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Heterogeneity of dsRNA-induced cell apoptosis

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by

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ABSTRACT OF THE THESIS

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Double-stranded RNA is now being identified as one of the most significant activators of antiviral responses. Once cytoplasmic sensors detect dsRNA, a series of antiviral responses will be initiated, including inhibition of protein synthesis, transcriptional induction of many types of cytokines and cell apoptosis. Among those antiviral responses, cell apoptosis might be the most

significant one because too much apoptosis will cause irreversible damage to infected organism. However, apoptosis can simultaneously bring many advantages when fighting against viral infection, which begs the question of how cells should choose between survival and death when they sense dsRNA. In fact, heterogeneity of dsRNA-induced apoptosis always exists in nature though it is often ignored in many previous experiments. In order to answer the question of what the sources of heterogeneity of dsRNA-induced apoptosis are, we gathered information and conclusions from previous studies and made several hypotheses. We suspected that heterogeneity of dsRNA-induced apoptosis is derived from different level of Interferon production or different cell cycle phases. We further designed corresponding cell lines and experiments to test our hypotheses.

Introduction

Since Covid-19 broke out in 2020 and have killed at least 1 million people in the whole world, virus is receiving more and more attention. During the process of viral infection, double-stranded RNA (dsRNA) signaling is considered to be indispensable because most viruses produce dsRNA during their replication cycles. For those RNA viruses, dsRNA is produced as replicative intermediate. Surprisingly, though genomes of dsRNA viruses are composed of dsRNA, many studies observed that dsRNA viruses have evolved to capsule their genome carefully during replication cycle, probably in order to avoid exposure of dsRNA to host cells (Gantier & Williams, 2007; Kumar & Carmichael, 1998). For DNA viruses, dsRNA is often generated from bidirectional transcription of overlapping genes (Jacobs & Langland, 1996). Additionally, there were many studies detected dsRNA in virus-infected cells directly using antibodies (Lee, Marshall, & Bowden, 1994). All the facts above proved existence of dsRNA in virus-infected cells.

Our cells are very sensitive to foreign dsRNA. Even one molecule of dsRNA per cell can have profound effect on cell physiology (Marcus & Sekellick, 1977). Cellular responses to dsRNA include inhibition of protein synthesis, induction of cytokines, and also cell apoptosis. Specifically, inhibition of protein synthesis is achieved by activation of dsRNA-activated protein kinase, which is also known as protein kinase R (PKR). Activation of PKR by dsRNA phosphorylates eukaryotic initiation factor 2 (elF2), which disrupts initiation phase of mRNA translation (Gal-Ben-Ari, Barrera, Ehrlich, & Rosenblum, 2018). A wide range of cytokines and chemokines can be stimulated by dsRNA, and Interferon (IFN) Type I might be the most critical one since dsRNA has also long time been recognized as a potent IFN inducer. Considering that IFN plays an important role in not only antiviral defense but also antitumor defense, dsRNA and its derivatives

have widely been used as an immunostimulant in clinical trials (Hafner, Corthesy, & Merkle, 2013; McNally, Willette, Ye, Partida-Sanchez, & Flano, 2012).

For human, dsRNA-induced apoptosis can either be our enemy or friend. On one hand, virally induced apoptosis may lead to irreversible damage to human's body. For example, dsRNA can induce apoptosis in pancreatic β cells, and lead to type I diabetes (Dogusan et al., 2008). It is also reported that infection of reovirus can damage central neural system (CNS) and heart through inducing apoptosis (Clarke et al., 2005). On the other hand, dsRNA-induced apoptosis is also a weapon for human to fight against viral infection. Theoretically, virally induced apoptosis can eliminate infected cells and prevent further spreading of viruses. Several studies already illustrated that higher sensitivity to apoptosis leads to less severe viral infection (Marques et al., 2005). Moreover, dsRNA-induced apoptosis can be utilized by human to fight against cancer. In Hirasawa's study, reovirus was used as an antihuman colon and ovarian agent to destroy cancer cells (Hirasawa et al., 2002).

Pathways of dsRNA-induced apoptosis seem to be very complicated, and a lot of things remain unclear. Basically, there are two pathways of cell apoptosis: extrinsic pathway and intrinsic pathway. Extrinsic pathway involves death receptors, which are transmembrane receptors and share a cytoplasmic domain called "death domain". All types of death receptors have their corresponding adaptor proteins recruited by death domains. Activation of adaptors causes formation of death-inducing signaling complex (DISC), and eventually Cysteinyl Aspartate Specific Proteinase 8 (Caspase 8) is activated. In contrast, intrinsic pathway is activated by intracellular signals which include, but not limit to absence of growth factor, radiation, toxins, hypoxia and hyperthermia. Intrinsic pathway is initiated with changes in the inner mitochondrial membrane and opening of the mitochondrial permeability transition pore. Proapoptotic proteins

are then released from mitochondrial into the cytosol, leading to formation of apoptosome and activation of Caspase 9. The two pathways converge on cleavage of Caspase 3, which lead to DNA fragmentation, degradation of cytoskeletal, and many other apoptotic events (Elmore, 2007). Both extrinsic pathway and intrinsic pathway are related with dsRNA-induced apoptosis. Extrinsic pathway is mainly activated by Toll-like Receptor 3 (TLR3). Though TLR3 is not a typical death receptor and lacks a death domain, it can still recruit Receptor-interacting Protein 1 (RIP1), leading to activation of Caspase 8 (Bianchi, Pretto, Tagliabue, Balsari, & Sfondrini, 2017). Besides, melanoma differentiation–associated antigen 5 (MDA-5) and retinoic acid–inducible gene I (RIG-1) are involved in dsRNA-induced apoptosis, mainly through activation of mitochondrial pathway. Activation of Caspase 9 was observed in MDA-5 & RIG-1 mediated dsRNA-induced apoptosis, and inhibition of Caspase 9 significantly reduced that kind of apoptosis (Besch et al., 2009). PKR was recognized as a key mediator for many cell responses to dsRNA, including several types of apoptosis. However, the function of PKR in dsRNA-induced pathway remains controversial. Several studies claimed that dsRNA-induced apoptosis occurred in a PKR-independent manner (Besch et al., 2009; Salaun, Coste, Rissoan, Lebecque, & Renno, 2006), which suggests that PKR might not mediate dsRNA-induced apoptosis in all types of cells (Figure 1a).

In addition to complexity of pathways of dsRNA-induced apoptosis, outcome of dsRNA infection is also unpredictable. Previous studies suggested that heterogeneity did exist in dsRNA-induced apoptosis, which means cells will not die equally under the pressure of viral infection. In Salaun's research paper, they reported that Poly(A:U) have apoptotic effect on breast cancer cells. Treatment of Poly(A:U) caused around 80% of Cama-1 cells death and still around 20% of cells survived after 48 hours (Salaun et al., 2006). In our time-lapse imaging experiment, heterogeneity of dsRNA-induced apoptosis was also observed. Polyinosinic-polycytidylic acid (Poly (I:C)) was

used to mimic dsRNA signal. Poly (I:C) is structurally similar to double-stranded RNA and can simulate viral infection. Treatment of Poly(I:C) caused that 33% HeLa cells died, and 67% HeLa cells survived after 10 hours.

Notably, there are some pathways stimulated by dsRNA that can potentially contribute to cell survival. The most significant evidence might be that many studies observed that nuclear factor kappa b (NF-kB) can be stimulated when cells get exposed to dsRNA (Jiang, Mak, Sen, & Li, 2004) (Figure 1b). NF-kB is a well-known transcription factor and activation of NF-kB is often related to cell survival and proliferation.

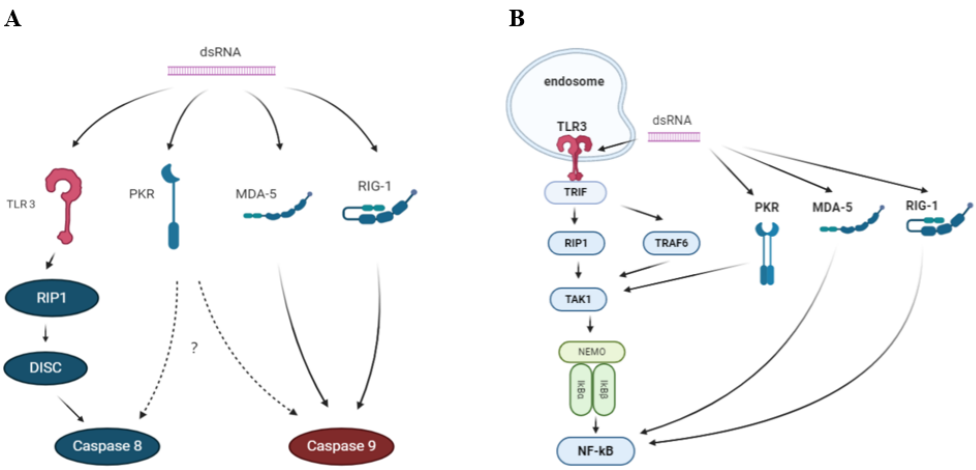

Figure 1. Summary of dsRNA-induced cell death and survival pathway
(A) Summary of dsRNA-induced apoptosis pathway. TLR3 is known to be able to activate Caspase 8, while MDA-5 and RIG-1 are known to be able to activate Caspase 9. Function of PKR remains controversial (B) Summary of dsRNA-induced NF-kB pathway. (Generated from BioGender.com)

In conclusion, considering that both apoptosis pathway and survival pathway can be stimulated by dsRNA, how fates of cells are determined become a significant question, since neither apoptosis nor cell survival is always beneficial for human. In order to tackle the question and identify sources of heterogeneity of dsRNA-induced apoptosis, we made two hypotheses based on information from previous studies – 1. Induction of IFN can potentiate dsRNA-induced apoptosis, 2. Cells in different cell cycle phases have different rate of dsRNA-induced apoptosis.

Induction of IFN might affect dsRNA-induced apoptosis

Back to 1970s, dsRNA and its derivatives were found to be able to strongly induce cytokines, especially IFN Type I. Interferons are classified into three distinct types based on their receptors: IFN Type I, type II and type III. Type I IFN includes IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω in human, and are mainly induced by viral infection. Type II IFN are also known as immune IFN and just have one member – IFN-γ. IFN Type II is mainly released by immune cells. IFN Type III is recently discovered IFN and is functionally similar to IFN Type I. However, though IFN Type I can be induced by dsRNA, this phenomenon is limited in epithelium cells (Samuel, 2001).

IFN plays an important role in virus defense, since the name "Interferon" was derived from the ability of "interfering" viral replication. First, IFN can mediate viral defense through activating immune system. Several kinds of immune cells are activated by interferon, especially nature killer cells and macrophages. IFN signaling can also stimulate antiviral responses in infected cells by stimulating ISGs (Interferon stimulating genes). There are already ~ 2000 ISG been identified and their complicated network may achieve almost any aspect of antiviral state, including inhibition of replication of viral genome, synthesis of protein, and also can induce apoptosis in infected cells.

Notably, IFN seems to have apoptotic effect on cells, but not in a direct way. Several studies claimed that IFN can potentiate cell apoptosis by upregulating expression of caspase 8 (Balachandran et al., 2000; Fulda & Debatin, 2002). More specifically, the function of IFN in dsRNA-induced apoptosis was highlighted in many studies. Salaun's study showed that IFN was necessary but not sufficient for dsRNA to induce apoptosis in breast cancer cell (Salaun et al., 2006), indicating the central role of IFN in dsRNA-induced apoptosis.

Though previous studies had proven that dsRNA is a potent IFN inducer overall, cell responses are still heterogenous and vibrant IFN production is sometimes not promised. First, in real cases, some viruses have evolved to disrupt induction of IFN to protect themselves. For example, hepatitis B virus can inhibit induction of IFN in hepatocytes to cause persistent infections (Whitten, Quets, & Schloemer, 1991). In addition, despite the fact of dsRNA can induce IFN Type I in almost any type of cells, it is reported that different cells have different abilities to activate IFN transcription when sensing dsRNA. Previous studies proved that many kinds of lung cancer cells have impaired virus-activated IFN responses, probably because of abolishment of dsRNA sensors and their corresponding signaling pathways (K. Li, Chen, Kato, Gale, & Lemon, 2005). Considering that production of IFN can strongly affect outcome of viral infection, we first suspected that different levels of dsRNA-induced IFN production contribute to heterogeneity of cell apoptosis.

In order to characterize dsRNA-induced IFN production, we made an IFN-β reporter cell line using lentiviral system. The cell line was made based on an existed cell line, which already carried a nucleus marker and Stat1 was tagged with mCherry. We inserted gene of cyan-fluorescent protein (CFP) which is driven by 2x IFN-β promotor (Figure 2a). Once phosphorylated IRF3 translocate to nucleus and bind to IFN-β promotor, CFP will express along with expression of IFN-β. Then we treated the cell line with 10 μg/ml Poly(I:C), and recorded cells using time-lapse imaging. We randomly picked two sample cells and analyzed their IFN production. Heterogeneity of IFN production was observed since the two cells produced significantly different amount of IFN Besides, difference of Stat1 translocation patterns suggested that IFN signaling does not correlate with IFN production. The cell that produced more IFN actually had lower Stat1 translocation. (Figure 2c). Thus, we will further consider IFN signaling as a separate factor.

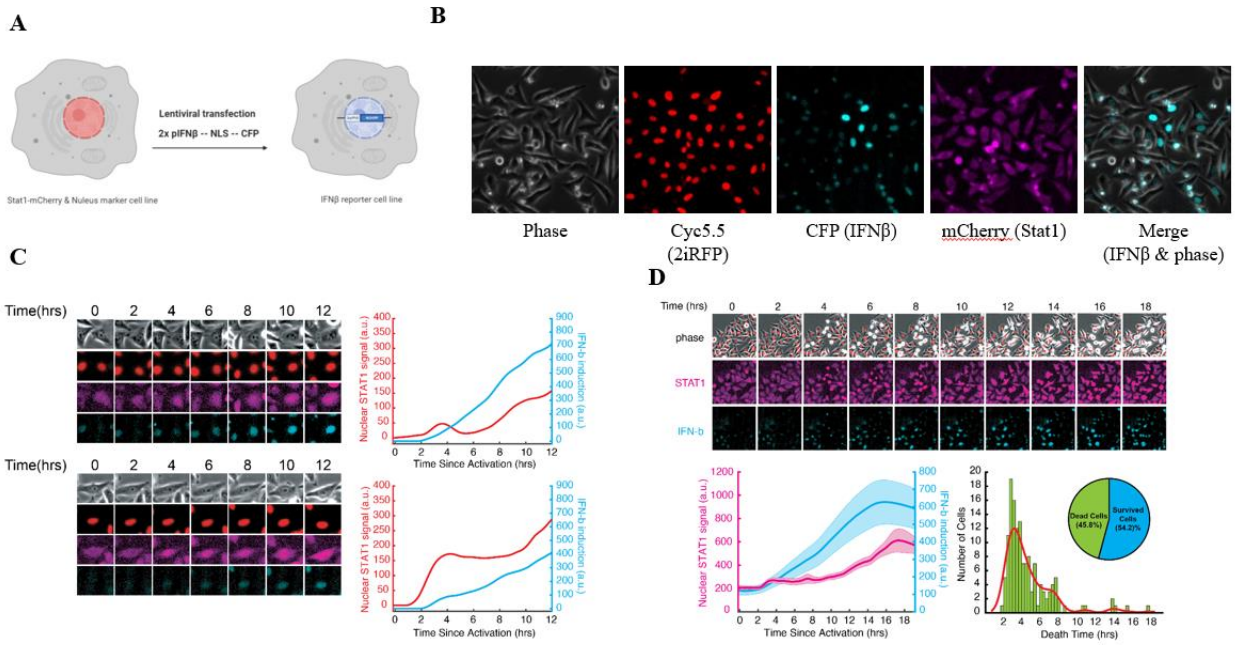

Figure 2. IFNβ reporter cell line construction and preliminary validation
(A) A diagram of IFNβ reporter cell line construction. The cell line was made based on a nucleus and Stat 1 tagged cell line. CFP along with promotor of IFNβ was inserted in cell using lentiviral system (B) The IFNβ reporter cell line was treated with 10 μg/ml Poly(I:C). (C) Two sample cells were chosen to be analyzed. The two cells showed different abilities of IFN production. Besides, Stat1 translocation seems to not correlate with IFN production. (D) Statistical analysis of IFN production, STAT1 translocation and cell death time distribution.

the cell line still needs be further validated. Since simple lentiviral transfection cannot make genotypes of cells to be homogenous, we are going to first make the cells line to be homogenous so that we can quantify induction of IFN based on intensity of fluorescent protein. Then we will treat the improved cell line with Poly(I:C) and do single-cell analysis so that we can correlate IFN production with cell apoptosis. Considering importance of IFN in dsRNA-induced apoptosis, I expect to see that cells which undergo apoptosis would have higher activities of IFN promotor.

Cell cycle phase-dependent dsRNA-induced apoptosis

Though the relationship between cell cycle and apoptosis has been emphasized in several studies, no one tried to correlate dsRNA-induced apoptosis with cell cycle yet. Apoptosis is affected by many cell cycle-dependent molecules and pathways. Cyclin-dependent kinases (Cdks) are known to have regulatory function in cell cycle. Depending on cyclins, activities of Cdks will increase or decrease at certain cell cycle phases (Pucci, Kasten, & Giordano, 2000). Some studies related activities of Cdks to apoptosis. Li's study had shown that activations of cyclin A/CDK2 and cyclin A/Cdc2 are required in HIV-1 Tat-induced apoptosis (C. J. Li, Friedman, Wang, Metelev, & Pardee, 1995). Similarly, it is reported that activation of cyclin A/CDK2 are necessary for thymocyte apoptosis (Gil-Gomez, Berns, & Brady, 1998). Not only CDKs, some other cell proliferation regulator, including p53, Myc or pRb, play an important role in promoting apoptosis. Those factors indicate that those cell cycle phase-dependent molecules should not be ignored when considering cell apoptosis.

Moreover, an interesting phenomenon was observed in Poly(I:C) treated HeLa cells, which potentially support the hypothesis. For those cells underwent apoptosis, they could be classified into three distinctive groups. The first one is that cells died before division after treatment of Poly(I:C). The second one is that cells divided first, and then two daughter cells died at similar time point after division. The third one is that cells divided first, and two daughter cells died at different time point. It is possible that one daughter cell died and the other survived (Figure 3a). Surprisingly, cells were very likely to undergo apoptosis with the second death pattern. For those daughter cells that underwent apoptosis, 58% of them died at similar time point while 42% of them died differently (Figure 3c). Since paired daughter cells are generated from one cell and they share

the same cell cycle phase, the second pattern of cell death suggested that cell cycle phase might be a determinant of cell apoptosis.

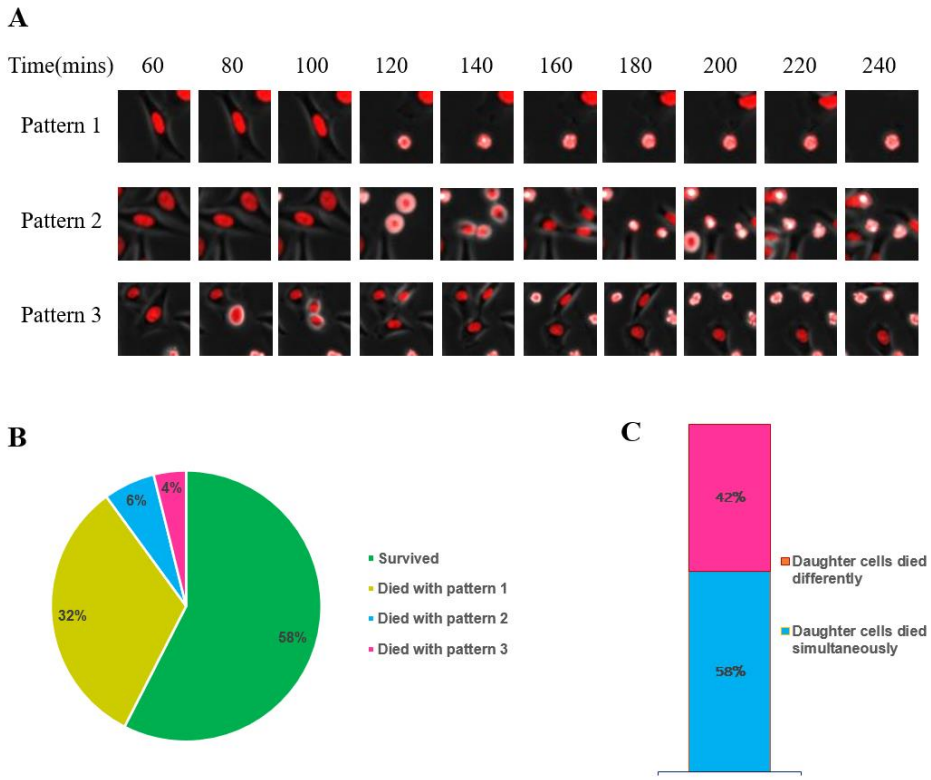

Figure 3. Three patterns of dsRNA-induced apoptosis
(A) Three different patterns of cell death were concluded from Poly(I:C) treatment experiment. Pattern 1: cell died before division. Pattern 2: cell divided, and two daughter cells died similarly. Pattern 3: cell divided, and two daughter cells died differently (e.g., one survived and one died). (B) Proportion of cell survival and each death pattern, n=259. (C) 58% of daughter cells died almost simultaneously on condition that cells divided first and at least one of daughter cells died.

In order to test the second hypothesis, we propose to use a cell cycle phase reporter cell line to elucidate the relationship between cell cycle phase and dsRNA-induced apoptosis. The cell line was already generated and can report cell cycle phases based on the fluorescent signals. Basically, mCherry was C-terminally tagged to amino acids 994-1084 of human DNA helicases B (DHB), which contains four CDK consensus phosphorylation sites, a nuclear export signal (NES), and a nuclear localization signal (NLS). This construct can be phosphorylated by CDK2. When activities of CDK2 are low, DHB are not phosphorylated and translocate to nucleus. When activities of CDK2 get higher, DHB are phosphorylated and exported to cytoplasm. Since activities of CDK2 change periodically along with cell cycle, we can tell corresponding cell cycle phase based on the fluorescence intensity in nucleus. Specially, cells in G1 have DHB in their nucleus and during S phase, DHB will be exported out of nucleus. Once cells enter G2 phase, level of DHB in nucleus will drop to the bottom (Spencer et al., 2013).

Treating this cell line with Poly(I:C) will allow us to identify if cells in different cell cycle phases respond to dsRNA differently. We expect to see cells within different cell cycle phases have different ratio of cell death, and also cell apoptosis happens not randomly in three cell cycle phases. Once we confirmed that cell apoptosis is more likely to happen in one certain cell cycle phase, potential mechanisms will be further investigated.

Besides, we would like to test effect of cell cycle inhibitors on dsRNA-induced apoptosis. Several kinds of cell cycle inhibitors were developed, in order to synchronize cell cycle phase of mammalian cells. Specially, we propose to test lovastatin, roscovitine and PD0332991. Lovastatin are found to be able to arrest normal cells and tumor cells in G1 phase reversibly (Keyomarsi, Sandoval, Band, & Pardee, 1991; Meikrantz, Gisselbrecht, Tam, & Schlegel, 1994). Roscovitine inhibits G1/S or G2/M Transition depending on the doses of the drug (Kolodziej et al., 2015).

PD0332991 is a drug that inhibits activities of CDK4 and CDK6 and also arrest cells in G1 phase (Fry et al., 2004). (Table 1)

By using those cell cycle inhibitors and let them synchronize in the same cell cycle phase, we can then treat cells with Poly(I:C) to check if those drugs can facilitate apoptosis or antagonize apoptotic effect of dsRNA. We expect that lovastatin and PD0332991 will lower the rate of dsRNA-induced apoptosis, while roscovitine will potentiate dsRNA-induced apoptosis. On one hand, roscovitine was reported to have pro-apoptotic effect on cells while the others have limited ability to induce apoptosis directly. On the other hand, cells which stay in G1 phase have low cyclin A activities, and activation of cyclin A/CDK2 seems to be required in many types of apoptosis.

Table 1. List of cell cycle inhibitors that can potentially affect dsRNA-induced apotposis

Name	**Effect on cells**	**Reference**
Lovastatin	Arrest in G1 phase; Little apoptotic effect on cells	(Keyomarsi et al., 1991; Meikrantz et al., 1994)
Roscovitine	Arrest in G1/S or G2/M depending on dose of the drug; Have proapoptotic effect on cells	(Kolodziej et al., 2015)
PD0332991	Arrest in G1 phase; have anti-proliferative effect on certain types of tumor	(Fry et al., 2004)

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