

## Minireview

# Chemical inhibitors: a tool for plant cell cycle studies

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**Abstract** Synchrony provides a large number of cells at defined points of the cell cycle. Highly synchronised cells are powerful and effective tools for molecular analyses and for studying the biochemical events of the cell cycle in plants. Usually, plant cell suspensions can be synchronised by chemical agents, which arrest the cell cycle by acting on the driving forces of the cell cycle engine such as cyclin-dependent kinase activity, enzymes involved in DNA synthesis or proteolysis of cell cycle regulators or by acting on the cell cycle apparatus (mitotic spindle). The specificity, reversibility and efficiency of each type of cell cycle inhibitor are described and related to their mode of action. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Synchronisation; Cell cycle; Plant; Inhibitor

## 1. Introduction

Plant development involves tight control and co-ordination of proliferative activity and growth in meristematic and differentiated tissues. To understand the regulation of plant growth, it is necessary to identify regulators of plant cell division. Our knowledge of the molecular events of the cell cycle has advanced considerably during the past few years through exploitation of highly synchronised plant cell suspensions, which provide a simple model to study proliferation in plants.

During the cell cycle, DNA replication is followed by the equitable distribution of the genetic material to the daughter cells. This process can be dissected into four steps: the S phase, where DNA replication takes place, preceded by the G1 phase and followed by the G2 phase and mitosis (M). A synchronous cell culture is characterised by a high proportion of cells proceeding to the same event of the cell cycle at the same time. While natural synchrony is observed in certain plant tissues which can be a source of synchronised cells, induction of synchrony can be achieved by the arrest of a cell population at a specific stage by growth factor starvation and/or addition of chemical agents. Chemical agents are most frequently used to achieve simple and highly reproducible synchrony of plant cells.

In this review, we discuss the most frequently used chemical agents, their advantages and drawbacks as well as their mode of action.

## 2. Synchronisation: general considerations

Chemical agents arrest the cell cycle by acting on the driving forces of the cell cycle engine (such as cyclin-dependent kinase (CDK) activity, enzymes involved in DNA synthesis or proteolysis of cell cycle regulators) or on the cell cycle apparatus (mitotic spindle).

### 2.1. Specificity and reversibility

A chemical inhibitor used as a synchronising agent will have special characteristics. The drug action has to be phase-specific and the chemical agent has to be efficient at low concentrations and rapidly effective, to avoid abnormalities in subsequent phases. To be sure that the cell division arrest is not a result of cellular death, the block must be reversed when the drug is removed.

### 2.2. Species specificity

The optimal concentration of a chemical inhibitor, the duration of the treatment and time to re-enter the cell cycle have to be calibrated for each plant species. The duration of the treatment is dependent on generation time and the cell cycle phase. For example, aphidicolin concentrations used to synchronise cells at the G1/S border are 10-fold larger in *Arabidopsis* cell suspensions [1] than in tobacco BY-2 cells [2] (Table 2).

### 2.3. Cell suspension characteristics

The characteristics of the cell suspension also determine the level of synchrony. This can be exemplified by two commonly used cell suspensions, tobacco BY-2 cells and *Arabidopsis thaliana* cell cultures. Tobacco BY-2 cell suspensions are composed of small clusters of cells with a relatively uniform cell size, and approximately the same growth rate for the cell population [2]. At stationary phase (7 days of culture), a majority of BY-2 cells have a 2C DNA content [3]. Since the cells are in the same state, refreshing them with new medium and applying a chemical treatment for 24 h is sufficient to block the cell suspension homogeneously. In contrast, *Arabidopsis* cell suspensions contain cells with a 2C and 4C DNA content at stationary phase (unpublished data). This cell suspension is formed of calli of different sizes and is composed of cell populations growing at different rates. The block will occur in a different way for each of these cell populations, and synchrony will be low. The quality of the synchrony depends on the features of the cell suspension. Therefore, a plant cell suspension developed for synchronisation has to grow as fast and homogeneously as possible.

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#### 2.4. Synchrony procedure

The balance between toxicity and efficacy prevents the use of high concentrations of chemical inhibitors. Synchrony gradually decreases from the moment the treatment is released. Therefore, a one-step treatment can be improved by combining different inhibitors in a two-step blocking method. The synchronised cells obtained after the first drug treatment are further treated with a second drug to induce an arrest in the subsequent phase. For example, after the release from a one-step aphidicolin block, a mitotic index (MI) of about 30–50% is obtained in tobacco BY-2 cells, whilst the MI is increased up to 90% by a treatment with an anti-tubulin drug after the release from an aphidicolin block [2,3].

### 3. Monitoring the synchrony of the cells

The proportion of synchronised cells is best estimated by combining various methods since each technique facilitates the monitoring of particular steps of the cell cycle.

#### 3.1. Flow cytometric analysis

Flow cytometry is used to monitor G1, S and G2 phases of the cell cycle. Flow cytometry allows rapid analysis of the plant nuclear DNA content of large populations of cells and is used to estimate the position of cells within the cell cycle and their proportion with respect to the total cell population [4]. However, in conventional univariate DNA-Hoechst flow cytometry, the G1 cells of the next cell cycle are indistinguishable from those of the original cell cycle. The discriminatory multiparametric analysis of Hoechst 33258 versus propidium iodide allows the detection of the cells that have progressed from one cell cycle to the next after incorporation of the thymidine analogue 5-bromodeoxyuridine (BrdU) [5]. This method has been used to monitor cell cycle progression and to distinguish cycling cells from resting cells in *Arabidopsis* cell suspensions [6]. In other species, this technique was used to study the induction or inhibition of DNA replication after treatment by plant growth factors in *Petunia hybrida* mesophyll protoplasts [7] or after treatment by salicylic acid and UV-light in tobacco BY-2 cell suspension [8]. The detection of BrdU incorporation with an anti-BrdU antibody is an alternative quantitative methodology [9].

#### 3.2. [<sup>3</sup>H]Thymidine labelling

This method is used to monitor DNA replication. After subculture in the presence of [<sup>3</sup>H]thymidine nucleotides ([<sup>3</sup>H]TTP), cells incorporate [<sup>3</sup>H]TTP in the newly synthesised DNA during replication. [<sup>3</sup>H] incorporation is estimated by

counting the radioactivity in a liquid scintillation counter [10]. The rate of [<sup>3</sup>H]thymidine incorporation is proportional to the quantity of cells engaged in DNA synthesis. This method is used in plant cell suspensions [11,12] and in plant tissues [13].

#### 3.3. Cytology

Cytology is mainly used to determine the proportion of cells in mitosis (MI), which is the number of mitotic figures related to the total number of cells. This number of dividing cells is estimated by UV-light microscopy observations after DNA staining with 4',6-diamidino-2-phenylindole (DAPI) or Hoechst 33342.

Microscopic analysis is complementary to flow cytometry in evaluating cells with 4C DNA content. This technique is particularly useful after an anti-tubulin drug treatment (colchicine, oryzalin or propyzamide). Indeed, such drugs disorganise the mitotic spindle and scatter the chromosomes in the cells. These metaphase-arrested cells cannot be counted by flow cytometry because the analysis of the DNA content requires entire nuclei. In this case, coupling flow cytometric analysis with the determination of the percentage of mitotic-like figures will give the exact number of 4C cells [3,14].

#### 3.4. Molecular markers

Expression of numerous cell cycle genes is cell cycle-regulated and occurs at different phases. The detection of gene expression by Northern blots gives information about the cell cycle stage. For example, in tobacco BY-2 cells, *CycD3;2* marks the entry of cells from G0 to G1 [15] and histone *H4* transcripts characterise the S phase [16]. The mitotic cyclin genes can be used to monitor the G2 and early M phases (for example, *Nicta;CycB1;1* gene [3,17], *Arath;CycB1;1*, [18]). The degree of synchrony may be checked by combining markers of different cell cycle phases.

### 4. Main inhibitors used in plant cell cycle studies

The specificity of action of different chemical agents is presented in Table 1. References are listed in a chronological order. Chemical agents and their specific inhibitory actions are illustrated during the cell cycle in Fig. 1.

#### 4.1. Blocking at G1/S and G2/M transitions

CDKs play a central role in the initiation, ordering and completion of cell cycle events (for review, see [27,28]). Inhibitors of CDK activity block the cell cycle and show anti-tumour activities (for review, see [29]). A specific inhibition of human CDK activity was shown by a purine derivative, called

Table 1

Name	Target/mechanism	Block	Reversibility <sup>a</sup>	References
HU	ribonucleotide reductase	G1/S progression	+/-	[19]
Aphidicolin	DNA polymerase $\alpha$ and $\delta$	G1/S progression	+	[20]
Colchicine	microtubule depolymerisation	metaphase	+/-	[21]
Oryzalin	microtubule depolymerisation	metaphase	+/-	[22]
Propyzamide	microtubule depolymerisation	metaphase	+/-	[23]
APM	microtubule depolymerisation	metaphase	+/-	[24]
Mimosine	ribonucleotide reductase	G1, before initiation of replication	+/-	[25]
Olomoucine	CDK activity	G1/S and G2/M	+	[6]
Roscovitine	CDK activity	G1/S and G2/M	+	[3]
MG132	proteasome	metaphase/anaphase	-	[26]

<sup>a</sup>+ reversible arrest; - irreversible arrest; +/- reversibility dependent on the duration of the treatment.

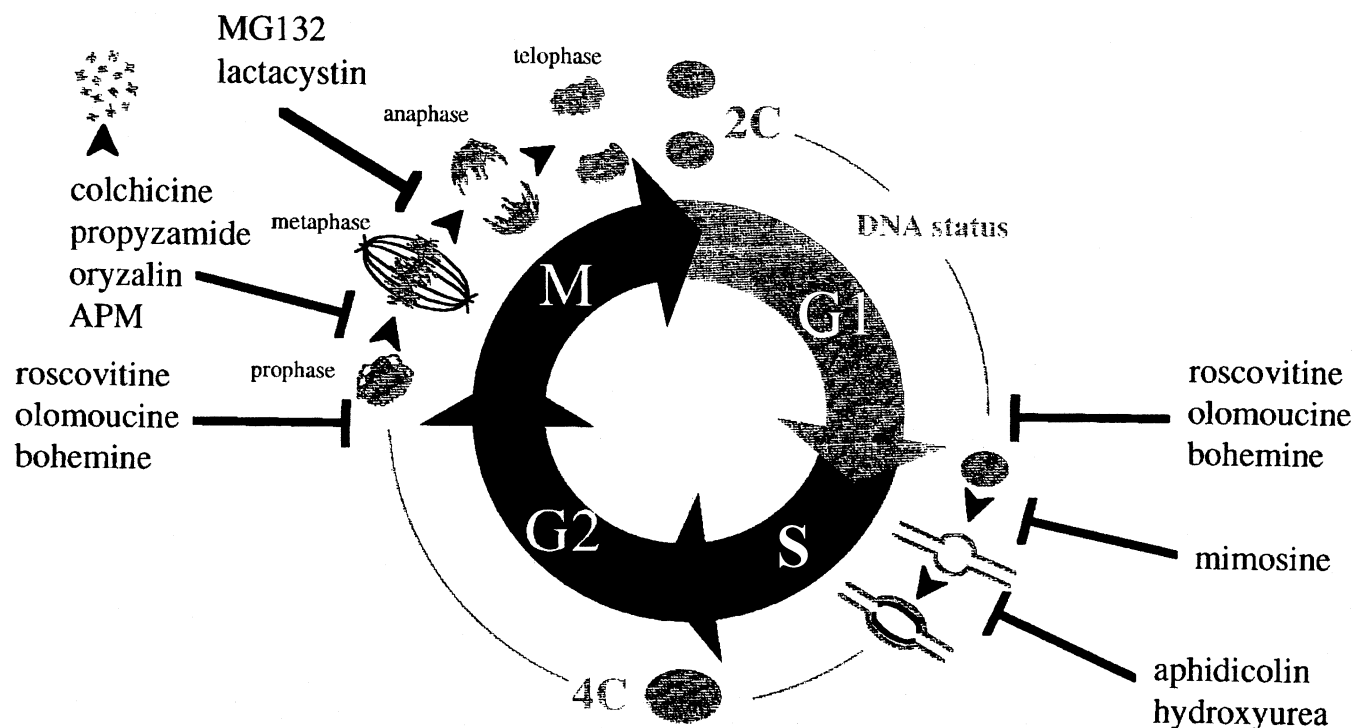


Fig. 1. Chemical agents arrest the cell cycle progression at different points.

olomoucine [30]. Two other purine derivatives, roscovitine and bohémine, were shown to inhibit human CDK1 and CDK2 activities at concentrations 10–100-fold less than olomoucine [31,32]. These purine derivatives were tested as potential synchronising agents and used to characterise the requirement of different CDK activities for cell cycle progression in plants [3,6,7,33]. Their reversibility and specificity make these inhibitors suitable for synchronisation.

In plant cells, olomoucine inhibits both the G1/S and the G2/M transitions [6]. Roscovitine and bohémine provide more specific tools to investigate the functions of CDKs in plant cells since roscovitine and bohémine are efficient at a lower concentration than olomoucine in mammals. The inhibitory effects of roscovitine act at two points in tobacco BY-2 cell suspension: the G1/S and G2/M transitions [3]. In *Arabidopsis* cell suspensions, 50  $\mu\text{M}$  of roscovitine is needed to block the cell cycle in G1. This arrest is reversible (unpublished data). Roscovitine and bohémine were used on *Vicia faba* root tip cells and were shown to disturb the spindle formation when applied to metaphase cells [33].

#### 4.2. DNA synthesis inhibitors

DNA replication depends on the synthesis of deoxyribonucleotides and the activity of the enzymes of the replication machinery. Different DNA synthesis inhibitors are efficient in plants: hydroxyurea (HU), mimosine and aphidicolin.

HU inhibits the activity of ribonucleotide diphosphate reductase, thus depriving the cells of newly synthesised deoxyribonucleotide triphosphates, consequently preventing DNA replication [19]. In mammal cells, HU does not lead to synchrony at the G1/S boundary at low concentrations whereas, at high concentrations, HU is toxic to S phase cells [34].

HU treatment is more efficient when applied to protoplasts than to entire plants. The cell wall, the diversity of cell-types and uptake of the drug by the root system are physical barriers for entry of the chemical agent. The quantity of inhibitor required to block activity in whole plant organs is often 10-fold higher than that required for plant cells in suspension culture. For instance, 5 mM HU is needed to synchronise alfalfa cell suspensions in S phase [35] whereas 100 mM HU

Table 2  
DNA synthesis inhibitors

	Aphidicolin	HU	Mimosine
Tobacco BY-2 <i>Nicotiana tabacum</i>	6 $\mu\text{g/ml}$ for 24 h [2]; 20 $\mu\text{g/ml}$ for 24 h [12]	from 30 to 60 mM for 24 h [12]	2 mM for 24 h [41]
<i>A. thaliana</i> cell suspension	from 20 to 50 $\mu\text{g/ml}$ [44]; 30 $\mu\text{M}$ or 10 $\mu\text{g/ml}$ for 20 h [6]		100 $\mu\text{M}$ for 28 h (irreversible arrest) [53]
<i>Medicago sativa</i>	10 or 20 $\mu\text{g/ml}$ for 36 h [52]	5 mM for 24–36 h [35]	
<i>Catharanthus roseus</i>	50 $\mu\text{M}$ or 17 $\mu\text{g/ml}$ [11]		
<i>A. thaliana</i> roots		100 mM for 48 h [36]; 2–100 mM for 48 h [13]	
<i>P. hybrida</i> mesophyll protoplasts			200 $\mu\text{M}$ for 24 h [25]
<i>Pisum sativum</i> roots	150 $\mu\text{M}$ or 50 $\mu\text{g/ml}$ for 15 h [54]		
<i>V. faba</i> roots		2.5 mM for 18 h [14]	

is needed for *Arabidopsis* roots [13,36] (Table 2). Moreover, the release from HU allows the synchronisation at subsequent phases. Eight hours after the release from a HU block, 55% of mitotic synchrony can be observed in root tips of *V. faba* [14].

Mimosine, a plant amino acid, is extracted from koa hoale seeds (*Acacia koa*). This inhibitor blocks cell cycle progression at the G1/S interface but the precise mechanism of mimosine action is still unknown. In mammals, some clues are emerging on mimosine action. It was first described as a suppressor in the formation of the rare amino acid, hypusine, in the eukaryotic translation factor 4D [37]. According to [38], the mimosine block occurs before the establishment of active DNA replication forks by altering deoxyribonucleotide metabolism. This inhibitor can be used in the analysis of initiation of DNA replication in mammals [39].

In the light of its effect in mammals, mimosine was tested on the plant cell cycle. Nuclei of *P. hybrida* mesophyll protoplasts predominantly have a 2C DNA content and can be induced to re-enter the cell cycle [40]. Mimosine is able to block *P. hybrida* mesophyll protoplasts in G1 [25]. Very low DNA synthesis and histone *H3* gene transcription were detected when a concentration of 2 mM of mimosine was applied for 24 h to tobacco BY-2 cells [41] (Table 2). Hence, mimosine blocks BY-2 cells before S phase entry.

Aphidicolin is a widely used inhibitor for blocking replication. Aphidicolin is extracted from the fungus *Cephalosporium aphidicola* and is a specific DNA polymerase  $\alpha$  and  $\delta$  inhibitor that blocks reversibly the replication machinery [42]. After the release from aphidicolin, the block is rapidly reversible because DNA polymerase  $\alpha$  is immediately available and active to continue DNA replication. This rapid reversibility is an advantage when compared to the effects of HU and mimosine, where de novo synthesis of deoxyribonucleotide triphosphates is required before replication can start. Since the initiation of replication is not blocked by aphidicolin, this inhibitor is only used to study progression from the S phase.

A G1/S block is observed with 20  $\mu\text{g/ml}$  of aphidicolin in tobacco BY-2 cell suspensions, whereas an enrichment in S phase cells (up to 80%) is obtained with a lower concentration, 4  $\mu\text{g/ml}$  [3] (Table 2). Histone *H4* gene is expressed in parallel with DNA synthesis with a low concentration aphidicolin treatment (3  $\mu\text{g/ml}$ ) in tobacco BY-2 cell suspensions [12]. This suggests that aphidicolin acts by slowing down replication. An aphidicolin treatment can induce inhibition of DNA repair in mammals [43], but the expression of a DNA repair gene (*AtRAD51*) is not induced by aphidicolin itself in *Arabidopsis* [1]. Aphidicolin has been used to study S phase-specific gene expression of histone genes [12,44,45], a proliferating cellular nuclear antigen gene [46] and the *AtRAD51* gene [1].

A large number of cells going through the M phase can be obtained from a cell population released from a G1/S arrest, as described previously with HU. The release from the aphidicolin

block allows the study of genes expressed at the subsequent G2/M transition, like cyclin genes [17,18,47–50]. Moreover, *cdc2* gene expression and measurement of CDK activities during the cell cycle was made possible by using aphidicolin and the subsequent release of the arrested cells [3,51,52].

These chemical agents efficiently deliver a G1/S block and after the progression of cells to mitosis, the release from this block can be studied.

#### 4.3. Blocking at mitosis

**4.3.1. Anti-tubulin drugs.** At the onset of mitosis, the microtubule network is essential for spindle organisation and chromosome movement. Microtubules are dynamic polymers composed of tubulin. Inhibition of microtubule polymerisation is one of the key actions of anti-mitotic drugs, which act at the metaphase/anaphase transition. The anti-tubulin drugs were widely used in studies of microtubule and microfilament dynamics [55]. A metaphase arrest can be obtained by the use of anti-tubulin drugs, like colchicine, oryzalin, propyzamide and amiprosphos-methyl (APM) [24] (for review, see [56]). Large quantities of plant chromosomes are obtained by the use of cell cultures that are synchronised at mitosis with chemical agents that disturb microtubule polymerisation [57].

To obtain higher synchrony, the cells are usually first synchronised with aphidicolin, washed and further treated with the mitotic agent [2]. Ninety percent of tobacco BY-2 cells are blocked at metaphase when treated with propyzamide or oryzalin [2,3] (Table 3). The mitotic agents do not block cells indefinitely in metaphase, since, after a few hours, the chromosomes decondense and form micronuclei [24].

Colchicine is an alkaloid extracted from *Colchicum autumnale* that binds to tubulin dimers and prevents the formation of the mitotic spindle. Colchicine has been shown to bind with high affinity in mammalian cells, and induces metaphase arrest at concentrations of  $10^{-7}$  M (for review, see [58]). This agent is effective at millimolar levels in plants ([21]). However, mitotic arrest of plant cells is preferably obtained with drugs having the same effect but at micromolar concentrations such as oryzalin, APM and propyzamide. The use of lower concentrations of inhibitor, which are easier to wash out, can avoid abnormal mitosis and micronuclei formation.

Oryzalin is a dinitroaniline herbicide that has strong binding affinity for plant tubulins and inhibits microtubule polymerisation [22]. This drug was shown to arrest cells at metaphase [24] (Table 3). Oryzalin was also used at low concentrations (from 0.17  $\mu\text{M}$  to 1  $\mu\text{M}$ ) to study microtubule organisation in *Arabidopsis* roots [59]. APM is a phosphoric amide that has a high microtubule depolymerising activity [24]. Prolonged APM treatments can induce micronucleation in plant cells [60]. Propyzamide is a herbicide that induces mitotic arrest [23]. Cells blocked for 13 h with propyzamide

Table 3  
Anti-tubulin drugs

	Oryzalin	Propyzamide
Tobacco BY-2	15 $\mu\text{M}$ for 24 h [18]	1.6 $\mu\text{g/ml}$ or 6 $\mu\text{M}$ for 4 h from cells released for 6 h from an aphidicolin block [2]; 3 $\mu\text{M}$ for 10–14 h [3]
<i>P. hybrida</i> mesophyll protoplasts	3 $\mu\text{M}$ for 14 h [6]	
<i>A. thaliana</i> roots	2–30 $\mu\text{M}$ for 48 h [13]	

or oryzalin after an aphidicolin block contain individual metaphase chromosomes [3] (Table 3). The crucial point with these drugs is that the treatment has to be short (less than 14 h), otherwise it leads to abnormal cell division figures and micro-nuclei formation (unpublished data).

**4.3.2. Proteasome inhibitors.** The events at late mitosis, from sister chromatid separation to cytokinesis, are governed by ubiquitin-dependent proteolysis of key regulatory proteins. A major step in this proteolysis is catalysed by a multimeric ubiquitin ligase known as the anaphase-promoting complex (APC). This complex targets B-type cyclins and other regulatory proteins for degradation to the 26S proteasome, allowing exit from mitosis [61]. Proteasome inhibition blocks APC action, preventing the destruction of cyclins and consequently escape from mitosis (for review, see [62]). MG132 is a peptide aldehyde (carboxybenzoxyl-leucinyll-leucinyll-leucinal) that functions as a substrate analogue and inhibits the 26S proteasome activity. MG132 was shown to arrest tobacco BY-2 cells at metaphase/anaphase transition [26]. Following a release from aphidicolin, a MG132 treatment leads to 50% metaphase cells [26]. However, the treatment is not completely reversible since aberrant anaphase figures are observed after the release from the MG132. Lactacystin is a more specific but very expensive APC inhibitor and could be particularly useful for investigations of the requirement for the APC at the metaphase to anaphase transition [62].

## 5. Conclusion

Several chemical agents are at present available to synchronise plant cells for experiments on plant cell cycle progression. The active search for chemical inhibitors of CDK activity is stimulated by their potential therapeutic applications in cancer treatment. The crystallisation of CDK-inhibitor complexes greatly improved knowledge about the shape complementarity between an inhibitor and its target enzyme. Structural data from active CDK-cyclin inhibitor complexes revealed novel inhibitory interactions (for review, see [29]). This research provides new tools to study the function of CDK in plants. The phase in which cells are arrested by these inhibitors is an indication of the function(s) of these key regulators in the cell cycle progression. In return, the highly synchronised suspensions obtained with this type of inhibitor can be used to study the expression of cell cycle-regulated functions. The pieces of the puzzle of plant cell cycle events begin to fall into place by the integration of various results obtained with highly synchronised cell suspensions [28].

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