newer chemotherapeutic agents that have targeted cell cycle effects. In this review, we introduce the emerging concept of cell cycle-mediated drug resistance and how this results in a further refinement of the administration of combination chemotherapy. Specifically, we describe the cell cycle and the various mechanisms by which chemotherapeutic agents impact on this cycle, thereby leading to drug resistance when used in combination. The concept of cell cycle mediated drug resistance is then illustrated with combinations of both standard and novel cell cycle-modulating chemotherapeutic agents. We also highlight the importance of sequence of administration of combination chemotherapy as a mechanism to overcome cell cycle-mediated resistance.

Cancer Resistance

The concept of cancer resistance is in part based on the work of Luria and Delbruck (8), who found that bacteria spontaneously developed mutations that made them resistant to bacteriophages (5, 8). When applying this concept to cancer, Goldie and Coldman (3, 9) proposed that the probability that a given tumor will contain resistant clones at the time of diagnosis would be a function of the mutation rate of that cancer and the size of the tumor at diagnosis. Even with low mutation rates of 1 in 10^6 mitoses, it would be virtually certain that drug-resistant mutants would populate the cells of a clinically detectable 1-cm tumor deposit (10^9 tumor cells; Refs. 3, 5, 9). Therefore, even at small tumor burdens, drug resistance would be a problem.

Biochemical synergy is a historical rationale for combination chemotherapy where, by choosing chemotherapeutic agents with different mechanisms of action, multiple sites in biosynthetic pathways can be attacked and/or several processes involved in the maintenance and function of essential macromolecules may be inhibited (4). Multidrug resistance is an important example of using drugs in combination to attack separate intracellular targets to have a greater than additive, or synergistic, antitumor effect. Multidrug-resistant cells avoid drug cytotoxicity by maintaining the intracellular drug concentration at an extremely low level, associated with an increase in the transporter protein P-glycoprotein (10). Verapamil is a calcium channel antagonist that reverses the multidrug-resistant phenotype *in*

Received 10/22/00; revised 4/5/01; accepted 4/15/01.

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[1] Supported by National Cancer Institute R01 CA67819.

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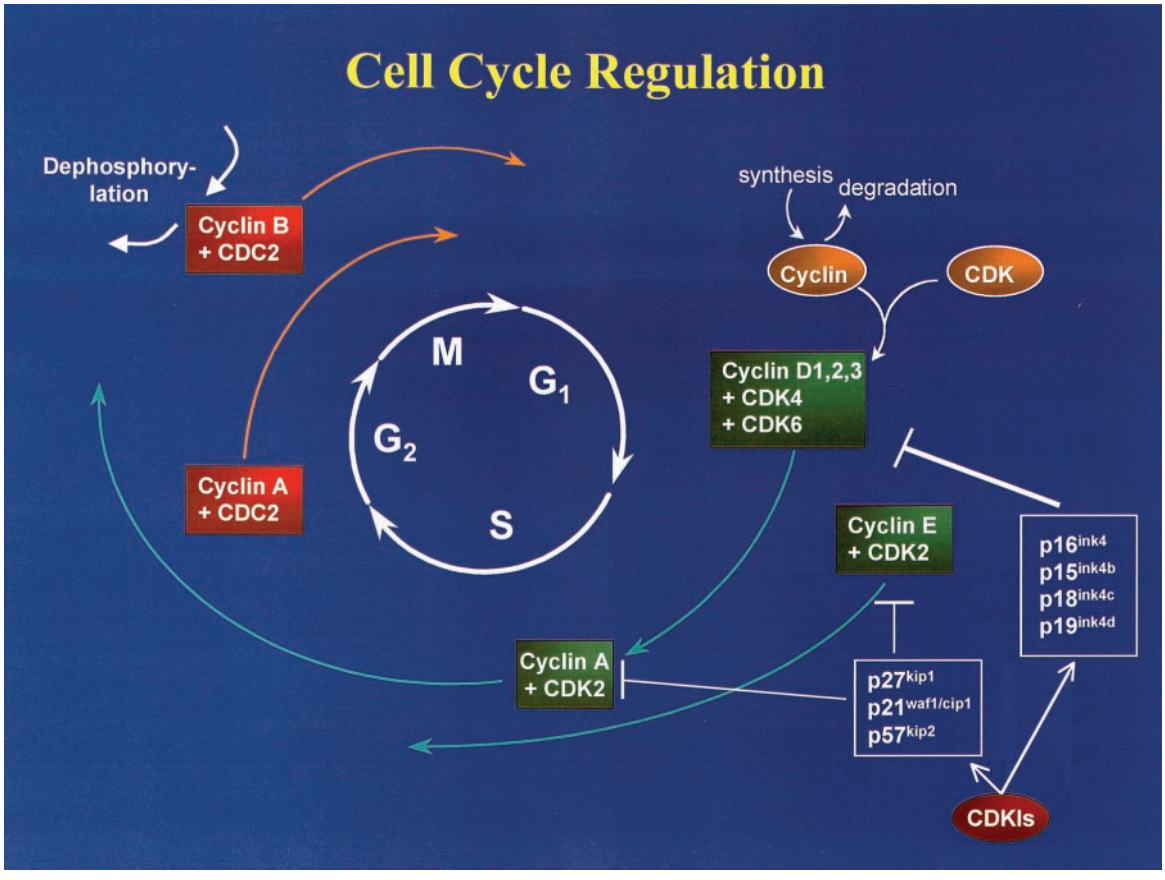

Fig. 1 The cell cycle and its regulation by cyclins, CDKs, and CDKIs. The cell cycle is divided into four distinct phases (G_1, S, G_2, and M). The progression of a cell through the cell cycle is promoted by CDKs, which are positively and negatively regulated by cyclins and CDKIs, respectively. As shown, cyclin D isoforms (cyclin D1–D3) interact with CDK4 and CDK6 to drive the progression of a cell through G_1. The association of cyclin E with CDK2 is active at the G_1-S transition and directs entry into S-phase. S-phase progression is directed by the cyclin A/CDK2 complex, and the complex of cyclin A with Cdc2 (also known as cdk1) is important in G_2. Cdc2/cyclin B is necessary for the entry into mitosis. The INK4 (for inhibitor of cdk4) class of CDKIs, notably $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$, bind and inhibit cyclin D-associated kinases (CDK4 and CDK6). The kinase inhibitor protein group of CDK inhibitors, $p21^{waf1}$, $p27^{kip1}$, and $p57^{kip2}$, negatively regulate cyclin E/CDK2 and cyclin A/CDK2 complexes.

vitro by interacting with P-glycoprotein and increasing intracellular chemotherapeutic drug levels, thereby increasing their effectiveness at their own intracellular targets (11, 12). Despite the notable preclinical data, however, the translation of multidrug-resistant inhibitors to clinical use has been unimpressive.

The Cell Cycle and Apoptosis

The fundamental processes of progression through the cell cycle and of programmed cell death involve the complex interaction of several families of proteins in a systematic and coordinated manner. They are separate, distinct processes that are intimately related and together play an important role in the sensitivity of malignant cells to chemotherapy.

The cell cycle is the mechanism by which cells divide. It is driven by a family of proteins called CDKs.[3] These kinases are positively regulated by cyclins (A, B, D, and E) and are negatively regulated by CDKIs (Fig. 1; Ref. 13). The pattern of cyclin expression varies with the progression of a cell through the cell cycle, and the specific cyclin expression patterns define the relative position of a cell in the cell cycle (14, 15). At least nine structurally related CDKs (CDK1–CDK9) have been identified, although not all have clearly defined cell cycle regulatory roles. A considerable number of cyclins have been identified to date (cyclin A–cyclin T). CDK/cyclin complexes themselves become activated by phosphorylation at specific sites on the CDK by CDK7/cyclin H, also referred to as the CDK-activating kinase (16). The retinoblastoma tumor suppressor gene product (pRb) closely regulates the G_1-S transition (Fig. 2).

Apoptosis is an active, energy-dependent process in which

[3] The abbreviations used are: CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; Apaf, apoptotic protease activating factor; PARP, poly-adenosine 5′-diphosphate-ribosyl polymerase; PKC, protein kinase C; QFM, quantitative fluorescent microscopy; TS, thymidylate synthase; DHFR, dihydrofolate reductase.

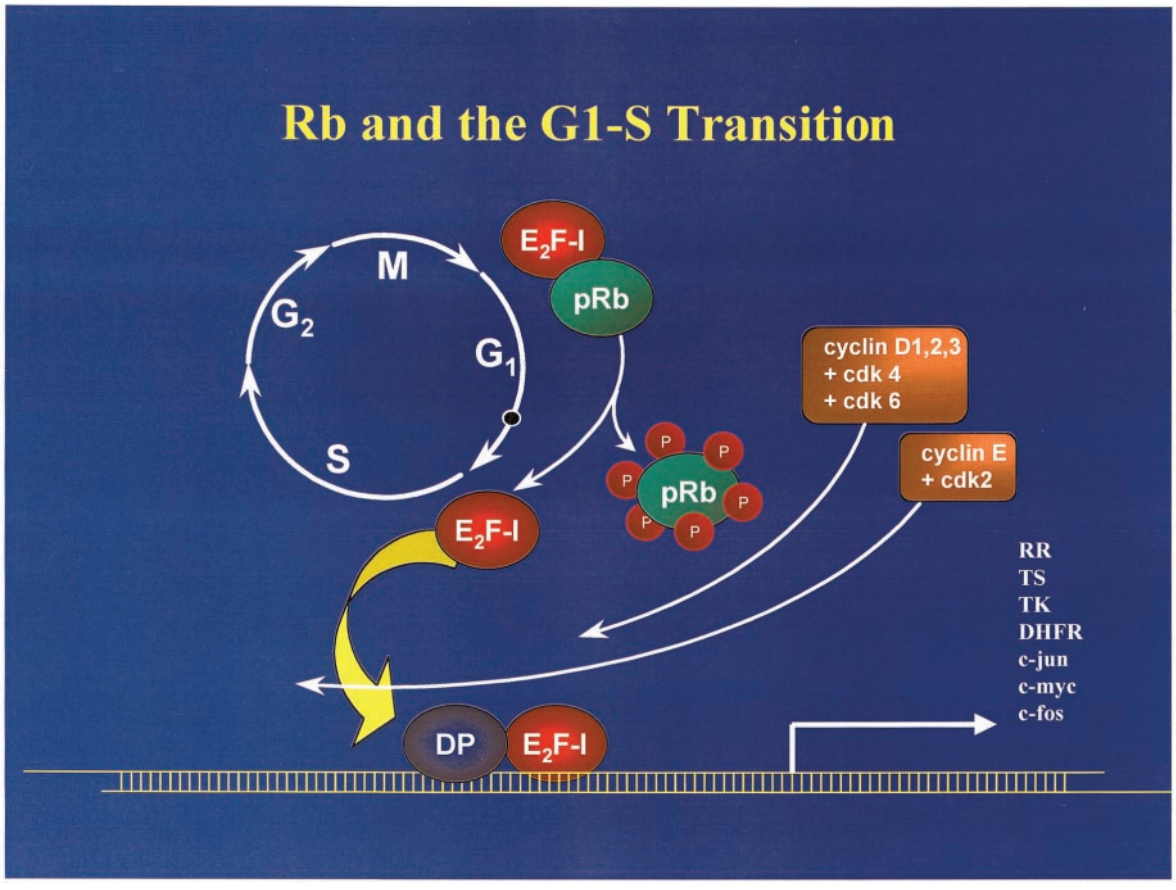

Fig. 2 Retinoblastoma gene product (Rb) and the G_1-S transition. In its active state, Rb is hypophosphorylated and forms a complex with a group of transcription factors known as E2F (E2F-1, E2F-2, and E2F-3). When pRb is inactivated by CDK2-, CDK4-, or CDK6-mediated phosphorylation, E2F transcription factors are released, resulting in progression into S-phase and transcription of a wide range of targets involved in chemotherapy sensitivity including ribonucleotide reductase (*RR*), thymidylate synthase (*TS*), thymidine kinase (*TK*), dihydrofolate reductase (*DHFR*), c-jun, c-myc, and c-fos.

the cell participates in its own destruction. The molecular cascade of apoptosis is characterized by the early release of mitochondrial cytochrome *c*, activation of Apaf-1, activation of caspase 9, and subsequent cleavage of downstream, or "effector" caspases in a self-amplifying cascade (Fig. 3). The caspases are cysteine proteases that represent the "executioners" of the apoptotic cascade. These effector caspases ultimately degrade a number of cellular proteins, such as PARP, laminin, and β-actin (17–21), which are hallmarks of programmed cell death.

The failure of many of our chemotherapeutic agents reflects, on a cellular level, an inability of these drugs to induce apoptosis (22–28). Neoplastic cells have acquired a number of cellular adaptations and mutations, which act as survival factors and thus act to prevent apoptosis. The tumor suppressor gene *p53* has a role in the regulation of the cell cycle, as well as in the initiation of apoptosis. Tumor cells with a mutation in the *p53* gene have shown resistance to undergo apoptosis in the presence of chemotherapy (29–34). Bcl-2 is an antiapoptotic signal (35), and overexpression of Bcl-2 correlates with resistance to cisplatin and paclitaxel (36). The Bcl-2 fusion protein, produced by chromosomal translocation t(14;18), is a feature of many non-Hodgkin's lymphomas (37) and confers a poor prognosis in a number of hematological malignancies. Indeed, chemotherapy itself may induce cellular survival signals. 1-β-D-Arabinofuranosylcytosine, for example, can activate PKC via diglyceride production, resulting in activation of mitogen-activated protein kinase, and an increase in transcription factor nuclear factor-κB activity, resulting in resistance to apoptosis induction (38–40).

The cell cycle and apoptosis are intimately related, as evidenced by the central role of p53, both in cell cycle arrest and in the induction of apoptosis. Another example of this intimate relation was demonstrated in human colon cancer cell lines that differ only in their p21 checkpoint status. Cells with wild-type p21, when irradiated with γ-radiation, underwent a cell cycle growth arrest followed by clonogenic survival, where as cells lacking p21, when irradiated with γ-radiation, did not undergo a cell cycle growth arrest and furthermore proceeded to apoptosis (41). Cells that undergo a growth arrest may be protected from apoptosis and may therefore be ultimately resistant to the cytotoxic agent.

Cell Cycle-mediated Resistance

Cell cycle-mediated drug resistance is best described as a relative insensitivity to a chemotherapeutic agent because of the position of the cells in the cell cycle. This is most prevalent in

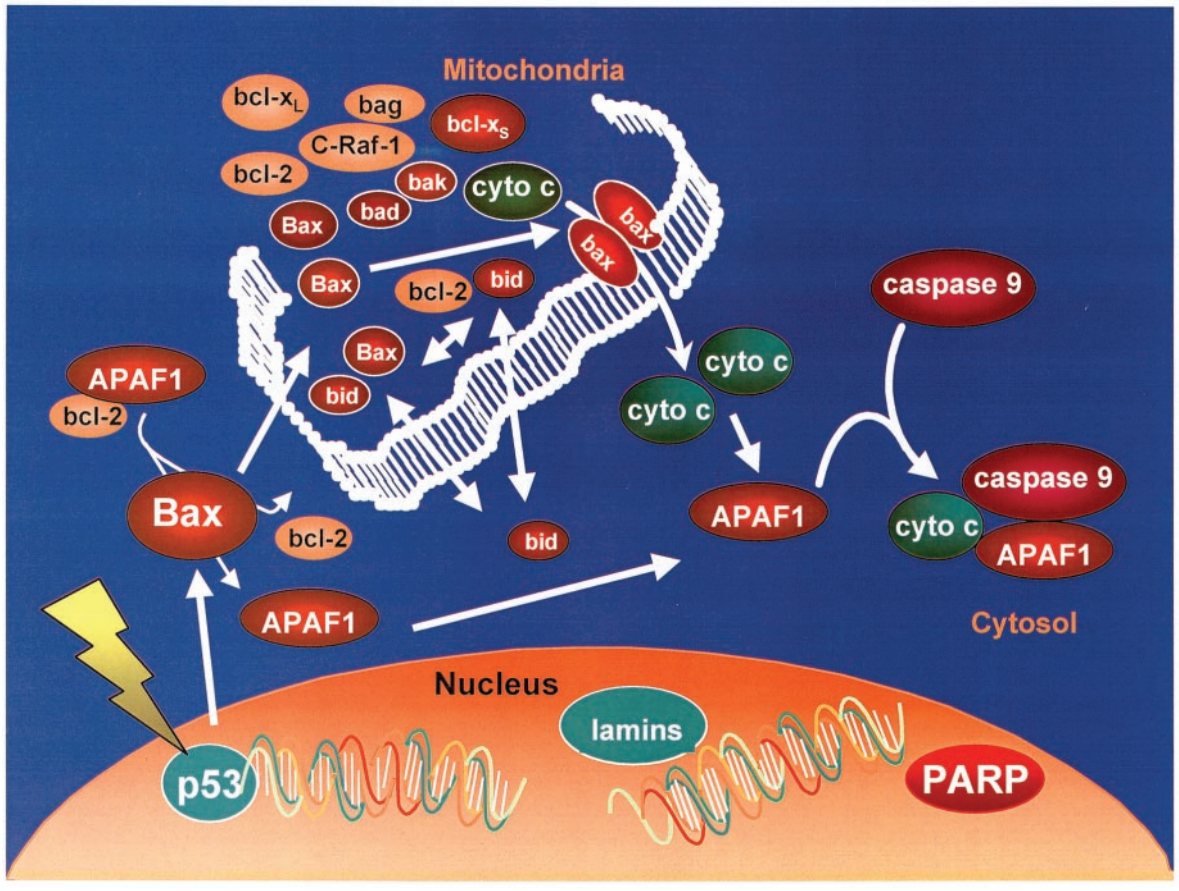

Fig. 3 Apoptosis. The biochemical cascade of apoptosis is subject to regulation at several levels. Members of the Bcl-2 family of proteins may be either antiapoptotic in nature (Bcl-2, Bcl-x_L, and Mcl-1) or proapoptotic, acting to enhance apoptosis (BAD, Bax, Bak, and others). Bcl-2 and Bcl-x_L bind and inhibit Apaf-1 and consequently prevent the activation of caspases. In the presence of excess Bax, however, Bcl-2 is displaced from Apaf-1, allowing caspase cleavage and activation. Bax further promotes apoptosis by mediating the release of cytochrome *c* from mitochondria.

combination chemotherapy, where one chemotherapeutic agent can impact the cell cycle such that the next chemotherapeutic agent given immediately in sequence becomes less effective. The best example demonstrating the relevance of the cell cycle in combination chemotherapy includes those combinations that involve taxanes, the prototypical class of cell cycle-specific chemotherapeutic agents. Using this as a model, we will review the impact of cell cycle-mediated resistance in combination chemotherapy, in particular as it relates to novel chemotherapeutic agents that target the cell cycle. We will also expand the discussion to include other chemotherapeutic combinations that involve camptothecins and fluorouracil.

The field is somewhat hampered by the fact that there is no single established methodology to examine synergy (*i.e.*, a greater than additive effect) or antagonism (*i.e.*, a less than additive effect). Several methodologies have been used including: isobologram method (42), isobologram method of Steel and Pecham (43), fractional product method of Webb (44), combination index method of Chou and Talalay (45), or more less mathematical methods like surviving fraction (46), percentage response to granulocyte/macrophage-colony forming unit compared with controls (47), or others (48, 49). For induction of apoptosis with drug combinations, there has been less reliance on these classical methods for testing for synergy and antagonism. Instead, investigators have used a series of techniques including QFM to detect morphological features of apoptosis, DNA "laddering," PARP cleavage, cytochrome *c* release, and activation of caspases. Of note, these varying preclinical and laboratory methods of determining the efficacy of combination chemotherapy may have disparate results, even within the same system. With these caveats in mind, we will summarize the existing data, from preclinical to clinical models, in support of the emerging concept of cell cycle-mediated drug resistance.

Taxanes

Cell Cycle Effects. The taxanes act by stabilizing microtubules, thereby causing a G_2-M arrest followed by apoptosis. Unlike other known mitotic spindle inhibitors (*Vinca* alkaloids, colchicine, and podophyllotoxin) that inhibit tubulin polymerization, taxanes markedly enhance microtubule assembly and disrupt the transition of a cell through mitosis. The two primary drugs in clinical use today include paclitaxel (Taxol) and docetaxel (Taxotere); however, significantly more preclinical and clinical information has been presented with paclitaxel.

Paclitaxel is an anticancer agent with a broad spectrum of

Table 1 Cell cycle events with chemotherapeutic agents

	Cell cycle events	Etiology
Taxanes	G_2/M arrest	Promotes microtubule assembly and stabilizes tubulin polymer formation (51)
		↓ destruction of cyclin B1 (59)
	G_1 arrest	↓ CDK4 (54)
	Apoptosis	Bcl-2 hyperphosphorylation (62, 63)
		c-Raf-1 phosphorylation (62, 63)
Platinum	Sensitive in G_1 and early S	(61)
	↑ duration of S phase and arrests cells in G_2	(86)
	Apoptosis	Unscheduled activation of cdc2 kinase in cisplatin-resistant cells (97)
Camptothecin	S-phase active	Topoisomerase I inhibitor (117, 118)
	G_1 and G_2 arrest	(122, 123)
	↑ bax from prolonged exposure	(124)
	Unscheduled cyclin B1/cdc2 activity	(125)
Fluorouracil	S-phase active	↓ TS activity (139)
		RNA incorporation (138)
	↑ p53 and ↑ p21	(141)
	Cells accumulate in early S-phase	↑ cyclin A, ↑ cyclin A/CDK2 activity (142)

activity, currently being used in patients with ovarian, breast, lung, head and neck, bladder, and esophageal cancers (50). Paclitaxel promotes microtubule assembly and stabilizes tubulin polymer formation (51), thereby interrupting the dynamic cellular reorganization necessary for mitosis (52), and resulting in a G_2-M arrest (53; Table 1). Paclitaxel is also associated with down-regulation of CDK4 (54) with concomitant G_1-S arrest. The primary effect of paclitaxel is to interfere with the assembly of the mitotic spindle, resulting in the failure of chromosomes to segregate (55). As a microtubule promoter, paclitaxel shifts the equilibrium in favor of the microtubule and thus decreases the concentration of tubulin necessary for subsequent assembly (56). Mitosis is initiated by the activation of the cyclin B1-CDK1 complex (also called cyclin B1-Cdc2 kinase), and as mitosis progresses, cyclin B is destroyed by ubiquitin-mediated proteolysis. Cyclin B and Cdc2 kinase activity are closely related to paclitaxel function. Expression of cyclin B and the activation of CDK1 occur coincidentally with paclitaxel-induced apoptosis (57, 58), and destruction of cyclin B1 can be inhibited by paclitaxel (59). Furthermore, a dominant-negative mutant of p34Cdc2 blocks paclitaxel-induced apoptosis (60).

Although cytotoxicity is maximal at G_2-M and minimal at G_1-S (61), paclitaxel may induce apoptosis by other mechanisms as well. In particular, paclitaxel exposure is also associated with hyperphosphorylation of bcl-2 and phosphorylation of c-Raf-1 (15, 62, 63), steps perhaps necessary for apoptosis. Loss of the Bcl-2 phosphorylation loop domain reduces the sensitivity of human leukemia cells (U937) to paclitaxel-mediated mitochondrial dysfunction and apoptosis (64). Overexpression of Bcl-x_L has been shown to inhibit apoptosis induced by ionizing radiation and by chemotherapeutic agents including paclitaxel (65, 66). However, the exact mechanism of programmed cell death induced by paclitaxel has been confounded by seemingly contradictory laboratory observations (67). These contradictions occur *in vitro* because of cell type specificity (68), as well as concentration and duration of exposure-related effects (67, 69). Nanomolar concentrations appear to be sufficient to polymerize tubulin, and micromolar concentrations have demonstrated tubulin-independent effects and may in fact be clinically irrelevant.

Paclitaxel and Novel Cell Cycle Modulators (Flavopiridol/Bryostatin-1). In view of the fact that paclitaxel is predominately an M-phase-specific drug, one would hypothesize that agents that arrest cells in G_1 before they enter M would antagonize paclitaxel effects. This issue is of clinical importance because cell cycle inhibitors are entering clinical trials in combination with chemotherapy, in particular with paclitaxel. Flavopiridol is a novel antineoplastic agent that originally was noted for its ability to inhibit the activity of a number of protein kinases. It is a synthetic flavone with a novel structure compared with that of polyhydroxylated flavones, including quercetin and genistein, and is identical to a compound obtained by derivation from a natural product obtained from *Dysoxylum benectariferum*, a plant indigenous to India (70). Flavopiridol is now best classified as a CDKI because of its considerable affinity for CDKs and its ability to induce cell cycle arrest in a number of cell lines (71–74). It has been shown to bind to and directly inhibit CDK1 (cyclin B1-Cdc2 kinase), CDK2, CDK4, and CDK6. Bryostatin-1 is an activator of PKC with a short duration of action by translocating it from the cell membrane, cytoskeleton, or nucleus. Over time, it leads to an overall decrease in activity thought to be secondary to down-regulation of PKC (75). Additionally, bryostatin-1 inhibits CDK2 kinase activity by inducing p21 (76) and down-regulating cyclin B1 (77).

The combination of each of these agents (flavopiridol or bryostatin-1) with paclitaxel demonstrates the concept of cell cycle-mediated drug resistance (Table 2). Flavopiridol was examined in combination with paclitaxel in various sequences in the MKN-74 human gastric cancer cell line as well as the MCF-7 human breast cancer cell line, which are both heterozygous for p53 (78, 79). Cell cycle-mediated resistance was demonstrated when flavopiridol exposure was followed by paclitaxel. The multiple cell cycle effects of flavopiridol [including the inhibition of CDK4, CDK6, and CDK2 at G_1 and the inhibition of cyclin B1-Cdc2 kinase activity at G_2 (78)], creates a cell cycle arrest. This prevents cells from entering M-phase,

Table 2 Cell cycle-mediated drug resistance in combination chemotherapy

Cell cycle-mediated drug resistance	Mechanism
Flavopiridol → Paclitaxel	Inhibition of CDK2, CDK4, and CDK6 at G_1 and inhibition of cyclin B1-Cdc2 kinase at G_2 (78) → cell cycle arrest
Bryostatin-1 → Paclitaxel	Suppression of cyclin B1 and decrease in cyclin B1-Cdc2 kinase activity (76) → cell cycle arrest in G_2
Cisplatin → Paclitaxel	G_2 cell cycle arrest by cisplatin (86, 92, 97) Cisplatin interference with tubulin-associated proteins (104)
Flavopiridol → Irinotecan	Inhibition of CDK2, CDK4, and CDK6 at G_1 and inhibition of cyclin B1-Cdc2 kinase and G_2 (78) → cell cycle arrest
Irinotecan + Fluorouracil	G_2 cell cycle arrest induced by irinotecan (133–135)
Fluorouracil → (no delay) → Irinotecan	G_1/early S accumulation induced by fluorouracil (142)
UCN-01 → Fluorouracil	G_1/S cell cycle arrest (162–164), associated with induction of p21 and dephosphorylation of CDK2 (165)

the phase during which paclitaxel is most active, and leads to a significant reduction in paclitaxel sensitivity in culture (78). Similarly, in a mouse mammary tumor xenograft system, treatment with bryostatin-1 followed by paclitaxel demonstrated bryostatin-1-mediated suppression of cyclin B1 and an associated decreased cyclin B1-Cdc2 kinase activity. This resulted in a significant reduction in paclitaxel sensitivity. In the mouse xenograft system, bryostatin-1 followed by paclitaxel was associated with a significant decrease in tumor doubling time as compared with paclitaxel alone (9.3 ± 1.9 days *versus* 22.7 ± 2.5 days, $P < 0.001$; Refs. 76, 80). Therefore, when either flavopiridol or bryostatin-1 is given first, as a consequence of cell cycle-mediated drug resistance, paclitaxel sensitivity is markedly reduced. In the case of flavopiridol, cells are arrested in the cell cycle and are insensitive to paclitaxel, which asserts its activity as cells enter M-phase. In the case of bryostatin-1, cyclin B1-Cdc2 kinase activity is reduced, resulting in cells arresting in G_2, because the cyclin B1-Cdc2 kinase is associated with the activity of the spindle assembly checkpoint (59), and is required to initiate entry into M-phase (14). Koutcher *et al.* (76) demonstrated the G_2 cell cycle arrest *in vitro*; treatment of human MKN-74 gastric cancer cells with bryostatin-1 followed by paclitaxel resulted in a decrease in cells entering M-phase (23% *versus* 56% with paclitaxel alone) and a concomitant increase in cells in G_2 (69% *versus* 21% with paclitaxel alone). As fewer cells enter M phase, the net effect is a significant decrease in paclitaxel sensitivity as a result of this cell cycle-mediated drug resistance.

Cell cycle-mediated drug resistance may be overcome by appropriate sequencing of the drug combination (Table 3). The reverse sequence of paclitaxel followed by flavopiridol is associated with an increased induction of apoptosis (78, 81), as evidenced by caspase-3 activation and PARP degradation (78). This sequence is associated with an accelerated exit of cells from mitosis; an event that may be critical for the sequence-dependent enhancement of paclitaxel-induced apoptosis by flavopiridol. In the case of paclitaxel followed by bryostatin-1, there is decreased tumor metabolism and blood flow (76), which may impact on tumor growth. The increased sensitivity to paclitaxel when followed by bryostatin-1 may be in part explained by Bcl-2:Bax, the heterodimeric pair that is closely associated with mitochondrial dysfunction and the initiation of apoptosis. Loss of the Bcl-2 phosphorylation loop domain (64) and ectopic expression of Bcl-x_L (82) can protect human leukemia cells (U937) from paclitaxel-mediated apoptosis. Administration of bryostatin-1 after paclitaxel can overcome paclitaxel resistance in U937 cells ectopically expressing Bcl-x_L (83) and is associated with an increase in the proapoptotic factor, Bax, with resultant increased sequence-dependent apoptosis (84).

The sequential combination of paclitaxel followed by flavopiridol has been evaluated in a Phase I study (NCI T96-0091; Ref. 85). The clinical results are remarkable for major responses in patients with chemotherapy refractory malignancies (*i.e.*, prostate and esophagus), including patients who have received prior paclitaxel therapy. In particular, five of seven patients with esophageal cancer responded to the combination treatment, and three of whom received prior paclitaxel therapy (85). In this clinical trial, there was no effect of flavopiridol on paclitaxel pharmacokinetics. We have also translated the preclinical studies demonstrating a sequence-dependent synergy between paclitaxel and bryostatin-1 to a Phase I clinical trial (NCI T97-0118). Patients were treated with a weekly dose of paclitaxel 80 mg/m^2, followed 24 h later with increasing doses bryostatin-1. Two partial responses were demonstrated, with 9 of 27 patients demonstrating stable disease, including a patient with metastatic pancreatic carcinoma whose disease remained radiographically stable for 15 months. Again, we found no pharmacokinetic effects on paclitaxel by bryostatin-1 (76, 80). Both studies are now in Phase II evaluations in patients with esophageal cancer: Phase II combination of paclitaxel followed by bryostatin-1 (NCI Protocol 250) for up-front treatment of esophagus cancer and followed by flavopiridol (NCI Protocol 1672) for paclitaxel-refractory patients with esophageal cancer.

Cisplatin (and Analogues)

Cell cycle-mediated resistance also plays an important role in combination therapies that do not include specific cell cycle modulators. Here, we describe the cell cycle effects of cisplatin and its role in cell cycle-mediated resistance in combination with paclitaxel.

Cell Cycle Effects. Cisplatin belongs to the alkylating agent group of chemotherapies. It binds to DNA bp, creating adducts, cross-links, and strand breaks that inhibit DNA replication. As such, cisplatin is not cell cycle specific, although cells appear to be maximally sensitive to cisplatin in G_1, just prior to

Table 3 Overcoming cell cycle-mediated drug resistance: Cell cycle effects

Overcoming resistance	Mechanism
Paclitaxel → Flavopiridol	Accelerated exit from mitosis (78)
Paclitaxel → Bryostatin-1	Overcomes paclitaxel resistance to ↑ Bcl-x_L (83) ↑ Bax (84)
Paclitaxel → Cisplatin	Increase intracellular uptake of cisplatin (106) Inhibition of repair of cisplatin-related DNA damage (98) Hasten mitotic exit (103) Cell synchronization (110)
Irinotecan → Flavopiridol	Decrease in p21 (133, 135, 137)
Irinotecan → Fluorouracil	Persistent inhibition of TS (123, 145, 147) S-phase delay (147)
Fluorouracil → (minimum 6-h delay) → Irinotecan	Synchronization of cells in S-phase (145)
Fluorouracil → UCN-01	Suppression of TS (169)

the onset of DNA synthesis, and minimally sensitive in peak DNA synthesis, with entry into S-phase resulting in a 2-fold decrease in sensitivity (Ref. 61; Table 1). Cells that remained blocked at the G_1-S-phase boundary during cisplatin treatment remain maximally sensitive after release (61). Detection of damaged DNA leads to the activation of CDKIs such as p21 and wee1/mik1, which subsequently arrest cells in either G_1 or G_2. Cisplatin exposure increases the duration of S-phase and blocks cells in G_2 in a dose-dependent manner (86). This arrest is accompanied by the accumulation of inactive, phosphorylated p34Cdc2 protein. After a protracted delay, the p34Cdc2 protein is dephosphorylated, and an aberrant mitosis occurs. In fact, a number of agents that abrogate the G_2 cell cycle checkpoint and induce premature mitosis have demonstrated enhancement of cisplatin-induced cytotoxicity (87–89). Resistance to cisplatin has been associated with increased glutathione levels, increased metallothioneins, decreased drug uptake, increased DNA repair, and the tolerance of the formation of platinum-DNA adducts (90).

Although the precise mechanism by which platinum-DNA damage results in cell death remains unknown, unrepairable DNA damage often results in activation of the apoptotic pathway (91, 92). p53 plays a significant role in DNA repair, proliferative arrest, and apoptosis (93) and has led to a correlation between p53 and cisplatin sensitivity (94–96). Apoptosis has been associated with an unscheduled activation of Cdc2 kinase in cisplatin-resistant cells and with the p53/p21Waf1 pathway in cisplatin-sensitive cells (97).

Taxanes and Cisplatin. Combination studies with paclitaxel and cisplatin have been pursued in the hopes of increased antitumor effects attributable to each individual drug's broad range of clinical activity and different mechanisms of action. However, this combination of standard chemotherapeutic agents doses demonstrates cell cycle-mediated drug resistance, particularly when cisplatin exposure precedes paclitaxel (Table 2). This sequence (cisplatin → paclitaxel) demonstrated antagonism in culture (92, 97–103), secondary to a G_2 arrest created by cisplatin treatment (86, 92, 97). Cisplatin may also interfere with tubulin or tubulin-associated proteins (104) limiting paclitaxel efficacy. Zaffaroni *et al.* (97) demonstrated the clearest example of cell cycle-mediated resistance, when cisplatin was followed by paclitaxel. With this sequence, they demonstrated an induction of p53 protein, an increase in the CDKI p21cip/waf1, and an increase in cyclin B1, with a concomitant transient G_2 arrest. The cell cycle arrest in G_2 attributable to cisplatin treatment caused a relative insensitivity to subsequent paclitaxel treatment. In both cell culture (92) and in an *in vivo* murine ovarian tumor model (103), the duration of time prior to administration of the second drug was important to antitumor efficacy. Judson *et al.* (92) demonstrated antagonism of paclitaxel effects when there was a 3-h time interval with either sequence of cisplatin → paclitaxel or reverse. Although cisplatin-resistant ovarian carcinoma cell lines retain sensitivity to paclitaxel, they found that concomitant exposure blocked paclitaxel-induced apoptosis but did not inhibit paclitaxel-induced stabilization of microtubules or Bcl-2 degradation (92). Prior treatment with cisplatin demonstrated a suppression of cells arrested in G_2-M, thereby creating a cell cycle-mediated drug resistance and a significantly decreased sensitivity to paclitaxel (103).

Again, the cell cycle-mediated drug resistance can be overcome by appropriate sequencing of this drug combination (Table 3). When paclitaxel precedes cisplatin, preclinical studies demonstrate synergistic antitumor efficacy in culture (92, 97–103, 105). These *in vitro* experiments were confirmed *in vivo* with the mouse mammary tumor preclinical experiments that demonstrated that the maximal antitumor effect occurred when paclitaxel preceded cisplatin (103). The reasons for the sequence-dependent synergy when paclitaxel precedes cisplatin may be multifactorial; paclitaxel given prior to cisplatin may increase intracellular uptake of cisplatin (106) and inhibit repair of cisplatin-induced DNA damage (98). Cisplatin may also hasten the exit from mitosis in paclitaxel-treated cells (103), thereby increasing cytotoxicity.

An *in vivo* M-109 murine lung carcinoma xenograft model further demonstrates the importance of sequence of administration of paclitaxel and cisplatin (107). In this model, i.p. therapy of cisplatin followed by paclitaxel resulted in toxic deaths of all mice treated, whereas the reverse sequence of paclitaxel followed by cisplatin demonstrated a significant prolongation of survival time compared with paclitaxel treatment alone (107). This significant differential toxicity is explained in part by a sequence-dependent pharmacokinetic drug interaction, such that if cisplatin is given prior to paclitaxel, there is a 33% reduction in paclitaxel clearance with a resultant increased myelotoxicity without improvement in antitumor efficacy (99, 108, 109). Because of this difference in paclitaxel clearance with associated

increased myelotoxicity, there have been no clinical studies comparing the sequences paclitaxel → cisplatin *versus* cisplatin → paclitaxel. However, one clinical study that increased the interval between paclitaxel and cisplatin to 12 h does demonstrate intriguing findings (110). In this study, patients with metastatic breast cancer who had failed prior chemotherapy received 135 mg/m^2 paclitaxel as a 3-h infusion followed by 75 mg/m^2 cisplatin as a 1-h infusion beginning 12 h later (6:00 a.m. paclitaxel → 6:00 p.m. cisplatin). The study was remarkable for 9 (22%) complete responses and 24 (59%) partial responses, for an overall response rate of 80% (95% confidence interval, 69–92%). When paclitaxel is followed immediately by cisplatin, in several other Phase II studies, the response rate ranges from 45% to 60% (111–115). One explanation for increased activity is that paclitaxel treatment may synchronize cells into the same phase of the cell cycle. The prolonged interval would then lead to an increased number of cells in G_1-S, just prior to DNA synthesis, where cisplatin is maximally active (61).

Camptothecins

Cell Cycle Effects. Camptothecins induce their primary cytotoxicity during the period of DNA synthesis. These agents form a class of chemotherapeutic drugs derived from the Chinese tree *Camptotheca acuminata* (116). They are alkaloids that are potent inhibitors of the nuclear enzyme topoisomerase I (117), an enzyme that functions primarily in the S-phase of the cell cycle. In fact, cells in S-phase are 100-1000 times more sensitive to camptothecin than cells in G_1 or G_2 (118). Topoisomerase I induces transient single-stranded breaks of DNA, relieving torsional strain and permitting DNA unwinding ahead of the replication fork during S-phase. Camptothecins stabilize the "cleavable complex" between topoisomerase I and DNA. When these cleavable complexes collide with the moving DNA replication fork, double-stranded DNA breaks, occur leading to cell death (119–121). This apoptotic cell death is mediated by caspase activation, and inhibition of this caspase activation shifts the cells from apoptosis to transient G_1 arrest followed by cell necrosis (122). Camptothecin treatment is associated with a G_2 cell cycle arrest (Ref. 123; Table 1). Apoptosis from short bolus exposure to camptothecin is not associated with changes in Bcl-2, Bax, p53, or p21 acutely; however, prolonged exposure (>72 h) is associated with increased expression of Bax (124). Finally, camptothecin treatment is associated with the transient and unscheduled stimulation of cyclin B1-Cdc2 kinase activity prior to apoptosis in HL60 cells (125). The clinically important members of this class of chemotherapeutic agents include irinotecan and topotecan. Irinotecan resistance, in part, appears to be mediated by improved DNA repair (126). Irinotecan has been approved for clinical use in the United States for colorectal cancer (127, 128), and both irinotecan and topotecan have been approved in Japan for small cell lung cancer, non-small cell lung cancer, uterine cancer, ovarian cancer, stomach cancer, colorectal cancer, breast cancer, skin cancer, and non-Hodgkin's lymphoma (129).

Irinotecan and Flavopiridol. Cell cycle-mediated drug resistance is again demonstrated in the human colon cancer cell line, HCT-116 (with an intact p53-p21 axis), both with irinotecan alone and with the combination of flavopiridol and irinotecan (Table 2). Although initially thought to be involved solely in the DNA damage checkpoint associated with G_1 cell cycle arrest (130), p21 has since demonstrated a crucial role in the G_2 DNA damage checkpoint as well (131, 132). p21 can sustain a stable G_2 arrest, possibly mediated by p21-associated inhibition of the activating phosphorylation of Cdc2 on Thr-161 in the cyclin B/Cdc2 complex (133). DNA damage is associated with an induction of p21, with a resultant arrest of cells in G_2 (131–133). These arrested cells are less sensitive to DNA-damaging agents than p21−/− cells that do not arrest in G_2 (132), thereby demonstrating the role of cell cycle-mediated inhibitors (CDKIs) in drug resistance. Camptothecins similarly induce p21 in a p53-dependent fashion, also associated with a G_2 arrest (133–135), and a similar relative insensitivity to the DNA damage caused by this chemotherapeutic agent (133, 135). This relative resistance to camptothecin has also been demonstrated in the KM12 colon cancer cell line, whereby irinotecan treatment results in a decrease in cyclin B/Cdc2 kinase activity and resultant G_2 arrest (136).

As a CDK inhibitor, flavopiridol itself induces a G_1 and G_2 cell cycle arrest; therefore, when flavopiridol precedes irinotecan, cell cycle-mediated drug resistance is again demonstrated. When HCT-116 cells were exposed to the drug sequence of flavopiridol followed by SN-38, QFM analysis demonstrated 15% ± 2% cell death. No induction of p21 by SN-38 was demonstrated, because of the cell cycle G_1 and G_2 arrest mediated by flavopiridol inhibition of CDK4, 6, and 2. However, this cell cycle-mediated resistance is overcome by appropriate drug sequencing (Table 3); SN-38 → flavopiridol resulted in significantly increased HCT-116 cell death at 44 ± 2% ($P < 0.001$; Ref. 135). This sequence (SN-38 → flavopiridol) demonstrated significant induction of apoptosis, as evidenced by PARP cleavage, caspase-3 activation, and DNA laddering. In a myeloid leukemia cell culture model, flavopiridol has been shown to block the induction of p21 by phorbol 12-myristate 13-acetate (137). We also have examined the role of specific cell cycle checkpoints in relation to the induction of apoptosis in the sequential administration of SN-38 → flavopiridol in our colon cancer cell model. We have found that flavopiridol suppresses p21 induction by SN-38, with resultant sensitization to SN-38-mediated DNA damage (135). These results suggest that suppression of p21 may present a novel target to increase sensitization to DNA-damaging agents, including irinotecan, and provide a mechanism to overcome this cell cycle-mediated drug resistance. We are now actively pursuing the combination of irinotecan followed by flavopiridol in a Phase I clinical trial. We are also currently investigating the relative importance of p21 in clinical tumor resistance to irinotecan.

Fluorouracil

Cell Cycle Effects. Fluorouracil is an antimetabolite with broad activity in epithelial tumors arising in the breast, head and neck, gastrointestinal, and ovarian cancers, with single-agent response rates ranging from 10 to 30% (138). Upon cell entry, fluorouracil is converted to floxuridine (FUdR) by thymidine phosphorylase, which is then again phosphorylated by thymidine kinase to its active form, 5-fluoro-2′-deoxyuridine monophosphate (FdUMP; Ref. 138). In the presence of a reduced

folate cofactor, this active metabolite forms a stable complex with TS, thereby limiting its ability to continually synthesize thymidine 5′-monophosphase (139), with resultant inhibition of DNA synthesis. Increased TS expression is associated with fluorouracil resistance in multiple fluorouracil-resistant cell lines (140). Fluorouracil also is extensively incorporated into both nuclear and cytoplasmic RNA species, interfering with normal RNA processing [reviewed by Allegra and Grem (138)]. It is purely an S-phase active chemotherapeutic agent, with no activity when cells are in G_0 or G_1 (Table 1). Twenty-four-h exposure to fluorouracil is associated with an accumulation of cells in S-phase, as well as a transient induction of p53 and p21 (141). The accumulation of cells in early S-phase is associated with expression of cyclin A and an increase in cyclin A-cdk2 kinase activity (142).

Camptothecin and Fluorouracil. The rationale for the combination of fluorouracil with camptothecins lies in their different mechanisms of action and different mechanisms of resistance; however, the combination again demonstrates cell cycle-mediated drug resistance (Table 2). Guichard *et al.* (143) examined various combinations of irinotecan and fluorouracil in HT-29 colon cancer cells, using the median effect principle to determine synergy *versus* antagonism. They found that the simultaneous combination of fluorouracil and irinotecan was antagonistic at low concentrations (143). Mans *et al.* (144) similarly demonstrated antagonism in HT-29 colon cancer cells when irinotecan and fluorouracil were combined simultaneously. The mechanism for the cell cycle-mediated drug resistance with this schedule is the induction of a G_2 arrest by irinotecan (143), thereby creating resistance to fluorouracil, an S-phase active chemotherapeutic. Cell cycle-mediated drug resistance was also demonstrated when exposure to fluorouracil was immediately followed by exposure to irinotecan (144). The mechanism for the cell cycle-mediated drug resistance with this schedule may be explained by a an accumulation of cells in early S-phase and a cell cycle arrest in G_1 induced by fluorouracil (131), making immediate irinotecan therapy ineffective.

The appropriate sequencing and scheduling of this combination of chemotherapeutics can again overcome the cell cycle-mediated drug resistance described above (Table 3). Guichard *et al.* (143, 145) demonstrated increased cytotoxicity when fluorouracil exposure was followed by a 6-h delay prior to irinotecan exposure. This increased sensitivity to irinotecan was explained by synchronization of cells in S-phase by fluorouracil therapy, where irinotecan exerts its primary cytotoxicity (143, 145). The increased cytotoxicity was associated with an increase in irinotecan and SN-38 cellular uptake, with a concomitant increase in topoisomerase I-DNA complexes (145). The reverse sequence (irinotecan → fluorouracil) also demonstrates synergistic cytotoxicity in culture and *in vivo* (143, 144, 146, 147). This sequence was associated with persistent inhibition of TS (perhaps because of a G_2 cell cycle arrest; Refs. 123, 145). Mullany *et al.* (147) confirmed that SN-38 sequentially induces diminished DNA synthesis, elevated dTTP pools (which may inhibit thymidylate synthase), but also demonstrated that SN-38 was associated with an S-phase delay.

In separate *in vivo* experiments using a chemically induced Ward colorectal carcinoma murine model, irinotecan followed by fluorouracil was the most active regimen, demonstrating complete tumor regression in 95% of the animals (146). When the drugs were administered simultaneously, together, or in the reverse sequence, the response rates were 62 and 38%, respectively, again demonstrating relative cell cycle-mediated resistance and confirming the preclinical *in vitro* data described above (146). Furthermore, this sequence also demonstrated significant potentiation, because synergy was seen with irinotecan and fluorouracil doses at a fraction of the maximum tolerated dose individually (146).

Although these preclinical data are quite compelling and although there have been several clinical studies examining the combination of irinotecan and fluorouracil, none of these studies has examined the combination of irinotecan and fluorouracil in a sequence-specific manner (127, 148–152). Irinotecan administered concurrently with fluorouracil has demonstrated increased responses compared with fluorouracil alone (127, 152), even conferring a survival advantage with the combination therapy (128, 152). No pharmacokinetic interactions have been demonstrated with the simultaneous combination (127). One study did alternate irinotecan with fluorouracil/leucovorin administered daily for 5 days; however, irinotecan was administered on day 1 and fluorouracil/leucovorin on days 22–26 repeated every 6 weeks (149). This lengthy interval between alternating agents would likely negate any potentiation by sequential administration of the two agents.

Fluorouracil and UCN-01. Appropriate sequencing of drug combinations is an important mechanism to overcome the cell cycle interactions creating cell cycle-mediated drug resistance. However, UCN-01 is a novel anticancer agent that, in addition to its cell cycle effects, increases chemosensitivity by suppressing the expression of critical events regulated in the cell cycle. UCN-01 (7-hydroxystaurosporine) is a staurosporine analogue isolated from the culture broth of *Streptomyces* species (153, 154) and is a selective inhibitor of PKC (154). UCN-01 abrogates the S-phase/G_2 checkpoint through a Cdc2-dependent pathway, resulting in premature activation of the mitosis-promoting kinase in DNA-damaged cells (155–157). UCN-01 thereby augments the induction of apoptosis by DNA-damaging agents, including cisplatin, mitomycin C, and irradiation (157–160) by causing a premature progression through mitosis. The molecular mechanism appears to be inhibition of Chk1 autophosphorylation (160, 161) and the loss of Cdc25c-serine 216 phosphorylation (161), which result in Cdc2 dephosphorylation and entry of cells into mitosis (160, 161). UCN-01 is also associated with a G_1-S cell cycle arrest (162–164), induction of p21CIP/Waf1, and dephosphorylation of CDK2 (165). Thus, we would predict the development of cell cycle-mediated drug resistance created by a cell cycle arrest in G_1 if UCN-01 treatment precedes fluorouracil (Table 2). UCN-01 treatment is also associated with dephosphorylation of the retinoblastoma gene product (pRb; Refs. 164, 165). Hypophosphorylated pRb remains tightly bound to E2F-1, thereby preventing cell cycle progression into S-phase (166). This inhibition of E2F-1 is associated with significant reduction in both *TS* and *DHFR* gene expression, which normally increase substantially during G_1-S-phase boundary of cell cycle (167, 168). Decreased expression of TS and DHFR as a result of the cell cycle effects of UCN-01 is relevant to its interaction with fluorouracil (169), with resultant increased antimetabolite chemosensitivity.

Both concepts of cell cycle-mediated drug resistance and augmentation of apoptosis by suppression of TS induction were demonstrated *in vitro* (169). In SK-GT5 cells, a human gastric adenocarcinoma cell line with a mutated *p53* gene, cell cycle-mediated drug resistance was demonstrated when fluorouracil followed UCN-01. This sequence demonstrated only 17 ± 1% apoptotic cells by QFM analysis. Maximal apoptosis was demonstrated with the reverse sequence of fluorouracil → UCN-01, with 46% ± 1% apoptotic cells by QFM analysis (Table 3; Ref. 169). Exposure to UCN-01 resulted in a dose-dependent decrease in TS protein expression, as well as a dose-dependent decrease in TS mRNA, with associated reductions in E2F-1 protein levels, and consequent increased sensitivity to fluorouracil as evidenced by increased apoptosis (169). Again, we are currently investigating this sequence-dependent combination in a Phase I clinical trial.

Conclusions

In this review, based on our current understanding of cellular physiology and the cell cycle effects of chemotherapeutic agents, we have proposed an emerging concept of cell cycle-mediated drug resistance, a concept that is best exemplified when cytotoxic agents are used in combination. We have reviewed the cell cycle effects of several classes of drugs, including standard drugs used for chemotherapy today as well as a novel class of cell cycle-specific modulators, which currently remain in investigational use. We have demonstrated that the combination of these chemotherapeutic agents can be associated with cell cycle-mediated drug resistance. Furthermore, we have learned that it is possible to overcome this resistance by the appropriate sequencing and scheduling of these drugs. Finally, as our understanding of cellular physiology grows, we would hope to use sequence-specific combination chemotherapy increasingly to our advantage in the ongoing fight against cancer.

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