

The Biology Behind

To Arrest or Not To G₂-M Cell-Cycle Arrest

Commentary re: A. K. Tyagi *et al.*, Silibinin Strongly Synergizes Human Prostate Carcinoma DU145 Cells to Doxorubicin-induced Growth Inhibition, G₂-M Arrest, and Apoptosis. *Clin. Cancer Res.*, 8: 3512–3519, 2002.

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Introduction

Cells transverse the cell-cycle in several well-controlled phases (1). In the G₁ phase, cells commit to enter the cell-cycle and prepare to duplicate their DNA in S phase. After S phase, cells enter the G₂ phase, where repair might occur along with preparation for mitosis in M phase. In the M phase, chromatids and daughter cells separate. After M phase, the cells can enter G₁ or G₀, a quiescent phase. Entry into each phase of the cell-cycle is carefully regulated by receptor collectives, termed cell-cycle checkpoints. One theme emerging in drug discovery is to develop agents that target the cell-cycle checkpoints that are responsible for the control of cell-cycle phase progression. It is clear that the cell-cycle checkpoints can regulate the quality and rate of cell division; agents are now under development that either increase or decrease the degree of checkpoint arrest (2–8). For example, defects in the G₁ arrest checkpoint may lead a cancer cell to enhanced proliferation, and efforts to correct these problems may slow growth and induce cell death. Defects in the G₂-M arrest checkpoint may allow a damaged cell to enter mitosis and undergo apoptosis, and efforts to enhance this effect may increase the cytotoxicity of chemotherapy. Alternatively, efforts to increase G₂-M arrest have also been associated with enhanced apoptosis. With a focus on the G₂-M checkpoint, Tyagi *et al.* (9) studied an agent capable of altering G₂-M cell-cycle checkpoint regulators and brought to light several questions, including the importance of enhancing cell-cycle checkpoint arrest compared with abrogation, what regulators should be targeted and the real contribution of checkpoint modulation to cytotoxicity and synergy.

The G₂-M Checkpoint

Cell cycle checkpoints help ensure the accuracy of DNA replication and division (1, 2). These checkpoints allow progression through the cell-cycle or arrest in response to DNA damage to allow time for DNA repair. The cell-cycle DNA damage checkpoints occur late in G₁, which prevents entry to S phase, and late in G₂, which prevents entry to mitosis. The checkpoint control system is regulated by a family of protein kinases, the

Cdks², which are in turn controlled by a complex array of proteins, including the cyclins. At the G₁ checkpoint in late G₁, the cell either exits to G₀ or commits to the cell-cycle and entry to S phase. The gene regulatory protein E2F is required for S-phase entry and is controlled by the cell-cycle inhibitor Rb. The active G₁ Cdk phosphorylates Rb and reduces its affinity for E2F, which then activates S-phase gene expression. In response to DNA damage, p53 stimulates the transcription of several genes, which inhibits G₁ Cdk. This in turn decreases Rb phosphorylation thereby stopping S-phase progression.

Analogous to the G₁ checkpoint before S phase, the G₂ checkpoint allows the cell to repair DNA damage before entering mitosis. In fact, DNA damage that occurs in a cancer cell with a defective G₁ checkpoint may result in more profound G₂-M arrest. Mitosis follows DNA replication in the G₂ phase of the cell-cycle after the mitotic Cdk, Cdk1(*cdc2*), is activated. As diagramed in Fig. 1, *cdc2* forms a complex with cyclin B1. Although the rise and fall of cyclin levels are the primary determinant of Cdk activity during the cell-cycle, several additional mechanisms are important. Regulation of the *cdc2*-B1 complex involves an activating phosphate by Cdk-activating enzyme and inhibitory phosphates at a pair of amino acids in the roof of the active site by Wee1. Dephosphorylation of these sites by the phosphatase Cdc25C increases Cdk activity (2). Chk1 inactivates Cdc25C through phosphorylation of *cdc25C*, as depicted in Fig. 1. This effect of Chk1 prevents dephosphorylation of *cdc2*, maintaining *cdc2*-B1 in an inactive state. DNA damage activates Chk1, which will then inactivate *cdc25C* and leave *cdc2*-B1 in an inactive phosphorylated state. Therefore, a crucial event in cell-cycle progression through the G₂-M checkpoint is the activation of the protein phosphatase Cdc25C, which removes *cdc2* inhibitory phosphates. Although this simple model of the G₂-M checkpoint suggests that cytotoxicity of DNA damaging agents will be enhanced with abrogation of this checkpoint, therefore driving the cell into mitosis before repair, other studies also suggest enhanced cytotoxicity associated with increased cell-cycle arrest (3–8). Therefore, both checkpoint abrogation or checkpoint arrest as a means to enhance cytotoxic effects of chemotherapy requires additional study.

G₂-M Checkpoint Abrogation

DNA damage is associated with many cellular events, including activation of Chk1, which in turn phosphorylates and inactivates *cdc25*, allowing inactivation of the *cdc2*-B1 complex and G₂-M arrest (3). Agents capable of overriding this G₂-M arrest were shown to enhance the cytotoxicity of DNA damag-

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² The abbreviations used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma protein.

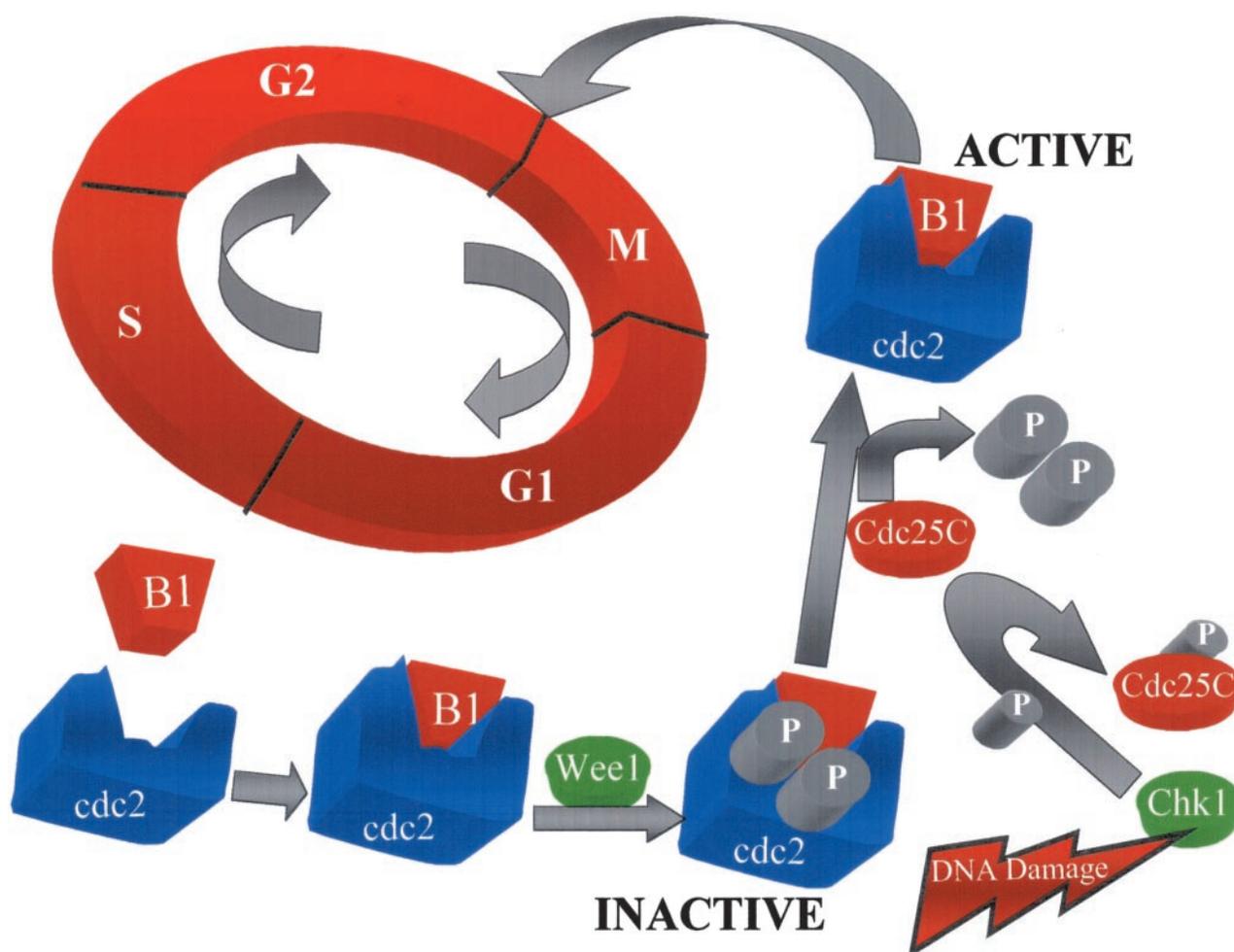


Fig. 1 The late G₂ checkpoint controlling cell-cycle progression from G₂ to M phase. Active Cdk1(cdc2) complexed to cyclin B1 is required for progression from G₂ to M phase as shown. Regulation of the cdc2-B1 complex involves inhibitory phosphates at a pair of amino acids in the roof of the active site by Wee1. Dephosphorylation of these sites by the phosphatase Cdc25C increases Cdk activity. DNA damage activates Chk1, which inactivates Cdc25C through phosphorylation of cdc25C, resulting in the phosphorylation and inactivity of cdc2-B1 and G₂-M arrest.

ing agents. For example, Jackson *et al.* (3) demonstrated that the Chk1 indolocarbazole inhibitor (SB-218078) abrogated gamma-irradiation and topotecan induced G₂-M arrest in HeLa cells and enhanced cytotoxicity. Hirose *et al.* (4) demonstrated that temozolamide induced G₂-M arrest in glioma cells associated with Chk1 activation and phosphorylation of cdc25. These events were inhibited, temozolamide cytotoxicity increased, and G₂-M arrest bypassed by a nonspecific Chk1 kinase inhibitor 7-hydroxystarospirine. 7-Hydroxystarospirine was recently studied in a Phase I clinical trial that demonstrated the drug was safe and had clinical activity (5). An interesting laboratory correlate using irradiated MCF-7 cells exposed to patient plasma after therapy demonstrated that plasma samples from patients decreased G₂-M checkpoint arrest (5). These data imply that G₂-M checkpoint abrogation may be an important target for enhancing cytotoxic agents. Despite these interesting associations, however, additional studies will be needed to determine the contribution of the observed changes in cell-cycle to cytotoxicity.

G₂-M Checkpoint Arrest

In contrast to the concept of bypassing a checkpoint, thought to drive a cell from a protective arrest after initial DNA damage into apoptosis, some agents are capable of enhancing cytotoxicity in association with enhanced checkpoint arrest (6–9). For example, flavopiridol, a semisynthetic derivative of the plant alkaloid rohitukine, is thought to inhibit Cdk-activating enzyme (cdk7), docking on cdk ATP-binding sites, and to decrease of cyclin D1 (6, 7). Flavopiridol causes arrest at both the G₁ and G₂ phases of the cell-cycle as would be expected from inhibition of cdk2, cdk4, and cdk1 (1, 2, 6, 7). Shapiro *et al.* (8) studied flavopiridol in patients with non-small cell lung cancer. Flavopiridol as a single agent was well tolerated but had little clinical activity. Bible *et al.* (7) demonstrated cytotoxic synergy with flavopiridol and with multiple chemotherapy agents in a lung cancer cell line, supporting additional studies of flavopiridol in combination with chemotherapy. Additional

studies will be needed to determine whether agents that enhance arrest can induce cytotoxicity or synergy because of effects on cell-cycle or if changes in the cell-cycle are a secondary effect.

In the report by Tyagi *et al.*, (9) silibinin, a derivative of milk thistle, induced increased G₂-M arrest in combination with doxorubicin and modulated G₂-M cell-cycle regulators. Silibinin, in combination with doxorubicin, decreased expression of cdc25C, cdc2/p34, and B1 protein levels compared with either compound alone. They also demonstrated inhibition of cdc2/p34 kinase activity assayed in histone H1 as substrate. The association of these changes with enhanced G₂-M arrest and synergy argues in favor of the importance of cell-cycle checkpoint arrest and synergy with doxorubicin. These data are hypothesis generating; this association suggests that down-regulation of the G₂-M cell-cycle regulators and G₂-M arrest could be a possible mechanism for the synergistic effect of silibinin combined with doxorubicin on cell growth and apoptosis. In support of the importance of cell-cycle arrest to doxorubicin cytotoxicity, Ling *et al.* (10) found that P388 cells synchronized in S and G₂-M phases were more sensitive to doxorubicin than cells in G₁ phase. Potter *et al.* (11) studied the cell-cycle importance on DNA damage. The damage to DNA by gamma radiation and hydrogen peroxide was not phase specific in HeLa and CEM cells. In contrast, doxorubicin-induced DNA damage was predominantly in the G₂ phase of the cell-cycle.

Differences in G₂-M Arrest

Other groups demonstrated G₂-M cell-cycle arrest using other herbal derivatives, but the mechanism of G₂-M arrest in many products may be secondary to effects on mitosis in contrast to the checkpoint modulation late in G₂, as was likely seen by Tyagi *et al.* (9). For example, Holy *et al.* (12) studied the effect of curcumin-induced G₂-M arrest in MCF-7 cells. In their studies G₂-M arrest was associated with problems in mitotic spindle structure, including assembly of aberrant monopolar mitotic spindles that lead to impaired segregation of chromosomes and likely represented mitotic arrest. In an effort to understand proven clinical antitumor activity of the herbal product PC-SPES, which was a commonly used herbal mixture for prostate cancer (before it was removed from the market secondary to quality control concerns), we demonstrated that the product had potent estrogenic and cytotoxic activity *in vitro*, *in vivo*, and in man (13). Additional studies of the herbal components of PC-SPES demonstrated that licorice root had estrogenic activity, cytotoxic activity, and induced G₂-M (14, 15). Using high-performance liquid chromatography, mass spectroscopy, and nuclear magnetic resonance, we identified and characterized cytotoxic chalcone derivatives from licorice root capable of causing G₂-M arrest, bcl-2 phosphorylation (a marker for mitosis), and microtubule bundling (15). Further analysis revealed that a chalcone 1-propanone,1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxyphenyl) and two glycosylated derivatives were responsible for this effect. Edwards *et al.* (16) also demonstrated that a series of chalcone structures had antimetabolic effects in tumor cell lines. Additional studies are needed to determine the effect of these derivatives on the checkpoint proteins such as B1, cdc2, and cdc25C. In contrast to the effect on mitosis by some of these agents, Frey *et al.* (17) demon-

strated that genistein, a soy isoflavone, decreased cdc2 and cdc25C in the nonneoplastic human mammary epithelial cell line MCF-10F, suggesting a late G₂ arrest. These data point out the difficulties in understanding if various herbal derivatives have similar targets (late G₂ checkpoint or mitosis). Prior studies of known antimicrotubule agents such as paclitaxel demonstrated increased cyclin B1 and stimulation of cdc2/cyclin B1 kinase activity at the same time as M-phase arrest and bcl-2 phosphorylation, suggesting that pharmaceutical or herbal agents that effect microtubules as a primary mechanism of cytotoxicity modulate G₂-M checkpoint proteins for entry into mitosis, in contrast to the late G₂ checkpoint arrest (9, 18, 19). Therefore, further study of silibinin and other agents that induce G₂-M arrest need to include not only activity and expression of checkpoint regulators but to clearly define if the cell is arrested in late G₂ or in mitosis.

Conclusions

Efforts to modulate cell-cycle arrest in G₂-M is the subject of laboratory and clinical studies. Both approaches to enhance arrest or abrogate arrest have been used to improve cytotoxicity of known agents. Although promising, these initial efforts have led to a number of questions that remain unanswered. Currently, the contribution of checkpoint regulation to synergy is largely unknown. Additionally, in contrast to the study of silibinin by Tyagi *et al.* (9), many other reported herbal derivatives capable of G₂-M arrest may be secondary to effects on microtubules, which induce mitotic arrest beyond the late G₂ checkpoint. The discovery of interesting agents like silibinin, with synergy in combination with chemotherapy and associated effects on cdk1 function, leads to the hypothesis that modulation of checkpoint regulators may or may not contribute to cytotoxicity and synergy with agents like doxorubicin and/or be useful as a markers of drug effect in clinical trials. Further study of these agents will be important to the development of novel clinical approaches and help increase our understanding of checkpoint modulation.

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