The Biology Behind

To Arrest or Not To G_2-M Cell-Cycle Arrest

Commentary re: A. K. Tyagi *et al.*, Silibinin Strongly Synergizes Human Prostate Carcinoma DU145 Cells to Doxorubicin-induced Growth Inhibition, G_2-M Arrest, and Apoptosis. Clin. Cancer Res., *8:* 3512–3519, 2002.

Robert S. DiPaola[1]

Department of Medicine, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, The Cancer Institute of New Jersey, and The Dean and Betty Gallo Prostate Cancer Center, New Brunswick, New Jersey 08901

Introduction

Cells transverse the cell-cycle in several well-controlled phases (1). In the G_1 phase, cells commit to enter the cell-cycle and prepare to duplicate their DNA in S phase. After S phase, cells enter the G_2 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 G_1 or G_0, 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 G_1 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 G_2-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 G_2-M arrest have also been associated with enhanced apoptosis. With a focus on the G_2-M checkpoint, Tyagi *et al.* (9) studied an agent capable of altering G_2-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 G_2-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 G_1, which prevents entry to S phase, and late in G_2, which prevents entry to mitosis. The checkpoint control system is regulated by a family of protein kinases, the Cdks[2], which are in turn controlled by a complex array of proteins, including the cyclins. At the G_1 checkpoint in late G_1, the cell either exits to G_0 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 G_1 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 G_1 Cdk. This in turn decreases Rb phosphorylation thereby stopping S-phase progression.

Analogous to the G_1 checkpoint before S phase, the G_2 checkpoint allows the cell to repair DNA damage before entering mitosis. In fact, DNA damage that occurs in a cancer cell with a defective G_1 checkpoint may result in more profound G_2-M arrest. Mitosis follows DNA replication in the G_2 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 G_2-M checkpoint is the activation of the protein phosphatase Cdc25C, which removes cdc2 inhibitory phosphates. Although this simple model of the G_2-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.

G_2-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 G_2-M arrest (3). Agents capable of overriding this G_2-M arrest were shown to enhance the cytotoxicity of DNA damag-

[1] To whom requests for reprints should be addressed, at Division of Medical Oncology, The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901.
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[2] The abbreviations used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma protein.

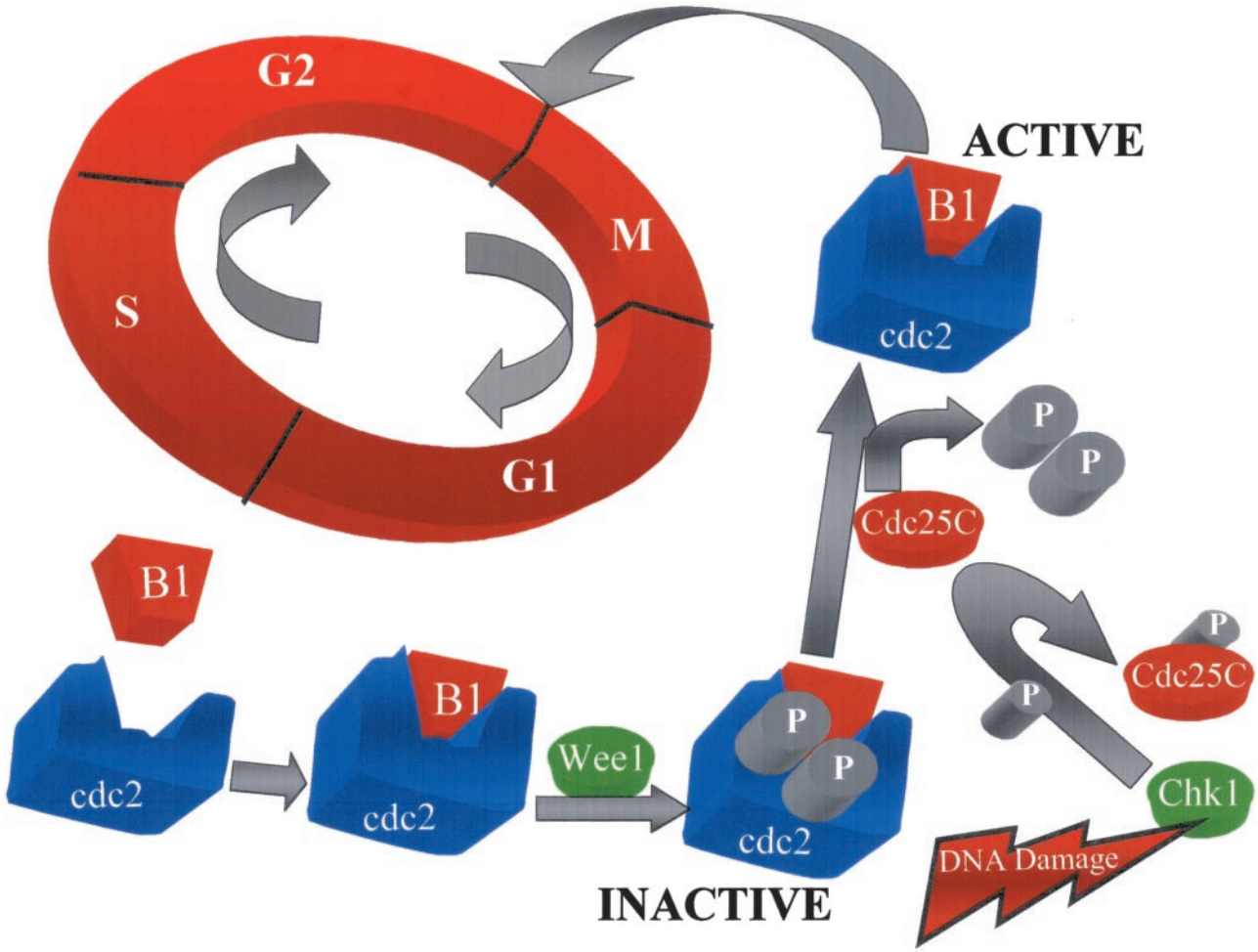

Fig. 1 The late G_2 checkpoint controlling cell-cycle progression from G_2 to M phase. Active Cdk1(cdc2) complexed to cyclin B1 is required for progression from G_2 to M phase as shown. Regulation of the cdc2-B1 complex involves inhibitory phosphates at a pair of amino acids in the roof of the active 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 G_2-M arrest.

ing agents. For example, Jackson *et al.* (3) demonstrated that the Chk1 indolocarbazole inhibitor (SB-218078) abrogated gamma-irradiation and topotecan induced G_2-M arrest in HeLa cells and enhanced cytotoxicity. Hirose *et al.* (4) demonstrated that temozolamide induced G_2-M arrest in glioma cells associated with Chk1 activation and phosphorylation of cdc25. These events were inhibited, temozolamide cytotoxicity increased, and G_2-M arrest bypassed by a nonspecific Chk1 kinase inhibitor 7-hydroxystarosporine. 7-Hydroxystarosporine 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 G_2-M checkpoint arrest (5). These data imply that G_2-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.

G_2-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 Cdks through multiple mechanisms, including the inhibition of Cdk-activating enzyme (cdk7), docking on cdk ATP-binding sites, and to decrease of cyclin D1 (6, 7). Flavopiridol causes arrest at both the G_1 and G_2 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 G_2-M arrest in combination with doxorubicin and modulated G_2-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 G_2-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 G_2-M cell-cycle regulators and G_2-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 G_2-M phases were more sensitive to doxorubicin than cells in G_1 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 G_2 phase of the cell-cycle.

Differences in G_2-M Arrest

Other groups demonstrated G_2-M cell-cycle arrest using other herbal derivatives, but the mechanism of G_2-M arrest in many products may be secondary to effects on mitosis in contrast to the checkpoint modulation late in G_2, as was likely seen by Tyagi *et al.* (9). For example, Holy *et al.* (12) studied the effect of curcumin-induced G_2-M arrest in MCF-7 cells. In their studies G_2-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 G_2-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 G_2-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 antimitotic 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) demonstrated that genistein, a soy isoflavone, decreased cdc2 and cdc25C in the nonneoplastic human mammary epithelial cell line MCF-10F, suggesting a late G_2 arrest. These data point out the difficulties in understanding if various herbal derivatives have similar targets (late G_2 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 G_2-M checkpoint proteins for entry into mitosis, in contrast to the late G_2 checkpoint arrest (9, 18, 19). Therefore, further study of silibinin and other agents that induce G_2-M arrest need to include not only activity and expression of checkpoint regulators but to clearly define if the cell is arrested in late G_2 or in mitosis.

Conclusions

Efforts to modulate cell-cycle arrest in G_2-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 G_2-M arrest may be secondary to effects on microtubules, which induce mitotic arrest beyond the late G_2 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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